Neuroprotective effects of Apigenin in Human Neuroblastoma SH-SY5Y cells Against Rotenone toxicity: a pilot study.

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Received on: 21-11-2013; Revised on: 12-12- 2013; Accepted on:18-12-2013

ABSTRACT

Parkinson’s disease (PD) is a degenerative disorder of the central nervous system that often impairs the sufferer’s motor skills, speech, and other functions. It is a debilitating neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra. The purpose of this study was to investigate the neuroprotective effects of apigenin (AGN) on rotenone (ROT) induced apoptosis in human dopaminergic SH-SY5Y cells. SH-SY5Y cells exposed to the ROT (10µM) significantly decreased the viability of SH-SY5Y cells, when examined by MTT assay and the cytotoxicity was examined by the lactate dehydrogenase released into the culture medium. DNA fragmentation was assayed as index of apoptosis. The nuclear damage and the cellular morphology were assessed by a fluorescent DNA-binding probe Hoechst 33258 and phase contrast microscopy, respectively. The results indicated that AGN increased the cell viability (MTT reduction), prevented the membrane damage (LDH release), in ROT induced SH-SY5Y cells. DNA laddering produced by ROT treatment was also prevented by apigenin. Apigenin pre-treatment attenuated the ROT induced cell shrinkage and nuclear condensation. These findings demonstrate that the flavonoid apigenin have neuroprotective effects against ROT induced neurotoxicity in SH-SY5Y cells and might provide a useful therapeutic strategy for the treatment of neurodegenerative disease like Parkinson’s disease.

Keywords Parkinson’s disease, SH-SY5Y cells, Apigenin, Rotenone, Neuroprotection.

1. INTRODUCTION

Parkinson’s disease (PD) is a chronic, progressive neurodegenerative movement disorder that affects more than six million people worldwide. The main pathological hallmark of idiopathic PD is a progressive loss of neuromelanin-containing dopaminergic neurons from the substantia nigra pars compacta (SNpc), a midbrain structure [1]. Cardinal signs of PD include tremor at rest, rigidity, akinesia (bradykinesia) and clinical features include secondary motor symptoms like postural instability and hypomimia, dystarthritis, dysphagia, slaorrhoea, micrographia, non-motor symptoms like autonomic dysfunction, cognitive/neurobehavioral abnormalities, sleep disorders and sensory abnormalities [2]. Dopaminergic cell loss is associated with the presence of cosinophilic intraneuronal inclusions, called Lewy bodies composed of neurofilament and ubiquitin [3]. The main mitochondrial defect observed in degenerating PD concerns complex I (nicotinamide adenine dinucleotide coenzyme Q reductase) of the mitochondrial respiratory chain [4]. Decreases in the activity and immunoreactivity of the reduced form of the complex I were observed in the SNpc of PD patients [3]. Rotenone (ROT) is a complex I inhibitor that is known to cause the degeneration of dopaminergic neurons and PD-related motor dysfunction [4]. Oxidative stress in PD arises from the metabolism of dopamine itself by both chemical and enzymatic mechanisms [5 & 6].

ROT is a naturally occurring complex ketone, derived from the roots of Lonchocarpus species [7 & 8] that has been used extensively as a prototypic mitochondrial toxin in cell cultures; exposure to it has been linked to a higher risk of PD [9 & 10]. Greanamyre [8] and colleagues reported that the administration of low-dose intravenous ROT to rats produces selective degeneration of nigrostriatal dopaminergic neurons accompanied by α-synuclein-positive LB-like inclusions [11]. ROT impairs oxidative phosphorylation in the mitochondria by inhibiting reduced nicotinamide adenine dinucleotide (NADH)-ubiquinone reductase activity through its binding to the PSST subunit of the multipolypeptide enzyme complex I of the electron transport chain [12].

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Flavonoids are naturally-occurring polyphenolic compounds presented in a variety of fruits, vegetables, and seeds. They can be classified into flavonols, flavanones, flavones, isoflavones, and anthocyanidins. Flavonoids have many biological, pharmacological activities including antioxidative, anti-inflammatory, and antitumor effects. Apigenin (4’, 5, 7-trihydroxyflavone) is a member of the flavones subclass of flavonoids present in fruits and vegetables such as onions, oranges, parsley, and chamomile [12-13]. Apigenin has long been considered to have various biological activities such as antioxidant, anti-inflammatory, anti-mutagenic, and anti-tumorigenic properties in various cell types.

In the present study, we evaluated the neuroprotective effects of AGN against ROT induced neurotoxicity in human neuroblastoma SH-SY5Y cells, a commonly used cellular model of PD.

2. MATERIALS AND METHODS

2.1. Cell culture and drug treatment

SH-SY5Y cells, a human dopaminergic neuroblastoma cell line were obtained from National centre for cell science (NCCS, Pune), and grown in Dulbecco’s modified Eagle’s medium/ Nutrient mixture F12 Ham (DMEM/F12, 1:1 mixture) (HiMedia, India) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma) and 100 U/ml penicillin/streptomycin. Cultures were maintained in a humidified incubator at 37°C with 5% CO2, and the medium was changed every 2 days. ROT (Sigma, India) was prepared fresh in dimethyl sulfoxide prior to each experiment. Apigenin (Sigma) was dissolved in DMSO and made up with DMEM. To evaluate the cell viability, AGN was added to the cultures 4 h prior to the ROT treatment.

2.2. Measurement of Cell viability by MTT assay

For the cell viability assay, the SH-SY5Y cells were plated in a 96 well plate at a density of 10 x 10^3 cells/ well and cultured for 24 h. ROT (Sigma, India) and apigenin (Sigma) were reconstituted fresh in dimethyl sulfoxide and distilled water, respectively, prior to each experiment. AGN was added 4 h prior to ROT treatment in cultures to evaluate its anti-apoptotic effect. Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as previously described [15]. After incubation with MTT solution (Sigma) for 4 h, the cells were exposed to an MTT-dissolving solution (DMSO) for 1 h. Cell viability was determined using an absorbance microplate reader (Thermo electron corporation) at a wavelength of 450 nm.

2.3. LDH release assay

Lactate dehydrogenase (LDH), which is a soluble cytosolic enzyme present in most eukaryotic cells, releases into culture medium upon cell death due to damage of plasma membrane. The increase of the LDH activity in culture supernatant is proportional to the number of lysed cells. To evaluate the general cytotoxicity, LDH release was measured by LDH cytotoxicity assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s protocol. The assay is based upon a coupled enzymatic conversion from tetrazolium salt (INT) to highly colored formazan which absorbs strongly at 490-520 nm. This enzymatic reaction is catalysed by the LDH released from the cells. Absorbance was measured at 490nm with a microplate reader (Thermo Electron Corporation).

2.4. Analysis of cellular morphology for phase contrast microscopy

Cells were incubated with 10 μM ROT in the presence or absence of AGN for 24 h and observed by phase contrast microscopy and fluorescence microscopy to analyse morphological changes. The medium was changed before the observation to remove the dead cells that had lost adherence to the plate. A 200 X objective was used.

2.5. Nuclear morphology by Hoechst 33342 staining

Changes in nuclear morphologies of apoptotic cells were investigated by labeling the cells with Hoechst 33342 nuclear stain, followed by fluorescent microscopy. Briefly, SH SY-5Y cells were pre-plated in 6 well plates (1.0×10^5 cells/ml) and treated with 10 μM ROT for 24 h after pre-treatment with AGN at 10μM for 1h. Cells were then stained with Hoechst 33342 (5 μg/ml), and the cells were washed with PBS and observed using fluorescence microscope (Zeiss Axio observer).

2.6. Measurement of mitochondrial membrane potential by rhodamine 123 staining

SH-SY5Y cells were plated in 6 well plates (1×10^5 cells/ml) and exposed to 10 μM ROT after the pre-treatment with 10 μM AGN for 1h. The cells were washed with PBS after 24 hours and fixed with methanol at RT for 20 minutes. The fixed cells were then incubated with 5 μg/ml of rhodamine 123 at 37°C for 30 mins. The stained cells were observed under fluorescent microscope (Zeiss Axio observer) with an excitation wavelength at 488 nm and emission wavelength at 525 nm.

2.7. Detection of DNA fragmentation due to apoptosis

SH SY-5Y cells (1.0×10^5 cells/ml) cells were pre-plated for 24 h and then treated with 10 μM ROT for 24 h after pre-treatment with 10 and 20 μM AGN for 1h. After incubation, 500 μl of lysis buffer (5mM Tris (pH 7.4) 5mM EDTA and 1% Triton X-100) was added and mixed for 3–4 minutes. The lysate was centrifuged at 13000 ×g for 10 minutes at 4°C. To the supernatant, equal volume of ice cold isopropanol was added, mixed well and centrifuged at 13,000 × g for 15 minutes at RT and the supernatant was discarded. To the pellet, 1 ml of 70% ethanol was added and centrifuged at 13,000 × g for 15 minutes at RT and supernatant was discarded. The pellet was dried at 37°C for 10 minutes, 50 μl of RNase free water was added to each vial and the DNA was suspended by placing the vial at 65°C for 15 minutes or at 4°C overnight DNA samples (10 μl) were electrophoresed on a 1.2% agarose gel in 450 mM Tris borate-EDTA buffer, pH 8. Then the gel was visualised under a UV transilluminator (Alpha imager, Innotech, Canada).

2.8. Statistical analysis

Data from at least three independent experiments were expressed as mean ± SD. Statistical evaluation was performed by one way analysis
of variance (ANOVA) followed by Duncan’s multiple range test (DMRT) using SPSS 17 version. A value of $p < 0.05$ was considered significant.

3. RESULTS

3.1. Apigenin attenuated rotenone induced cytotoxicity

Cell viability was measured by MTT assay. After exposure to ROT from 5 µM to 50 µM for 48 h, the cell viability decreased significantly. The survival of SH SY5Y cells exposed to ROT and AGN is shown in (Fig 1 a and b). The 50% inhibitory concentration(IC 50) was noted at 10 µM ROT and this concentration was used for subsequent experiments. Cells were treated with different concentrations of apigenin and it was found that the IC 50 of apigenin was around 60 µM.

3.2. Effect of apigenin on LDH release

Lactate dehydrogenase (LDH) is a soluble enzyme located in the cytosol. The enzyme is released into the surrounding culture medium upon cell damage or lysis, process that occur during both apoptosis and necrosis. The results showed that the treatment with 10µM ROT for 24 h showed a significant increase in LDH release of 47% (Fig 2). Preincubation with AGN (10, 20 and 30 µM) for 1 h significantly decreased the LDH release to 36 – 41 % in SH SY5Y cells.

Fig. 1 MTT Cell viability assay.

(a) Effect of different concentration of ROT on cell viability in normal SHSY-5Y cells after treatment for up to 24 h. (b) cells were treated with different concentrations of AGN for 24 h. All data are expressed as percent of values in untreated control cultures and are means ± SD of four experiments. For statistical evaluations, one way ANOVA analysis followed by Duncan’s multiple range test were used. *$P < 0.01$, versus control.

3.3. Effect of apigenin on cellular morphology

To assess the cellular morphology, SH SY5Y cells were treated with ROT for 24 hrs and observed under inverted phase contrast microscope. In the ROT induced case, the cells showed retraction of cytoplasm and shrinkage (Fig 3c). The effect was partially reverted when the cells were pre-treated with apigenin (AGN) at a dose of 10 µM (Fig 3c).
3.4. Apigenin suppressed the apoptosis and necrosis in rotenone induced SH SY5Y cells
To assess the protective effect of AGN against neurotoxicity of ROT in qualitative term, we investigated the effect on the nuclear morphological changes observed in the presence or absence of AGN. Hoechst 33342 assay revealed the appearance of a collection of multiple chromatin and fragmented apoptotic nuclei upon ROT treatment at a concentration of 10 µM for 48 h (Fig. 3b1). However, when SH SY5Y cells were synchronously treated with 10 µM AGN in the presence of ROT, a significant reduction in the apoptotic nuclei was observed (Fig. 3c1).

3.5. Apigenin attenuates the rotenone induced decrease in mitochondrial membrane potential
Mitochondrial dysfunction plays an important role in the induction of apoptosis and the mitochondrial membrane potential is affected by apoptotic factors, the earliest recorded change is the reduction of mitochondrial membrane potential, and then cells enter the irreversible apoptotic process. The loss in mitochondrial membrane potential was visualised by rhodamine 123 staining. Rhodamine 123 is a cationic fluoroscent dye retained functional mitochondria with high mitochondrial membrane potential. When there is a decrease in mem-

Fig. 3 Photomicrograph of Hoechst 33342 and Rhodamine staining of SH-SY5Y cells. Cells incubated with ROT and / or AGN and visualised and photographed under 200 x magnification of fluorescence microscopy. a - d shows phase contrast microscopy, a1 - d1 shows the Hoechst staining, a2 - d2 shows the rhodamine 123 staining, a3 – d3 shows the merged photograph of both stains. a, a1, a2, a3 – Control cells. B, b1, b2, b3- 10 µM ROT, c, c1, c2, c3- 10 µM ROT+ 10 µM AGN. d, d1, d2, d3- 10 µM AGN. The arrows in b1, b2 and b3 indicate the chromatin condensation in the cells treated with ROT (10 µM). The arrows in c1, c2 and c3 indicate the retrieval of nuclear morphology in the AGN treated cells. AGN was administered 1h prior addition of 10 µM ROT to SH-SY5Y cells.
brane potential, the dye is released into the cytosol and can be detected further. The results showed that there is a decrease in the fluorescent intensity representing a fall in the mitochondrial membrane potential after the cells were treated with ROT. (Fig. 3a2-d2). The treatment with 10 µM AGN to the cells exposed to ROT, significantly inhibited the fall in mitochondrial membrane potential caused by ROT.

3.6. Effect of apigenin on DNA fragmentation

The DNA laddering was observed in the cells treated with 10µM ROT. The figure shows the results of DNA electrophoresis in the cells pre-treated with 10 µM and 20 µM AGN, 1 hr before exposure to ROT. (Fig 4) shows that, AGN at these concentrations prevented the ROT induced cell injury and death and also reduced the formation of DNA ladder.

MTT cell viability assay showed that AGN antagonized ROT induced cell death, which may be due to the ability of AGN in increasing the cell viability of normal cells. Through phase contrast microscopy and fluorescence microscopy, our result confirmed that in SH-SY5Y cells, ROT induced cell death by apoptotic mechanism and that AGN pre-treatment markedly mitigated these distinct morphological changes. The nuclear damage in ROT treated cells and the AGN pre-treated cells was evaluated by Hoechst 33342 staining. Condensed and fragmented nuclei were brightly stained and these were considered as apoptotic cells.

DNA fragmentation is one of the main hallmarks of apoptotic death. ROT induced SH-SY5Y cells exhibited DNA fragmentation at a dose of 10 µM. Pre-treatment of SH-SY5Y cells with AGN dose dependently reduced the ROT induced DNA laddering, resulting from fragmentation of internucleosomal DNA into specific oligonucleosomal DNA and hence it is considered as a biochemical hallmark of apoptosis.

For Hoechst 33342 and Rhodamine staining, SHSY5Y cells were pre-treated with 10µM AGN for 2 hrs and then treated with 10 µM ROT for 24 hrs. Microscopic imaging showed that AGN decreases ROT induced cell shrinkage, irregular morphology and cellular detachment in ROT induced cells. AGN treated cells showed greater improvement in cellular morphology similar to the control cells.

It has been shown that ROT induces apoptosis in the dopaminergic SH-SY5Y cells that requires the activation of the P38 MAP kinases, caspases and JNK. The p38 kinase is a member of the mitogen-activated protein (MAP) kinase superfamily activated by stress signals and is implicated in cellular processes involving inflammation and apoptosis. c-Jun N-terminal kinase (JNK) is another well-known MAP kinase involved in many apoptotic signaling events and it has been implicated DA-induced apoptosis as evidenced by increased JNK activity, phosphorylation of c-Jun and in primary neonatal rat striatal neuronal cultures. It has been suggested that these pathways may be viable drug targets for slowing down the disease progression of Parkinson’s disease by preserving dopamine synthesizing neurons that have not yet been lost to the disease.

Flavonoids can protect the brain by their ability to modulate intracel-
The mechanism behind the neuroprotective effect of AGN remains to be outlined. Studies show that AGN suppressed p38 mitogen activated protein kinase (MAPK), c-jun N-terminal kinase (JNK) phosphorylation without affecting the extracellular signal related kinase [14]. AGN also showed its anti-amnesic and protective effects against Aβ25-35-induced toxicity and the underlying mechanisms in the cerebral cortex of mice [34]. Although the precise mechanism by which AGN exerts its beneficial action is unclear, antioxidant activity could not be ruled out.

5. CONCLUSION
To conclude, our results show that AGN protects SH SY5Y cells against ROT induced neurotoxicity via ameliorating the mitochondrial dysfunction that is associated with oxidative stress. The present results support that AGN is a potent neuroprotective agent against ROT induced apoptosis in human neuroblastoma SH- SY5Y cells.

ACKNOWLEDGEMENTS
This study was supported by University of Madras, Department of Medical Biochemistry, Taramani Campus in the form of University research fellowship (URF). The authors are thankful to the Virology Department, King Institute of Preventive Medicine and Research, Guindy, Chennai – 600032, Tamil Nadu, India for carrying out the in vitro work.

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Source of support: Nil, Conflict of interest: None Declared