

# Comparable neuroprotective effect of rapamycin against low and high rotenone concentrations in primary dopaminergic cell culture

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## ABSTRACT

The present study was carried out to investigate neuroprotective effects of the autophagy inducer rapamycin against low and high concentrations of rotenone in primary dopaminergic cell culture. Cultures prepared from embryonic mouse mesencephala were treated on the 10<sup>th</sup> day in vitro (DIV) with different concentrations of rotenone (10, 20 nM) and rapamycin (1, 10, 100, 1000 nM) for 48 h. On the 12<sup>th</sup> DIV, cultures were stained immunocytochemically using tyrosine hydroxylase antibody. Rotenone significantly reduced the number of dopamine neurons/their neurites by 22/33% and 40/60% at the concentrations of 10 and 20 nM, respectively. On the other hand, rapamycin was seen to completely reverse rotenone's effect on the number of dopamine neurons at the 10 nM rotenone while it rescued only 17% in cultures treated with 20 nM of rotenone. Moreover, rapamycin caused much more attenuation in the loss of cell neurites in cultures treated with 10 nM rotenone (57%) than those treated with 20 nM rotenone (48%). In conclusion, rapamycin produces much protection against low rotenone concentration on dopamine neurons. This effect might be attributed to the efficacy of the autophagy process induced by rapamycin in repairing slightly damaged dopamine neurons by low rotenone concentration.

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## INTRODUCTION

Parkinson's disease (PD) is a chronic neurodegenerative disorder with a global prevalence of 1 - 3% in the population over 55 years (Fahn and Sulzer, 2004). The disease is characterized by preferential death of dopamine neurons in substantia nigra pars compacta (SNpc) and subsequent depletion of dopamine levels in the striatum (Li *et al.*, 2015). This leads to the cardinal motor symptoms including resting tremors, bradykinesia, muscle rigidity and postural instability. These motor signs may be accompanied by some non-motor symptoms such as constipation, urinary symptoms, sleep disorders and dementia

(Fahn, 2003). Although the etiology of PD is still elusive, two forms of the disease are recognized, genetic and sporadic forms. Genetic form represents approximately 5 - 10% of the cases and results from mutations in some gene loci including alpha-synuclein, parkin, PINK, LRRK2 and some other genes (Singleton *et al.*, 2013). Sporadic PD affects 90% of the cases and is thought to be associated with environmental exposures, most notably to pesticides (de Lau and Breteler, 2006; Lees *et al.*, 2009). Liu *et al.*, (2015) reported that there is a strongest association between pesticides and the incidence of PD. Rotenone is a naturally occurring insecticide that derived from the roots of *Derris* and *Lanchorcarpus* plant species. It is used as an active ingredient in hundreds of pesticide products worldwide (Betarbet *et al.*, 2000). Upon exposure, rotenone as a hydrophobe easily crosses biological membranes and reaching mitochondria where it produces inhibition of complex I of respiratory chain (NADH-dehydrogenase) (Worth *et al.*, 2014).

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Since 2000 when Betarbet and her colleagues (Betarbet *et al.*, 2000) used rotenone to reproduce pathological features characteristic of PD in rats, rotenone has been suggested to play an important role in the development of the disease (Brown *et al.*, 2006). At present, levodopa therapy remains the most effective symptomatic treatment for PD patients (Singer, 2012). With time, levodopa therapy becomes inadequate in controlling motor fluctuation and dyskinesias (Worth, 2013). Moreover, levodopa undergoes auto-oxidation and forms reactive oxygen species (ROS) which could be toxic to surviving neurons (Lipski *et al.*, 2011). Dopamine agonists are used for symptomatic treatment of early PD patients. They are beneficial over levodopa in minimizing the risk of the development of dyskinesias due to their longer half-life (Stathis *et al.*, 2015).

Neuroprotective strategies that can protect dopamine neurons from progressive damage have received much attention in the last two decades. So far, just only preclinical advances have been proposed to rescue dopamine neurons in PD but none of them has been proved in clinical trials (More and Choi, 2015). Most recently, autophagy dysfunction is linked to several neurodegenerative diseases including PD (Hu *et al.*, 2015). For instance, Dehay *et al.*, (2010) reported that the number of autophagosomes increased and autolysosomes decreased in PD brains.

In accordance, our current study was carried out to investigate comparable neuroprotective effects of the autophagy inducer rapamycin against low and high rotenone concentrations in primary dopaminergic cell culture relevant to PD.

## MATERIALS AND METHODS

### Preparation of primary mesencephalic cell culture

All experimental procedures in the present study were done in accordance with the guidelines of the European Union Council (86/609/EU). Primary dopaminergic cell cultures were prepared from OF1/SPF embryos according to Radad *et al.*, (2015). In brief, embryonic mouse mesencephala were dissected on the 14<sup>th</sup> day of gestation and cut into small pieces in a drop of DPBS (Invitrogen, Germany), 2 ml of 0.2% trypsin solution (Invitrogen, Germany) and 2 ml of 0.02% DNase I solution (Roche, Germany) were added and the tissue was subsequently incubated in a water bath at 37°C for 7 min. Then, 2 ml of trypsin inhibitor (0.125 mg/ml) (Invitrogen, Germany) were added, the tissue was centrifuged at 100x g for 4 min and the supernatant was aspirated. The tissue pellet was triturated 2–3 times with a fire-polished Pasteur pipette, each time 0.02% DNase I (Invitrogen, Germany) was included in the medium. Dissociated cells were plated at a density of 257.000 cells/cm<sup>2</sup> in DMEM (Sigma, Germany) supplemented with 4 mM glutamine, 10 mM HEPES buffer, 30 mM glucose, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 10% heat-inactivated fetal calf serum (Sigma, Germany).

The medium was exchanged on the 1<sup>st</sup> day in vitro (DIV) and on the 3<sup>rd</sup> DIV. On the 5<sup>th</sup> DIV half of the medium was

replaced by serum-free DMEM containing 0.02 ml of B-27/ml (Invitrogen, Germany) DMEM. Serum-free supplemented DMEM was used for feeding from the 6<sup>th</sup> DIV and subsequently replaced every 2<sup>nd</sup> day.

### Treatment of cultures with rapamycin

A stock solution of 1 mM of rapamycin (Invitrogen, USA) was prepared in dimethyl sulfoxide (DMSO). The solution was further diluted in DMEM to final concentrations. To investigate whether rapamycin alone affects the survival of dopamine neurons, cultures were treated with different concentrations of rapamycin (1, 10, 100 and 1000 nM) on the 10<sup>th</sup> DIV for 48 h.

### Treatment of cultures with rapamycin and rotenone

In each treatment, a fresh stock solution (1 μM) of rotenone (Sigma-Aldrich, Germany) was prepared in DMSO. The solution was further diluted in DMEM to final concentrations. To investigate the neurotoxic effect of rotenone, two cultures were separately treated with 10 and 20 nM rotenone on the 10<sup>th</sup> DIV for 48 h. On the other hand, another two cultures were concomitantly treated with rapamycin (1, 10, 100, 1000 nM), and 10 and 20 nM rotenone, respectively, on the 10<sup>th</sup> DIV for 48 h to investigate the effect of rapamycin against rotenone neurotoxicity.

### Identification of dopaminergic neurons

Dopaminergic neurons were identified immunocytochemically using anti-tyrosine hydroxylase (TH) antibody. On the 12<sup>th</sup> DIV, cultures were rinsed carefully with phosphate buffered saline (PBS, pH 7.2) and fixed in 4% paraformaldehyde for 45 min at 4°C. After washing with PBS, cells were permeabilized with 0.4% Triton X-100 for 30 min at room temperature. Cultures were washed 3 times with PBS and incubated with 5% horse serum (Vectastain ABC Elite kit) for 90 min to block nonspecific binding sites. To determine the number of TH<sup>+</sup> cells, cultures were sequentially incubated with anti-TH primary antibody overnight at 4°C, biotinylated secondary antibody (Vectastain) and avidin-biotin-horseradish peroxidase complex (Vectastain) for 90 min at room temperature and washed with PBS between stages.

The reaction product was developed in a solution of diaminobenzidine (1.4 mM) in PBS containing 3.3 mM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and stained cells were counted with a Nikon inverted microscope in 10 randomly selected fields per well at 10x magnification.

### Statistics

Each experiment was run in triplicate with four wells in each treatment. Data were expressed as mean ± standard error of mean (SEM). Comparisons were made using ANOVA and post-hoc Duncan's test using IBM SPSS statistics 22. P < 0.05 was considered as statistically significant.

## RESULTS

### Effect of rapamycin on the survival of dopamine neurons

Treatment of cultures with rapamycin alone (1, 10, 100, 1000 nM) on the 10<sup>th</sup> DIV for 48 h affected neither the survival (Figure 1) nor the morphology of dopamine neurons (data not shown).

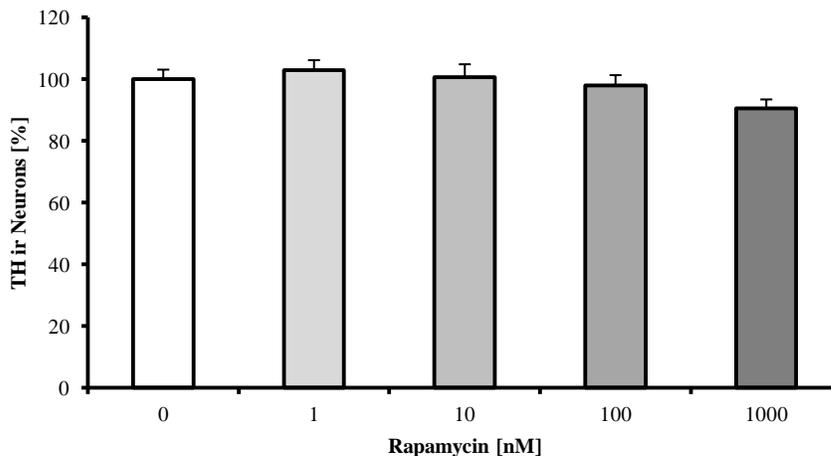
### Effect of rotenone on dopamine neurons

Treatment of cultures with rotenone significantly decreased the survival and reduced the neurites number of dopamine neurons, and altered their morphology. At 10 nM, rotenone decreased the survival of dopamine neurons and their neurite number by 22% and 33%, respectively, compared to untreated control (Fig. 2,3), and slightly altered their morphology (Fig. 4). While at 20 nM, rotenone produced much more decrease in the survival and the neurite number of dopamine neurons by

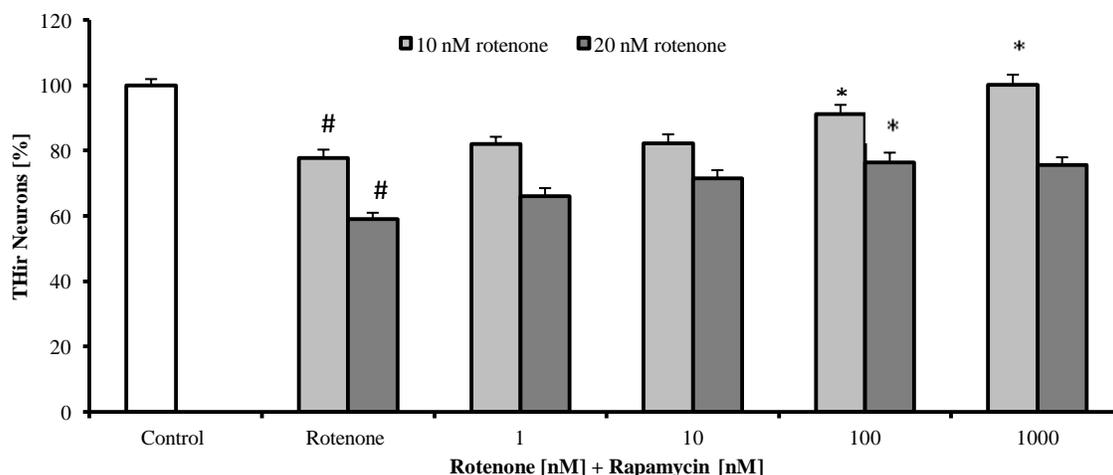
about 40% and 60%, respectively, than the cultures treated with 10 nM (Fig. 2,3). Moreover, such concentration markedly altered the morphology of dopamine neurons in the form of dysmorphic and shortened neurites (Fig. 4).

### Effect of rapamycin against rotenone-treated dopamine neurons

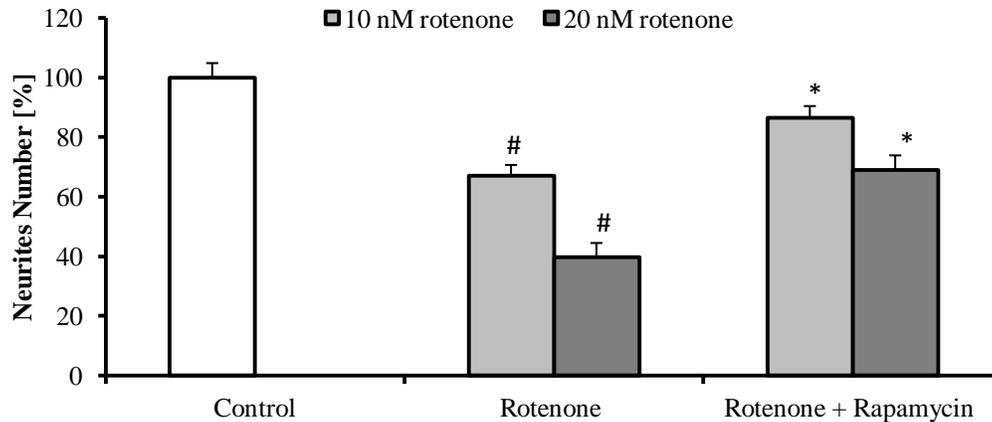
Co-treatment of cultures with rapamycin (100 nM) and rotenone completely attenuated rotenone's effect on the number of dopamine neurons in the 10 nM rotenone-treated cultures compared to rescuing only 17% of dopamine neurons in 20 nM rotenone-treated cultures (Fig. 2). Rapamycin (100 nM) was also seen to produce much attenuation in the loss of cell neurites in cultures treated with 10 nM rotenone (57%) than those treated with 20 nM rotenone (48%) (Fig. 3). Moreover, rapamycin markedly improved the morphology of dopamine neurons in the form of increasing their neurite lengths (Fig. 4).



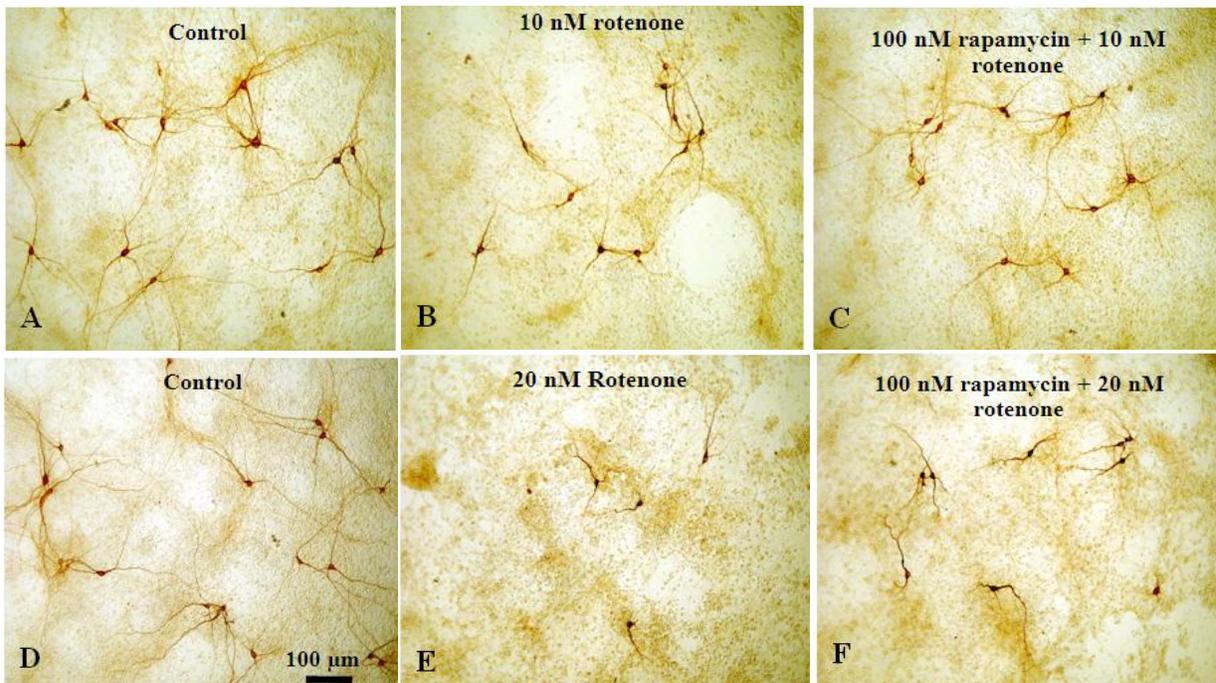
**Fig. 1:** Treatment of cultures with rapamycin on the 10<sup>th</sup> DIV for 48 h. 100% corresponds to the total number of dopamine neurons (the average number of dopamine neurons was 26 cells/field) after 12 DIV in untreated controls. Values represent the mean  $\pm$  SEM of three independent experiments with four wells in each treatment. In each well, dopamine neurons were counted in 10 randomly selected fields.



**Fig. 2:** Survival of dopamine neurons after concomitant treatment of cultures with rapamycin and rotenone on the 10<sup>th</sup> DIV for 48 h. 100% corresponds to the total number of dopamine neurons (the average number of dopamine neurons was 24 cells/field) after 12 DIV in untreated controls. Values represent the mean  $\pm$  SEM of three independent experiments with four wells in each treatment. In each well, dopamine neurons were counted in 10 randomly selected fields. ( $p^{#,*} < 0.001$ ).



**Fig. 3.** The neurites number of dopamine neurons after concomitant treatment of cultures with rapamycin and rotenone on the 10<sup>th</sup> DIV for 48 h. 100% corresponds to the average number of neurites (4.12 neurites/cell) in untreated control cultures after 12 DIV. Values represent the mean  $\pm$  SEM of three independent experiments with four wells in each treatment. In each well, neurites number were counted for 5 dopamine neurons in 10 randomly selected fields (200 cell/experiment). ( $p^{*,*} < 0.001$ )



**Fig. 4.** Representative micrographs of dopamine neurons after 12 DIV. Untreated control cultures show dopamine neurons with long and branched neurites. Rotenone-treated cultures show much more morphological alteration in the 20 nM-treated cultures than 10 nM-treated ones. Co-treatment of cultures with rapamycin and rotenone markedly attenuated morphological alterations particularly in cultures treated with 20 nM rotenone.

## DISCUSSION

In the present study, dopaminergic cell culture was used to investigate the comparable neuroprotective effects of rapamycin against low and high rotenone concentrations relevant to PD. In which, neurotoxicity of the well-known and powerful complex I blocker rotenone to dopaminergic neurons was concentration-dependent. It decreased the survival of dopamine neurons and their neurites by 22% and 33%, respectively at 10 nM compared to 40% and 60% at 20 nM. Moreover, morphological alterations in the form of dysmorphic and shortened neurites were markedly seen at the concentration of 20 nM more than at 10 nM of rotenone.

Similarly, Radad *et al.*, (2006) reported that rotenone challenged primary cultured dopamine neurons in a dose-dependent manner as it decreased survival of dopamine neurons by about 16% and 50% at the concentrations of 5 and 20 nM of rotenone, respectively, compared to untreated controls. Moon *et al.*, (2005) also observed that the cytotoxic damage after 24 h of rotenone treatment gradually increased from 5 to 20 nM in neuron-enriched primary dopaminergic cultures. Similarly, rotenone decreased the viability of SH-SY5Y in a dose-dependent manner (Xiong *et al.*, 2013). Majority of literature attributed rotenone's neurotoxicity to: (1) accumulation of  $\alpha$ -synuclein, (2) enhancement production of ROS and (3) activating caspase-3-mediated apoptosis. In this context,

Radad *et al.*, (2006) found that treatment of primary dopaminergic cell culture with 20 nM of rotenone significantly decreased mitochondrial membrane potential ( $\Delta\psi_m$ ) leading to increasing superoxide and ROS production, and enhancing apoptotic cell death. Xiong *et al.* (2013) reported that rotenone induced apoptotic cell death of SH-SY5Y through decreasing  $\Delta\psi_m$  and increasing ROS. Satish Bollimpelli and Kondapi (2015) found that rotenone produced  $\alpha$ -synuclein aggregation, caspase-3 activation and ROS production in primary cultured mature dopamine neurons. Against rotenone, the autophagy inducer rapamycin significantly protected dopamine neurons and their neurites. In 10 nM rotenone-treated cultures, rapamycin completely blocked rotenone effect on the number of dopamine neurons and produced much more attenuation in neurite loss compared to rescuing only 17% of dopamine neurons and producing less attenuation in neurite loss at 20 nM of rotenone. Rapamycin was similarly seen to protect dopaminergic neurons in some in *in vitro* and *in vivo* experimental models. For instance, Pan *et al.*, (2009) reported that rapamycin alleviated rotenone-induced death in SH-SY5Y cells. Jiang *et al.*, (2013) found that rapamycin protected against the loss of dopamine neurons in the 6-hydroxydopamine-induced rat model of PD. Neuroprotective effect of rapamycin was reported to be mediated by inactivation of the mammalian target of rapamycin (mTOR) leading to autophagy activation (Ding *et al.*, 2015). In consistent, rapamycin was seen to increase the signal of Lysotracker Deep Red fluorescent dye in rotenone-treated primary dopaminergic cell culture leading to protection of  $\Delta\psi_m$  and decreasing apoptotic cell death (Radad *et al.*, 2015). Besides neuroprotection, our findings indicated that rapamycin's neuroprotective effect on dopamine neurons is conversely proportional to the damaging effects of rotenone. i.e. rapamycin neuroprotection depends on how much is the cellular damage? The effect which needs further clarification in future research, to determine to which point autophagy is protecting against neurodegeneration? In conclusion, rapamycin is much more effective against low rotenone concentration on dopamine neurons. This effect seems to depend on to what extent autophagy process by rapamycin could repair rotenone-induced damage on dopamine neurons.

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