

Rapamycin protects dopaminergic neurons against rotenone-induced cell death in primary mesencephalic cell culture

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Abstract

Introduction: Parkinson's disease is the most common movement disorder, characterized by a progressive and extensive loss of dopaminergic neurons in the substantia nigra pars compacta and their terminals in the striatum. So far, only symptomatic treatment is available, and no cure or disease-modifying drugs exist. The present study was designed to investigate the neuroprotective effect of rapamycin, an autophagy inducer, on dopaminergic neurons against rotenone-induced cell death in primary mesencephalic cell culture.

Material and methods: Primary mesencephalic cell cultures were prepared from embryonic mouse mesencephala (OF1/SPF, Vienna, Austria) at gestation day 14. Four sets of cultures were treated as follows: one was run as an untreated control, a second one was treated with 20 nM rotenone on the 10th day in vitro (DIV) for 48 h, a third one was co-treated with 20 nM rotenone and rapamycin (1, 10, 100, 1000 nM) on the 10th DIV for 48 h, and a fourth one was treated with rapamycin alone (1, 10, 100, 1000 nM) on the 10th DIV for 48 h. On the 12th DIV, cultures were subjected to immunohistochemistry against tyrosine hydroxylase and to fluorescence staining using LysoTracker Deep Red, JC-1 and DAPI stains.

Results: Exposure of such cultures to 20 nM rotenone on the 10th DIV for 48 h reduced the number of dopaminergic neurons by 41% and increased the release of lactate dehydrogenase (LDH) by 178% above untreated controls. Rapamycin (1, 10, 100, 1000 nM) added together with rotenone from the 10th to 12th DIV spared dopaminergic neurons by 17% and reduced the release of LDH by 64% at the concentration of 100 nM compared to rotenone-treated cultures. Activation of an autophagic process by rapamycin was demonstrated by LysoTracker Deep Red fluorescent dye, as indicated by a shift to increased red fluorescence. Rapamycin also significantly elevated the mitochondrial membrane potential ($\Delta\psi_m$), as shown by an increase of the red:green fluorescence ratio of JC-1. Increased apoptotic cell death due to rotenone was lowered by rapamycin, as shown by the blue-fluorescent DAPI nucleic acid stain.

Conclusions: Our study indicates for the first time that rapamycin, known as an autophagy inducer, protected dopaminergic neurons against rotenone-induced cell death in primary mesencephalic cell culture.

Key words: apoptosis, autophagy, dopaminergic neurons, neuroprotection, Parkinson's disease, rapamycin.

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Introduction

Parkinson's disease (PD) is a common progressive neurodegenerative disorder affecting more than 6 million people worldwide [28]. The disease is characterized clinically by a triad of cardinal motor symptoms including bradykinesia, tremors and rigidity [13], and pathologically by the selective loss of dopamine neurons in the substantia nigra pars compacta (SNpc) and the formation of Lewy bodies [27]. Although the etiology of PD is still unclear, there is growing evidence indicating that intracellularly oxidative stress, mitochondrial damage, lysosomal dysfunction, neuroinflammatory changes and formation of pathologic inclusions contribute to the pathology of the disease [9].

Treatment of PD is generally symptomatic, where levodopa remains the most effective agent [34]. However with disease progression, levodopa medication becomes increasingly inadequate for the management of motor fluctuations and dyskinesias [40]. Levodopa given clinically undergoes autooxidation and forms reactive oxygen species (ROS) which could be toxic to remaining dopamine neurons [20]. Dopamine agonists and monoamine oxidase B inhibitors are also prescribed for the treatment of early PD as in addition to their symptomatic benefits they postpone the onset of levodopa therapy [11].

During the last two decades, researchers have shown increased interest in developing neuroprotective substances that can slow or stop the clinical progression of PD. As a result, significant numbers of compounds have been identified as neuroprotective in preclinical studies [8]. For instance, the D3/D2/D1 dopamine receptor agonist rotigotine was shown to protect dopaminergic neurons against glutamate, MPP⁺ and rotenone in primary mesencephalic cell culture [23,32]. The active principal of *Nigella sativa* seed thymoquinone protected dopaminergic neurons against MPP⁺-induced cell death in primary mesencephalic cell culture [30]. Inhibition of sphingosine kinase (Sphk1), a regulator of bioactive sphingolipid homeostasis, by SKI II protected the dopaminergic SH-SY5Y cells through an anti-apoptotic pathway [29]. However, to date, there is no drug that has provided neuroprotection against dopaminergic cell loss in clinical trials [4].

Rapamycin is a lipophilic macrocyclic antibiotic produced by the bacterium *Streptomyces hygroscopicus* indigenous to Easter Island [38]. Rapamycin

was shown first to exhibit antibacterial, antifungal, immunosuppressive and anticancer effects [33]. Recently, rapamycin has been reported to reduce cytotoxic injury in different models of neurodegenerative disorders [5]. For instance, Park *et al.* [26] reported that rapamycin protected human neuroblastoma SH-SY5Y cells against fipronil-induced apoptotic cell death. Kanno *et al.* [18] found that rapamycin reduced locomotor impairment and neuronal death after spinal cord injury in mice. This effect of rapamycin was reported to be mediated through activation of autophagy by inhibiting the mammalian target of rapamycin (mTOR) signaling pathways [36].

More recently, studies using post-mortem human tissues and genetic and toxin-induced animal and cellular models have implicated autophagy dysfunction as an important issue in PD pathogenesis [1]. For instance, Dehay *et al.* [7] reported that the number of undegraded autophagosomes increased and the number of autophagolysosomes decreased in post-mortem PD brain samples. Park *et al.* [25] showed that MPP⁺ inhibited autophagosome formation and increased α -synuclein expression in mice. Parganlija *et al.* [24] found that SH-SY5Y cells with PINK1 knockdown showed down-regulation of key autophagic genes including Beclin, LC3 and LAMP-2. Accordingly, our present study was designed to investigate the neuroprotective potential of the autophagy inducer rapamycin against rotenone-induced dopaminergic cell death relevant to PD. To date, there have been no reports describing the potential neuroprotective role of rapamycin on dopamine neurons in primary mesencephalic cell culture relevant to PD.

Material and methods

Preparation of primary mesencephalic cell culture

Primary mesencephalic cell cultures were prepared from OF1/SPF embryos according to Meinel *et al.* [22]. In brief, embryonic mouse mesencephala were dissected on the 14th 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, Ger-

many) were added, the tissue was centrifuged at $100 \times g$ 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/cm² 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 1st day *in vitro* (DIV) and on the 3rd DIV. On the 5th DIV half of the medium was replaced with serum-free DMEM containing 0.02 ml of B-27/ml (Invitrogen, Germany) DMEM. Serum-free supplemented DMEM was used for feeding from the 6th DIV and subsequently replaced every 2nd day.

Treatment of cultures with rapamycin

A stock solution of 1 mM rapamycin (Invitrogen, USA) was prepared in dimethyl sulfoxide (DMSO) and further diluted in DMEM to final concentrations. On the 10th DIV, cultures were treated with rapamycin (1, 10, 100, 1000 nM) for 48 h to investigate its effect on the survival of dopaminergic cells.

Treatment of cultures with rapamycin and rotenone

A stock solution of 1 μ M of rotenone (Sigma-Aldrich, Germany) was prepared in DMSO and then diluted in DMEM to final concentrations. For each treatment, fresh rotenone solutions were used to avoid breakdown of rotenone by storage. To investigate the neuroprotective potential of rapamycin against rotenone-induced dopaminergic cell death, cultures were co-administered with both rotenone (20 nM) and rapamycin (1, 10, 100, 1000 nM) on the 10th DIV for 48 h.

Identification of dopaminergic neurons

Dopaminergic neurons were identified immunocytochemically by tyrosine hydroxylase staining. On the 12th 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 tyrosine hydroxylase immunoreactive (THir) 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 (H₂O₂), and stained cells were counted with a Nikon inverted microscope in 10 randomly selected fields per well at 10 \times magnification.

Measurement of LDH activity

Cellular injury was quantitatively assessed by measuring the activity of lactate dehydrogenase (LDH) released from damaged cells into the culture medium. The reaction was initiated by mixing 0.2 ml of cell-free supernatant (diluted 1 : 1 with distilled water) with potassium phosphate buffer containing β -nicotinamide adenine dinucleotide (NADH) and sodium pyruvate (0.18 and 0.62 mM in potassium phosphate buffer, respectively) in a final volume of 0.5 ml in 1 ml cuvettes. The decrease of NADH was spectrophotometrically (NOVASPEC1 II) monitored. Reagent blanks were subtracted. LDH activity was calculated from the slope of the decrease in optical density at 334 nm over a 3 min time period. The LDH release is proportional to the number of damaged or destroyed cells [10,19].

Staining of cultured cells with LysoTracker Deep Red fluorescent dye

LysoTracker dye is a highly soluble small molecule that is retained in acidic subcellular organelles such as lysosomes. It is used to investigate the biosynthesis of lysosomes. Here, 100 nM rapamycin (the concentration significantly protected dopaminergic neurons in rotenone-treated cultures) was added together with 20 nM rotenone on the 10th DIV for 48 h. On the 12th DIV, culture media were aspirated and cells were incubated with a new medium containing 100 nM LysoTracker Deep Red fluorescent dye (Life Technologies, Invitrogen, USA) for 15-30 min at 37°C. After washing with DPBS, stained cells were photographed on a Nikon inverted microscope equipped with an epifluorescence attachment using a rhodamine filter set with an excitation wavelength

of 580 and an emission wavelength of 590, G-2A and a Coolpix 990 digital camera (Nikon, Japan). Six photos were taken randomly from each well (24 photos per experiment). All photos were analyzed densitometrically using Adobe Photoshop software.

Measurement of $\Delta\psi_m$ by JC-1 fluorescent dye

JC-1 is a lipophilic cationic dye that selectively enters mitochondria. In healthy cells with high mitochondrial $\Delta\psi_m$, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. In the case of apoptotic cells the dye remains in its monomeric form with green fluorescence. The JC-1 red:green ratio has been used as a tool to estimate changes in $\Delta\psi_m$ [39]. JC-1 was dissolved in DMSO and further diluted in DMEM (10 $\mu\text{g}/\text{ml}$ final concentration). After removal of the culture medium cells were loaded with JC-1 for 15 min at 37°C, rinsed twice with PBS and photographed on a Nikon inverted microscope equipped with an epifluorescence attachment using a rhodamine filter set with an excitation wavelength of 510 nm and an emission wavelength of 520 nm and a Coolpix 990 digital camera (Nikon, Japan). Six photos were taken randomly from each well (24 photos per experiment). Fluorescence intensity of the red:green ratio was determined semiquantitatively using Adobe Photoshop software.

Counting of apoptotic cells by blue-fluorescent DAPI nucleic acid stain

DAPI is a fluorescent stain that binds strongly to DNA. It passes through intact membranes of live and fixed cells. Cells were fixed with 4% paraformaldehyde for 45 min at 4°C. After washing with PBS (pH 7.2), cells were permeabilized with 0.4% Triton X-100 for 30 min at room temperature. DAPI solution (2 μM final concentration) was added to the cultures at room temperature for 5 min in the dark. After washing with DPBS, six photos were taken randomly from each well (24 photos per experiment) with a Coolpix 990 digital camera connected to an inverted microscope with an epifluorescence attachment using an ultraviolet (UV) filter (Nikon, Japan). Nuclei with condensed and fragmented chromatin were counted when the photos were reviewed with Adobe Photoshop software.

Statistics

Each experiment was run in triplicate with four wells in each treatment. Data were expressed as mean \pm standard error of mean (SEM). Comparisons were made using ANOVA and post-hoc Duncan's test using the statistical program SAS 1998. $P < 0.05$ was considered as statistically significant.

Results

Rapamycin did not affect the survival of dopaminergic neurons

Treatment of cultures with rapamycin (1, 10, 100, 1000 nM) on the 10th DIV for 48 h did not affect the survival (Fig. 1) or the morphology of dopaminergic neurons (data not shown).

Rapamycin rescued dopaminergic neurons from rotenone-induced cell death

Treatment of cultures with 20 nM rotenone on the 10th DIV for 48 h decreased the number of dopaminergic neurons by 41% and altered the morphology of surviving neurons compared to untreated controls (Fig. 2A,B). On the other hand, co-administration of rapamycin and rotenone on the 10th DIV for 48 h significantly increased the survival of dopaminergic

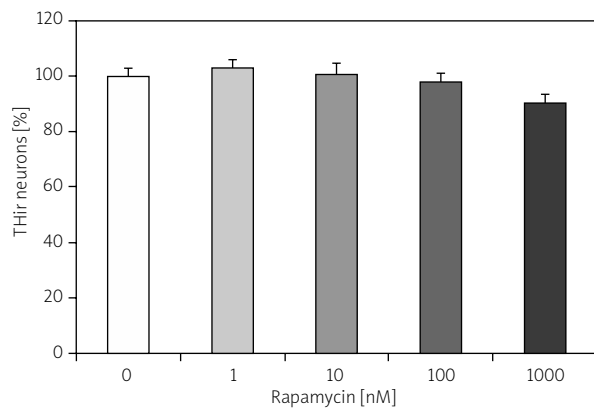


Fig. 1. Treatment of primary mesencephalic cell cultures with rapamycin on the 10th DIV for 48 h. 100% corresponds to the total number of THir neurons (the average number of THir neurons was 26 cells/field) after 12 DIV in untreated controls. Values represent the mean \pm SEM of three independent experiments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

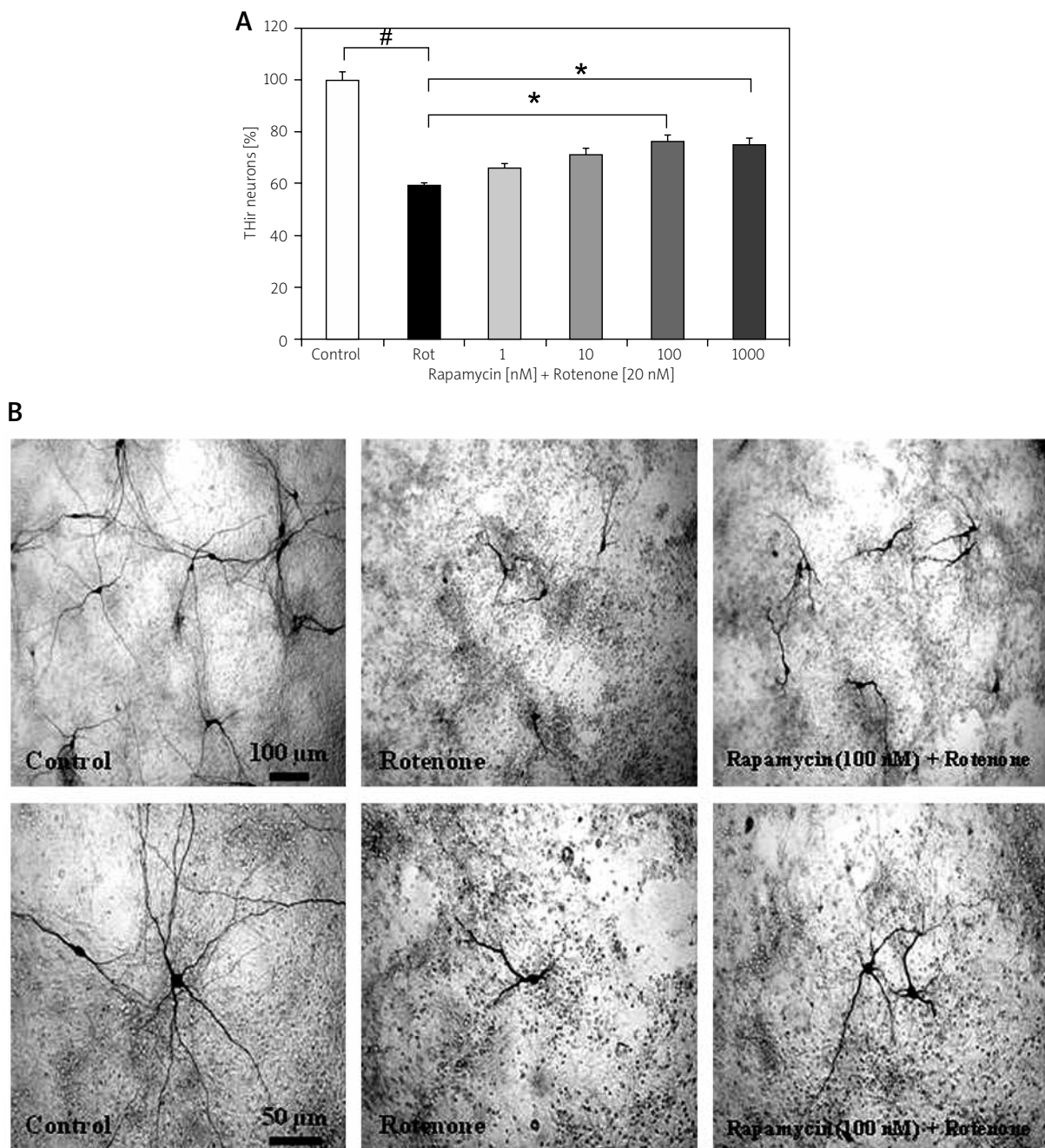


Fig. 2. A) Concomitant treatment of primary mesencephalic cell cultures with rotenone (20 nM) and rapamycin (1, 10, 100, 1000 nM) on the 10th DIV for 48 h. 100% corresponds to the total number of THir neurons (the average number of THir neurons was 23 cells/field) after 12 DIV in untreated controls. Values represent the mean ± SEM of three independent experiments with four wells in each treatment. In each well THir neurons were counted in ten randomly selected fields ([#]*p* < 0.001, ^{*}*p* < 0.001). **B)** Representative micrographs of THir neurons after 12 DIV. Untreated control cultures showed THir neurons with long and branched processes. Rotenone-treated cultures showed THir neurons with very few, shortened and thickened neurites. Treatment with rapamycin improves the morphology of THir neurons compared to rotenone-treated cultures.

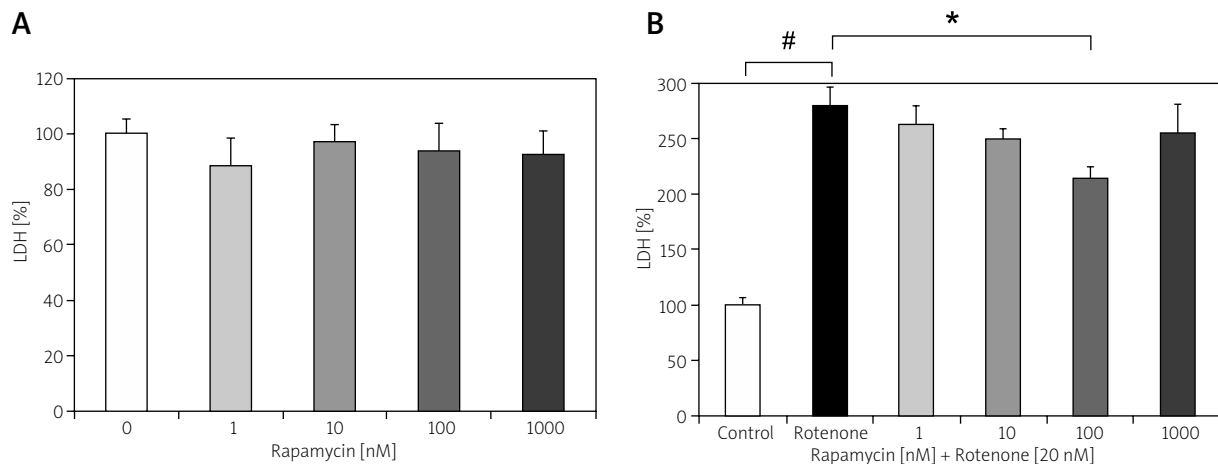


Fig. 3. Release of LDH into the culture medium in primary mesencephalic cell cultures. **A)** Treatment of cultures with rapamycin on the 10th DIV for 48 h. **B)** Concomitant treatment of cultures with rotenone (20 nM) and rapamycin (1, 10, 100, 1000 nM) on the 10th DIV for 48 h. 100% corresponds to the amount of LDH in the culture medium after 12 DIV. Values represent the mean \pm SEM of three independent experiments with four wells in each treatment ([#] $p < 0.001$, ^{*} $p < 0.001$).

neurons by 17% compared to rotenone-treated cultures (Fig. 2A). Also, rapamycin reduced degenerative changes in dopaminergic neurons seen with rotenone exposure (Fig. 2B).

Rapamycin attenuated rotenone-induced LDH release

Treatment of cultures with rapamycin on the 10th DIV for 48 h did not affect the level of LDH in the culture medium (Fig. 3A). Consistent with the cytotoxic effect of rotenone on dopaminergic neurons, 20 nM rotenone led to an increase in release of LDH into the culture medium by 178% compared to untreated controls (Fig. 4B). Against rotenone, rapamycin (100 nM) reduced the release of LDH by 64% compared to rotenone-treated cultures (Fig. 3B).

Rapamycin increased LysoTracker Deep Red fluorescence

Co-treatment of cultures with rapamycin (100 nM) and rotenone (20 nM) on the 10th DIV for 48 h significantly increased the fluorescent intensity of LysoTracker Deep Red by about 63% compared to rotenone-treated cultures (Fig. 4A). In parallel, Fig. 4B shows higher red fluorescence in the cultures co-treated with rapamycin and rotenone compared to cultures treated with rotenone alone. Rapamycin alone did not produce a significant increase in the

fluorescent intensity of LysoTracker Deep Red compared to untreated control culture (Fig. 4A,B).

Rapamycin increased red: green fluorescence ratio of JC-1

Treatment of cultures with rotenone (20 nM) on the 10th DIV for 48 h decreased the red:green fluorescent ratio of JC-1 by about 29% compared to untreated controls (Fig. 5A). On the other hand, concomitant treatment of cultures with 100 nM rapamycin and 20 nM rotenone on the 10th DIV for 48 h significantly restored the red:green fluorescent ratio of JC-1 by 19% compared to rotenone-treated cultures (Fig. 5A). Figure 5B showed that cultures co-administered with rapamycin and rotenone displayed much higher red fluorescence than the cultures treated with rotenone alone. Rapamycin alone did not significantly affect the red:green fluorescent ratio of JC compared to untreated control culture (Fig. 5A,B).

Rapamycin decreased rotenone-induced apoptotic cell death

Staining of cultured cells with the nuclear fluorescence dye DAPI revealed that rotenone (20 nM on the 10th DIV for 48 h) increased the number of nuclei showing apoptotic features by 119% compared to untreated cultures (Fig. 6A). Against rotenone, rapamycin was found to decrease the number of apoptotic

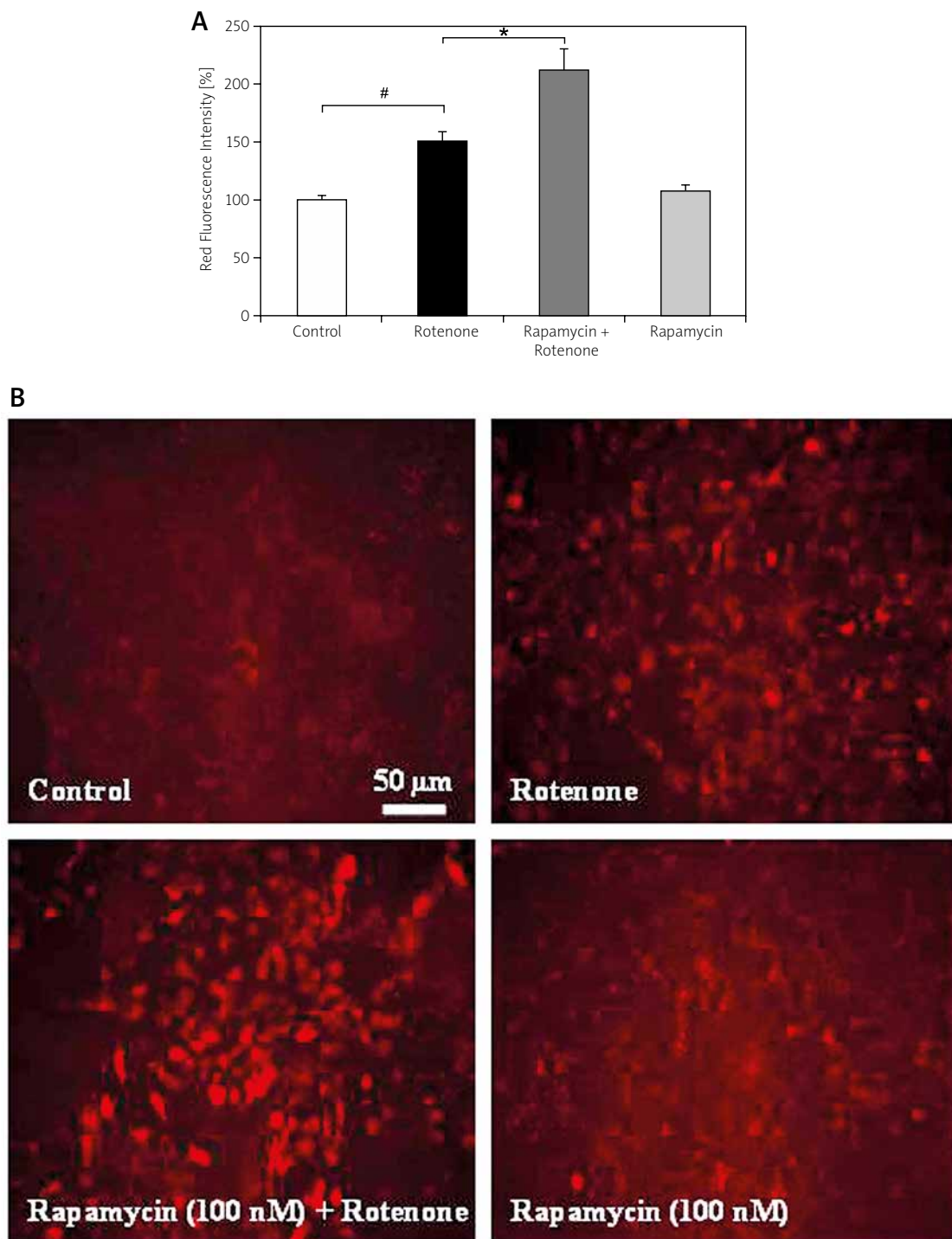


Fig. 4. A) LysoTracker Deep Red fluorescence intensity in primary mesencephalic cell cultures. 100% corresponds to the intensity of LysoTracker Deep Red in primary mesencephalic cell cultures after 12 DIV. Values represent the mean \pm SEM of three independent experiments with four wells in each treatment. Fluorescence intensity was determined densitometrically from 24 randomly selected micrographs in each experiment (6 photos from each well) ($\#p < 0.01$, $*p < 0.001$). **B)** Representative micrographs showing that treatment of cultures with rapamycin increased LysoTracker Deep Red fluorescence intensity compared to rotenone-treated cultures.

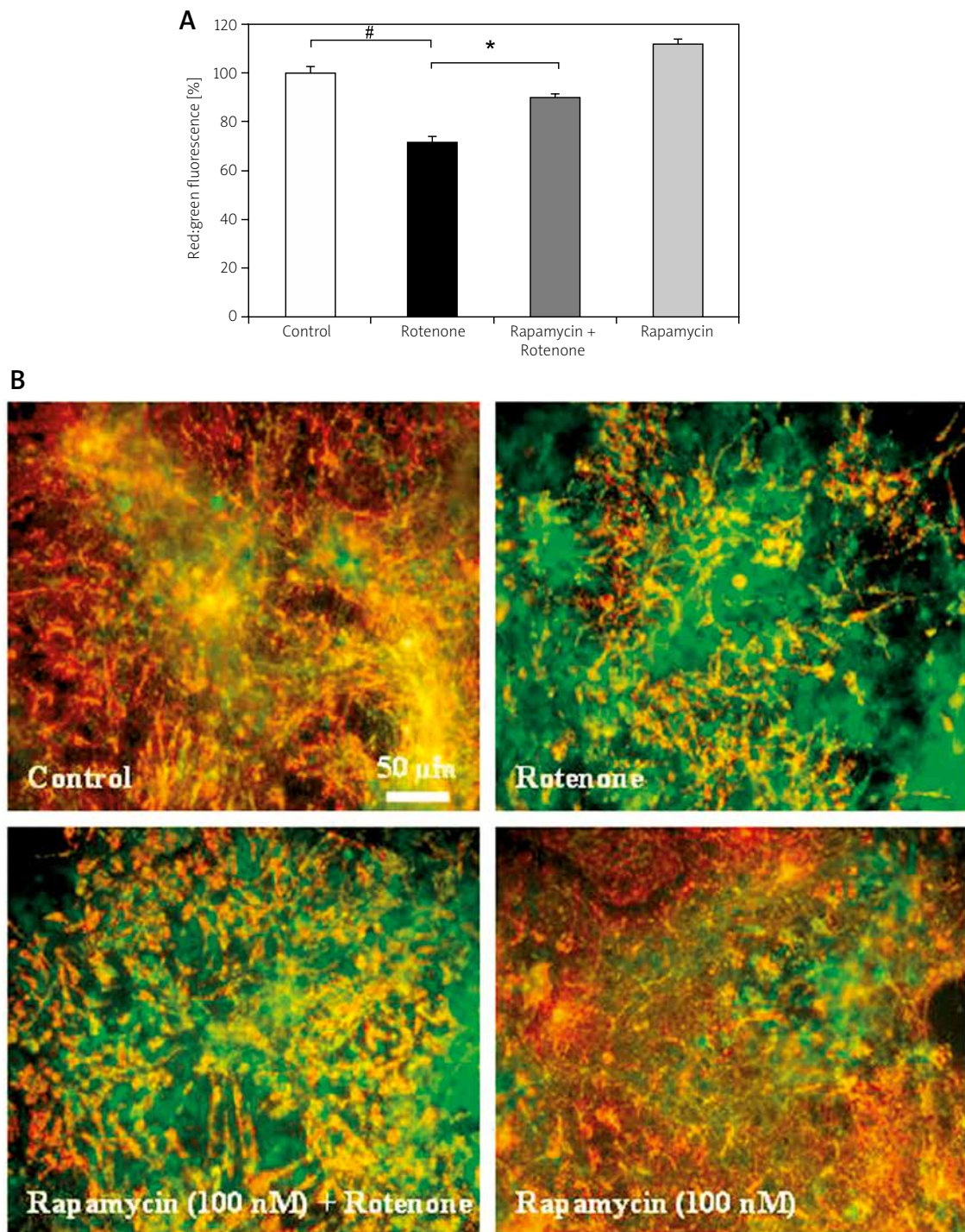


Fig. 5. A) Red:green fluorescence ratio of JC-1 in primary mesencephalic cell cultures. 100% corresponds to the red:green fluorescence ratio of JC-1 in primary mesencephalic cell cultures after 12 DIV. Values represent the mean \pm SEM of three independent experiments with four wells in each treatment. Red:green fluorescence ratio of JC-1 was determined densitometrically from 24 randomly selected micrographs in each experiment (6 photos from each well) ($\#p < 0.001$, $*p < 0.001$). **(B)** Representative micrographs showing that treatment of cultures with rapamycin increased red fluorescence compared to rotenone-treated cultures which exhibit marked green fluorescence.

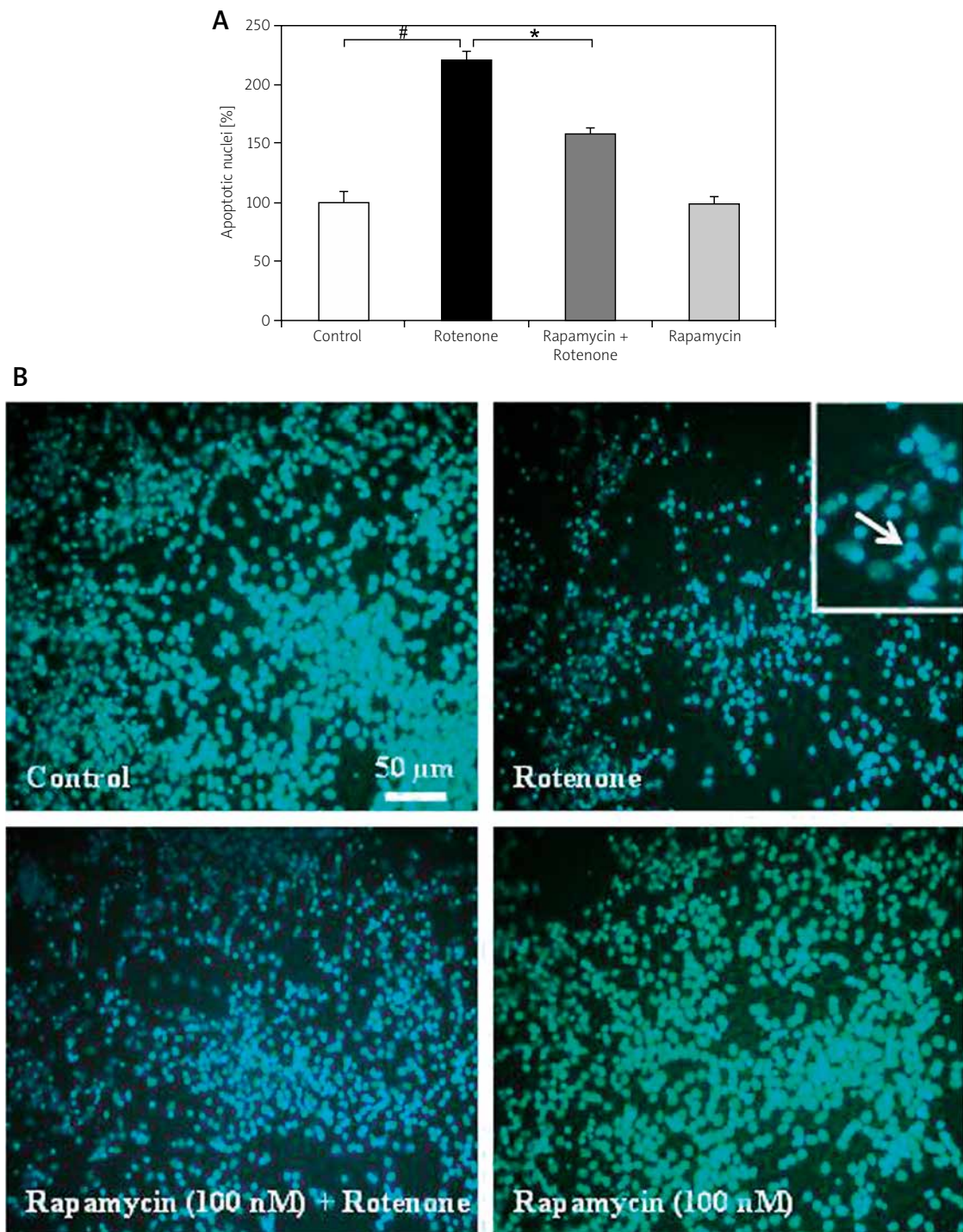


Fig. 6. (A) Number of nuclei showing apoptotic features with condensed and fragmented chromatin in primary mesencephalic cell cultures. 100% corresponds to the number of apoptotic nuclei (the average number of apoptotic nuclei was 38 nuclei/photo) in untreated control cultures after 12 DIV. Values represent the mean \pm SEM of three independent experiments with four wells in each treatment. Twenty-four photos were taken from each experiment (6 photos from each well) ($\#p < 0.001$, $*p < 0.001$). **(B)** Representative micrographs showing that treatment of cultures with rapamycin decreased the number of apoptotic nuclei compared to rotenone-treated cultures. Insert shows apoptotic nuclei at 20 \times magnification.

nuclei by about 100% compared to rotenone-treated cultures (Fig. 6A). Apoptotic nuclei showed highly condensed and fragmented chromatin (Fig. 6B). Rapamycin alone did not affect the number of apoptotic nuclei compared to untreated control culture (Fig. 6A,B).

Discussion

In the present study, rotenone-treated primary mesencephalic cell culture was used as a neurotoxicity model to investigate the neuroprotective potential of the autophagy inducer rapamycin on dopaminergic neurons relevant to PD. In summary, rotenone was shown to 1) decrease the survival of dopaminergic neurons; 2) increase the release of LDH into the culture medium; 3) disrupt the mitochondrial $\Delta\psi_m$ of cultured cells and 4) increase the features of apoptotic cell death in primary mesencephalic cell culture. This neurotoxic effect of rotenone has been reported in different *in vitro* and *in vivo* models since Betarbet and her colleagues [3] described rotenone as a PD neurotoxin in 2000. For instance, Radad *et al.* [31] found that exposure of primary mesencephalic cell culture to 20 nM rotenone destroyed dopaminergic neurons and resulted in a higher level of LDH in the culture medium. Im *et al.* [15] reported that rotenone decreased the viability and survival of PC12 cells. Tapias *et al.* [37] described a loss of dopamine neurons and nigrostriatal terminals as a result of rotenone treatment in rats. Using JC-1 and DAPI fluorescence dyes we showed that rotenone decreased the $\Delta\psi_m$ and induced apoptotic cell death in our mesencephalic cell cultures, as indicated by the decreased red:green fluorescence ratio of JC-1 and increased number of nuclei with condensed and fragmented chromatin, respectively. Similarly, Hu *et al.* [14] demonstrated that rotenone caused a loss of $\Delta\psi_m$ and induced apoptotic cell death in SH-SY5Y cells. Mitochondrial damage by rotenone played a central role in apoptotic cell death through interrupting cellular energy metabolism, increasing ROS production and the release of apoptotic factors into the cytosol [2].

Our results showed that rapamycin rescued a significant number of dopaminergic neurons and decreased the release of LDH into the culture medium when concomitantly added with rotenone to primary mesencephalic cell cultures. Likewise, similar neuroprotective effects of rapamycin have been reported in some *in vitro* and *in vivo* models of neurodegeneration. For example, Malagelada *et al.* [21] found that

rapamycin protected PC12 cells from 6-OHDA toxicity. Jiang *et al.* [16] observed that rapamycin provided behavioral improvements and protected against the loss of dopaminergic neurons in a rat model of PD.

Rapamycin increased cellular fluorescence of LysoTracker Deep Red compared to rotenone-treated cultures, indicating that rapamycin upregulated an autophagic process in cultured cells. In line with our results, Chikte *et al.* [6] reported that the signals of LysoTracker Deep Red were increased as the result of rapamycin treatment and could be used as a marker for autophagy in Jurkat T-cell and K562 erythro-myeloid cell lines. He and Klionskey [12] also correlated the fluorescence signals of LysoTracker with the upregulation of autophagy in zebrafish. Staining of cultures co-administered with rapamycin and rotenone with JC-1 fluorescent dye showed that rapamycin enhanced $\Delta\psi_m$ as it increased the red:green fluorescence ratio of JC-1 compared to cultures treated with rotenone alone. Rapamycin was similarly reported to enhance $\Delta\psi_m$ in the human breast cancer cell line MCF-7, as shown by the JC-1 potentiometric dye [24]. Moreover, rapamycin was reported to ameliorate mitochondrial defects in cells from individuals with the PARK2 mutation through rescuing $\Delta\psi_m$ [35]. Counting of apoptotic nuclei using blue-fluorescent DAPI nucleic acid stain indicated that rapamycin decreased rotenone-induced apoptotic cell death in primary mesencephalic cell cultures. Yin *et al.* [41] and Jing *et al.* [17] similarly reported an antiapoptotic effect for rapamycin against transient focal cerebral ischemia/reperfusion and subarachnoid hemorrhage in mice and rats, respectively. The antiapoptotic effect of rapamycin was reported to be mediated by decreasing Bax production and the downstream release of cytochrome c from mitochondria to the cytosol [17]. In addition to aforementioned mechanisms of neuroprotection, rapamycin was found to protect neuronal cells through some other pathways. In this context, Tain *et al.* [35] found that activation of the translation inhibitor 4E-BP protected dopaminergic neurons in parkin and PINK1 mutant *Drosophila*. Jiang *et al.* [16] reported that reduction of oxidative stress is one of the underlying mechanisms that mediate neuroprotection in a 6-OHDA rat model of PD.

Taking all the data together, our study shows that rapamycin protected dopaminergic neurons against rotenone-induced cell death in primary mesencephalic cell culture. This neuroprotection might be attributed to increasing autophagy, enhancing mitochondrial

membrane potential and decreasing apoptotic cell death in primary mesencephalic cell culture.

Disclosure

The authors report no conflict of interest.

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