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Neuroprotective Effects of Delta-9-Tetrahydrocannabinol against $FeSO_4$- and H_2O_2-Induced Cell Damage on Dopaminergic Neurons in Primary Mesencephalic Cell Culture

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ABSTRACT

Delta-9-Tetrahydrocannabinol and other phytocannabinoids have been previously demonstrated to possess neuroprotective effects in murine mesencephalic cell culture models of Parkinson's disease, in which increased levels of superoxide radicals led to the loss of dopaminergic neurons. In these models, delta-9-tetrahydrocannabinol did not scavenge these radicals but displayed antioxidative capacity by increasing glutathione levels. Based on these findings, in the present study, we investigated whether the neuroprotective effect of delta-9-tetrahydrocannabinol can also be detected in $FeSO_4$- and H_2O_2-stressed cells. Mesencephalic cultures were concomitantly treated with $FeSO_4$ (350 µM) or H_2O_2 (150 µM) and delta-9-tetrahydrocannabinol (0.01, 0.1, 1, 10 µM) on the 12th *days in vitro* for 48 h. On the 14th DIV, dopaminergic neurons were stained immunocytochemically by tyrosine hydroxylase, and fluorescently using crystal violet, Hoechst 33342, and JC-1. $FeSO_4$ and H_2O_2 significantly reduced the number of dopaminergic neurons by 33 and 36 %, respectively, and adversely affected the morphology of surviving neurons. Moreover, $FeSO_4$, but not H_2O_2, significantly decreased the fluorescence intensity of crystal violet and Hoechst 33342, and reduced the red/green ratio of JC-1. Co-treatment with delta-9-tetrahydrocannabinol at the concentrations 0.01 and 0.1 µM significantly rescued dopaminergic neurons in $FeSO_4$ and H_2O_2-treated cultures by 16 and 30 %, respectively. delta-9-Tetrahydrocannabinol treatment also led to a higher fluorescence intensity of crystal violet and Hoechst 33342, and increased the red/green fluorescence ratio of JC-1 when concomitantly administered with $FeSO_4$ but not H_2O_2. To conclude, delta-9-tetrahydrocannabinol rescues dopaminergic neurons against $FeSO_4$- and H_2O_2-induced neurotoxicity. Using fluorescence dyes, this effect seems to be mediated partially by restoring mitochondrial integrity and decreasing cell death, particularly in $FeSO_4$-treated cultures.

* These two authors contributed equally to this work.

ABBREVIATIONS

Δ9-THC	delta-9-tetrahydrocannabinol
Δψm	mitochondrial membrane potential
CNS	central nervous system
CV	crystal violet
DIV	days in vitro
DPBS	D-phosphate buffered saline
MPP+	1-methy-4-phenylpyridinium
NMDA	N-methy-D-aspartate
PD	Parkinson's disease
ROS	reactive oxygen species
SN	substantia nigra
TH	tyrosine hydroxylase

Introduction

Cannabis sativa L. (Cannabaceae) is one of the oldest cultivated plants originating in Central Asia [1]. It has a long history as a medicinal plant. In 2600 BC, it was prescribed by the Chinese emperor Huang Ti to relief cramps and rheumatic and menstrual pain [2]. The plant has become a subject of increasing scientific interest and, nowadays, about 100 Cannabis-derived phytocannabinoids are characterized [3]. In drug hemp, Δ9-THC is among the major phytocannabinoids accounting for about 40 % of the plant's extract [4]. This drug acts as a partial agonist on both cannabinoid CB1 and CB2 receptors [5]. Activation of these receptors by Δ9-THC triggers many different physiological processes, for instance, neuromodulatory action by presynaptic inhibition of transmitter release [6]. More recently, Δ9-THC has been reported to be utilized as an adjunct treatment for various neurological diseases including PD [4]. Since in PD, mitochondrial impairment plays a crucial role in cell degeneration, the use of phytocannabinoids is not quite obvious, as studies demonstrated mitochondrial toxicity of phytocannabinoids leading to mitochondrial dysfunction and an increase of oxidative stress [7, 8]. But in a PD cell culture model, phytocannabinoids, especially Δ9-THC, showed antioxidant properties against MPP^+ and rotenone. Both compounds led to decreased oxidative phosphorylation by inhibiting mitochondrial complex I [9, 10]. Consequently, these compounds increased the levels of superoxide radical levels and ATP depletion. Therewith, they are suitable PD models in cell culture and animal studies. Superoxide, the first ROS intermediate, is produced by the reduction of molecular oxygen and leaks from mitochondria and serves as a precursor for other types of ROS. In these models, the beneficial effect of Δ9-THC was mediated by an increase of glutathione levels rather than the scavenging of superoxide radicals.

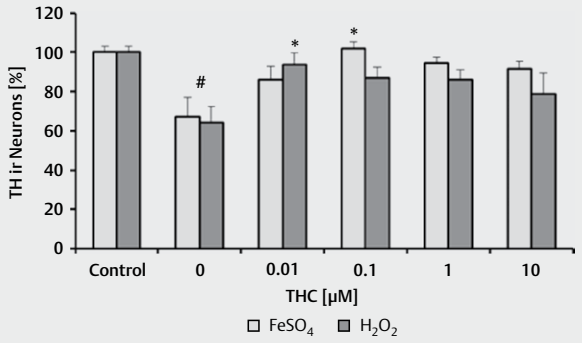
PD is the second most prevalent neurodegenerative disorder that affects 2–3 % of population above 65 years of age. The pathological hallmarks of the disease are the loss of dopaminergic neurons in SN pars compacta and presence of α-synuclein-containing intracellular inclusions in the surviving cells. This results in a deficiency of striatal dopamine, leading to cardinal motor symptoms, including bradykinesia, resting tremor, rigidity, and loss of postural reflexes [11]. As other cell types through the CNS and peripheral nervous system are involved, some characteristic non-motor signs occur, including olfactory deficits, sleep behavior disorders, gastrointestinal disturbances, and cognitive impairment [12]. The exact etiology of PD remains unclear and seems to be multifactorial, including both environmental and genetic factors. Environmental factors such as consumption of well water and exposure to pesticides are reported to be linked with a higher PD risk [13]. Genetic-induced PD accounts for ~10–15 % of all PD cases and constitutes what is called the familial form of PD. Family-linked cases are associated with at least seven causal genes, including leucine-rich repeat kinase 2, alpha-synuclein, parkin RBR E3 ubiquitin protein ligase, phosphatase- and tensing homolog-induced kinase 1, and further with Parkinson protein 7, glucocerebrosidase, and the vacuolar protein sorting-associated protein 35 [14]. Increasing evidence implicates the generation of ROS, mitochondrial dysfunction, excitotoxicity, inflammation, accumulation of misfolded proteins, and ubiquitin-proteasome system dysfunction [15], of which oxidative stress has been shown to play a fundamental role in the pathogenesis of PD [16]. This is supported by both experimental and postmortem studies that showed higher levels of ROS in the SN [17]. The plausible sources for the generation of ROS are the metabolism of dopamine itself, mitochondrial dysfunction, iron, inflammatory cells, excessive intracellular Ca^{2+} levels, and aging [18].

Iron is an essential element [19] and plays an important role in some biological processes, including oxidative phosphorylation, oxygen transportation, myelin production, and the synthesis and metabolism of neurotransmitters [20]. Abnormal brain iron homeostasis can lead to pathological changes in the CNS [19], and increasing iron levels in the brain can result in cellular damage through production of hydroxyl radicals by the Fenton reaction [20]. The coexistence of excessive iron and different types of ROS in mitochondria can lead to neurotoxic effects and, therefore, long-term cellular damage. This neurotoxicity was associated with cell death [21] and neurodegenerative diseases, in particular PD [22].

H_2O_2 is produced by peroxisomes and can be converted to water by catalases, which prevent its accumulation. Upon peroxisomal damage, H_2O_2 is released in large amounts to the cytosol and induces oxidative stress [23]. Moreover, H_2O_2 is largely produced by damaged mitochondria besides other ROS such as superoxide anion, hydroxyl radical, and singlet oxygen [24]. In dopaminergic neurons, auto-oxidation of dopamine into ROS, including H_2O_2, is a significant contributor to cellular damage in PD brains [15]. Additionally, the metabolism of dopamine by monoamine oxidases and catechol-O-methyltransferases leads to the formation of H_2O_2 [15]. Furthermore, neuromelanin is biosynthesized from the dopamine precursor L-DOPA and is able to reduce ferric iron to ferrous iron to complete the vicious circle. Due to that, the dopaminergic neurons are highly vulnerable to oxidative stress. Accordingly, the present study aimed to investigate the neuroprotective potential of Δ9-THC on dopaminergic neurons in mesencephalic cell culture stressed by high levels of Fe^{2+} and H_2O_2.

Results

$FeSO_4$ (450 µM) and H_2O_2 (150 µM) significantly decreased the number of dopaminergic neurons by 33 and 36 %, respectively



▶ **Fig. 1** Effect of Δ9-THC on the survival of dopaminergic neurons in $FeSO_4$- and H_2O_2-treated primary mesencephalic cell cultures. Two sets of cultures were separately co-treated with Δ9-THC (0.01, 0.1, 1, 10 µM) and $FeSO_4$ (350 µM) or H_2O_2 (150 µM) on the 12th DIV for 48 h. The 100 % corresponds to the number of dopaminergic neurons in untreated control cultures after 14 days. Values represent the mean ± SEM of three independent experiments with four wells in each treatment. In each well, dopaminergic neurons were counted in 10 randomly selected fields; ($^{\#, *}$ $p < 0.5$).

(▶ **Fig. 1**). Compared to untreated control cultures, which show intact dopaminergic neurons with long and branched neuritis (▶ **Fig. 2a,b**), treatment of cultures with FeSO4 and H_2O_2 also altered the neurites of surviving cells (▶ **Fig. 2c,d**). On the other hand, co-treatment of cultures with Δ-9-THC and $FeSO_4$ or H_2O_2 significantly rescued dopaminergic neurons by 34 and 29 % at the concentrations 0.1 and 0.01 µM, respectively (▶ **Fig. 1**). Moreover, Δ-9-THC markedly improved the morphology of the surviving neurons (▶ **Fig. 2e,f**) compared to $FeSO_4$- and H_2O_2-tretaed cultures (▶ **Fig. 2c,d**).

To investigate the total numbers of cells in primary mesencephalic cell cultures, they were semiquantified by measuring the intensity of CV and Hoechst 33342 fluorescence. $FeSO_4$, but not H_2O_2, significantly decreased the intensity of CV stain when added to the cultures on the 12th DIV for 48 h (▶ **Fig. 3**). Δ-9-THC relieved such an effect by 19 and 12 % when coadministered with $FeSO_4$ at the concentrations 1 and 10 µM, therewith reaching values above the respective control levels (▶ **Fig. 3**). In Hoechst 33342 fluorescence photometry, $FeSO_4$, but not H_2O_2, significantly decreased the fluorescence intensity of Hoechst 33342 fluorescence by about 12 % compared to untreated controls (▶ **Fig. 4**). Co-treatment of cultures with Δ-9-THC and $FeSO_4$ on the 12th DIV for 48 h markedly, but insignificantly, mitigated such a decrease in the intensity of Hoechst 33342 fluorescence by 17 % at a concentration of 10 µM (▶ **Fig. 4**).

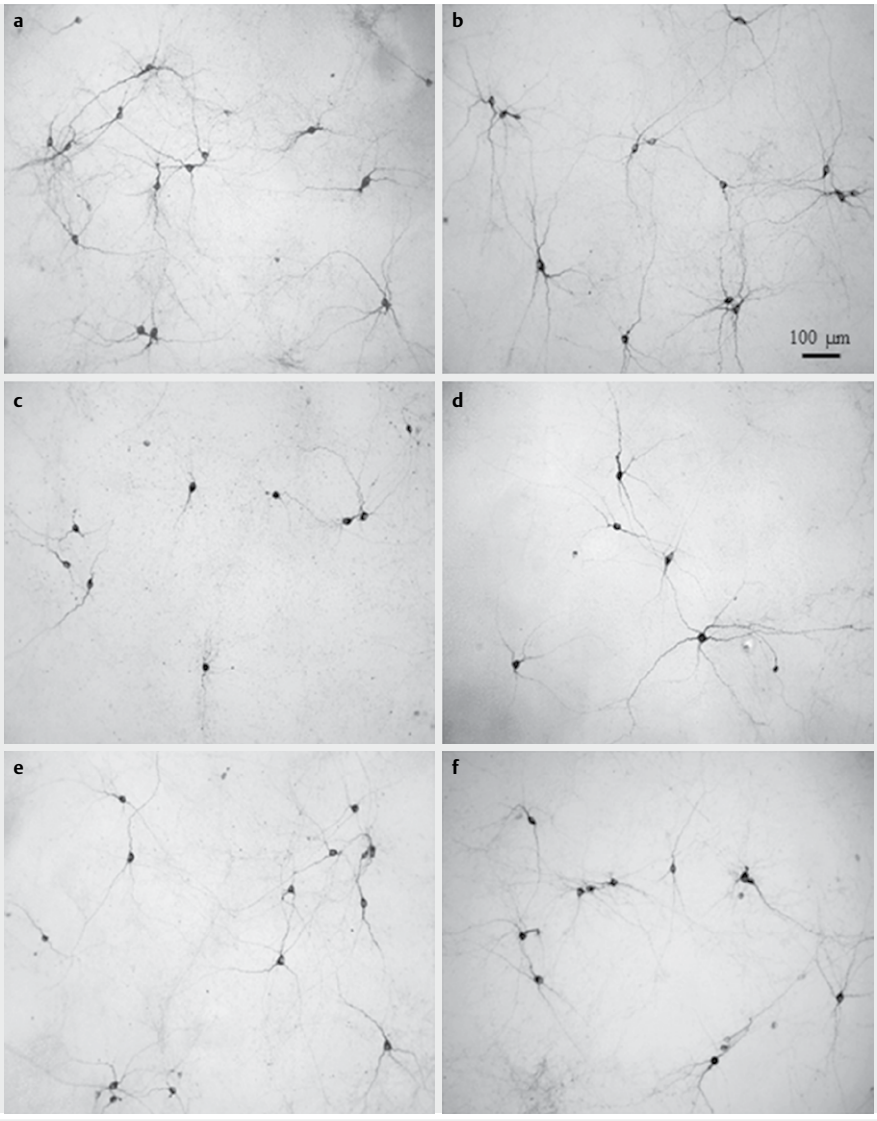
JC-1 fluorescence dye selectively enters the mitochondria forming J-aggregates with intense red fluorescence or remains in its monomeric form with green fluorescence according to the status of the Δψm. $FeSO_4$, but not H_2O_2, significantly decreased red/green fluorescence ratio by 19 % compared to untreated controls (▶ **Fig. 5**). When cultures were concomitantly treated with Δ-9-THC and $FeSO_4$ on the 12th DIV for 48 h, red/green fluorescence of JC-1 was markedly, but not significantly, increased by about 15 % at the concentration of 1 µM (▶ **Fig. 5**).

Discussion

In the present study, primary mesencephalic cell cultures oxidatively stressed with $FeSO_4$ and H_2O_2 were utilized to investigate the potential neuroprotective effects of Δ-9-THC, which might be relevant to PD. Treatment of cultures with $FeSO_4$ significantly resulted in a decrease of dopaminergic neurons by 33 % and altered the number and morphology of neurites of surviving neurons. Consistent with our results, Michel et al. [25] reported that exposure of dissociated rat mesencephalic cultures to iron significantly caused a loss of 50 % of dopaminergic neurons. The authors also revealed that iron impaired dopamine uptake, which reflects the disintegrity of dopaminergic nerve terminals [25]. Reichelt et al. [26] found that $FeSO_4$ produced a significant reduction of dopaminergic neurons in primary mesencephalic cell culture, with an IC_{50} of 450 µM. Besides dopaminergic neurons, $FeSO_4$ was seen to significantly reduce the intensity of CV and Hoechst 33342 staining, indicating an additional damaging effect on other cell types. Similarly, Michel et al. [25] found that the toxic effect of $FeSO_4$ was not restricted to dopaminergic neurons and affected other types of cells, as evaluated by the trypan blue dye exclusion index and gamma-aminobutyric acid uptake, pointing to the damage of other types of cells in the cultures. The neurotoxic effects of $FeSO_4$ were also reported in some other cellular and animal models. For instance, Du et al. [27] showed that $FeSO_4$ decreased the viability of human neuroblastoma SKN-SH cells. Liu et al. [28] found that $FeSO_4$ damaged mature hippocampal slices concentration- and time-dependently. Ayton et al. [29] observed that iron accumulation contributed to dopaminergic cell death in MPTP-intoxicated rats. The toxic effects of $FeSO_4$ on dopaminergic neurons and other cell types in primary mesencephalic cell cultures appeared to be mediated by decreasing the Δψm and subsequent apoptotic cell death. These effects seem to be a consequence of increasing ROS production, the core events produced by $FeSO_4$, and the concomitant driving of the Fenton reaction. In this context, Reichelt et al. [26] showed increasing oxidative stress in $FeSO_4$-treated primary mesencephalic cell cultures, as evaluated by an increased formation of thiobarbituric acid reactive substances and decreased glutathione concentration in cultured cells. Campos et al. [30] observed that incubation of N27 dopaminergic neurons with $FeSO_4$ resulted in higher levels of ROS and lipid peroxides.

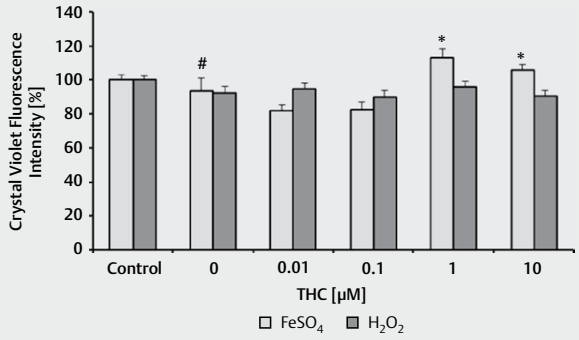
Likewise to $FeSO_4$, H_2O_2 significantly decreased the survival of dopaminergic neurons in our primary mesencephalic cell cultures by 36 % compared to untreated controls, but it did not affect CV, Hoechst 33342, and JC-1 fluorescence intensities. In accordance, such a toxic effect of H_2O_2 was reported by Ismail et al. [31] in human SH-SY5Y cells. The authors reported that exposure of human SH-SY5Y cells to 250 µM H_2O_2 for 24 h led to 50 % cell cytotoxicity [31]. Athanasiou and colleagues [7] could show that production of H_2O_2 was significantly triggered by µM range concentrations of THC. The same effects were found by Wolff and her colleagues [8]. Nonetheless, it is noteworthy that in these experiments, THC was administered in concentrations up to 100 µM. Further, higher levels of H_2O_2 in the SN were reported to be involved in the pathogenesis of PD.

Against the oxidative insult that was produced by $FeSO_4$ and H_2O_2, Δ-9-THC significantly rescued dopaminergic neurons and improved the morphology of the surviving ones in primary mesence-

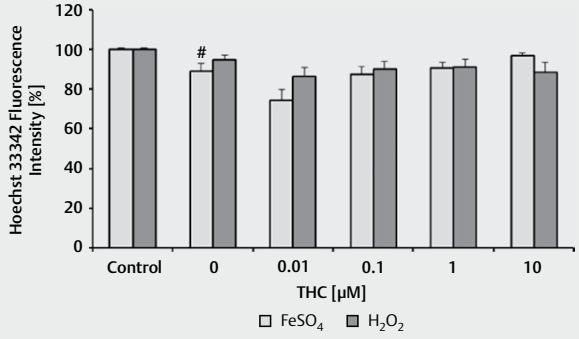


▶ **Fig. 2** Representative micrographs of dopaminergic neurons (TH immunoreactive neurons) after 14 days *in vitro*. **a, b** Untreated control cultures show many dopaminergic neurons with intact, long, and branched neurites, while **c** $FeSO_4$-treated cultures possess only a few survived dopaminergic neurons with severely degenerated neurites compared to untreated controls. **d** In H_2O_2-treated cultures, the number of dopaminergic neurons decreased and the surviving cells exhibited shortened neurites compared to the untreated controls. **e** Co-treatment of cultures with 0.1 µM of Δ9-THC and $FeSO_4$ markedly rescued a considerable number of dopaminergic neurons and their neurites compared to $FeSO_4$-treated cultures. **f** Likewise, concomitant treatment of culture with 0.01 µM of Δ9-THC and H_2O_2 markedly increased the survival of dopaminergic neurons and preserved their neurites compared to H_2O_2-treated cultures.

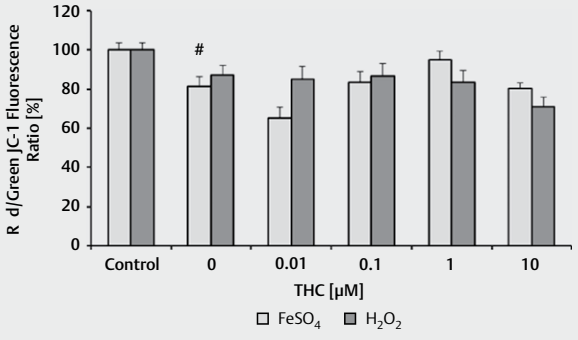
phalic cell cultures. In addition, Δ-9-THC significantly increased the number of other cell types in the cultures, as evaluated by CV and Hoechst 33342 staining. Consistent with our findings, Δ-9-THC has been shown to have a neuroprotective effect in an increasing number of studies. In this context, Nguyen et al. [32] reported that Δ-9-THC protected dopaminergic neurons and other cell types against glutamate-induced neurotoxicity in primary dissociated cultures. Moldzio et al. [9] found that Δ-9-THC counteracted the toxic effect of MPP^+ on dopaminergic neurons in primary mesencephalic cell cultures. Lastres-Becker et al. [33] could show that Δ-9-THC significantly alleviated 6-hydroxydopamine-induced dopamine depletion and reduced TH activity in the striatum and TH-mRNA levels in the SN of rats. Using JC-1 and Hoechst 33342 fluorescence staining, it has been shown that Δ-9-THC exerted its neuroprotective action through increasing mitochondrial polarization and decreasing cell death, particularly in $FeSO_4$-treated cultures. Similar to these effects, Nguyen et al. [32] found that Δ-9-THC counteracted mitochondrial membrane depolarization and apoptotic changes in glutamate-treated primary mesencephalic cell cultures. Still under discussion, some scientists attributed the effect of Δ-9-THC to its direct antioxidant activity against oxidative damage. For example, Hampson et al. [34] and Chen et al. [35] reported that Δ-9-THC acted as an antioxidant against ROS- and NMDA-induced neurotoxicity in rat cortical neuronal cultures and the AF5 cell line, respectively. It seems that these characteristics are connected to the



▶ **Fig. 3** Effect of Δ9-THC on CV staining in $FeSO_4$- and H_2O_2-treated primary mesencephalic cell cultures. Two sets of cultures were separately co-treated with Δ9-THC (0.01, 0.1, 1, 10 µM) and $FeSO_4$ (350 µM) or H_2O_2 (150 µM) on the 12th DIV for 48 h. The 100 % corresponds to the fluorescence intensity of CV in untreated control cultures after 14 days. Data are expressed as means ± SEM of eight independent experiments, whereas each condition was carried out in duplicate; ($^{\#,*}p<0.5$).



▶ **Fig. 4** Effect of Δ9-THC on Hoechst 33342 fluorescence intensity in $FeSO_4$- and H_2O_2-treated primary mesencephalic cell cultures. Two sets of cultures were separately co-treated with Δ9-THC (0.01, 0.1, 1, 10 µM) and $FeSO_4$ (350 µM) or H_2O_2 (150 µM) on the 12th DIV for 48 h. The 100 % corresponds to the fluorescence intensity of Hoechst 33342 in untreated control cultures after 14 days. Data are expressed as means ± SEM of eight independent experiments, whereas each condition was carried out in duplicate; ($^{\#}p<0.5$).



▶ **Fig. 5** Effect of Δ9-THC on the red/green fluorescence ratio of JC-1 fluorescent dye in $FeSO_4$- and H_2O_2-treated primary mesencephalic cell cultures. Two sets of cultures were separately co-treated with Δ9-THC (0.01, 0.1, 1, 10 µM) and $FeSO_4$ (350 µM) or H_2O_2 (150 µM) on the 12th DIV for 48 h. The 100 % corresponds to the red/green fluorescence ratio of JC-1 in untreated control cultures after 14 days. Data are expressed as means ± SEM of eight independent experiments, whereas each condition was carried out in duplicate; ($^{\#}p<0.5$).

molecular structure of THC and are not mediated by the CB1 receptor [36, 37].

Besides its antioxidant properties, Δ-9-THC was shown to induce neuroprotection through a CB1 receptor-dependent mechanism. In this context, Nguyen et al. [32] reported that the CB1 receptor antagonist, SR141716A, blocked the protective effect of Δ-9-THC against glutamate-induced excitotoxicity in primary mesencephalic cell cultures, indicating a CB1 receptor-dependent mechanism. On the other hand, Chen et al. [35] demonstrated that Δ-9-THC protected the AF5 CNS cell line against NMDA-induced cytotoxicity through a CB1-independent mechanism. The authors showed that NMDA toxicity was reduced by Δ-9-THC, but not by cannabinoid receptor agonist WIN55,212-2. In their study, the cannabinoid antagonist SR141716A did not inhibit the neuroprotection induced by Δ-9-THC or alter the effect of the WIN55,212-2 agonist [35]. Using cerebellar granule cell cultures prepared from CB1-knockout mice, Marsicano et al. [38] also confirmed that Δ-9-THC antioxidant effect is receptor-independent.

Taken together, our study demonstrates the neurorescuing effects of Δ-9-THC on dopaminergic neurons in primary mesencephalic cell culture that were oxidatively stressed by $FeSO_4$ and H_2O_2. This effect was seen to be mediated by increasing mitochondrial polarization and decreasing cell death, particularly against $FeSO_4$-treated cultures. The study suggests a promising role for Δ-9-THC as a therapeutic agent against neurodegenerative diseases, in which oxidative stress and ROS formation are crucial for the neuronal decline.

Materials and Methods

Preparation of primary mesencephalic cell cultures

Pregnant mice (OF1/SPF) at gestation day 14 were obtained from the Institute for Laboratory Zoology and Veterinary Genetics, Himberg, Austria. Animals were cared and handled in accordance with the guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals. Primary mesencephalic cell cultures were prepared from embryonic mesencephala as described previously by Radad et al. [39]. In brief, embryos were collected under aseptic condition in DPBS (Invitrogen). Under a stereoscope, brains were released from the skulls and the mesencephala were carefully cleaned from the meninges. They were enzymatically and mechanically dissociated using 0.2 % trypsin solution (Invitrogen) and fire-polished Pasteur pipettes, respectively. Then, obtained cells were grown in DMEM supplemented with 10 mM HEPES buffer, 10 % heat-inactivated FCS, 32 mM glucose, and 4 mM glutamine. On the

1st and 3rd DIV, the culture medium was exchanged with DMEM supplemented with FCS. Half of the medium was replaced with serum-free DMEM supplemented with 2 % B-27 (Invitrogen) on the 5th DIV. From the 6th DIV, serum-free DMEM with 2 % B-27 serum supplement was used for feeding of the cultured cells and subsequently replaced every 2nd day.

Treatment of cultures with $FeSO_4$ and H_2O_2

Two sets of cultures were prepared on 48- or 96-well plates according to the different analytical procedures. The first set of cultures were treated with different concentrations of $FeSO_4$ (50, 150, 250, 350, 450 µM) on the 12th DIV for 48 h. $FeSO_4$ was dissolved in H_2O and final concentrations were prepared in DMEM. The second set of cultures was treated with H_2O_2 (10, 25, 50, 100, 150 µM) on the 13th DIV for 1 h. Afterwards, the cultures were kept for a further 24 h. Hydrogen peroxide was dissolved in DPBS, and the final concentrations were prepared in DMEM. $FeSO_4$ and H_2O_2 at the concentrations 350 µM and 150 µM, respectively, were found to induce a higher loss of dopaminergic neurons in primary mesencephalic cell cultures (**Figs. 1S and 2S**, Supplementary Material).

Treatment of cultures with delta-9-tetrahydrocannabinol, $FeSO_4$ and H_2O_2

To investigate the effect of Δ9-THC against $FeSO_4$- or H_2O_2-induced cellular damage, a set of cultures was co-treated with different concentrations of Δ9-THC (0.01, 0.1, 1, 10 µM) and 350 µM of $FeSO_4$ on the 12th DIV for 48 h. Another set of cultures was first treated with Δ9-THC (0.01, 0.1, 1, 10 µM) on the 12th DIV for 24 h. On the 13th DIV, 150 µM of H_2O_2 were added for 1 h followed by a culture medium change with freshly prepared medium containing the same concentrations of Δ9-THC as before for another 24 h.

Identification of dopaminergic neurons

Dopaminergic neurons in primary mesencephalic cell culture were identified by immunocytochemistry against TH. On the 14th DIV, cultured cells were rinsed carefully with PBS (pH 7.2) and fixed with Histochoice (Sigma-Aldrich) for 15 min at room temperature. Cultured cells were then rinsed with PBS and permeabilized with 0.4 % Triton X-100 for 30 min at room temperature. After washing with PBS, cultured cells were incubated with 5 % horse serum (Vectastain) for 90 min to block nonspecific binding sites. Then, cells were incubated with anti-TH primary antibody overnight at 4 °C, biotinylated secondary antibody (Vectastain, ABC kite), and avidin-biotin-horseradish peroxidase complex (Vectastain) for 90 min at room temperature. The reaction product was developed in a solution of diaminobenzidine (1.4 mM) in PBS containing 3.3 mM hydrogen peroxide (H_2O_2). Dopaminergic neurons were counted with a Nikon inverted microscope in 10 randomly selected fields/well at 10× magnification.

Crystal violet assay and Hoechst 33342 fluorescence staining

Both methods are used for the evaluation of cell survival. CV and Hoechst 33342 fluorescence are relatively quick and easy methods for cell number determinations. On the one hand, CV gives a good overview of the cell layer uniformity, but the high number of washing steps removes most of incompletely fixed cells and therefore falsifies the result. On the other hand, Hoechst 33342 is only modestly toxic to cells, which allow the staining and measurement of living cells, while no distinction between living or dead cells can be made.

As CV, a triphenylmethane dye, can bind to proteins and DNA, it is used to quantitatively assess the number of cells in whole cell cultures [40]. On the 14th DIV, culture medium was aspirated, and the cells were fixed with pure ethanol (96 %; Sigma-Aldrich) for 15 min at −20 °C. Then, cells were incubated with CV (0.1 % in 10 % ethanol) in the dark for 15 min. The dye was removed, and cells were washed three times with distilled water. Plates were left in the dark at room temperature for a minimum of 24 h to dry. Afterwards, the stained cells were solubilized in 300 µL acetic acid (10 %). From each well, 200 µL were transferred to a 96-well microplate and the OD extinction of CV was measured by a microplate reader at a wavelength of 590 nm [41].

Hoechst 33342 (bisbenzimide) is a low cytotoxic DNA dye that binds to triplet adenine and thymine base pairs in the minor groove [42]. Therefore, Hoechst 33342 can be additionally used to detect nuclear condensation and fragmentation that indicate apoptotic changes [43]. For measurement of fluorescence intensity of Hoechst 33342, cultures were prepared and treated in black 96-well microtiter plates. On the 14th DIV, cells were incubated with Hoechst solution (1:2000 in DMEM) for 10 min at 37 °C. After washing with DPBS, cells were covered with 100 µL DPBS and the optical intensity of Hoechst 33342 was measured using an Enspire multilabel plate reader at 497 nm with an excitation wavelength of 360 nm. The intensity of Hoechst 33342 corresponds to the number of living cells.

JC-1 fluorescence staining

JC-1 is a lipophilic cationic dye that selectively enters into mitochondria. In normal cells with high Δψm, JC-1 forms J-aggregates with intense red fluorescence. In apoptotic cells, the dye remains in its monomeric form with green fluorescence. Accordingly, the JC-1 red/green ratio has been used as an indicator of the changes in Δψm [44]. Cultures were prepared and treated in black 96-well microtiter plates. After treatment protocols on the 14th DIV, cells were incubated with 15 µL of JC-1 (1:10 in DMEM) for 30 min at 37 °C. Measurement of the JC-1 monomers was performed using an Enspire multilabel plate reader at 485 nm and 535 nm as excitation and 530 nm and 590 nm as emission wavelengths, respectively.

Statistics

All experiments were repeated at least four times and data are presented as the mean ± SEM. Statistical significance was evaluated using the Kruskal-Wallis (H)-test followed by a $\chi 2$-test. Comparison between independent groups was done by the Mann-Whitney U test. Both the Kruskal-Wallis (H)-test and Mann-Whitney U test were performed in StatView, whereas the $\chi 2$-test was performed in Microsoft Excel 2016. Differences with $p < 0.05$ were regarded as statistically significant.

The toxic effects of $FeSO_4$ and H_2O_2 on dopaminergic neurons in murine mesencephalic primary cultures are available as Supporting Information.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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