The neuroprotective effects of (-)-deprenyl in an in vivo stroke model and in an in vitro hypoxia model

**Introduction**

(-)-Deprenyl, the N-propargyl analogue of (-)-methamphetamine, is a monoamine oxidase-B antagonist having an antiparkinson effects. (-)-Deprenyl is considered to be antiapoptotic. (-)Deprenyl significantly reduced the lesion size in a permanent middle cerebral artery occlusion model of stroke, following a 7-day treatment.

It was demonstrated in a PC12 cell culture system that (-)-deprenyl upregulates Bcl-2 and reduces the number of apoptotic cells. Bcl-2 expression keeps mitochondria functioning. Bcl-2 is an antiapoptotic protein interfering with the actions of proapoptotic proteins of the same protein family (e.g. Bax, Bad, Bid). Bcl-2 blocks cytochrome c release from mitochondria, preventing the activation of the apoptotic machinery.

The presence of Bcl-2 is characteristic of axonal growth cones. The upregulation of GAP-43 signals enhanced synaptic plasticity and is characteristic for neuronal plasticity following middle cerebral artery occlusion. GAP-43 has been used as an index of axonal sprouting and reflects enhancement of neuronal plasticity.

Our primary target was to examine the neuroprotective effectiveness of (-)-deprenyl in an in vivo stroke model in the rat. We used the determination of the infarct volume as an endpoint. Our secondary target
was to consider if (-)deprenyl could improve or induce post-stroke plasticity processes. In this case our endpoints were Bcl-2, GAP-43 and synapsin expression just around the ischaemic lesion. (-)Deprenyl dose-dependently modulates $\Delta \Psi_M$ and the production of reactive oxygen species (ROS) by altering the respiratory function of mitochondria. Our next move was to investigate the extent of neuroprotective efficacy of (-)deprenyl, as well as the possible cytoprotective mechanisms of its actions following hypoxia/re-oxygenization in nerve growth factor-differentiated PC12 cell culture. In this experiment our first endpoint was the number of dead cells in hypoxic, (-)deprenyl-treated hypoxic, (-)deprenyl-treated normoxic and normoxic cultures. Our second endpoint was the investigation of the mitochondrial transmembrane potential changes present in the same cell cultures, that is, the investigation of viability by assessing the energy supply of the cells. Our third endpoint was the individual free radical content of cells.

Aims:
We tried to attenuate the infarct volume by means of reducing the number of apoptotic cells. In this approach (-)deprenyl was used as an antiapoptotic agent.

Our aims were

1., to test whether (-)deprenyl, a pharmacological agent of anti-apoptotic properties, which is known as a permanent blocker of monoamine oxidase B, could reduce infarct volume at a dose not inhibiting monoamine oxidase B.

2., to test whether (-)deprenyl, a pharmacological agent, which is known to induce Bcl-2, an anti-apoptotic gene, could change neuronal cell death patterns in the cortical peripheral region of the infarct following permanent middle cerebral artery occlusion.

3., to characterise the effect of (-)deprenyl in PC12 cells following hypoxia/reoxygenization via using the combination of the following methods: propidium iodide staining (for the assessment of cell death), modified JC-1 staining method (for the characterization of mitochondrial membrane potential) and cerium staining (for the in situ quantitative measurement of free radical production) provide us with a useful data set to evaluate the cytoprotective features of a selected drug.

4., to characterize our novel JC-1-cerium double staining method

Methods:
Permanent middle cerebral artery occlusion
A craniectomy hole about 3mm in diameter was made just above the left middle cerebral artery. The dura mater was removed. Subsequently, the middle cerebral artery was identified and a bipolar coagulator was applied to it in order to obstruct the distal branches of the middle cerebral artery.
**Intraperitoneal treatment of animals using osmotic minipumps**

For intraperitoneal placement, a small midline incision was made in the skin below the rib cage of a rat. Another small incision in the abdominal muscle was made directly under the cutaneous incision. The pump was inserted, flow moderator first, into the peritoneal cavity. The muscle incision was closed with sutures. The skin incision could be closed with either a wound clip or sutures. The animals were infused with 0.2 mg/kg/day of (-)deprenyl in a vehicle of 0.9% physiological saline, delivered via osmotic minipumps intraperitoneally for two days. The control rats with permanent middle cerebral artery occlusion were infused with the vehicle only.

**Computerized planimetry of TTC-stained rat brain slices**

Rat brains were removed, sliced and the 2 mm-thick fresh brain slices were stained with TTC (2, 3, 5-triphenyltetrazolium chloride) for delineation of the tissue damage. The TTC reaction stains functional mitochondrial dehydrogenases red. The unstained area was identified as an infarct area. The TTC-stained brain slices were photographed, digitised and the territory of the lesion was defined using computer-aided morphometry in each slice. The infarct volumes in mm³ were then calculated.

**Fluorescent TUNEL method (In Situ Cell Death Detection Kit)**

DNA strand breaks can be identified by labeling free 3'-OH termini with modified nucleotides in an enzymatic reaction. In this kit terminal deoxynucleotidyl transferase (TdT), which catalyses polymerization of nucleotides to free 3'-OH DNA ends in a template-independent manner, is used to label DNA strand breaks. The incorporated fluorescein is detected using fluorescent or laser scanning confocal microscopy.

**Immunofluorescent labelling and double labelling (TUNEL, caspase-3 and Neu N staining)**

For the identification of apoptotic cells in the peri-infarct region, broken DNA (deoxyribonucleic acid)-ends were visualized using an in situ fluorescent TUNEL (Terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate (conjugated to the green fluorophore fluorescein isothiocyanate) DNA nick end labelling) kit, and caspase-3 fluorescence immunohistochemistry was also performed. The enhanced expression of caspase-3 after middle cerebral artery occlusion could be localized. For the identification of neurons, NeuN mouse anti-neuronal nuclei antibodies were used. TUNEL-NeuN and TUNEL-caspase -3 double-labelling was performed. GAP-43 was also visualized to characterize neuronal plasticity.
Fluorescence detection (CLSM)

A BIO-RAD MRC 1024 confocal system was used, installed on a Nikon DIAPHOT inverted microscope (Donsanto Corp.) For excitation 488 and 568nm lines of a Krypton-Argon laser were applied sequentially. Detection was performed with a standard filter set.

Culturing and NGF differentiation of PC12 cells

Rat phaeochromocytoma (PC-12) cells were maintained in Dulbecco’s modified Eagle’s medium, supplemented with 10% (vol/vol) calf serum, 2 mM L-glutamine, penicillin, streptomycin. PC-12 cells were predifferentiated on round cover glasses covered with a collagen membrane prepared from acid soluble collagen isolated from rat tail by adding 50 ng/ml nerve growth factor (NGF) in a humidified incubator aerated with 5% CO$_2$ at 37°C.

Hypoxia/re-oxygenation of PC12 cells

Cultures were placed on the bottom of an open chamber. Subsequently, it was filled with Argon gas and then the chamber was closed. A blood gas analyser was used to control the partial O$_2$ pressure in the cell culture medium. After 1 h of oxygen deprivation, the cultures were returned to the incubator (re-oxygenation) for 24 h. Control cultures were maintained in the incubator under normal conditions (normoxia).

Assessment of cell death in PC12 cell culture with propidium iodide staining

The extent of cell death was determined by staining the cultures with 1,5 μg/ml propidium iodide dissolved in physiological saline for 2 minutes. The procedure was the following: the DMEM was removed and 300 μl of 1,5 μg/ml propidium iodide solution was added to the cell cultures. The numbers of viable and dead cells were counted with a fluorescence microscope using 450-490 nm excitation and 520 nm barrier filters. Cellular death was expressed as a mean percentage of dead cells in three separate cultures, in twelve samples.

Combined staining procedure (JC-1+cerium)

The DMEM was removed and 300 μl of 10 μg/ml JC-1 solution was added to the cultures for 10 minutes. The solution was removed and the cultures were rinsed with physiological saline for 2 minutes. Then, 300 μl of 20 mmol/l CeCl$_3$ solution (in lactated Ringer) was applied for 2 minutes. Following the removal of theCeCl$_3$ solution the cell cultures were rinsed again with physiological saline for 2 minutes and subsequently the cells were fixed in 0,25% (vol/vol) glutaraldehyde solution for 2 minutes.
Data analysis and statistics

(-)Deprenyl in a rat stroke model
The data obtained from the rat stroke model were statistically evaluated using Students’ t test.

(-)Deprenyl in a PC12 hypoxia/reoxygenation model
The data were statistically evaluated using ANOVA and a post-hoc Duncan test. The results were considered to be statistically significant if p<0.05. Data are shown as mean±SEM.

Characterization of JC-1-cerium double staining in PC12 cells following hypoxia/reoxygenation
All data was statistically evaluated using the Mann–Whitney test and the Kolgomorov-Smirnov test. The intensity curve was made by SPSS 12.0 (LEAD Technologies, US) statistical program. Data was considered to be statistically significant if \( p < 0.05 \).

Results:

(-)Deprenyl’s effects in a rat stroke model
The average lesion size in (-)deprenyl-treated animals was 36.5 mm\(^3\), while it was 65.8 mm\(^3\) in control rats.

The number of TUNEL-labelled cells averaged over 60 samples was 17±12 in treated rats, while it was 28±25 in control rats (\( P = 0.002 \)). The number of TUNEL-caspase-3 double-labelled cells averaged over 30 samples was 3±3 in treated rats, while it was 8±6 in control rats (\( P = 0.0003 \)).

Samples stained with GAP-43 showed a slight upregulation of GAP-43 in (-)deprenyl-treated rat brains after permanent middle cerebral artery occlusion as compared to that in control rat brains after middle cerebral artery occlusion.

Reverse transcriptase-polymerase chain reaction analysis showed that in (-)deprenyl-treated control rat temporal cortices Bcl-2 mRNA expression was increased by about 100 % as compared to that in physiological saline-treated control rat, and in (-)deprenyl-treated control rat temporal cortices GAP-43 mRNA expression increased only slightly as compared to that in physiological saline-treated control rat temporal cortices.

Argon hypoxia and re-oxigenation of PC-12 cell cultures
The partial \( O_2 \) pressure in the cell culture medium was 154.65±1.35 mm Hg (\( n = 6 \)) in normoxic conditions and it was 131.96±2.29 mm Hg (\( n = 6 \)) (mean±SEM) following the hypoxic period.

Right after 1 h of hypoxia and 24 h of re-oxygenation the number of propidium iodide-positive (PI+) cells increased as compared to the normoxic control (36.66±3.25 %) (\( n = 12 \)) versus (PI+:19.5±2.18 %) (\( n = 12 \)) (\( p < 0.001 \)), and the cells had decreased and relatively uniform JC-1 red/green ratios (0.681±0.013) (\( n = 50 \)) versus 1.07±0.18 (\( n = 50 \)) (\( p < 0.01 \)) and they showed an increased cytoplasmic levels of peroxyl radicals (28.99±0.65) (\( n = 100 \)) versus 23.78±0.78 (\( n = 100 \)) (\( p < 0.001 \)).
Treatment with (-)deprenyl decreased the number of PI+ cells in oxygen-deprived and reperfused PC12 cell cultures at $10^{-12}$ M (21.41±3.97 %) (n=12) (p<0.01) and $10^{-8}$ M (21.54±2.33 %) (n=12) (p<0.001) significantly, while $10^{-3}$ M (-)deprenyl, the highest concentration, increased the number of PI+ cells (44.25±5.76 %) (n=12) (p<0.05) as compared to the hypoxic control (36.66±3.25 %) (p<0.01). The average JC-1 red/green signal intensity ratio (the representative of mitochondrial activity) of oxygen deprived/re-oxygenated cultures was elevated by (-)deprenyl treatment in a concentration dependent fashion, the $10^{-12}$ M being the most potent membrane potential booster and the $10^{-3}$ M being the least potent. The average JC-1 red/green signal intensity ratio was 0.68±0.013 (n=50) in hypoxic control cultures, 2.83±0.1 (n=50) (p<0.001) at $10^{-12}$ M, 2.33±0.074 (n=50) (p<0.001) at $10^{-8}$ M and 1.68±0.095 (n=50) (p<0.001) at $10^{-3}$ M. The cerium signal intensity in oxygen deprived/re-oxygenated PC12 cultures also changed in a concentration dependent fashion. At $10^{-12}$ M (20.84±0.58) (n=100) (p<0.001) (-)deprenyl decreased the cerium signal level in hypoxic/re-oxygenated PC12 cultures below that of the normoxic control (23.78±0.78) (n=100), on the other hand, at $10^{-8}$ M (28.86±0.78) (n=100) (-)deprenyl did not affect the cerium signal, while at $10^{-3}$ M (32.15±0.75) (n=100) (p<0.01) (-)deprenyl considerably increased cerium reflectance intensities as compared to the hypoxic control (28.99±0.65) (n=100).

In the normoxic control cells treated with (-)deprenyl the number of PI+ cells was not affected at $10^{-8}$ M (19.67±2.82 %) (n=12), contrary to the concentration of $10^{-12}$ M which considerably decreased the number of PI+ cells (10.18±0.79 %) (n=12) and slightly decreased it at $10^{-3}$ M (14.83±1.06 %) (p>0.05) (n=12) as compared to the normoxic control (19.5±2.18 %) (n=12). JC-1 red/green pixel intensity ratio was significantly increased in (-)deprenyl-treated normoxic control PC12 cultures at all (-)deprenyl concentrations. It was 1.07±0.025 (n=50) in normoxic control cultures, 2.15±0.088 (n=50) (p<0.001) at $10^{-12}$ M, 2.17±0.077 (n=50) (p<0.001) at $10^{-8}$ M and 2.83±0.18 (n=50) (p<0.001) at $10^{-3}$ M. The cerium signal level, however, in the same (-)deprenyl-treated normoxic PC12 cultures was the lowest at $10^{-8}$ M (16.76±1.11) (n=100) (p<0.001). At $10^{-3}$ M of (-)deprenyl concentration it was slightly increased (26.86±1.05) (n=100) (p<0.05), while at $10^{-12}$ M (24.63±0.93) (n=100) it was at the level of normoxic absolute control (23.78±0.78) (n=100).

Characterization of JC-1-cerium double staining in PC12 cells following hypoxia/reoxygenation
In the normoxic group, the red/green ratio was 18.2±9.3, while in the hypoxia/reoxygenization group this ratio was as low as 1.65±0.9. This difference was significant (p < 0.05). The cerium reflectance appeared as fine red signals in the cells. The distribution and location of ROS was evaluated in the same cells where JC-1 signals were detected. The
average signal intensity was 2.5±1.2 \((n = 30)\) in the control group while it was as high as 5.8±3.1 \((n = 30)\) in the hypoxia/re-oxygenation group \((p < 0.05)\). There was an inverse relationship between ROS signal and JC-1 ratio. A low JC-1 ratio means that there will be a low amount of the aggregated form of JC-1 in the mitochondria and this correlates with a high amount of ROS. Our double-labeling data correlated well with the PI staining which represents the cell injury level. The percentage of the PI positive cells after hypoxia/re-oxygenization was about doubled as compared to the normoxic group \((43.5±3.2\% \text{ versus } 18.7±2.3\% \ (n = 12, p < 0.05))\).

**Conclusions:**

1. (-)-Deprenyl significantly decreases the infarct volume in rats following middle cerebral artery occlusion in a dose as low as 0.2 mg/kg/day.

2. (-)-Deprenyl significantly increases the expression of GAP-43 in the peri-lesional zone of the cortex following a middle cerebral artery occlusion.

3. (-)-Deprenyl increases the expression of synapsin I in the peri-lesional zone of the cortex following a middle cerebral artery occlusion.

4. Mitochondrial lesion is prevented and reversed by (-)-deprenyl treatment at the concentration of \(10^{-12}\) M. However, (-)-deprenyl at the concentration of 10-3 M increases cell death and ROS production, showing a characteristic biphasic action.

5. JC-1-cerium double staining is reproducible, and it is a good tool for screening ROS-blocking cytoprotective molecules.

**Publications and manuscripts**


**Other publications**


Book chapter: