DOI 10.1007/s00702-003-0063-1
J Neural Transm (2004) 111: 37–45

Journal of Neural Transmission
Printed in Austria

Ginsenosides Rb1 and Rg1 effects on survival and neurite growth of MPP^+-affected mesencephalic dopaminergic cells

K. Radad[1], **G. Gille**[2], **R. Moldzio**[1], **H. Saito**[3], **K. Ishige**[3], and **W.-D. Rausch**[1]

[1] Institute for Medical Chemistry, Veterinary Medical University, Vienna, Austria
[2] Department of Neurology, Technical University, Dresden, Germany
[3] Department of Pharmacology, College of Pharmacy, Nihon University, Chiba, Japan

Received May 26, 2003; accepted September 10, 2003
Published online December 3, 2003;

Summary. Ginsenosides Rb1 and Rg1 are the main active ingredients of Panax ginseng C.A. Meyer (Araliaceae). They appear to exert protection against ischaemia and anoxic damage in animal models, suggesting an antioxidative and cytoprotective role. In our study, primary cultures from embryonic mouse mesencephalon are applied to examine the effects of these two ginsenosides on neuritic growth of dopaminergic cells and their survival affected by 1-methyl-4-phenylpyridinium-iodide (MPP^+). Ginsenoside Rb1 (at 10 μM) enhanced the survival of dopaminergic neurons by 19% compared to untreated control. MPP^+ (at 1 μM) significantly reduced the number of dopaminergic neurons and severely affected neuronal processes. Both ginsenosides counteracted these degenerations and significantly protected lengths and numbers of neurites of TH^+ cells. Both compounds however could not prevent the cell loss caused by MPP^+. Our study thus indicates partial neurotrophic and neuroprotective actions of ginsenosides Rb1 and Rg1 in dopaminergic cell culture.

Keywords: Ginsenosides, MPP^+, dopaminergic, Parkinson's disease.

Introduction

Ginseng, the root of Panax ginseng C.A. Meyer (Araliaceae), is a well known herbal medicine (Rhim et al., 2002). It has been used as a constituent of many prescriptions in Chinese Traditional medicine and is now popular in the world as a natural medicine (Himi et al., 1989). The molecular components responsible for the action of ginseng are ginsenosides, which are also known as ginseng saponins (Lee et al., 2002). Among more than 30 different ginsenosides, Rb1 and Rg1 are the main active ingredients of Panax ginseng (Liu and Zhang, 1995). The chemical structures of ginsenosides Rb1 and Rg1 were clarified by Wen et al. (1996). Jiang et al. (2000)

and Liao et al. (2002) reported that ginsenosides Rb1 and Rg1 reduced neuronal death following transient cerebral ischaemia and promoted survival and outgrowth of spinal cord neurons, respectively. The beneficial effects of ginsenosides are mediated through scavenging of free radicals (Lim et al., 1997), improving energy metabolism and preserving structural integrity of the neurons (Jiang and Qian, 1995). Moreover, ginsenosides Rb1 and Rg1 block calcium over-influx into neuronal cells (Liu and Zhang, 1995) and inhibit Na^+ channel activity (Liu et al., 2001).

MPP^+, the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), is highly selective and toxic for nigrostriatal dopaminergic neurons either in vivo or in vitro (Michel et al., 1990; Bai et al., 2002; Li et al., 2002). The structural analogy of MPP^+ to dopamine and its high affinity for the dopamine reuptake system seem to explain its selective toxic properties for dopaminergic cells (Michel et al., 1990). However mechanisms by which MPP^+ kills dopaminergic cells are still a matter of debate, though numerous concepts have been proposed. MPP^+ inhibits NADH-dehydrogenase (complex I) activity of the mitochondrial respiratory chain, which leads to increased production of free radicals and finally to impaired production of ATP thus causing damage to dopaminergic neurons (Li et al., 2002). MPP^+ also increases intracellular calcium concentration in mesencephalic culture and this could involve biochemical processes leading to malfunctioning of mitochondria, energy failure and ultimately cell death (Chen et al., 1995). MPP^+ elevates extracellular dopamine levels with a consequent increase in H_2O_2 by monamine oxidase (MAO) via deamination and non-enzymatically by autooxidation (Matarredona et al., 1997). According to Bai et al. (2002), MPP^+ stimulates the production of superoxide radicals in vitro and induces cell death in PC12 cells. Apoptotic pathways contribute to cell death in mesencephalic dopaminergic cells (Hochizuki et al., 1994).

Although recent studies have shown that ginsenosides exert beneficial actions on neuronal cells, there is no report concerning a possible effectivity of these ginsenosides against MPP^+ neurotoxicity as a cellular model of Parkinson's disease. Therefore, we examined the effect of ginsenosides Rb1 and Rg1 on MPP^+ induced neurotoxicity in primary mesencephalic dopaminergic cell culture.

Materials and methods

Materials

Pregnant OF1/SPF mice at gestation day (GD) 14 were purchased from the Institute for Laboratory Zoology and Veterinary Genetics in Himberg (Austria). Dulbecco's Modified Eagles Medium (DMEM), fetal calf serum (FCS), glutamine, putrescine, triiodo-L-thyronine, corticosterone, progesterone, bovine serum albumin, diaminobenzidine and paraformaldehyde were purchased from Sigma (USA). Insulin-transferrin-sodium selenite supplement, transferrin, penicillin-streptomycin and anti-tyrosine hydroxylase antibody (anti-TH antibody) were purchased from Roche Molecular Biochemicals (Germany). Vectastain ABC Elite Kit (Mouse IgG) was purchased from Vector Laboratories (USA). 1-methyl-4-phenylpyridinium (MPP^+) was purchased from RBI (USA).

Methods

Preparation of mesencephalic dopaminergic cell cultures

Pregnant mice were sacrified on GD 14, the uteri were dissected and the embryos were carefully removed and transferred to a Petri dish containing phosphate buffered saline (pH 7.2)

for dissection. Under a stereoscope (Nikon SMZ-1B, 10× magnification), the brains were then dissected, the ventral mesencephala were excised and primary cultures prepared according to Koutsilieri et al. (1995). Briefly, after careful removal of the meninges, tissues were mechanically cut into small pieces in PBS (pH 7.2) and subsequently triturated with a fire-polished Pasteur pipette in DMEM. Dissociated cells were resuspended in DMEM supplemented with Hepes buffer (25 mM), glucose (30 mM), glutamine (2 mM), penicillin-streptomycin (10 U/ml and 10 μg/ml, respectively) and heat-inactivated FCS (10%). The cell suspension was plated into 4-wells multidishes (Nunclon) precoated with poly-D-lysine (50 μg/ml). Cultures were grown at 37°C in an atmosphere of 5% CO_2/95% air and 100% relative humidity. On the 6th day in vitro (DIV), the medium was changed to a serum free DMEM growth medium containing additionally transferrin (100 μg/ml), insulin-transferrin-Na-selenite (10 μg/ml, 100 μg/ml and 60 nM respectively) and N3-additives (200 μM putrescine, 40 nM progesterone, 570 nM corticosterone, 30 nM triiodothyronine (T3) and 0.001% bovine serum albumin).

Treatment of the cultures with MPP^+ and ginsenosides

Stock solutions of either ginsenosides (100 μM) were prepared in DMEM and added to the culture medium on the 6th DIV (final concentrations 10 μM). The culture medium was changed every 2 days with the same concentration of ginsenosides. MPP^+ iodide (10 μM) was added directly to the medium (final concentration 1 μM). MPP^+ was added on 10th DIV for 48 hours either alone or with the ginsenosides. Control cultures were kept in parallel. Three independent experiments were performed for each compound tested.

Identification of tyrosine hydroxylase immunoreactive (TH ir) neurons

Cultures were rinsed carefully with PBS (pH 7.2) on the 12th DIV and fixed in 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. Cultures were washed 3 times with PBS and incubated with 5% horse serum (Vectastain ABC Kit) for 90 min to block non-specific binding sites. Cells were sequentially incubated with anti-TH 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 (pH 7.2) between stages. The reaction product was developed in a solution of diaminobenzidine (1.4 mM) in PBS (pH 7.2) containing 3.3 mM hydrogen peroxide (H_2O_2). Total TH ir cell numbers were counted in 10 randomly selected fields (0.01 mm^2/field) at 100× magnification with a Nikon inverted microscope. Also, TH ir cells with rudimentary ($\leq$10 μm) or without processes were counted in 10 randomly selected fields at 100× magnification. Lengths (the longest neurite/30 TH ir cells in each experiment) and numbers (number of neurites/30 TH ir cells in each experiment) of the processes of TH ir cells were performed with computerized video imaging technique (NIH Image). Cellular processes were counted at 200× magnification.

Statistics

Data were expressed as mean ± standard error of mean (SEM). Statistical differences were determined using the Kruskal-Wallis (H)-test followed by χ^2 test. Differences with $^*p < 0.05$ were regarded as statistically significant.

Results

Effect of MPP^+ on mesencephalic dopaminergic cells

Treatment of mesencephalic dopaminergic cells with 1 μM MPP^+ on the 10th DIV for 48 hours significantly decreased the number of TH ir cells by 36%

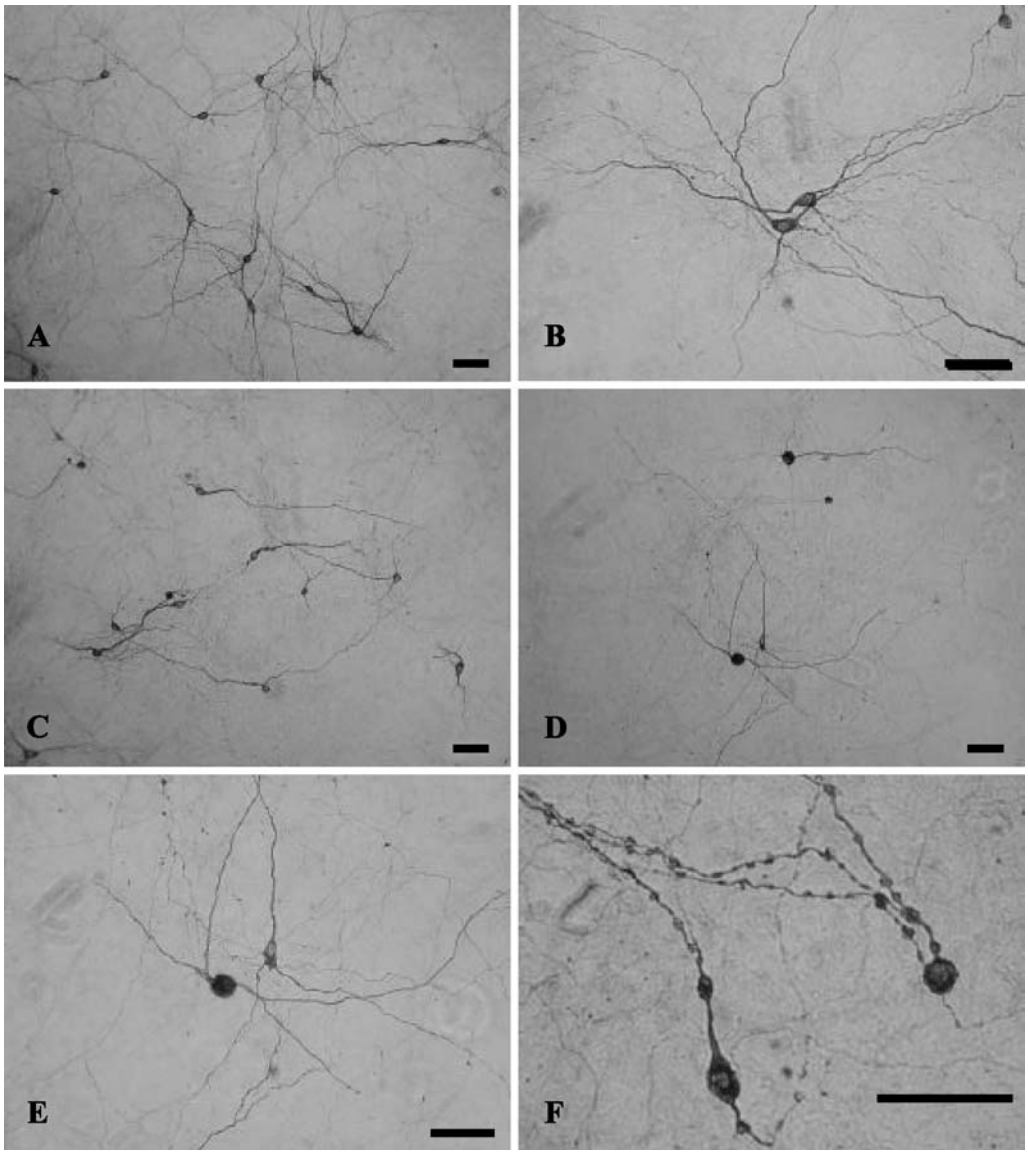

Fig. 1. Representative fields for dopaminergic neurons after 12 DIV. TH-immunocytochemistry. **A** Untreated control dopaminergic neurons. **B** Untreated control dopaminergic neurons showing triangular cell bodies and long branched processes. **C** MPP^+ treated neurons showing loss of neurites. **D** MPP^+ treated neurons showing swollen cell bodies. **E** Same as D at higher magnification. **F** MPP^+ treated neurons showing many TH^+ bead-like structures along the course of neuronal processes. (bar = 100 μm)

compared to untreated controls (Fig. 2). The surviving cells showed many degenerative changes as evaluated by the length of the longest neurites, the number of the neurites and the general morphological appearance of TH ir cells. MPP^+ significantly decreased the lengths and numbers of the neuronal processes of the surviving cells. The lengths of the neurites were reduced by 16% (Fig. 3) and the numbers were lowered by 64% (Fig. 4). In addition, the

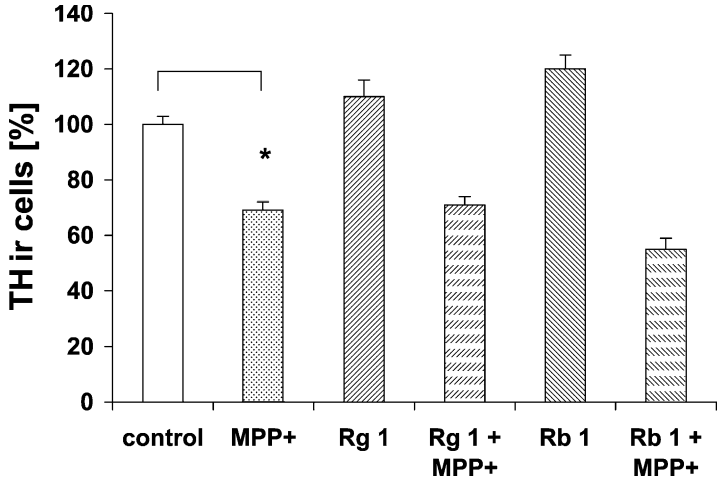

Fig. 2. Effects of ginsenosides Rb1 and Rg1 on survival and MPP^+ induced cell death in dopaminergic neurons. 100% corresponds to the number of TH ir cells after 12 DIV in untreated controls (Cell numbers of TH^+ cells for different control cultures range from 15 ± 3 to 33 ± 8.4 cells/field). Values represent the mean $\pm$ SEM for three independent experiments. Values of each experiment are the means of 40 randomly selected fields in four different wells. Statistical differences were determined as described in Materials and methods ($^*p < 0.05$)

number of cells with rudimentary or without processes increased by 5,27 folds (Fig. 5). Cell bodies usually appeared round, swollen and with eccentric nuclei. Neuronal processes were fragmented, dysmorphic and showing many TH^+ bead-like structures along their courses (Fig. 1).

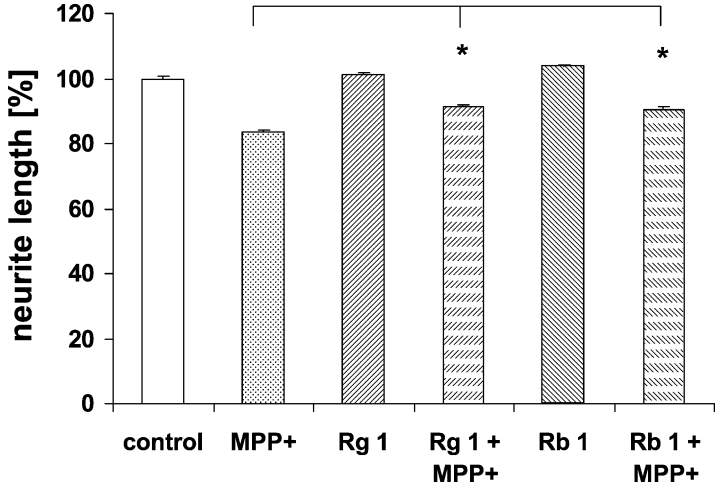

Fig. 3. Effects of ginsenosides Rb1 and Rg1 on neurite growth of MPP^+ treated dopaminergic cells. 100% corresponds to neurite lengths (longest neurite/cell) of TH ir cells after 12 DIV in untreated control (Length of the longest neurite for different control cultures, 0.49 ± 0.01 mm/longest neurite). Values represent the mean $\pm$ SEM for three independent experiments. Value of each experiment is the mean of the longest neurite of 30 cells in four different wells. Statistical differences were determined as described in Materials and methods ($^*p < 0.05$)

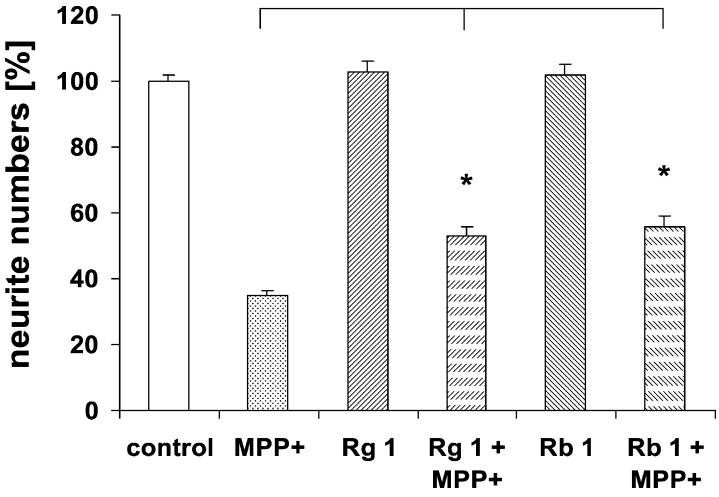

Fig. 4. Effects of ginsenosides Rb1 and Rg1 on neurite numbers of MPP^+ treated dopaminergic neurons. Values represent the mean ± SEM for three independent experiments. The value of each experiment is the mean of 30 cells in four different wells. 100% corresponds to the number of neurites/cell in controls (number of neurites for different control cultures range from 4 ± 0.06 to 4.3 ± 0.03 neurite/cell). Statistical differences were determined as described in Materials and methods ($^*p < 0.05$)

Effect of ginsenosides Rb1 and Rg1 on mesencephalic dopaminergic cells

Both ginsenosides Rb1 and Rg1 partially attenuated MPP^+ neurotoxicity. When Rb1 or Rg1 were added to mesencephalic dopaminergic cell cultures at a

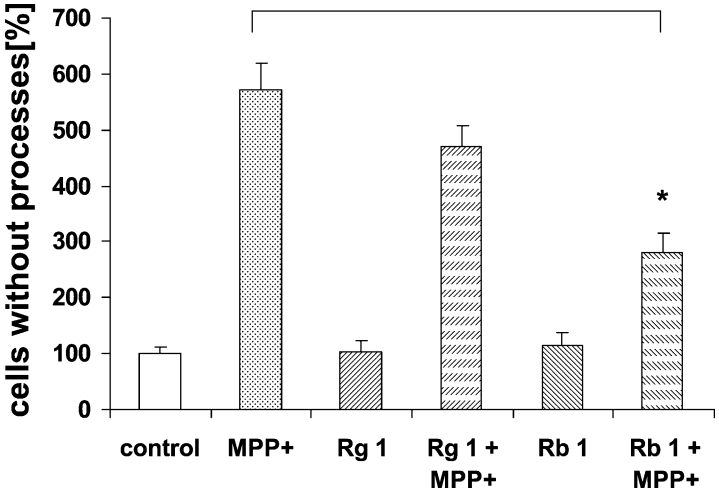

Fig. 5. Ginsenosides Rb1 and Rg1 prevent cellular degeneration of MPP^+ treated dopaminergic neurons. 100% corresponds to the number of cells without processes of TH ir cells after 12 DIV in untreated control (number of cells without processes for different control cultures range from 2.6 ± 0.3 to 2.8 ± 0.3 cells/field). Values represent the mean ± SEM for three independent experiments. The value of each experiment is the mean of 40 randomly selected fields in four different wells. Statistical differences were determined as described in Materials and methods ($^*p < 0.05$)

concentration of 10 μM on the 6th DIV for 6 consecutive days, ginsenoside Rb1 appeared to increase the survival rate of dopaminergic cells by 19% compared to untreated control, whereas Rg1 increased it by 14% (Fig. 2). On the other hand, both ginsenosides Rb1 and Rg1 could not prevent cell loss produced by MPP^+ in mesencephalic dopaminergic cell cultures (Fig. 2). At the level of surviving cells in MPP^+ exposed cultures, both ginsenosides significantly decreased the reduction produced by MPP^+ in neurite lengths ($Rb1 + MPP^+$ vs MPP^+, 41%; $Rg1 + MPP^+$ vs MPP^+, 47%, Fig. 3) and neurite numbers ($Rb1 + MPP^+$ vs MPP^+, 28%; $Rg1 + MPP^+$ vs MPP^+, 30%, Fig. 4). Cell cultures exposed to MPP^+ were screened for cells with loss of neurites. The appearance of deteriorated cells was highly increased by MPP^+ (MPP^+ vs control, 5,27 folds). The addition of ginsenoside Rb1 significantly attenuated these degenerative changes ($Rb1 + MPP^+$ vs MPP^+, 55%, Fig. 5).

Discussion

Recent studies in neuronal cells show that ginsenosides, the active ingredients of Panax ginseng roots, exert beneficial actions on neuronal cells. However there are no reports concerning a possible effectivity against MPP^+ neurotoxicity. In this present study, ginsenoside Rb1 increased the survival of dopaminergic cells in mesencephalic culture by 19%. This result is in agreement with Himi et al. (1989) who reported that ginsenoside Rb1 promoted survival of cortex neurons in chick and rat embryonic cell culture. Given that the overall pharmacology of ginsenosides is complex (Attele et al., 1999), the stimulatory effects of both ginsenosides on survival of dopaminergic cells may be mediated through improving the energy metabolism and preserving the structural integrity of neurons (Jiang and Qian, 1995). Induction of antioxidant enzymes which are important for maintaining cell viability may equally contribute by lowering free radicals generated from intracellular metabolism either by neuronal or other cells in culture (Nishiyama et al., 1994; Chang et al., 1999). Similar to the study of Rudakewich et al. (2001) who reported that ginsenosides Rb1 and Rg1 alone did not enhance neurite growth of PC12 cells, there was no significant enhancement of neurite outgrowth by both ginsenosides when added on 6th DIV for six consecutive days in our dopaminergic cell culture. Consequent with previous studies where MPP^+ resulted in highly selective and irreversible toxicity for cultured dopaminergic neurons (Michel et al., 1990; Gille et al., 2002), addition of 1 μM MPP^+ to our cultures on 10th DIV for 48 hours caused a cell loss by 36%. On the other hand, both ginsenosides could not prevent cell loss by MPP^+. The failure of these ginsenosides to antagonize cell loss by MPP^+ may be attributed to the inability of these ginsenosides to block the selective uptake of MPP^+ by dopamine neurons via a high affinity dopamine (DA) transporter or to overcome its inhibitory effects on mitochondria and production of overt free radicals (Rudakewich et al., 2001). At the level of surviving cells in MPP^+ exposed cultures, ginsenosides Rb1 and Rg1 partially promoted neurite lengths and numbers as well as ameliorated the degenerative changes such as cell swelling and loss of neurites (cell without processes). This ameliorative effect may be primarily attributed to preserving the structural integrity of neurons, improving energy metabolism and antioxidative properties of these

ginsenosides. These criteria were clearly stated in some recent studies which have been done with other neurodegenerative disease models. Deng and Chang (1991) reported that ginsenosides Rb1 and Rg1 inhibit lipid peroxidation within liver and brain by upregulating catalase and glutathione peroxidase. Using ischaemia reperfusion neurodegenerative model, ginsenoside Rb1 protects hippocampal CA1 neurons aganist lethal ischaemia possibly by scavenging free radicals (Wen et al., 1996; Lim et al., 1997). In addition to aforementioned mechanistic actions of ginsenosides, growth promoting effects have been implicated through increasing the expression of nerve growth factor by ginsenosides (Jiang et al., 2000; Tohda et al., 2002).

To summarize, ginsenoside Rb1 increased the survival rate of dopaminergic cells when given at 10 μM on 6th DIV for 6 consecutive days. Against MPP^+ neurotoxicity, ginsenoside Rb1 significantly promoted neurite lengths and numbers and ameliorated degenerative changes such as cell swelling and loss of the neurites. On the other hand, ginsenoside Rg1 exerted lower effect on dopaminergic cells than ginsenoside Rb1. This partial neurotrophic and neuroprotective effect of ginsenosides Rb1 and Rg1 against MPP^+ might be related to support of energy metabolism, antioxidant properties and preserving the structural integrity of the dopaminergic neurons.

References

Attele AS, Wu AT, Yuan CS (1999) Ginseng pharmacology: multiple constituents and multiple actions. Biochem Pharm 58: 1685–1693

Bai J, Nakamura H, Hattori I, Tanito M, Yodoi J (2002) Thioredoxin suppresses 1-methyl-4-phenylpyridinium induced neurotoxicity in rat PC12 cells. Neurosci Lett 321: 81–84

Chang MS, Lee SC, Rho HM (1999) Transcriptional activation of Cu/Zn superoxide dismutase and catalase genes by panaxadiol ginsenosides extracted from Panax ginseng. Phytother Res 13: 641–644

Chen TS, Koutsilieri E, Rausch WD (1995) MPP^+ selectively affects calcium homostasis in mesencephalic cell cultures from embryonal C57/B6 mice. J Neural Transm 100: 153–163

Deng H, Zhang J (1991) Anti-lipid peroxidase effect of ginsenoside Rb1 and Rg1. Chin Med J 104: 395–398

Gille G, Rausch WD, Hung ST, Moldzio R, Janetzky B, Hundemer HP, Kolter T, Reichmann H (2002) Pergolide protects dopaminergic neurons in primary culture under stress condition. J Neural Transm 109: 633–643

Himi T, Saito H, Nishiyama N (1989) Effect of ginseng saponins on the survival of cerebral cortex neurons in cell cultures. Chem Pharm Bull 37: 481–484

Hochizuki H, Nakamura N, Nishi K, Mizuno Y (1994) Apoptosis is induced by 1-methyl-4-phenylpyridinium ion (MPP^+) in ventral mesencephalic-striatal co-culture in rat. Neurosci Lett 70: 191–194

Jiang F, Desilva S, Turnbull J (2000) Beneficial effect of ginseng root in SOD-1 (G93A) transgenic mice. J Neurol Sci 180: 52–54

Jiang KY, Qian ZN (1995) Effects of Panax notoginseng saponins on posthypoxic cell damage of neurons in vitro. Zhongguo Yao Li Xue Bao 16: 399–402

Koutsilieri E, Chen TS, Kruzik P, Rausch WD (1995) A morphometeric analysis of bipolar and multipolar TH-IR neurons treated with the neurotoxin MPP^+ in co-culture from mesencephalon and striatum of embryonic C57/B6 mice. J Neurosci Res 40: 197–205

Lee JH, Kim SR, Bae CS, Kim D, Hong HN, Nah SY (2002) Protective effect of ginsenosides, active ingredients of Panax ginseng, on kainic acid-induced neurotoxicity in rat hippocampus. Neurosci Lett 325: 129–133

Li XJ, Gu J, Lu SD, Sun FY (2002) Melatonin attenuates MPTP-induced dopaminergic neuronal injury associated with scavenging hydroxyl radical. J Pineal Res 32: 47–52

Liao B, Newmark H, Zhou R (2002) Neuroprotective effects of ginseng total saponins and ginsenosides Rb1 and Rg1 on spinal cord neurons in vitro. Exp Neurol 173: 224–234

Lim JH, Wen TC, Matsuda S, Tanaka J, Maeda N, Peng H, Aburaya J, Ishihara K, Sakanaka M (1997) Protection of ischaemic hippocampal neurons by ginsenoside Rb1, a main ingredient of ginseng root. Neurosci Res 28: 191–200

Liu D, Li B, Liu Y, Attele AS, Kyle JW, Yuan CS (2001) Voltage-dependent inhibition of brain Na(+) channels by American ginseng. Eur J Pharmacol 413: 47–54

Liu M, Zhang JT (1995) Effects of ginsenosides Rb1 and Rg1 on synaptosomal free calcium level, ATPase and calmodulin in rat hipocampus. Chin Med J (Engl) 108: 544–547

Matarredona ER, Santiago M, Cano J, Machado A (1997) Involvement of iron in MPP^+ toxicity in substantia nigra: protection by desferrioxamine. Brain Res 773: 76–81

Michel PP, Dandapani BK, Knusel B, Sanchez-Ramos J, Hefti F (1990) Toxicity of 1-methyl-4-phenylpyridinium for rat dopaminergic neurons in culture: selectivity and irreversibility. J Neurochem 54: 1102–1105

Nishiyama N, Cho SI, Kitagawa I, Saito H (1994) Malonylginsenoside Rb1 potentiates nerve growth factor (NGF)-induced neurite outgrowth of cultured chick embryonic dorsal root ganglia. Biol Pharm Bull 17: 509–513

Rhim H, Kim H, Lee DY, Tae HO, Nah SY (2002) Ginseng and ginsenosides Rg3, a newly identified active ingredient of ginseng, modulate Ca^{2+} channel currents in rat sensory neurons. Eur J Pharm 436: 151–158

Rudakewich M, Ba F, Benishin CG (2001) Neurotrophic and neuroprotective actions of ginsenosides Rb1 and Rg1. Planta Med 6: 533–537

Tohda C, Matsumoto N, Zou K, Meselhy MR, Komatsu K (2002) Axonal and dendritic extension by protopanaxadiol-type saponins from ginseng drugs in SK-N-Sh cells. Jpn J Pharm 99: 254–262

Wen T-C, Yoshimura H, Matsuda S, Lim J-H, Sakanaka M (1996) Ginseng root prevents learning disability and neuronal loss in gerbils with 5-minutes forebrain ischaemia. Acta Neuropathol 91: 15–22

Authors' address: Univ.-Prof. Dr. W.-D. Rausch, Institute for Medical Chemistry, Veterinary Medical University Vienna, Veterinärplatz 1, A-1210 Vienna, Austria, e-mail: wolf.rausch@vu-wien.ac.at