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Additive Protective Effects of Luteolin and Pyruvate against 6-Hydroxydopamine and 3-Hydroxykynurenine Induced Neurotoxicity in SH-SY5Y Cells[*]

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ABSTRACT

Oxidative stress has been implicated as one of the causes in cell death in many neurodegenerative disorders. Due to antioxidative properties *in vitro*, the use of flavonoids and other polyphenolic compounds synthesised by plants are considered to be a promising strategy to prevent Alzheimer's disease and Parkinsons's disease. In the present study, we tested protective effects of some polyphenols and sodium pyruvate on 6-hydroxydopamine (6-OHDA), salsolinol and 3-hydroxykynurenine (3-HK) induced neurotoxicity in human neuroblastoma SH-SY5Y cells. We found that luteolin prevented from 6-OHDA and 3-HK induced cell viability reduction and that one of the mechanisms involved in the neuroprotective process was the ability to increase the level of cellular ATP. However, luteolin was ineffective against salsolinol-induced toxicity. Neither pre-treatment with flavonoids nor simultaneous addition had any protective effects on 6-OHDA, salsolinol or 3-HK induced neurotoxicity. Interestingly, both pre-treatment and co-treatment with pyruvate provided protection against 6-OHDA, salsolinol or 3-HK induced toxicity. Moreover, luteolin and sodium pyruvate, administered together, acted additively, so to achieve the same effect, lower concentrations were needed. The ability of luteolin and sodium pyruvate to reduce toxicity of 6-OHDA and 3-HK in SH-SY5Y cells may be related to two different neuroprotective mechanisms and the capability to penetrate into the cell.

Keywords: Flavonoids; Oxidative Stress; Pyruvate; ATP; Luteolin; Neurotoxicity

1. Introduction

Oxidative stress is induced by an imbalance between the production of reactive oxygen species (ROS) and the detoxification of reactive intermediates in mitochondria and has been strongly implicated as one of the causes and triggering factors of cell death. Accumulation of ROS and decreased antioxidant capability can lead to damage of cell lipids, proteins, nucleic acids and might disturb mitochondrial functions, which results in initiating apoptotic and necrotic cell death. There is extensive literature on mitochondrial dysfunction and its association with different neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) or Huntington's disease [1,2]. ROS are highly reactive and toxic reduced oxygen forms such as hydrogen peroxide (H_2O_2), superoxide radical (O_2^-) and hydroxyl radical (OH^-) continuously generate in the mitochondrial respiratory chain,

in which energy is produced in the form of adenosine-5'-triphosphate (ATP). Moreover, neurons are especially vulnerable to elevated ROS or decreased ATP level, due to the fact that those cells are rich in mitochondria and have a high demand for ATP. Intracellular ROS generation can be induced by a number of diverse insults, e.g. salsolinol, 6-OHDA, 3-HK, Aβ, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-(MPTP) or rotenone [3-7]. Physiologically the only available defence against ROS in mitochondria are enzymes (for example superoxide dismutase, catalase, peroxidase) or low molecular weight oxidants (e.g. chelating agents, glutathione, NADPH). Externally generated oxidative stress may be reduced through agents from dietary sources. However, in order to reach equilibrium between ROS production and detoxification in brain cells the antioxidant strategies have been proposed. Since flavonoids, polyhydroxylated phytochemicals and common dietary components present in fruits, vegetables and beverages like tea or wine, have

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been proven to posses strong anti-oxidative, anti-inflammatory and other biological properties *in vitro* [8], the use of flavonoids and other polyphenolic compounds synthesised by plants has been considered to be a promising strategy to protect from neurodegenerative diseases including AD and PD.

On the other hand, it has been noted that several anti-oxidants, such as catalase, vitamin E, ascorbic acid, pyruvate, glutathione, N-acetyl cysteine, protected from 6-OHDA-induced toxicity in cells [4,9-11] and that their bioavailability were reported higher than flavonoids.

Sodium pyruvate, an endogenous low molecular weight antioxidant and energy substrate was found to protect neuronal cells through direct acting in mitochondria by decreasing generation of ROS and stabilization of mitochondrial membrane potential [12]. In various experimental cell models it was also found to protect against such insults as *β*-amyloid and 6-OHDA [10,13].

In the present study, we tested potential protective effects of apigenin, amentoflavone, chrysine, (-) epigallocatechin-3-gallate, isorhamnetin, kaempferol, luteolin, luteolin-7-glucoside, myricetin, naringenin, quercetin, resveratrol, rosmarinic acid, rutin, saponarin, sinensetin and sodium pyruvate in human neuroblastoma cells SH-SY5Y exposed to different neurotoxic agents such as salsolinol, 6-OHDA and 3-HK.

2. Materials and Methods

2.1. Reagents and Tested Substances

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), Trypsin-EDTA, sodium pyruvate were purchased from Biochrom, Berlin, Germany. Amentoflavone, apigenin, isorhamnetin, kaempferol, luteolin, luteolin-7-glucosid, naringenin, sinensetin, rosmarinic acid and were obtained from Roth, Karlsruhe, Germany; chrysine, epigallocatechin-3-gallate (EGCG), myricetin, quercetin, resveratrol, 3-hydroxykynurenine, 6-hydroxydopamine, (±) salsolinol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma, St. Louis, MO. Saponarin was a generous gift from Maya Yotova (Medical University of Sofia, Bulgaria).

Flavonoids and rosmarinic acid were solubilised in dimethyl sulfoxide (DMSO), stored in the dark at −20°C, and diluted in medium before use. The final concentration of DMSO was limited to 0.5% and did not affect cell viability.

2.2. Cell Cultures

Human neuroblastoma cell line SH-SY5Y was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DMSZ), Braunschweig, Germany. SH-SY5Y cell cultures were grown in DMEM supplemented with 15% FBS. All cells were maintained at 37°C in a humidified 5% CO_2/air atmosphere. The cells were transferred to low serum media (1% FBS) 2 hours prior to the treatment. Cells were weekly passaged (Trypsin-EDTA, Biochrom, Germany). Cell number was measured using CASY cell counter (Schärfe System, Reutlingen, Germany). Tests were carried out in 96-well microplates, and the density of 5×10^4 (SH-SY5Y) cells per well was considered in the screening experiment. Cells were incubated 24 h before the drug was added, and the drug remained in contact with the cells for 24 h or 48 h.

2.3. Cell Viability Assay/(MTT) Reduction Assay

For the viability assay, cells were plated at a density of 5×10^4 SH-SY5Y cells per well on 96-well plates in 100 μl of DMEM containing 1% FBS. One day after plating, amentoflavone, apigenin, chrysine, epigallocatechin 3-gallate, isorhamnetin, kaempferol, luteolin, luteolin-7-glucosid, saponarin, sinensetin, naringenin, myricetin, quercetin, rosmarinic acid, rutin and sodium pyruvate were added at various concentrations to the cells for 24 hours. 3-HK (500 μM), 6-OHDA (75 μM) or salsolinol (500 μM) were given to the medium simultaneously or after 24 hours of pre-treatment with tested compounds for 24 h. As control, cells were incubated with 0.5% of DMSO, which did not influence cell viability, assessed using the modified MTT assay [14]. Briefly, MTT was added to the cells after treatment in 96-well plates. Cells were incubated for 2 h with MTT solution (5 mg/ml). The formazan formed was dissolved in DMSO and the absorbance was measured by a multiplate reader at 570 nm wavelength and at 620 nm as reference wavelength (Tecan, Austria). All experiments were done in triplicate. Results were expressed as percentage of control.

2.4. Measurement of Cellular ATP Content

Cellular ATP concentrations were assessed by the Cell-Titer-Glo cell viability assay, according to the manufacturer's instructions. Briefly, the assay is based on measurement of luminescence generated by the reaction of ATP and luciferin. Cells were plated at 5×10^4 cells per well on white 96-well microplates. Polyphenolics, pyruvate and toxic compound were added 24 before measurements. The final media volume was 200 μl. Luminescence levels were measured using a Tecan microplate reader (Tecan, Austria). All experiments were done in triplicate. Luciferase activity of untreated cells was arbitrarily set to 100%. Data were expressed as % of control ATP.

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3. Results

Firstly, to examine whether tested polyphenols and pyruvate possess potential in protection against chosen neurotoxins, we established dose-and time-response curves for 6-OHDA, 3-HK and salsolinol in cultures of SH-SY5Y neuroblastoma cells. 6-OHDA, 3-HK and salsolinol induced cell death in a concentration-and a time-dependent manner, as measured by MTT assay and Cell-Titer-Glo cell viability assay. An effective toxic dose of 6-OHDA after 24 hours treatment was observed at about 75 µM in MTT assay and 50 µM in ATP and Cell-Titer-Glo cell viability assay resulted in about 50% cell death. At 500 µM concentration in MTT assay and 300 µM in CellTiter-Glo cell viability assay, 3-HK decreased cell viability to ~50% of controls after exposure for 24 hours. Treatment of SH-SY5Y with salsolinol (500 µM or 250 µM, in MTT and CellTiter-Glo cell viability assay, respectively) for 24 hours significantly decreases cell viability to 50%. Afterwards, SH-SY5Y cells were treated for 24 hours with wide range concentrations of each polyphenol and pyruvate. To determine whether tested substances need time to be able to penetrate into the cells, we also performed an additional experiment in which we added the phenolic compound or pyruvate to the culture cells for 24 hours before the toxic compound treatment.

The results of MTT cell viability assay demonstrated that luteolin at concentrations from 6 µM to 13 µM was the only phenolic compound that mitigated 6-OHDA and 3-HK induced cell death when added simultaneously. Luteolin at 6 µM restored the cell availability by 15 and at 13 µM about 30% in both 6-OHDA and 3-HK toxicity model. Protective effects of luteolin against 3-HK and 6-OHDA are presented in **Figure 1**. Cytotoxic effect induced by salsolinol, wasn't inhibited by luteolin at none of the tested concentrations. Also preincubation with luteolin did not prevent 6-OHDA, 3-HK and salsolinol toxicity in the cells at any concentration. Furthermore, luteolin was found to be cytotoxic at concentrations above 25 µM after 24 hours of treatment. After incubation with luteolin 25 µM about 15% of dead cells were observed. It may suggest toxic effect of luteolin to the cells. Neither pre-treatment with various concentrations of other phenolic compounds nor simultaneous addition to SH-SY5Y cells had any protective effects in the 6-OHDA, salsolinol and 3-HK model of neurotoxicity. Additionally, sodium pyruvate was able to reverse the cytotoxicity caused by 6-OHDA, 3-HK and salsolinol in a dose-dependent manner when pre-treated and co-treated. Treatment with pyruvate alone did not cause any viability loss up to the highest concentration (1000 µM). As shown in **Figure 2**, treatment of SH-SY5Y cells with 100, 500 and 1000 µM of pyruvate reduced the 6-OHDA
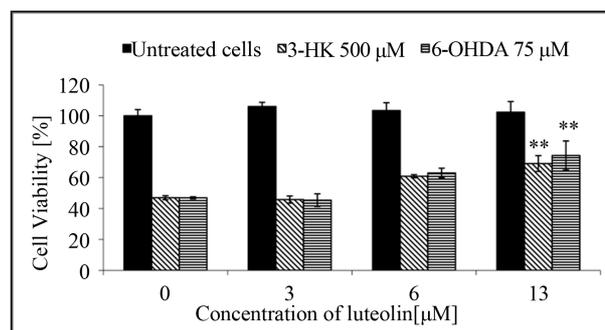

Figure 1. Luteolin protected SH-SY5Y neuroblastoma cells from 3-hydroxykynurenine (3-HK) and 6-hydroxydopamine (6-OHDA). SH-SY5Y human neuroblastoma cultures were exposed to 3-HK or 6-OHDA with or without various concentrations of luteolin for 24 h (3-HK, 6-OHDA), and cell viability was measured by the MTT assay and presented as untreated control. Significant neuroprotection was observed at 6 and 13 µM of luteolin. Data points represent the mean ± SEM of three independent experiments performed in triplicates. **: p < 0.01 compared to control cell death.
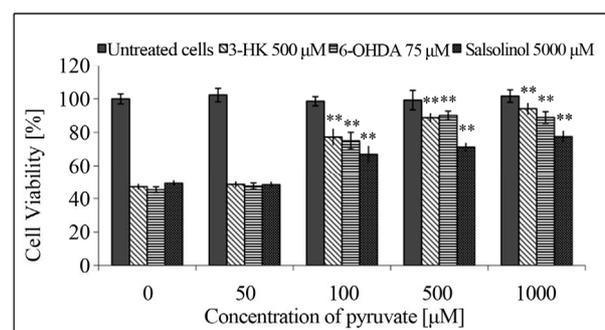

Figure 2. Pyruvate protected SH-SY5Y neuroblastoma cells from 3-hydroxykynurenine (3-HK), 6-hydroxydopamine (6-OHDA) and salsolinol toxicity. SH-SY5Y human neuroblastoma cultures were exposed to 3-HK, 6-OHDA and salsolinol with or without various concentrations of pyruvate for 24 h (3-HK, 6-OHDA, salsolinol) and cell viability was measured by the MTT assay and presented as untreated control. Significant neuroprotection was observed already at 100 µM of pyruvate. Data points represent the mean ± SEM of three independent experiments performed in triplicates. **: p < 0.01 compared to control cell death.

and 3-HK cytotoxicity to about 77%, 75%, 89%, 90%, 94% and 89% of the control group, respectively. Administration of pyruvate provided significant protection also against the toxicity of salsolinol, however 100, 500 and 1000 µM pyruvate restored the MTT reduction from 49% to 67%, 71% and 78% of baseline, respectively. Interestingly, simultaneous addition of luteolin and pyruvate in the presence of toxic compound potentiated the protective effect. 3-HK (500 µM) alone decreased cell viability to 47%, with luteolin (3 µM) and pyruvate (100 µM) cell viability increased to 89%, whereas pyruvate

(100 μM) alone increased to cell viability to 77% and luteolin (3 μM) wasn't found to be able to restore cell viability at all. At higher concentrations of pyruvate (500 μM) and luteolin (6 μM) cell viability was almost completely restored. This finding we report for the first time. Effect of pyruvate on 3-HK toxicity on neuroblastoma cells is presented in **Figure 3**.

The effects of 6-OHDA, 3-HK and salsolinol on intracellular ATP content in SH-SY5Y cells were assessed using CellTiter-Glo cell viability assay after 24 hours. As expected, 6-OHDA, 3-HK and salsolinol induced a dose-dependent decrease in cellular ATP content. The decrease in intracellular level of cellular ATP induced by 6-OHDA and 3-HK was attenuated after 24 h pre-treatment with similar concentrations of luteolin (6 - 13 μM) and pyruvate (100 μM, 500 μM, 1000 μM) to those found in MTT-assay, as shown in **Figures 4** and **5**, respectively. However, salsolinol induced toxicity was also reduced in the presence of pyruvate but not by luteolin. Interestingly, after SH-SY5Y cells were treated for 24 hours with various concentrations of luteolin alone, increase in the mitochondrial ATP level was observed as compared to untreated cells. At concentration of 3 μM and 6 μM luteolin ATP-levels were 113% and 117%. However, ATP-level after luteolin treatment at concentration 13 μM returned to 106%.

Co-treatment of 6 μM luteolin and 100 μM pyruvate increased cellular ATP concentration in the presence of 6-OHDA and 3-HK (**Figure 6**). Moreover, 6 μM luteolin and 500 μM pyruvate elevated ATP level in SH-SY5Y to

Figure 4. Luteolin increased mitochondrial ATP level in 3-hydroxykynurenine (3-HK) and 6-hydroxydopamine (6-OHDA) in SH-SY5Y neuroblastoma cells induced neurotoxicity. SH-SY5Y human neuroblastoma cultures were exposed to 3-HK or 6-OHDA with or without various concentrations of luteolin for 24 h (3-HK, 6-OHDA), and ATP-level was measured by the CellTiter-Glo cell viability assay and presented as untreated control. Significant ATP-level increase was observed at 6 and 13 μM of luteolin. Data points represent the mean ± SEM of three independent experiments performed in triplicates. **: p < 0.01 compared to control cell death.
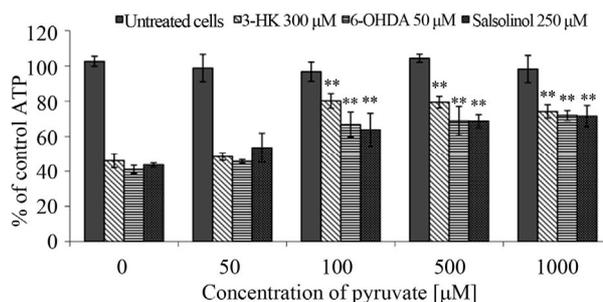
Figure 5. Pyruvate increased mitochondrial ATP level in 3-hydroxykynurenine (3-HK) and 6-hydroxydopamine (6-OHDA) in SH-SY5Y neuroblastoma cells induced neurotoxicity. SH-SY5Y human neuroblastoma cultures were exposed to 3-HK, 6-OHDA and salsolinol with or without various concentrations of pyruvate for 24 h (3-HK, 6-OHDA, salsolinol), and ATP-level was measured by the CellTiter-Glo cell viability assay and presented as untreated control. Significant ATP-level was observed already at 100 μM of pyruvate. Data points represent the mean ± SEM of three independent experiments performed in triplicates. **: p < 0.01 compared to control cell death.
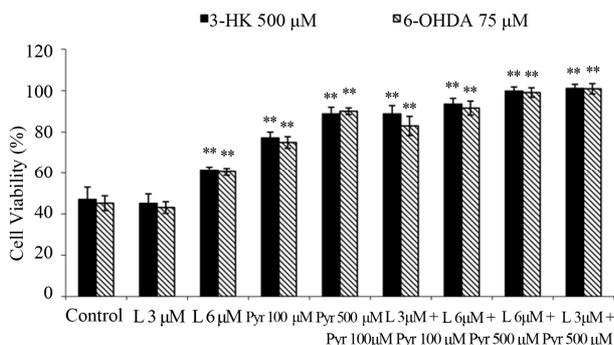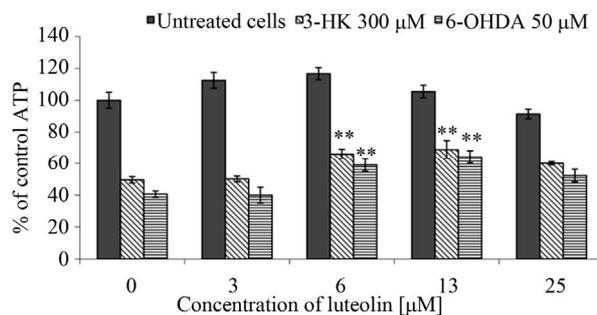
Figure 3. Coadministration of luteolin and pyruvate enhanced protection of SH-SY5Y neuroblastoma cells from 3-hydroxykynurenine (3-HK) and 6-hydroxydopamine (6-OHDA) toxicity. SH-SY5Y human neuroblastoma cultures were exposed to 3-HK and 6-OHDA with or without various concentrations of luteolin (L) and pyruvate (Pyr) for 24 h (3-HK, 6-OHDA), and cell viability was measured by the MTT assay and presented as untreated controls. Significant neuroprotection was observed already at 100 μM of pyruvate and 3 μM luteolin. Data points represent the mean ± SEM of three independent experiments performed in triplicates. **: p < 0.01 compared to control cell death.

near 100% and completely blocked the 3-HK and 6-OHDA toxicity.

4. Discussion

In the present study we intended to evaluate structurally diverse plant phenolics for their abilities to reduce neurotoxicity induced by substances with different mode of action in SH-SY5Y human neuroblastoma cell line. The results of MTT and CellTiter-Glo cell viability assay show that most of the tested polyphenolic compounds

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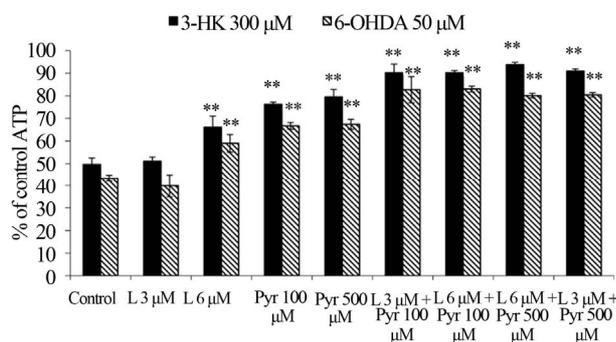and 3-Hydroxykynurenine Induced Neurotoxicity in SH-SY5Y Cells

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Figure 6. Coadministration of luteolin and pyruvate enhanced mitochondrial ATP-level in SH-SY5Y neuroblastoma cells induced 3-hydroxykynurenine (3-HK) and 6-hydroxydopamine (6-OHDA) toxicity. SH-SY5Y human neuroblastoma cultures were exposed to 3-HK and 6-OHDA with or without various concentrations of luteolin (L) and pyruvate (Pyr) for 24 h (3-HK, 6-OHDA), and ATP-level was measured by the CellTiter-Glo cell viability assay and presented as untreated controls. Significant neuroprotection was observed already at 100 μM of pyruvate and 3 μM luteolin. Data points represent the mean ± SEM of three independent experiments performed in triplicates. **: p < 0.01 compared to control cell death.

failed to protect against 6-OHDA, 3-HK and salsolinol induced toxicity. Antioxidative efficacy of flavonoids and polyphenolic acids *in vitro* are well described in the literature [15-17]. Ishige *et al.*, 2001 suggested that in the protective properties of flavonoids three different mechanisms are implicated, namely increasing intracellular glutathione, lowering levels of ROS and preventing the influx of Ca^{2+} [18]. However, in order to display neuroprotective activity a potent protectant need to enter a neuronal cell through the cell membrane. Our results would indicate that most of the tested polyphenolics enter the cell rather poorly, if at all and due to this fact is not able to achieve a protective level of concentration. Among the tested phenolic compounds, only luteolin exhibited neuroprotection against oxidative stress-induced by 6-OHDA and 3-HK by increasing cell survival and intracellular ATP level in SH-SY5Y human neuroblastoma cell line. However, no cell survival and increase in ATP level was observed in the presence of salsolinol. In this investigation several neurotoxins were chosen as oxidative stressors. Salsolinol has been reported to cause oxidative stress by increasing ROS levels, decreasing ATP and glutathione levels in SH-SY5Y cell line by inhibiting mitochondrial complex-I and complex-II enzyme activities, tyrosine hydroxylase and monoamine oxidase [7,19,20]. Cell death was not inhibited by the addition of antioxidants such as α-tocopherol or the water-soluble vitamin E analogue-Trolox C, however nicotine and donepezil restored cell survival, indicating that in the salsolinol toxicity nicotinic receptors could be involved

[21,22]. Another toxic insult used in this investigation is the widely used dopamine derivative 6-OHDA, which similarly to salsolinol induces ROS generation and inhibits mitochondrial complex I and, additionally, mitochondrial complex IV [23]. On the other hand, 3-HK is a kynurenine pathway intermediate, which neurotoxicity results from the intracellular accumulation of hydrogen peroxide [24]. Based on available elusive evidences, it's difficult to explain the lack of neuroprotective activity of luteolin against salsolinol induced cell death. Probably in the cell death induced by salsolinol different signaling pathways are involved and luteolin is not able to interact with cytotoxic mechanisms caused by this neurotoxin.

In our study, when the cells were preincubated with luteolin and then exposed to 3-HK or 6-OHDA, we could not observe protection against these toxic compounds. Probably, the hydroxyl groups present in the luteolin molecule were metabolized or oxidized. Luteolin (3', 4', 5, 7-tetrahydroxyflavone) is a widespread flavonoid aglycone found in high concentrations in celery (*Apium graveolens*), peppers (*Capsicum annuum*), perilla (*Perilla frutescens*) and has been shown to posses many biological activities including antioxidant [25,26] and anti-inflammatory [27,28]. Luteolin bears an electrophilic α-β-unsaturated carbonyl moiety with intermediate polarity and solubility in DMSO. Furthermore, also tested sinensetin—a flavone of similar structure to luteolin but fully methylated was not found to be neuroprotective in none of the experiments. On the other hand, apigenin and chrysin belonged to the same large group of flavones and were not found to be protective. Moreover, also tested cynaroside, a 7-O-glucoside of luteolin has not been found to reduce induced toxicity. These findings suggest that presence of free hydroxyl group on C7 and in the position C3' and C4' on the B-ring play an important role in acting as neuroprotectant and would support our hypothesis that the ability of luteolin to exhibit neuroprotection in the 6-OHDA, 3-HK and induced cell death may be related to the capability to penetrate into the cell. This result was in agreement with previous studies, where the authors suggested that presence of catechol-o-dihydroxy structure on the B-ring build by C'3 and C'4 is crucial for scavenger properties [15].

On the other hand, numerous findings indicated that neither lowering ROS nor increasing antioxidant capacity were not able to bring health-beneficial effects [29,30]. On the contrary, it has been postulated that ROS formation might induce cellular signalling, transmit messages between mitochondria and other compartments and increase activities of phase II response enzymes that protect from ROS damage [31,32]. This theory could partly explain the lack of cell death protecting activities of polyphenols presented in this investigation. However, it also has to be mentioned that perhaps the benefits of

ROS formation might not be true for neurons, which contain more mitochondria than other cells.

Our study has also revealed that pyruvate could act as a survival factor protecting SH-SY5Y cells from 6-OHDA and 3-HK and salsolinol injury. Enhancing of neuronal defense of pyruvate against 6-OHDA induced cell death has been reported before [12], however, reduced neurotoxic action by pyruvate against 3-HK and salsolinol induced apoptosis we report for the first time. Interestingly, increasing concentrations of pyruvate showed no further improvement of protective effects against 3-HK, 6-OHDA and salsolinol. Furthermore, in our study administration of both pyruvate and luteolin resulted unexpectedly in additive neuroprotective effect against 6-OHDA and 3-HK-toxicity. This finding suggests that luteolin and pyruvate posses different cell survival-increasing activities. Further investigations are required to determine what is responsible for the observed additive effect of luteolin and pyruvate. Briefly, pyruvate is a final product of glycolysis, which enters the Krebs cycle and is a source of ATP generation. Moreover, pyruvate has been reported to upregulate glutathione peroxidase expression and to activate the Akt signaling pathway [10].

We couldn't confirm actions of EGCG against 3-HK induced neurocytotoxicity in SH-SY5Y cell line reported earlier by Jeong *et al.* [33]. At the same reported concentration of 10 μM no cytoprotective effects were observed [33]. Neuroprotection had been also described for estrogens [34]. However, kaempferol—a flavonol with estrogenic and antiestrogenic properties was not found to be protective against neurotoxicity in our investigation as it was in the study conducted by Roth *et al.*, on PC12 rat pheochromocytoma cell line [35]. Also rosmarinic acid was not observed to reduce cell death in 6-OHDA treated MES23.5 dopaminergic cells. These may be due to the fact that in those studies different cell lines as models of neurotoxicity were used.

In conclusion, we found that the combined administration of luteolin and pyruvate exerted neuroprotective effects against 6-OHDA and 3-HK-toxicity by increasing cellular ATP levels and number of surviving cells. However, only pyruvate was found to enhance neuronal defence against salsolinol-induced cell-death. Therefore, luteolin in combination with pyruvate can become interesting candidate for possible treatment of neuronal injury. Further studies are necessary to determine what is responsible for the observed additive protective effect of luteolin and pyruvate.

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