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Exploration of the Protective Effects of *Aronia Melanocarpa* Extract in an *in vitro* Model of Parkinson's Disease

Istraživanje protektivnih učinaka ekstrakta crnoplodne aronije u modelu Parkinsonove bolesti *in vitro*

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Abstract. ***Aim***: In this study, we aimed to explore whether the *Aronia melanocarpa* (chokeberry) extract pre-treatment protects primary mouse astrocytes in an *in vitro* model of Parkinson's disease (PD) induced by the application of mitochondrial toxin 6-hydroxydopamine (OHDA) and if this effect was related to its antioxidant properties. ***Material and methods***: Experiments were performed on primary mouse astrocytes that were exposed for 24 h to different concentrations of chokeberry extract (10, 50 or 100 µg/mL). OHDA was added to the cell culture medium for the next 72 h. Cell culture media were prepared for the lactate dehydrogenase (LDH) cell cytotoxicity assay and the cell lysates for the western blot analyses. ***Results***: We determined that the pre-treatment of astrocytes with different concentrations of chokeberry extract alone does not significantly affect the LDH activity in the cell media samples. Application of OHDA, however, significantly increased the LDH activity while the pre-treatment of cells with chokeberry extract in concentrations of 50 and 100 µg/mL decreased the intensity of cell damage. Neither OHDA application, nor aronia pre-treatment affected the expression level of antioxidant enzyme superoxide dismutase 1, and the OHDA-induced decrease in glutathione peroxidase 1 protein expression remained unchanged by the tested antioxidant application. ***Conclusions***: This study points to possible protective effects of chokeberry extract in an *in vitro* model of PD. The exact mechanism of the protective effect is still unclear and could be the subject of further research.

Keywords: Astrocytes; Glutathione Peroxidase; L-Lactate Dehydrogenase; Parkinson Disease; Photinia; Superoxide Dismutase

Sažetak. ***Cilj:*** U ovom istraživanju cilj je bio ispitati štiti li predtretman ekstraktom crnoplodne aronije (*Aronia melanocarpa*) primarne mišje astrocite u modelu Parkinsonove bolesti (PB) *in vitro,* izazvane primjenom mitohondrijskog toksina 6-hidroksidopamina (OHDA) i je li ovaj učinak povezan s antioksidativnim svojstvima istraživane biljke. ***Materijal i metode:*** Pokusi su provedeni na primarnim mišjim astrocitima koji su 24 sata bili izloženi različitim koncentracijama ekstrakta aronije (10, 50 ili 100 µg/mL). OHDA je dodan u medij za stanične kulture tijekom sljedećih 72 sata. Medij za stanične kulture korišten je za ispitivanje stanične citotoksičnosti mjerenjem aktivnosti laktat-dehidrogenaze (LDH), a stanični su lizati pripremljeni za analizu Western blot. ***Rezultati:*** Utvrdili smo da tretman astrocita različitim koncentracijama ekstrakta aronije ne utječe značajno na aktivnost LDH u uzorcima staničnih medija. Primjena OHDA je, međutim, značajno povećala aktivnost LDH, dok je predtretman stanica ekstraktom aronije u koncentracijama od 50 i 100 µg/mL smanjio intenzitet oštećenja stanica. Ni primjena OHDA, kao ni predtretman aronijom nisu utjecali na razinu izražaja antioksidativnog enzima superoksid-dismutaze 1, a smanjenje izražaja proteina glutation-peroksidaze 1 izazvanog primjenom OHDA ostalo je nepromijenjeno i nakon primjene istraživanog antioksidansa. ***Zaključci:*** Ova studija ukazuje na moguće zaštitne učinke ekstrakta aronije u modelu PB-a *in vitro*. Točan mehanizam zaštitnog učinka još je nejasan i mogao bi biti predmetom daljnjih istraživanja.

Ključne riječi: astrociti; fotinija; glutation-peroksidaza; L-laktat dehidrogenaza; Parkinsonova bolest; superoksid-dismutaza

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INTRODUCTION

Parkinson's disease (PD) is the most common movement disorder, and it is the second most common chronic, progressive neurodegenerative disease in the world. It was first described in James Parkinson's study in 1817, in which the motor signs of the disease were recorded[1]. The main symptoms of PD are the tremors, bradykinesia, and rigidity. PD is usually diagnosed in patients between the ages of 40 and 70, while the estimated prevalence and incidence in Europe range between 65 and 12500/100000 and between 5 and 346/100000 people per year, respectively. Although it is most commonly that PD is classified as idiopathic, one of the possible causes of the disease is exposure to 1-methyl-4-phenyl tetrahydropyridine (MPTP), also known as the "synthetic heroin", which causes the death of nigrostriatal dopaminergic cells. Other risk factors include the use of neuroleptics, and exposure to toxic levels of manganese, trichloroethylene or carbon monoxide[2]. In addition, history of brain traumas, infections as well as metabolic disorders, atherosclerosis and degenerative brain diseases can also lead to the development of parkinsonism. It is also necessary to mention the genetic predisposition that is most often identified in patients with the juvenile and familial types of the disease in the amount of 20% in the immediate family[2].

The neuropathology of PD is based on the loss of neurons or depigmentation of the *substantia nigra* and increased accumulation of the intracellular protein α-synuclein. Namely, in the earlier stages of the disease, the loss of dopaminergic neurons can be observed mostly in the ventrolateral parts of the *substantia nigra*, and as the disease progresses, it dramatically spreads to other parts of the midbrain. In addition, the abnormal deposition of α-synuclein in the cytoplasm of neurons causes the aggregation of the protein and the appearance of Lewy bodies. Formed inclusions, in asymptomatic cases, occur in cholinergic and monoaminergic neurons of the lower part of the brain stem, while in patients with developed motor symptoms of PD, they infiltrate neurons of the midbrain and forebrain. In the last stages of the disease, Lewy pathology affects the limbic and neocortical brain regions. In addition to the above, numerous studies have highlighted the role of oxidative stress, mitochondrial dysfunction and neuroinflammation in the development of the pathogenesis of PD[3].

The mainstays in today's treatment of PD disease are preparations based on levodopa, intended for replenishing dopamine in the depleted striatum. Although it is considered the most effective drug, levodopa has a limited duration of effectiveness (3-5 years). Most PD patients start with low doses of 150-1000 mg/day to avoid adverse effects. That is because the more the dose is increased, the greater the toxicity and occurrence of side effects. For this reason, it is most often combined with other drugs such as carbidopa, which inhibits peripheral DOPA carboxylase, does not cross the blood-brain barrier (BBB) and does not reach the central nervous system (CNS). Other therapeutic options include dopaminergic receptor agonists, monoamine oxidase B inhibitors, anticholinergics, and amantadine, but none of these pharmaceuticals are capable of preventing disease progression and are only somewhat successful in treating PD symptoms. Therefore, it is still very much needed to explore other pharmacological options for PD treatment, including the prevention of this condition in at-risk individuals.

In Parkinson's disease, oxidative stress plays a role in the death of dopamine-producing neurons. Astrocytes have been shown to play a role in protecting neurons from oxidative stress. However, in Parkinson's disease, astrocytes themselves may become damaged by oxidative stress, contributing to the progression of the disease.

Aronia melanocarpa, or chokeberry, belongs to the *Rosaceae* family and originates from North America. In addition to being known for its high antioxidant activity, it stands out for its hepatoprotective and anti-inflammatory effects and strong bacteriostatic and antiviral properties[4]. It is a plant with black fruits that are rich in polyphenols, especially anthocyanins, which have been proven to have antimutagenic and immunomodulating activity in cell cultures and in

patients with breast cancer. Anthocyanins cross the BBB and inhabit a wide range of brain regions, including the cortex, cerebellum, hippocampus, and striatum[5, 6]. Their main component is cyanidin-3-O-glucoside, which is associated with a reduction in the risk of brain disorders[7]. The neuroprotective action of anthocyanins is based on the suppression of neuroinflammation and oxidative stress and the protection of neurons from cellular toxicity induced by MPTP or 6-hydroxydopamine (OHDA) toxins[8]. OHDA is an experimental dopaminergic neurotoxin and the oldest and most commonly used chemical in PD modelling. Thanks to this model, numerous data were obtained on the behavioural, biochemical, and physiological effects of dopamine depletion and the loss of dopaminergic cells in the brain. OHDA can enter catecholaminergic neurons via dopamine receptors, where it causes autoxidation and cell injury through oxidative stress.

Several cell culture systems have been developed to study the pathogenesis of Parkinson's disease or to determine the effectiveness and side effects of certain drugs. Traditional *in vitro* cell cultures are most often based on cell lines such as human embryonic kidney cells 293 (HEK293), human neuroglioma H4 cells or PC12 pheochromocytoma cells derived from the marrow of the adrenal gland[9]. In addition, the SH-SY5Y neuroblastoma cell line is often used because it reproduces the dopaminergic phenotype of PD pathology[10]. Other than the use of immortalized cell lines, primary neuronal cultures, pluripotent stem cells and neurological stem/progenitor cells have also been employed in modelling PD *in vitro*. However, these cell lines all focus on exploring the pathophysiology of PD in neurons even though other CNS cells, namely astrocytes and microglia, also carry important roles in the disease pathology and progression.

The main hypothesis of this research was that the pre-treatment of astrocytes with chokeberry extract affects the survival of cells exposed to the action of the OHDA toxin. The specific objectives of the research were to examine how the treatment with different concentrations of aronia affects the survival of astrocytes and to determine whether OHDA causes astrocyte injury. Mainly, we tested if the chokeberry pre-treatment affects the survival of astrocytes exposed to OHDA. Additionally, the effects of OHDA and chokeberry extract application on the expression levels of antioxidant enzymes superoxide dismutase (Cu-Zn) (superoxide dismutase 1, SOD1) and glutathione peroxidase 1 (GPX1) were examined.

Table 1. Nutritional declaration of the chokeberry powder used in the experiments.

AVERAGE NUTRITIONAL VALUE IN 100 g	
Energy value	1672 kJ/394 kcal
Fats	< 0.5 g
Saturated fatty acids	<0.1 g
Carbohydrates	85 g
Sugars	5.1 g
Proteins	6.9 g
Salt	0.04 g

MATERIAL AND METHODS

Chemicals

For the analyses, we used the reagents obtained from the following manufacturers: Sigma-Aldrich, Taufkirchen, Germany (6-hydroxydopamine, OHDA; antibiotic and antimycotic solution), PAN-Biotech, Germany (Dulbecco's Modified Eagle Medium, DMEM; bovine serum albumin, BSA; fetal bovine serum, FBS). All the other commonly used laboratory reagents and chemicals were obtained from Carl Roth, Germany, unless stated otherwise.

Aronia samples

For the experiments, we used 100% aronia powder produced and generously provided to us by Bobica d.o.o. (Novska, Croatia). The fruit powder was obtained by shredding the bark and seeds of chokeberry and the declared nutritional declaration is shown in Table 1. Values of total phenolic compounds and the antioxidant capacity have been previously determined by Tolić et al[11].

Primary mouse astrocytes

To perform the *in vitro* experiments, three separate isolations of astrocytes were done from pups (0-2 days old) of C57BL/6 mice of both sexes. Experiments were performed in cultures prepared

from three isolation procedures which makes three biological replicates. For each isolation procedure, we used five mice to obtain the desired number of astrocytes for the culture, and total number of mice used were therefore 15. The sectioned mouse cortices were separated from the meninges and the white matter, and then transferred to 50 mL sterile tubes with transparent Dulbecco's Modified Eagle Medium (DMEM). The cortices were homogenized with an automatic pipette and passed through sterile filters with 230 and 140 µm diameters. The purified cell suspension was subjected to centrifugation at 12000 rpm for 5 min, and resuspended in cell growth medium (DMEM, 10% fetal bovine serum, 1% antibiotic/antimycotic). The resulting suspension was passed through a filter with a diameter of 73 µm, and the number of cells per mL medium was determined using Trypan blue dye and a Neubauer chamber. In 75 cm^2 flasks (T75), based on calculations, cells were planted and incubated under conditions of 37°C, 5% CO_2 and relative humidity of 95% for one week. After the incubation, when the cells had reached confluence, the flasks were placed onto an orbital shaker for 30 min at 180 rpm, for microglia to detach from them. The medium containing microglia was replaced with a fresh medium and put on the shaker for 6 h at 240 rpm, for the precursors of oligodendrocytes to be removed. After 6 h, the medium was replaced with a fresh one and the flasks were put into the incubator and allowed to proliferate. For the experiments, astrocytes were seeded on 96-well or 6-well plates (depending on the protocol) until reaching around 80-90 % confluence.

Experimental protocol

The prepared stock solution of chokeberry powder (2 mg/mL) was diluted to concentrations of 100, 50 and 10 µg/mL using DMEM medium (ARO100, ARO50 and ARO10). In addition, a toxin sample, OHDA, was prepared at a concentration of 120 µM.

The effect of prepared chokeberry samples of different concentrations was investigated by pretreating astrocytes with chokeberry powder solutions of the specified concentrations for 24 h, after which some of the cells were exposed to OHDA for 72 h (for the cell survival analyses) or 24 h (for the analyses of protein expressions) (Figure 1).

Figure 1. Schematic representation of the timeline of aronia (ARO) pre-treatment and 6-hydroxydopamine (OHDA) application in the protocols related to lactate dehydrogenase (LDH) and western blot analyses.

Measurement of lactate dehydrogenase activity

A colorimetric test was used to determine the activity levels of lactate dehydrogenase (LDH), a cytosolic enzyme that is released as a result of cell membrane disruptions. We used the commercially available kit for this test (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega Corporation, Madison, WI, USA). Released LDH causes the conversion of the violet-coloured tetrazolium salt (iodonitrotetrazolium violet, INT) into a red compound, formazan. The intensity of the resulting red coloration is proportional to the amount of damaged cells. Based on the scheme shown in Figure 1, within the first 24 hours, the treatment of cells planted on 96-well plates with prepared samples ARO10, ARO50 and ARO100 was carried out.

When testing the effects of aronia extract on the cells exposed to OHDA toxin, cell culture media with chokeberry was removed following 24 h pre-treatment, and 120 µM OHDA was added to the wells. After 72 h, 50 µL aliquots of cell growth medium were separated and transferred to microtiter plates, and LDH activity was detected spectrophotometrically at 490 nm by adding the LDH kit substrate. The plates were analyzed by the spectrophotometric method using the BioTek EL808 Microplate Reader (Agilent Technologies, Santa Clara, CA, USA).

Western blot analysis

For the Western blot analyses, astrocytes were pre-treated for 24 h with aronia powder solution,

and then exposed to OHDA for 24 h. At the end of the experiment, the medium was removed and 100 µL of radioimmunoprecipitation assay (RIPA) buffer was added to the cells, and the astrocytes were then scraped off the wells. After 20 min on ice with occasional mixing, cell lysates were collected and centrifuged at 14000 rpm at 4°C for 10 min. The separated supernatants were stored at -80 °C, and the protein concentration was determined by the Bradford method[12].

Proteins at a concentration of ~1 µg/µL were added to a 10 % polyacrylamide gel. Then, electrophoresis was started at 200 V for 40 min, and proteins were transferred from the gel to the nitrocellulose membrane using the appropriate transfer system (Bio-Rad Laboratories, USA). After blocking with 5 % milk in Tris-buffered saline with 0.1 % Tween 20 (TBST) for 1 h, membranes were incubated overnight at 4 °C with primary antibodies: SOD1 (1:200; Santa Cruz Biotechnology, Dallas, TX, USA), GPX1 (1:1000; Abcam, Cambridge, UK), GAPDH (1:5000, Proteintech, Rosemont, IL, USA). Then, the membranes were washed with TBST for 30 min and treated with biotin-conjugated secondary antibodies: biotinylated goat anti-rabbit IgG (H+L), (1:2000; Invitrogen, Waltham, Massachusetts, USA), biotinylated goat anti-mouse, (1:3000; Invitrogen, Waltham, Massachusetts, USA). After incubation for 1 h, the membranes were incubated with streptavidin and horseradish peroxidase (HRP) conjugate for 30 min (1:10000). Protein visualization was performed using the chemiluminescent kit and Kodak Image Station 440 (Eastman Kodak, Rochester, NY, USA) with Kodak 1D analysis program (v.3.6.5, Kodak Scientific Imaging Systems, Rochester, NY, USA).

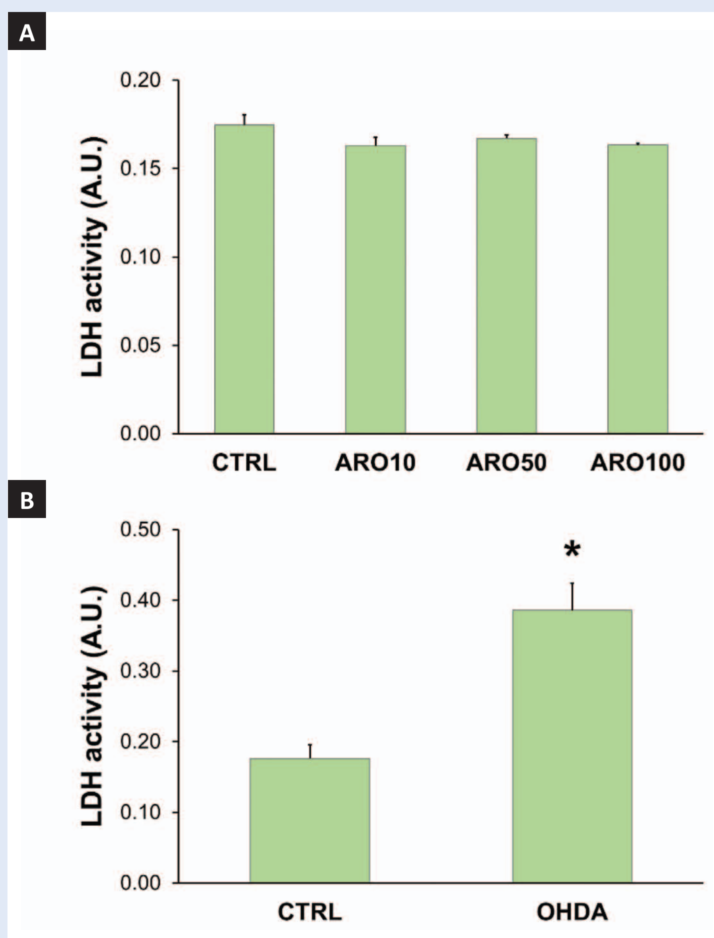

Figure 2. The effects of aronia extract pre-treatment (A) and OHDA exposure (B) on the LDH activity in cell culture media conditioned by primary mouse astrocytes.

Data collection and statistical analysis

The Microsoft Office Excel® program (Microsoft Corp., Redmond, WA, USA) was used to store data and create histograms. For statistical data processing, parametric procedures of statistical analysis, student T-test analysis or one-way analysis of variance (ANOVA) with Duncan Multiple Range *post-hoc* test were used. A level of $P < 0.05$ was considered statistically significant. Statistical analysis procedures were performed using the computer software STATISTICA® version 13 (StatSoft Inc., Tulsa, OK, USA).

RESULTS

Aronia at concentrations of 50 and 100 µg/ml reduces the LDH activity levels in astrocytes exposed to the OHDA toxin

The intensity of astrocyte injury induced by OHDA was monitored by measuring the activity of the intracellular enzyme LDH. This enzyme is released from the cytoplasm into the extracellular space in response to cell injury. Due to its stability, measuring the amount of released LDH enzyme enables a quick and reliable quantification of cell survival in culture.

In our initial experiment (Figure 2A), we wanted to determine if the incubation of cells with aronia extract alone might cause cell toxicity. Based on the results of the LDH test, it was observed that

the addition of chokeberry extract, in all the used concentrations, to wells with astrocytes, over the period of 24 h, did not affect the levels of LDH activity, measured at 72 h following the pre-treatment cessation (ANOVA: $F(3;8)=2.065$; $P=0.183$). Next, we wanted to confirm, in our own testing conditions, that the exposure of astrocytes to the toxic compound OHDA at a concentration of 120 μM affects the LDH activity compared to control cells, as was previously published[13]. From Figure 2B it is evident that the 72-h exposure of astrocytes to OHDA seriously damages the integrity of their cell membranes as the LDH levels in OHDA samples were more than double of those determined in media aliquots from the control cells (t-test: $P=0.008$).Finally, in the cell culture media samples conditioned by astrocytes pre-treated with chokeberry extract followed by 72-h OHDA exposure (Figure 3), lower levels of LDH activity were measured compared to the levels recorded in cells treated only with the investigated toxin ($F(3;12) = 7.172$; $P = 0.005$). Although LDH activity levels were lower in the samples from all three pre-treatment procedures, significantly lower activity levels of this enzyme were recorded in treatments at chokeberry extract concentrations of 50 and 100 μg/mL.

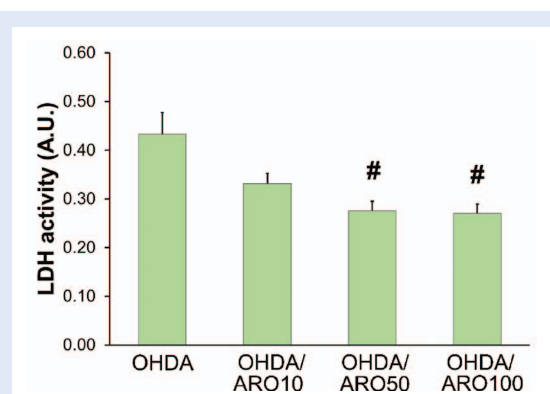

Figure 3. The effects of aronia (ARO) extract pre-treatment on the LDH activity levels in primary mouse astrocytes exposed to OHDA mitochondrial toxin.

Effects of OHDA and the pre-treatment with aronia extract on the expression of antioxidant enzymes SOD1 and GPX1

Given that the beneficial effect of chokeberry pre-treatment on cells exposed to the action of the OHDA toxin was observed when concentrations of 50 and 100 μg/mL were applied, samples of astrocytes treated with the highest concentration of the studied antioxidant were used for further analyses.

Figure 4 shows representative blots and associated densitometric analysis of the protein expres-

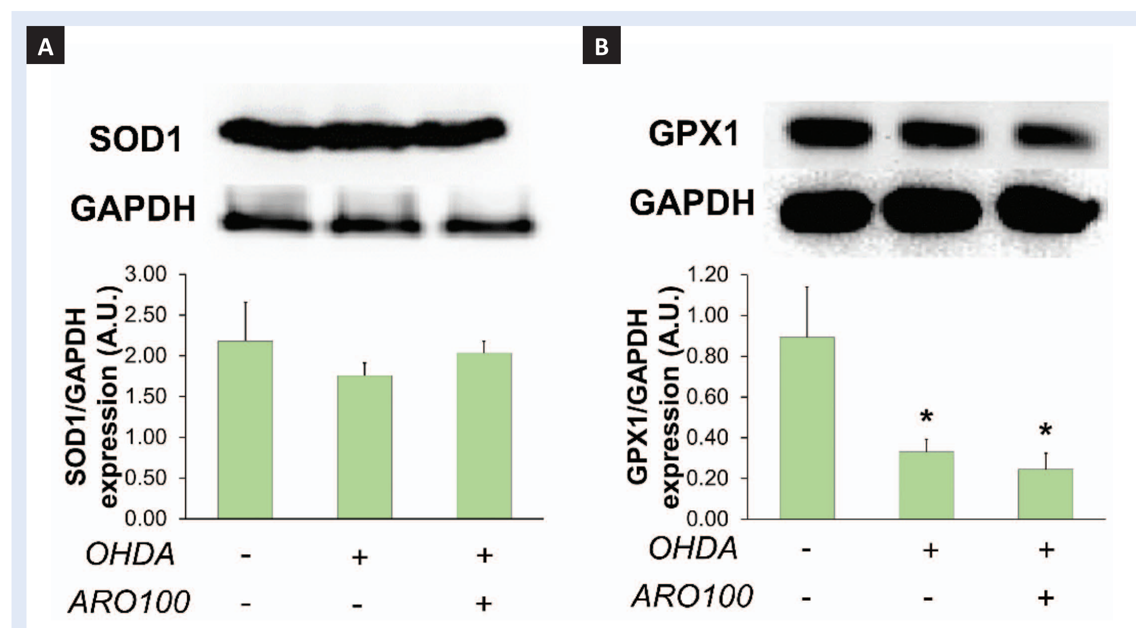

Figure 4. The effects of aronia (ARO) extract pre-treatment on the SOD1 and GPX1 antioxidant enzymes' protein expression levels in primary mouse astrocytes injured with neurotoxin OHDA.

sion analyses for the antioxidant enzymes SOD1 and GPX1.

The expression of the antioxidant enzyme SOD1 was analysed by western blotting (Figure 4A) and it was also observed that the expression of this protein does not significantly change with either OHDA exposure or the pre-treatment of cells with chokeberry extract (ANOVA: $F(2;6) = 0.515$; $P = 0.622$). The effect of chokeberry pre-treatment on the expression of GPX1 after exposure of astrocytes to the toxin OHDA was also investigated (Figure 4B). It is evident that the 72-h exposure to the toxic compound OHDA caused a significant decrease in the expression of this antioxidant enzyme's activity, but also that the aronia pre-application failed to counteract this OHDA effect (ANOVA: $F(2;6) = 5.267$; $P = 0.048$).

DISCUSSION

In this study, we investigated the effects of pre-treating primary mouse astrocytes with chokeberry extract before exposing them to the mitochondrial toxin OHDA, commonly used compound for modelling PD both *in vivo* and *in vitro*. The main results of our experiments are that by treating astrocytes with chokeberry extract, we were able to salvage them from the toxicity of OHDA and this effect was significant with 50 and 100 μg/mL concentrations. We then hypothesized that this protective effect might be related to the changes in the expression of antioxidant enzymes SOD1 and GPX1, but we found no connection between the treatments and the expression of the aforementioned proteins.

In PD *in vitro* models, it is mostly neuronal cell cultures that are used, but we decided to focus on another type of CNS cells that have an important role in PD pathogenesis and progression. Astroglia or astrocytes are a class of nerve cells of neuroepithelial origin that are responsible for homeostasis and protection of the CNS. Thanks to their high heterogeneity in form and function, astrocytes are responsible for maintaining the CNS during development and aging. These are cells that are tightly entwined in neural networks and whose basic activity is realized at all levels of the organization, from molecules to organs. Namely, by transferring ions and protons, removing and catabolizing neurotransmitters and releasing neurotransmitter precursors, astrocytes achieve molecular homeostasis, while by stimulating neurogenesis, synaptogenesis and defining cytoarchitecture, they contribute to the homeostasis of other CNS cells. At the organ level, astroglia controls the BBB and the local lymphatic system, and achieves systemic balance by acting as a chemosensor, creating neuro-glia-vascular units, regulating food intake, local blood flow and glycogenolysis. There is now clear evidence that some PD-related genes such as DJ-1, α-synuclein, iPLA2, ATP13A2, PINK1, and Parkin, are involved in astrocyte-specific functions, including inflammatory responses, glutamate transport, and neurotrophic capacity. Still, only a minority of investigations have thus far covered the role of astrocytes in PD even though this may have important implications for the development of new treatments[14].

The results of our experiments, specifically those related to the ameliorative effect of chokeberry pre-treatment, point to the protective potential of this plant extract in an *in vitro* model of PD. We observed better results of the LDH cytotoxicity test with all the tested aronia extract concentrations, however only 50 and 100 μg/mL concentrations showed significant results.

The components present in black chokeberry juice are rich sources of anthocyanin and polyphenolic substances that reduce free radicals. Because of this, aronia is considered, compared to other fruits, the strongest antioxidant that has a positive effect on almost all organic systems. Previously, studies have shown that different fruit extracts rich in anthocyanins, including chokeberry extract, managed to protect neuronal cells from hydrogen peroxide toxicity[15–18]. Moreover, in a study using paraquat, another toxin that induces apoptotic cell death through oxidative stress, treatment of cells from a neuroblastoma cell line with chokeberry concentrate provided ameliorative outcomes[19]. The general belief is that the protection provided by chokeberry, but also other anthocyanin-rich fruit extracts, is related to multiple mechanisms of action. These compounds are able to modulate several pathological pathways including oxidative

stress, calcium homeostasis disturbances, inflammation, and imbalance of the pro-survival versus the pro-apoptotic signalling pathways[20]. Since the antioxidant properties of chokeberry are widely thought to be most significant in regard to their beneficial effects, we wanted to test if this is also true in the *in vitro* PD model used in our study.

Oxidative stress can be defined as an imbalance between the biochemical processes of production of reactive oxygen species and cellular antioxidative capacity, which leads to critical failures in biological functions. In PD, the described changes caused by free radicals are responsible for the damage to neuronal membrane lipids, proteins and other components of brain tissue. The greatest losses are recorded within the *substantia nigra*, a region sensitive to oxidative stress with a large deficit in defense mechanisms, and this is probably due to increased oxidation of dopamine and neuromelanin, increased concentration of free iron and decreased concentration of ferritin as well as decreased reduction and increased oxidation of glutathione. Oxidative/nitrosative stress of astrocytes has been associated with PD pathogenesis as the aberrant astrocytic function can be responsible for the chronic production of reactive oxygen and nitrogen species and thus contribute to PD progression. That is why experimental models utilizing environmental toxicants, such as OHDA, provide important information regarding the mechanisms of astrocytic impact on PD pathogenesis.

Prior studies proved that the treatment with dietary antioxidants resulted in improved mitochondrial and neuronal function in *in vitro* PD models. For example, baicalein[21], a large bioactive flavone, was observed to reduce muscle tremors and increase the number of tyrosine hydroxylase neurons in rotenone-induced parkinsonism in rats. Hesperidin is also considered an effective antioxidant[22], which showed a neuroprotective effect in rotenone-induced neuroblastoma of cellular PD models. In addition to contributing to mitochondrial function, this citrus flavonol improved neuronal and behavioral parameters in OHDA-induced PD mouse models. Several human cohort studies have shown a connection between the use of antioxidants in the diet and a reduction in the risk and symptoms of Parkinson's disease. Specifically, in 85000 patients, the synergistic effect of vitamin E and β-carotene in reducing the risk of disease was proven[23], while creatine was prominent in delaying the onset of cognitive disorders in PD patients[24]. Conversely, a meta-analysis of several studies that examined the association between antioxidants (vitamin A and carotenoids) and PD progression did not show statistically significant differences[25, 26].

The results of our study suggest that there is potential role of using Aronia melanocarpa for Parkinson's disease prevention. While some studies have suggested that its antioxidant properties may be beneficial in reducing oxidative stress, more research is needed to fully understand its effects.

We detected that the OHDA exposure caused a significant decline in GPX1 antioxidant enzyme expression, contrary to SOD1 whose expression level did not change upon the 72-h toxin application. In our experimental conditions, aronia extract pretreatment had no detected effect on the GPX1 and SOD1 expression levels. To the best of our knowledge, this is the first research in which the effects of chokeberry extract were studied in an *in vitro* model of PD and in the astrocytes as CNS type of cells. Our results regarding the effects of aronia on the antioxidant enzymes' expression or activity in the CNS differ from some previous studies. For example, in a model of accelerated aging of mice by D-galactose supplementation, anthocyanins from aronia retained the levels of total SOD and GPX, as well as additionally inhibited the accumulation of pro-inflammatory cytokines[27]. The extended longevity and improved locomotion in the fruit fly *Drosophila melanogaster* due to supplementation with aronia extract were probably owing to increases in the level of antioxidant enzymes (SOD, GPX and catalase)[28]. However, in those *in vivo* studies, subjects were given aronia extract for a prolonged period, which is probably the reason for the pronounced changes in their anti-oxidant defences. In future studies, it is necessary to do some additional tests regarding the possible

mechanism of protective actions of aronia extract in the PD model that we used in our experiments. Firstly, we measured only the SOD1 and GPX1 expression levels at 72 h following the OHDA application. It is quite possible that the activity of these enzymes changed following treatments, but unrelatedly to the protein expression level. In addition, we suggest that it is possible that the ameliorative effects of chokeberry were expressed at earlier time points, and through the activation of other antioxidant mechanisms. Namely, chokeberry polyphenols are considered to have scavenger effects on the reactive oxygen species, they are believed to be involved in the regulation of glutathione synthesis, inhibition of NFκB pathway activation, and inhibition of expression of enzymes such as inducible nitric oxide synthase and cyclooxygenase-2[29].

CONCLUSION

In this research, the influence of the prepared extract of chokeberry powder, in different concentrations, on primary mouse astrocytes exposed to the neurotoxin OHDA was examined. The obtained results led to the following conclusions. Pre-treatment with different concentrations of chokeberry solutions did not affect the survival of uninjured astrocytes. Exposure of astrocytes to the toxin OHDA causes their injury, i.e. cell lysis that leads to the release of significantly higher levels of LDH. Most importantly, the pre-application of chokeberry extract in concentrations of 50 and 100 μg/mL improves the survival of astrocytes exposed to OHDA. In this study, we did not confirm our hypothesis that these protective effects were related to the changes in the expression levels of antioxidant proteins SOD1 and GPX1. As the exact glioprotective mechanisms of this plant extract are still unclear, further studies are necessary to determine what are the precise cellular, biochemical or molecular changes induced by *Aronia melanocarpa* that stopped the OHDA cytotoxic effects in astrocytes. Overall, there is limited research on the effects of aronia on astrocytes, which makes further studies necessary to determine the potential role of this fruit extract in supporting the health and function of these cells.

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Conflicts of Interest: Authors declare no conflicts of interest.

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