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Regular paper

Effect of 6-hydroxydopamine increase the glutathione level in SH-SY5Y human neuroblastoma cells

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Treatment of human neuroblastoma SH-SY5Y cells with a catecholaminergic neurotoxin, 6-hydroxydopamine (6-OHDA) is an acknowledged *in vitro* experimental model of Parkinson disease (PD). A decrease in the glutathione content occurs in PD. Higher concentrations of 6-OHDA lowered the glutathione level in SH-SY5Y cells, nonetheless, we and other authors found a considerable increase in these cells' glutathione content after 24 h treatment with 60 μM 6-OHDA. A synthetic antioxidant, 4-aminotetramethylpiperidine-1-oxyl (4-AT) exerted a similar effect. The aim of the present study was to explain this surprising effect by monitoring the time course of changes in the levels of reduced (GSH) and oxidized glutathione (GSSG), total antioxidant activity (TAC) of human neuroblastoma cell SH-SY5Y extracts as well as the level of reactive oxygen species and activities of enzymes of glutathione metabolism after treatment of the cells with 60 μM 6-OHDA and/or 4-AT for 30 min – 24 h. A transient decrease in the level of GSH and TAC of cell extracts, increase in the level of GSSG, and decrease in the activities of glutathione peroxidase, glutathione reductase, glutathione *S*-transferase and γ-glutamyl-cysteine ligase activities were found followed by normalization or overshoot of the GSH level, TAC and enzyme activities. Increased activity of γ-glutamyl-cysteine ligase activity starting after 4-6 h was responsible for the elevation of the level of GSH and TAC in cells treated with 6-OHDA, 4-AT, and both compounds. The 6-OHDA-induced increase in the GSH content is a result of an overcompensatory response. The antioxidant 4-AT may be useful for the induction of an increase in the level of GSH in neural cells, without the negative effect of 6-OHDA.

Key words: Parkinson disease, 6-OHDA, 4-amino-TEMPO, glutathione, γ-glutamyl-cysteine ligase, oxidative stress

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Abbreviations: ABTS', 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid radical; 4-AT, 4-amino-(2,2,6,6-tetramethylpiperidine)-1-oxyl; DHE, dihydroethidium; GCL, γ-glutamyl-cysteine ligase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; GST, glutathione *S*-transferase; NEM, N-ethylmaleimide; OPA, ortho-phtalaldehyde; PD, Parkinson disease; 6-OHDA, 6-hydroxydopamine; PBS, phosphate-buffered saline; ROS, reactive oxygen species; TAC, total antioxidant capacity

INTRODUCTION

Glutathione (GSH) is the most abundant intracellular antioxidant playing a crucial role in the maintenance of redox homeostasis in cells, including neurons (Aoyama, 2021). A decrease in the GSH content was observed in the substantia nigra of patients with Parkinson disease (PD) and attributed mainly to its reactions with dopamine and its degradation products (Smeyne & Smeyne, 2013; Bjørklund *et al*., 2021).

The treatment of human neuroblastoma SH-SY5Y cells with 6-hydroxydopamine (6-OHDA) is an acknowledged cellular model of PD (Lopes *et al*., 2010; Tsai *et al*., 2020; Xicoy *et al*., 2020; Pichla *et al*., 2020; Elyasi *et al*., 2021; Pichla *et al*., 2021). 6-Hydroxydopamine induces oxidative stress in the neuroblastoma cells, as demonstrated by increased levels of reactive oxygen species (ROS), decreased activity of superoxide dismutase and catalase, decreased total antioxidant capacity (TAC) as well as increased total oxidant status and heightened levels of oxidative DNA damage, lipid peroxidation, and protein carbonylation (Pichla *et al*., 2020; Cirmi *et al*., 2021; Lee *et al*., 2020; Rashidi *et al*., 2021; Sun *et al*., 2020; Ko *et al*., 2019; Betharia *et al*., 2019; Hara *et al*., 2003a; Chen *et al*., 2022; Ferak Okkay *et al*., 2021; Ryu *et al*., 2013; Zhang *et al*., 2014). Although 6-OHDA is able to cause dopaminergic neurodegeneration in experimental models of PD by an oxidative stress-mediated process, the underlying molecular mechanism remains unclear. There are divergent data on the effect of 6-OHDA on the GSH level in the SH-SY5Y human neuroblastoma cell line. Sun and others (Sun *et al*., 2020) observed about a 50% decrease in the GSH content after the treatment of SH-SY5Y cells with 80 μM 6-OHDA for 21 h. In turn, Urano and others (Urano *et al*., 2018) found an about 50% decrease in the GSH content after 3 h treatment of the SH-SY5Y neuroblastoma cell line with 100 μM 6-OHDA. Ko and others (Ko *et al*., 2019) reported an about 40% decrease in GSH content, while Jing and others (Jing *et al*., 2016) a 30% decrease in GSH content after 24 h treatment of SH-SY5Y cells with 100 μM 6-OHDA. SH-SY5Y cells treated with 250 μM 6-OHDA for 24 h showed a decrease in the GSH content to about 60% (Cirmi *et al*., 2021) and to about 20% of the control value (Rashidi *et al*., 2021). Treatment with 500 μM 6-OHDA for 4 h decreased the GSH level by 40% in neuroblastoma cells (Barrachina *et al*., 2003), whereas treatment with 600 μM 6-OHDA induced a progressive decrease of the GSH content in SH-SY5Y cells during 3 h incubation (Miyama *et al*., 2011).

In contrast, other authors reported increases in the GSH content after treatment of SH-SY5Ycells with lower concentrations of 6-OHDA. Betharia *et al*. (2019)

observed a 6-fold increase in total glutathione after 24-h SH-SY5Y cells treatment with 40 μM 6-OHDA. Tirmenstein and others (Tirmenstein *et al.*, 2005) found a 12-fold increase in the GSH concentration after 24 h treatment of SH-SY5Ycells with 50 μM 6-OHDA noting no change in the GSH: GSSG ratio. We observed no change and an about 2.6-fold increase in the GSH content after 1 h and 24 h treatment of SH-SY5Y human neuroblastoma cells with 65 μM 6-OHDA, respectively (Pichla *et al.*, 2020).

The level of glutathione seems to be critical for the survival of SH-SY5Y cells treated with 6-OHDA since a pretreatment with compounds increasing the level of glutathione ameliorated the toxicity of 6-OHDA to the cells. Pretreatment of SH-SY5Y cells with ^{3}H-1,2-dithiole-3-thione (Jia *et al.*, 2008), *t*-butyl hydroquinone (Hara *et al.*, 2003b), dithiolethiones (Brown *et al.*, 2014, 2016) and 3,4-dihydroxybenzalacetone (Gunjima *et al.*, 2014) protected the cells against 6-OHDA toxicity; all these compounds increased the glutathione content of the cells. Buthionine sulfoximine, an inhibitor of glutathione synthesis, prevented this effect (Gunjima *et al.*, 2014, Brown *et al.*, 2016).

The increase in the GSH content of SH-SY5Y cells induced by lower concentrations of 6-OHDA is intriguing. We decided to study the kinetics of changes in the activities of glutathione metabolism enzymes and glutathione levels in order to understand the mechanism of alterations in the GSH level evoked by 60 μM 6-OHDA, a concentration causing 50% inhibition of cell proliferation and an over 2-fold increase in the GSH content, as found in our previous study (Pichla *et al.*, 2020). We observed previously the protection of SH-SY5Y cells by nitroxides and nitroxide-containing redox nanoparticles from oxidative stress so in this study, we checked also the effect of one of the previously employed nitroxides, 4-amino-(2,2,6,6-tetramethylpiperidine)-1-oxyl (4-amino-TEMPO, 4-AT), applied alone and in combination with 6-OHDA on the glutathione metabolism enzymes. The concentration of 4-AT of 75 μM was employed; this concentration offered significant (ca 50%) protection against 6-OHDA (Pichla *et al.*, 2020). Not differentiated cells were used, like in the previous study (Pichla *et al.*, 2020) and in most studies on the effects of 6-OHDA on the glutathione level in SH-SY5Y cells. We examined also the dynamics of changes in the TAC of SH-SY5Y cell extracts and in the level of ROS in the treated cells.

MATERIALS AND METHODS

Materials and equipment

Human neuroblast cell line from neural tissue SH-SY5Y (CRL-2266) was obtained from the American Cell Culture Collection. This cell line was derived from a metastatic bone tumor of a 4-year-old cancer patient.

Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 without phenol red (cat. no. 21041025), Dulbecco's Phosphate Buffered Saline (DPBS) (cat. no. 14040-117), and Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (cat. no. A1413202) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Fetal Bovine Serum (cat. no. S1813), Penicillin-Streptomycin solution (cat. no. L0022), and Trypsin-EDTA solution (10×) (cat. no. X0930) were obtained from Biowest (Nuaillé, France). Dihydroethidium (DHE) (cat. no. 37291), 0.4% Trypan Blue solution (cat. no. T8154), 4-amino-TEMPO (4-AT; cat. no. 163945), *N*-ethylmaleimide (NEM) (cat. no. E3876), dithiothreitol (DTT) (cat. no. D9760), trichloroacetic acid (TCA) (cat. no. T4885), diethylenetriaminepentaacetic acid (DTPA), ethylenediamineteraacetic acid (EDTA) (cat. no. D1133), L-ascorbic acid (cat. no. A0278), dimethyl sulfoxide (DMSO) (cat. no. D2438), *o*-phtaldialdehyde (OPA) (cat. no. P1378), 6-hydroxydopamine hydrobromide (6-OHDA) (cat. no. 162957), Triton X-100 (cat. no. X-100), monosodium phosphate (cat nos. 567545), disodium phosphate (cat. no. 106580), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (cat. no. 10102946001), potassium persulfate (cat. no. 216224), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox; cat. no. 238813), reduced glutathione (GSH) (cat. no. G4251), oxidized glutathione (GSSG) (cat. no. G4376), NADPH (cat. no. 10107824001), 1-chloro-2,4-dinitrobenzene (CDNB) (cat. no. 237329), the Folin-Ciocalteu reagent (cat. no. F9252), sodium carbonate (cat no. 223530), sodium hydroxide (cat. no. S5881), copper sulfate (cat. no. 209198), sodium tartrate (cat. no. 217255), bovine serum albumin (cat. no. A7030), and methanol (cat. no. 322415) were provided by Merck (Poznań, Poland). Hydrochloric acid was purchased from Chempur (Piekary Śląskie, Poland; cat. no. 115752837). Glutathione Peroxidase Assay Kit was obtained from BioAssay Systems, (Hayward, CA, USA; cat. no. EGPX-100). γ-Glutamylcysteine Ligase (GCL) Activity Assay Kit was from Solarbio Life Sciences (Beijing, China; cat. no. BC1210).

Cell culture T25 flasks (cat. no. 156367) were provided by Thermofisher Scientific (Waltham, MA, USA). Transparent 96-well Advanced TCTM culture plates (cat. no 655980), black 96-well flat bottom μClear® Advanced TCTM plates (cat. no. 655986), transparent 96-well plates (cat. no. 655101), black 96-well flat bottom plates (cat. no. 655209) and 24-well cell culture transparent plates (cat. no. 662160) were obtained from Greiner Bio-One (Kremsmünster, Austria). Other sterile cell culture materials were provided by Nerbe (Winsen, Germany) or Greiner Bio-One (Kremsmünster, Austria).

4-amino-TEMPO was dissolved in PBS, filtered through a 0.22 μm filter before each experiment, and diluted in the cell medium. 6-Hydroxydopamine hydrobromide was freshly prepared and stabilized with 0.01% L-ascorbic acid and filtered using a 0.22 μm syringe filter for each experiment. Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Fluorometric and absorptiometric measurements were performed in a Tecan Infinite 200 PRO multimode reader or a Spark multimode microplate reader (Tecan Group Ltd., Männedorf, Switzerland). Measurements were performed in nine repetitions (GSH and GSSG content) or in triplicate (ROS level, enzyme activities). Transmission light microscope observations were done in an inverted Olympus CKX53 microscope (OLYMPUS, Tokyo, Japan).

SH-SY5Y cell culture

SH-SY5Y cells were cultured in DMEM/F12 without phenol red (Dulbecco's Modified Eagle Medium Nutrient Mixture F-12), supplemented with 10% v/v heat-inactivated fetal bovine serum (hi-FBS) and 1% v/v penicillin/streptomycin solution. Cells were maintained at 37°C under 5% carbon dioxide and 95% humidity. The medium was changed twice a week, and the cells were passaged at about 80% confluence. For all studies, cells up to 14 passages were used. The morphology was examined under an inverted microscope with phase con-

trast Zeiss Primo Vert (Oberkochen, Germany).Cell viability was estimated by the Trypan Blue exclusion test. Cells were counted using a Thoma hemocytometer (Marienfeld Superior, Lauda-Königshofen, Germany).

Cell treatment

Cells were seeded into wells of transparent 96-well plates, previously covered with 1% Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix, according to the manufacturer's protocol, at an amount of 3.5×10^4 cells/well in 100 μL culture medium. After 48-hour incubation, the medium was gently removed and replaced with 100 μL/well of fresh culture medium or culture medium supplemented with adequate compounds: 60 μM 6-OHDA, 75 μM 4-AT, or both compounds together and placed in an incubator. Alternatively, when more cells were needed, 0.5×10^6 cells were grown in T-25 flasks for 48 h and added with proportionally higher volumes of the medium. After various times (30 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 24 h) the plates were withdrawn and analyses were performed immediately.

Estimation of the content of reduced and oxidized glutathione (GSH and GSSG)

The content of GSH was assayed with OPA using a slightly modified method of Senft and others (Senft *et al.*, 2000). Briefly, the medium was gently removed and cells were washed with 150 μL/well of phosphate-buffered saline (PBS). PBS was gently removed by aspiration. Subsequently, 60 μL/well of freshly prepared cold lysis buffer (RQB buffer: 20 mM HCl, 5% TCA, 5 mM DTPA, and 10 mM L-ascorbic acid was added; then, the plates were shaken at 900 rpm for 5 minutes, and centrifuged at 4,000 rpm (5 min, room temperature).

Cell lysates were transferred into two separate black 96-well plates ("+ NEM"' and "– NEM") with a black bottom in a volume of 25 μL/well. Into the first plate, "+NEM", 4 μL/well of freshly prepared 7.5 mM NEM in ice-cold RQB buffer were added. Then, 40 μL/well of 1 M phosphate buffer, pH 7.0, was pipetted into both plates, which were shaken for 5 minutes at 900 rpm. Then, 160 μL/well of ice-cold 0.1 M phosphate buffer (pH 6.8) and 25 μL/well of newly prepared 0.5% OPA in methanol were added into "+NEM" and "–NEM" plates. Then, the plates were shaken at 900 rpm for 30 minutes. Fluorescence was measured with a TECAN Spark® multimode plate reader at 355/430 nm. GSH concentration was obtained by subtracting the fluorescence of the plate without NEM from the fluorescence of the NEM-containing plate and the GSH content was calculated, respectively, with reference to protein content in each well.

To determine the content of GSSG, 100 μL/well of the cell lysate was added to the "–DTT" and "+DTT" black 96-well plates followed by 4 μL/well of freshly prepared 7.5 mM NEM and 40 μL/well of 1 M phosphate buffer, pH 7.0. After 5-min shaking, 10 μL of 10 mM DTT was added to the "+DTT" plate, and the plates were incubated for 60 min at room temperature with shaking. Then, 80 μL/well of 0.2 M phosphate buffer (pH 6.8) and 25 μL/well of newly prepared 0.5% OPA were added and the procedure was run as above. The fluorescence of "–DTT" plate was subtracted from that of the "+DTT" plate and the GSSG content was calculated, respectively, with reference to protein content in each well.

Estimation of total antioxidant capacity

The cells (5×10^5) were seeded in T25 flasks and cultured for 48 h; after this time the flask contained about 10^6 cells. Then the cells were subjected to respective treatments and after 24 h, detached by trypsinization, centrifuged (1 000 rpm, 5 min) washed with PBS, centrifuged, and added with 500 μL of ice-cold 10 mM TCA to lyse the cells and prevent oxidation of cell constituents by acidification of the mixture, and frozen at –80°C. After thawing, the lysates were centrifuged (5 min, 13,000 rpm). The total antioxidant activity of the supernatants was assayed using a modified ABTS• decolorization assay (Kut *et al.*, 2022). Briefly, stock ABTS• solution prepared by oxidation of ABTS solution with potassium persulfate was diluted so that absorbance of 200 μin a well of a 96-well plate at 734 nm was equal to 1. To this solution, 25 μL of the supernatants were added and a decrease of absorbance was measured after 1 min and after 30 min at room temperature and corrected for the ABTS• self-decomposition. Total antioxidant capacity was calculated using Trolox with reference to the protein content in each well.

Estimation of the level of reactive oxygen species (ROS)

Cells were seeded on black 96-well plates, cultured, and treated as described above. 100 μL/well of freshly prepared DHE working solution in PBS was added; the final concentration of DHE was 10 μM. The fluorescence was measured immediately at 37°C, at 405/570 nm for 30 min at 1 min intervals. The "area under the curve"(sum of the fluorescence values measured at successive times) was assumed as a measure of the level of ROS.

Estimation of glutathione peroxidase activity

The cells were grown in T25 flasks as above, and after 24 h of the treatments, were detached by trypsinization, centrifuged (1 000 rpm, 5 min) washed with PBS, centrifuged, and added with 500 μL of ice-cold 20 mM phosphate buffer, frozen and thawed. Glutathione peroxidase activity was determined in the cell lysates using the EnzyChrom Glutathione Peroxidase Assay Kit according to the manufacturer's instructions.

Estimation of glutathione reductase activity

Glutathione reductase was assayed by a modification of the method of Carlberg and Mannervik (1975). Aliquots (50 μL) of lysates prepared as above were pipetted to wells of a transparent 96-well plate, added with 110 μL of 0.1 M phosphate buffer, pH 7.6, 20 μL of 1 mM NADPH and 20 μL of 1 mM GSSG. Kinetics of absorbance decrease at 340 nm was measured in a plate reader at 37°C for 5 min. On the basis of the rate of absorbance decrease, the enzyme activity was calculated with respect to the protein content of a well.

Estimation of glutathione *S*-transferase activity

Glutathione *S*-transferase activity was assayed by a modification of the procedure proposed by Habig *et al.* (1974). Cell lysates (50 μL were pipetted to wells of a transparent 96-well plate and added with 130 μL of 0.1 M phosphate buffer, pH 6.5, 10 μL of 20 mM GSH, and 10 μL of 20 mM CDNB in ethanol. An increase in absorbance at 340 nm was monitored for 10 min at room temperature. On the basis of the rate of absorb-

ance increase, the enzyme activity was calculated with respect to the protein content of a well.

Estimation of γ-glutamyl-cysteine ligase activity

Gamma-glutamyl-cysteine ligase activity was assayed using the γ-Glutamylcysteine Ligase (GCL) Activity Assay Kit according to the manufacturer's instructions. The cells were grown in T25 flasks, subjected to the treatments, detached by trypsinization, and washed with PBS. They were lysed by sonication (3 min on ice) as recommended in the instruction.

Protein assay

Protein content was determined according to Lowry *et al.* (Lowry *et al.*, 1951) using bovine serum albumin as a standard.

Statistical analysis

The results are expressed as a percent of values obtained for control (not treated cells) cultured in parallel for the same time. The significance of differences between the control and treated cells was estimated using the Student's "*t*" test.

RESULTS

The time course of the contents of reduced and oxidized glutathione in SH-SY5Y cells treated with 6-OHDA, the nitroxide 4-AT, and both compounds together is shown in Fig. 1. After an initial decrease in the GSH content in cells subjected to all treatments after 30 min, the GSH content increased progressively with respect to control after all treatments. This increase reached statistical significance after 6, 8, and 24 h in cells treated with 6-OHDA, and after 2, 4, 6, 8, and 24 h in cells treated with 4-AT and treated with 6-OHDA and 4-AT. Changes at the "zero time" with respect to control observed in cells treated with 6-OHDA were apparently due to the fact that the minimal manipulation of the cells till complete stopping the reactions took at least a minute; during this time, the reagents applied could react with glutathione and other cell components.

There was an initial small increase in the GSSG content of the cells treated with 6-OHDA or 4-AT after 30 min. Then the GSSG level remained constant, except for a small decrease for 4-AT-treated cells after 8 hours.

Total antioxidant capacity of cell extracts measured by the reduction of the ABTS• radical after 1 min reflects mainly the content of "fast" (rapidly reacting) antioxidants while the measurement of the ABTS• reduction after 30 min reflects the content of the sum of "fast" and "slow" antioxidants ("summary" TAC). The values of TAC obtained after 1 min reduction by the cell extracts were about 67% of the values of TAC obtained after 30 min; this percentage was not affected by the treatments applied. Changes in TAC of cell extracts measured after both 1 min and 30 min were similar to those of the GSH content. There was an initial (for the 0 time) decrease in the content of "fast antioxidants" for cells treated with 4-AT and after 30 min for cells treated with 6-OHDA and a decrease of the "summary TAC" at the 0 time for all types of treatment, maintained after 30 min in cells treated with 6-OHDA. After 4, 6, 8, and 24 h the content of "fast" antioxidants increased over the control levels in cells treated with 6-OHDA, 4-AT, and both compounds, reaching the highest values after 24 h for cells treated with 6-OHDA and 6-OHDA plus 4-AT. Statistically significant elevation of the "summary TAC" was observed after 6, 8 and 24 h for all types of treatment (Fig. 2).

The level of ROS was elevated in cells treated with 6-OHDA over the entire experiment, after 30 min in cells treated with 4-AT, and at the 0 time and after 30 min and 1 h in cells treated with 6-OHDA and 4-AT simultaneously. Later on, the ROS level decreased in cells treated with 4-AT after 8 and 24 h, and in cells treated with 6-OHDA and 4-AT simultaneously after 24 h (Fig. 3).

The patterns of changes in the activities of glutathione peroxidase (GPx; EC 1.11.1.9) and glutathione reductase (GR; EC 1.6.4.2) were similar. Initially, the GPx activity was decreased in cells treated with 6-OHDA after 30 min, 1 h, and 2 h, and increased over the control level for cells treated with 6-OHDA and with 6-OHDA and 4-AT simultaneously after 8 h. GR activity decreased after 30 min for all types of treatment, and after 1 h in cells treated with 6-OHDA and 6-OHDA and 4-AT simultaneously. Later on, an increase in the GR activity was noted, peaking after 6 and 8 h for types of treatment. This increase was maintained after 8 h for cells

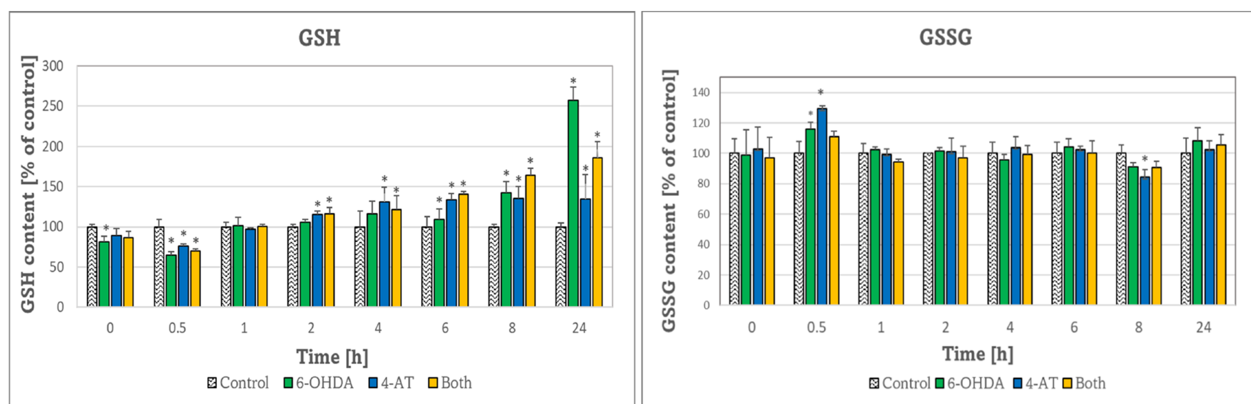

Figure 1. Time course of changes in the GSH and GSSG content of SH-SY5Y cells treated with 50 µM 6-OHDA, 75 µM 4-AT, and both compounds together.
**p*≤0.05 *vs*. control (not treated) cells (Student's "*t*" test); n=9.

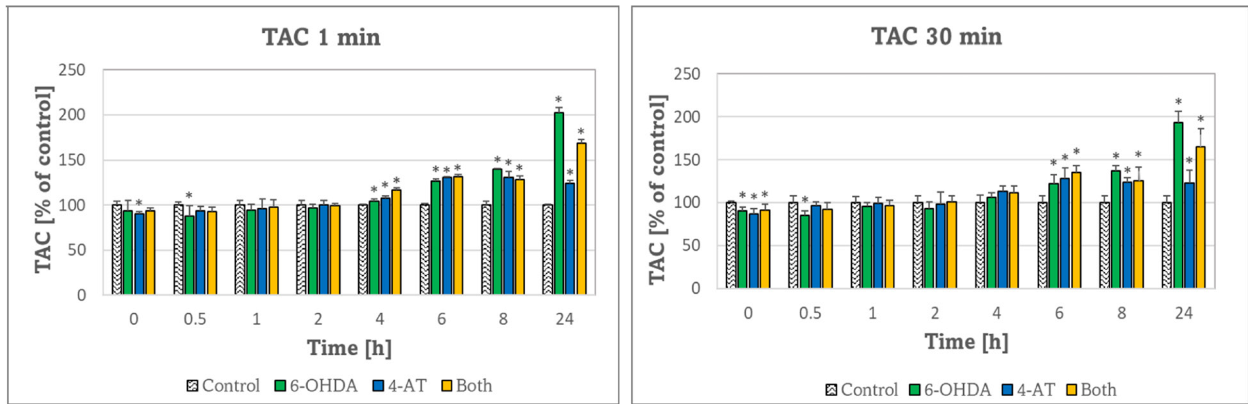

Figure 2. Time course of changes in the total antioxidant capacity of extracts of SH-SY5Y cells treated with 50 μM 6-OHDA, 75 μM 4-AT, and both compounds together.
*$p \leq 0.05$ *vs*. control (not treated) cells (Student's "*t*" test); n=3.

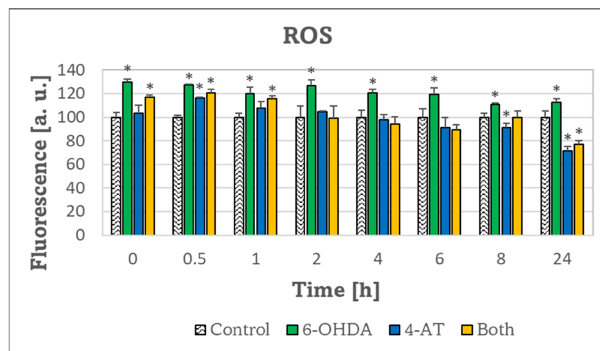

Figure 3. Time course of changes in the level of ROS in SH-SY5Y cells treated with 50 μM 6-OHDA, 75 μM 4-AT, and both compounds together.
*$p \leq 0.05$ *vs*. control (not treated) cells (Student's "t" test); n=3.

treated with 6-OHDA and with 4-AT, coming back to control values after 24 h (Fig. 4).

The activities of glutathione *S*-transferase (GST; EC 2.5.1.18) and γ-glutamyl-cysteine ligase (GCL; EC 6.3.2.2) changed in a similar way though the amplitude of changes was greater for the latter enzyme. The activity of GST was decreased after 30 min in cells treated with 6-OHDA, after 0.5 and 1 h for cells treated with 4-AT and after 30 min, 1 h and 2 h for cells treated with 6-OHDA and 4-AT simultaneously. Then its activity was significantly increased after 8 h in cells treated with 4-AT, and after 8 and 24 h in cells treated with 6-OHDA and 4-AT simultaneously. The activity of GCL was decreased after 0.5 h and elevated after 4, 6, 8 and 24 h in cells treated with 6-OHDA, decreased after 0.5 h, and increased after 2, 4, 8 and 24 h in cells treated with 4-AT, and increased after 6, 8 and 24 h in cells treated with 6-OHDA and 4-AT simultaneously (Fig. 5).

DISCUSSION

It was speculated that supplementation with N-acetylcysteine to restore the level of glutathione may inhibit the progression of PD (Aaseth *et al*., 2018; Tardiolo *et al*., 2018). The results of animal experiments are encouraging (Tardiolo *et al*., 2018).

A decrease in the GSH level was observed in SH-SY5Y cells treated with relatively high concentrations of 6-OHDA as a cellular model of PD. Interestingly, cell treatment with lower concentrations of 6-OHDA induced a profound increase in the cellular GSH content (Tirmenstein *et al*., 2005; Betharia *et al*., 2019; Pichla *et al*., 2020). Exact comparison of data of various authors

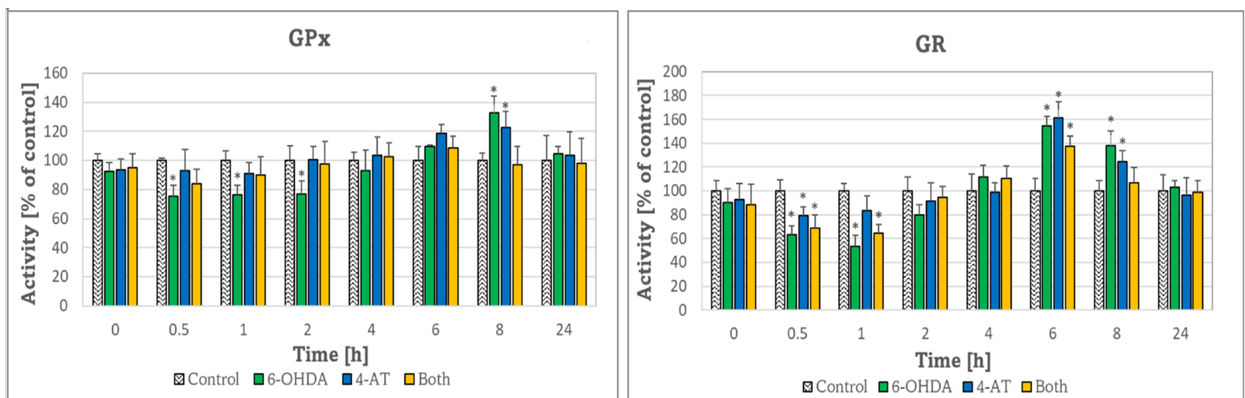

Figure 4. Time course of changes in the activities of glutathione peroxidase (GPx) and glutathione reductase (GR) in SH-SY5Y cells treated with 50 μM 6-OHDA, 75 μM 4-AT and both compounds together.
*$p \leq 0.05$ *vs*. control (not treated) cells (Student's "*t*" test); n=3.

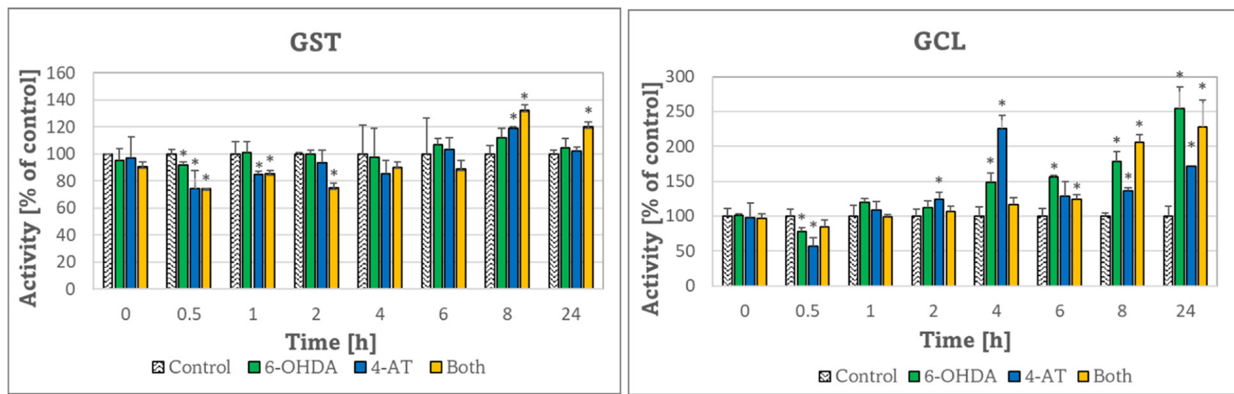

Figure5. Time course of changes in the activities of glutathione *S*-transferase (GST) and γ-glutamyl-cysteine ligase (GCL) in SH-SY5Y cells treated with 50 μM 6-OHDA, 75 μM 4-AT and both compounds together.
*$p \leq 0.05$ *vs*. control (not treated) cells (Student's "*t*" test); n=3.

may be not easy due to subtle differences in conditions. Inter alia, concentrations of the treating agents are usually reported while a description of exposure conditions in the mole per cell metric would be more relevant (Doskey *et al*., 2015; Bartosz *et al*., 2022). In any case, treatment with lower amounts of 6-OHDA induced an increase rather than a decrease in the glutathione content of SH-SY5Y cells. This phenomenon is intriguing as it may point to the ways of elevation of the glutathione content in neuronal cells subjected to oxidative stress.

Results of the present study strongly suggest that this increase in the GSH content is of adaptive nature and is preceded by a transient decrease in the GSH level in the cells shortly after the addition of 6-OHDA, 4-AT, and both compounds together. There was an initial small increase in the content of GSSG in cells treated with 6-OHDA and 4-AT alone and, with one exception, no significant changes afterward. The lack of significant changes in the GSSG level in SH-SY5Y cells challenged with a similar concentration of 6-OHDA after 4 h and 24 h was reported previously (Tirmenstein *et al*., 2005).

The small increase in the GSSG content being a result of GSH oxidation after 30 min can contribute to but cannot account for the decrease in the GSH content occurring at this time as the GSSG constituted not more than 2% of the total glutathione content in SH-SY5Y cells. The main reason for the transient decrease in the content of GSH is most probably the formation of conjugates with products of 6-OHDA oxidation, especially *p*-quinone (Izumi *et al*., 2005). Nitroxides are known to oxidize glutathione (Głebska *et al*., 2003) the relatively low decrease in the GSH content and the relatively high increase of the GSSG content in cells treated with 4-AT are compatible with the assumption that in this case, GSH loss was mainly due to the GSH oxidation by 4-AT. 4-AT is a nitroxide, an antioxidant able to decompose superoxide radical anions in a pseudo-superoxide dismutase reaction and to react with other free radicals (Lewandowski & Gwozdzinski, 2017; Prescot & Bottle, 2017); reactions with free radicals can contribute to the GSH loss as well. It is therefore not surprising that the loss of the GSH content observed after 30 min in cells treated with 6-OHDA and 4-AT together is not higher than those observed for cells treated with any of these agents alone and the increase of the GSSG content observed after this time is even lower than that those found after the action of the single compounds. Changes in TAC of cell extracts generally coincided with those observed for GSH content, in agreement with the view that thiols are the main determinants of cellular TAC.

The permanent elevation of the ROS level in cells treated with 6-OHDA evidences permanent oxidative stress induced by this compound, not counteracted efficiently by increased levels of GSH and TAC. The ROS level was not increased and even decreased by 4-AT at longer incubation times. The presence of 4-AT decreased the ROS level in cells treated with 6-OHDA, evidencing the antioxidant action of this compound.

Cell treatment with 6-OHDA, 4-AT, and both compounds together brought about transient decreases of activity of all enzymes of glutathione metabolism studied, starting at 30 min of incubation and followed by a recovery or even an overshoot several hours later. The loss of enzyme activity may be due to a non-covalent inhibition and (probably mostly) to the damage to enzymatic proteins by thiol oxidation (6-OHDA, 4-AT) and reactions of free radicals and products of 6-OHDA degradation.

Of particular importance are the changes in the GCL activity since it is the rate-limiting enzyme of glutathione biosynthesis. The decrease in GCL activity coincided with the loss of GSH level, apparently evidencing the inability of the enzyme to secure the normal level of GSH consumed at this stage of incubation. The activity of the enzyme was restored relatively quickly; later on, an overshoot took place lasting until 24 h and apparently was responsible for the elevation of GSH content over the control level. It was reported previously that exposure of SH-SY5Y cells to 6-OHDA results increased the expression of the Nrf-2-dependent genes coding for the GCL modifier and catalytic subunits (Tirmenstein *et al*., 2005). Our results demonstrate that an increase in the activity of this enzyme indeed takes place under these conditions and leads to an overcompensatory increase in the GSH content.

An increase in the expression of increased genes coding for the GCL modifier and catalytic subunits was reported in SH-SY5Y cells treated with a similar concentration of 6-OHDA (Tirmenstein *et al*., 2005). Genes coding for other enzymes studied (GPx , GR, and GST) are also under the control of the Nrf2 transcription factor (He *et al*., 2020), which explains the common pattern of activation of all enzymes covered by this study. Semiquinones and quinones react with the Keap protein and activate Nrf2; such products of 6-OHDA metabolism are formed in the cells (Kang *et al*., 2005).

4-amino-TEMPO increased the level of GSH and TAC, especially after 2-24 h (GSH content) and 4-24 h (TAC). Nitroxides can also activate Nrf2 (Greenwald *et al.*, 2014), which explains the similar pattern of enzyme activation by 6-OHDA and 4-AT. The common mechanism of Nrf2 activation may explain the lack of synergy or additivity of the response.

CONCLUSION

Elevation of the level of cellular GSH is apparently an adaptive reaction to oxidative stress imposed by 6-OHDA, occurring if the cell viability is not compromised too strongly and seems to be advantageous for the cells. Mounting of the Nrf2 response by 4-AT may be of potential therapeutic interest since in PD the level of GSH is decreased in substantia nigra and application of a nitroxide, which has beneficial antioxidant properties *per se*, might be an acceptable way in attempts to increase the level of this key cellular antioxidant.

Declarations

Conflict of interest statement. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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