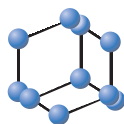


RESEARCH ARTICLE



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SCIENCE**

A Quantitative Proteomic Analysis to Reveal Effects of N-acetylcysteine on H₂O₂-induced Cytotoxicity



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Abstract: Aims: This study aimed to perform a quantitative analysis of the proteomic changes in neural cells under H₂O₂ exposure and N-acetylcysteine (NAC) treatment.

Background: NAC is a potent antioxidant and an effective free radical scavenger that has been used as a potential treatment of several neurological disorders. Although the molecular mechanisms of neuroprotective action by NAC have been studied, more efforts are still needed for the elucidation of the mechanisms through a quantitative proteomic analysis.

Objective: This study aimed to identify differentially expressed proteins among control, H₂O₂-treated group, and NAC+H₂O₂-treated group as well as reveal proteins involved in the protection of neural cells from H₂O₂-induced toxicity.

Methods: SK-N-MC cells were untreated (control), treated with H₂O₂ (disease group), pretreated with NAC, and then treated with H₂O₂ (NAC group). Proteins were digested to peptides and analyzed using liquid chromatography-tandem mass spectrometry with the data-independent acquisition. Skyline was used to quantify peptides and proteins. MSstats was used for statistical analysis. Gene ontology and protein-protein interactions were performed using the Differentially Expressed Proteins (DEPs).

Results: Cytoprotective effects of NAC on the cell against H₂O₂-induced toxicity were first proven using a cell viability study and lactate dehydrogenase assay. The proteomic analysis found 93 DEPs in three comparisons. Among them, 37 proteins were differentially expressed under H₂O₂ exposure. Only 10 DEPs were rescued in the case of NAC pretreatment. Aspartate aminotransferase and L-lactate dehydrogenase B chain were two DEPs involved in the cysteine and methionine metabolism pathway, which might relate to the mechanisms of NAC protective effects.

Conclusion: The findings of cell studies and proteomic analysis were in agreement with previous results, confirming the cytoprotective effects of NAC on neural cells against oxidative stress.

Keywords: Proteomics, N-acetylcysteine, H₂O₂, cytotoxicity, data-independent acquisition, LC-MS/MS, gene ontology.

1. INTRODUCTION

Oxidative stress is one of the common factors involved in many neurodegenerative diseases, such as Parkinson's disease [1-3], Alzheimer's disease [4, 5], stroke [6], spinal cord injury [7], multiple sclerosis [8], and amyotrophic lateral sclerosis [9]. It occurs as a result of the imbalance between antioxidant defense systems and the accumulation of reactive oxygen species (ROS), including hydrogen peroxide

(H₂O₂), superoxide anion (O₂⁻), (OH•) [10]. In normal conditions, ROS plays a crucial role in various cellular signaling pathways and transcription factors. However, their excess accumulation in the body can be harmful to DNA, lipids, and proteins, resulting in severe damage in various cellular organelles [11]. ROS can induce cell death through the activation of signaling pathways within the apoptotic cascade, including mitochondrial apoptosis, mitogen-activated protein kinases, and phosphoinositide 3-kinase [12, 13]. The brain is highly susceptible to oxidative damage of ROS since the unsaturated fatty acids and enriched iron in brain are easily peroxidized [14]. Furthermore, the brain has increased oxidative metabolisms and fewer antioxidative enzymes as compared to other organs [15]. Given that oxidative stress is one

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of the principal causes of neurodegenerative diseases, various antioxidants have been proven to be effective in neurotoxicity protection [16, 17].

N-acetylcysteine (NAC) is a potent antioxidant and an effective free radical scavenger. It has emerged as a potential treatment of several neurological disorders [18, 19]. The antioxidant ability of NAC is associated with direct interactions with ROS [20]. In addition, NAC is a precursor of glutathione (GSH), a principal antioxidant in preventing neurodegenerative diseases and central nervous system injuries. NAC greatly increases the intracellular levels of GSH and thereby minimizes the oxidative effects of ROS [21]. In addition, NAC exerts neuroprotective effects by maintaining the redox state and improving the immune responses under the oxidative condition [22, 23]. Empirically, numerous studies have reported the neuroprotective activity of NAC and its potential for application with neurodegenerative disease therapies. In patients with Parkinson's disease, NAC positively affects the dopaminergic system, increases dopamine transporter binding, and significantly improves symptoms of the disease [24]. In human neuroblastoma cells, NAC can reduce amyloid- β peptide, a neurotoxic compound of Alzheimer's disease [25, 26]. Also, NAC shows effective neuroprotection in ischemic stroke through increasing hypoxia-inducible factor-1 α [27]. Although the molecular mechanisms of neuroprotective action by NAC have been studied, more efforts are still needed for the elucidation of the mechanisms through *in vitro* and *in vivo* studies. Notably, there have not been many studies on NAC neuroprotective effects using proteomics, an approach that allows large scale identification of proteins in samples and evaluation of protein level changes under stimuli.

Proteomics is the study of entire proteins in a given sample, including protein functions, modifications, and interactions [28-30]. By identification of changes in protein level, proteomics is one of the potent strategies to investigate the current status of cells, and thereby elucidate mechanisms related to diseases and treatment therapies [31, 32]. Two major analytical strategies in proteomics are top-down and bottom-up approaches. While top-down proteomics directly separates and identifies intact proteins, bottom-up proteomics analyzes peptides resulted from the enzymatic proteolysis of proteins [33]. Mass spectrometry (MS)-based bottom-up proteomics is one of the most frequently used strategies nowadays. It can be performed using data-dependent acquisition (DDA) or data-independent acquisition (DIA). In DDA mode, the most intense precursor ions are selected in the first stage of MS and subsequently fragmented and analyzed in the next stages. In contrast, DIA MS divides the precursor mass window into narrow ones and consecutively acquires MS/MS data from all precursors detected within each window [34]. Until now, DIA has been regarded as a powerful strategy for both protein identification and quantification [35].

In this study, a quantitative proteomic analysis was performed using DIA MS. SK-N-MC human neuroblastoma cell line was evaluated in three conditions: control, H₂O₂ treatment (disease group), and NAC + H₂O₂ treatment (NAC group) to investigate the cytotoxicity induced by hydrogen

peroxide (H₂O₂) as well as the cytoprotective effect of NAC. DDA MS was carried out to build a spectra library prior to DIA MS. Protein quantification was conducted using Skyline to determine differentially expressed proteins (DEPs) between groups. Further analyses were also performed, including the gene ontology (GO), protein-protein interaction, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Iodoacetamide (IAA), ammonium bicarbonate (ABC), trifluoroacetic acid (TFA), NAC (for cell culture, $\geq 99\%$), H₂O₂ solution (in water, 29.0-32.0%), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tris (2-carboxyethyl) phosphine (TCEP) was purchased from Thermo Fisher Scientific (Rockford, IL, USA). Acetonitrile (ACN), formic acid (FA), and high-performance liquid chromatography (HPLC) grade water were supplied by J.T. Baker (Philipsburg, NJ, USA). Sequencing-grade modified trypsin was from Promega (Madison, WI, USA). cOmplete™ Protease inhibitor cocktail (tablet type) was from Roche Biochemicals (Basel, Switzerland). Methanol was purchased from Fisher Scientific (Pittsburgh, PA, USA). Eagle's Minimum Essential Medium (EMEM) and fetal bovine serum (FBS) were purchased from American Type Culture Collection (Manassas, VA, USA). Penicillin, streptomycin, and trypsin-EDTA solutions were obtained from GIBCO-BRL (Grand Island, NY, USA).

2.2. Cell Culture and Treatments

The human neuroblastoma SK-N-MC cells (Korean Cell Line Bank, Seoul, Korea) were grown in a humidified 5% CO₂ atmosphere at 37°C in EMEM containing 10% (v/v) heat-inactivated FBS, penicillin (100 unit/mL), and streptomycin (100 μ g/mL). For the experiment, the cells were plated at a density of 1×10^5 cells/well in triplicate in 96-well plates and incubated for 24 h. There were three groups: control, H₂O₂, and NAC. H₂O₂ group was treated with H₂O₂ (200 μ M) for 24 h. NAC group was pretreated with 1 mM of NAC for 2 h and then treated with H₂O₂ (200 μ M) for 24 h. NAC was dissolved in phosphate buffer saline (PBS) for the experiments and stored at -20 °C until use. Cell pellets were collected for proteomic analysis.

2.3. MTT Assay for Cell Viability

The cell viability was determined by the MTT reduction assay. After treatment with H₂O₂ (0-800 μ M), the medium was removed in the plate, and then the cells were incubated in an MTT solution containing EMEM (final concentration of 1 mg/ml in PBS) at 37°C with 5% CO₂ for 4 h. Next, the insoluble formazan crystals were dissolved in 200 μ L of DMSO. Consequently, the amount of MTT reduction was determined by measuring the absorbance at 570 nm using a microplate reader (Bio Tek, Winooski, VT, USA).

2.4. Lactate Dehydrogenase (LDH) Assay for Cytotoxicity

The cytotoxicity induced by H_2O_2 treatment was determined using the LDH cytotoxicity detection kit (Takara Bio Inc, Tokyo, Japan), according to the manufacturer's instructions. Briefly, the cells (control, H_2O_2 , and NAC groups) were centrifuged at $250 \times g$ for 10 min at $4^\circ C$, and then 100 μL of supernatants were collected and mixed with 100 μL of the reaction mixture (catalyst and Dye solution) in a 96-well plate. After incubation for 30 min at room temperature, the LDH release was measured at 490 nm using a microplate reader (Bio Tek, Winooski, VT, USA) [36].

2.5. Sample Preparation for Proteomic Analysis

2.5.1. Cell Lysis

The cells were used in 3 groups as follows: (1) non treated group (Control), (2) only 200 μM H_2O_2 treated group (Disease), and (3) pretreatment with 1 mM NAC + 200 μM H_2O_2 treated group (NAC). The cell pellets (3 groups $\times$ 3 biological replicates) were mixed with 1 mL of lysis solution (8M Urea, 0.1 M Tris-HCl buffer, pH 8.5) and 40 μL of protease inhibitor cocktail (stock solution 25X) in glass tubes. Cell lysis was performed using Covaris S2 Focused-Ultrasonicator (Covaris, Woburn, MA, USA) for 15 min. The protein concentration in samples was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

2.5.2. Automated Filter-Aided Sample Preparation (a-FASP)

The a-FASP was performed on a liquid handling robotic system (Agilent Technologies, Santa Clara, CA, USA) controlled by VWorks software [37, 38]. The vacuum was supplied during sample preparation using Multiscreen Vacuum ManifoldTM (Millipore, Billerica, MA, USA). Protein samples (100 μg) were loaded onto a 96-well filter plate (Acro-Prep Advance Filter Plates, 350 μL , Omega 30K MWCO, PALL, NY, USA). Proteins were reduced with TCEP ($37^\circ C$, 30 min) and alkylated with IAA ($25^\circ C$, 30 min, in the dark). After washing with 50 mM ABC, the proteins were digested with trypsin at an enzyme: protein ratio of 1:50 (w/w) ($37^\circ C$, 18 h). After digestion, peptide mixtures were collected. FA was added to inactivate the trypsin. The samples were then desalted by using Sep-Pak tC18 96-well plate (Waters, Milford, MA, USA).

2.5.3. Fractionation

The peptides from nine samples were pooled and fractionated using high-pH reversed-phase liquid chromatography. The sample was loaded on a Sep-Pak Vac 1cc C18 cartridge (Waters, Milford, MA, USA). Peptides were eluted with a series of 16 eluting solvents containing acetonitrile (2.5-50% v/v) and trimethylamine (0.1%) in water. The acetonitrile concentration was increased stepwise. The eluted samples were pooled to make 5 final fractions, followed by drying in

the ScanSpeed 40 centrifugal evaporator (1,800 rpm, 3 h). The dried samples were reconstituted in solvent A (0.1% formic acid in water) prior to LC-MS/MS analysis [39].

2.6. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analysis

Analysis of the peptide mixtures was performed on an LC-MS/MS system consisting of a Dionex Ultimate 3000 HPLC coupled with a Q ExactiveTM Hybrid Quadrupole-Orbitrap MS (Thermo Fisher Scientific, MA, USA). An AcclaimTM PepMapTM 100 C18 nano-trap column ($75 \mu m \times 2$ cm, 3 μm particles, 100- $\text{\AA}$ pores, Thermo Fisher Scientific) was used to load the samples. The loading step was carried out using solvent A at a flow rate of 2.5 $\mu L/min$ for 5 min. Subsequently, an AcclaimTM PepMapTM C18 100A RSLC nano-column ($75 \mu m \times 50$ cm, 2 μm particles, 100- $\text{\AA}$ pores, Thermo Fisher Scientific) was used to separate the peptide mixtures. The solvent consisted of solvent A and solvent B (0.1% formic acid in 80% ACN). The flow rate was fixed at 300 nL/min. The gradient set up for solvent B was as follows: 4% in 4 min, 4-40% in 116 min, 40-96% in 10 min, 96-4% in 0.1 min, and 4% B for 19.9 min. The nano-electrospray ionization source was operated under positive mode with a spray voltage of 2.0 kV. The capillary temperature was $320^\circ C$ [31]. The isolation width was ± 2 m/z, and the scan range was 400 to 2000 m/z. The resolution in full-MS scans and in MS/MS scans at 200 m/z was 70,000 and 17,500, respectively. For DIA MS, 20 non-overlapping 20-m/z-wide windows between 500 and 900 m/z were used, and 9 samples were analyzed in triplicates.

2.7. Data Analysis

Raw MS/MS files of 5 analyses in DDA MS were converted to .mzXML using MSConvert. Comet (ver. 2017.01 rev.0) was used to search MS/MS spectra against the human proteome database obtained from Uniprot. The search was set up as follows: maximum of two missed cleavages with trypsin; 10 ppm and 0.02 Da tolerances of precursor ion masses and fragment ion mass, respectively; static carbamidomethylation of cysteine (+57.012 Da); and variable modifications including methionine oxidation (+15.995 Da) and carbamylation of protein in N-term (+43.0006 Da). The output files (.pepXML) were subjected to the Trans-Proteomic Pipeline (TPP) [40] with PeptideProphet [41] and ProteinProphet [42]. An FDR $\leq 1\%$ was used to filter the data. Proteins were identified with a minimum of 2 unique peptides. Protein quantification was performed using Skyline (ver. 4.2.0) [43]. DDA data were acquired to build a spectral library with a cut-off score of 0.99 prior to the analysis of DIA data. The parameters used in Skyline were as follows: precursor charges, 1-4; ion charges, 1-2; ion types, y, b, p; ion match tolerance, 0.05 m/z; maximum missed cleavages; 2; and a minimum number of peptide per protein, 2. MSstats (ver. 3.13.6) was used for statistical quantification of the results [44]. Quantile normalization was applied. DEPs were defined with a fold-change (FC) ≥ 2 ($-\log_2 FC \geq 1$) or FC ≤ 0.5 ($-\log_2 FC \leq -1$) and p-value ≤ 0.05 was considered [45].

2.8. Gene Ontology, Protein-Protein Interaction, and Pathway Analysis

DEPs were used for gene ontology (GO) analysis (<http://geneontology.org/>). Protein-protein interaction (PPI) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were analyzed through the String database (<http://string-db.org/>) [46] and visualized with Cytoscape ver. 3.7.1 (National Institute of General Medical Sciences, MD, USA). KEGG pathway maps were obtained from www.kegg.jp [47-49] with permission to re-use.

3. RESULTS

3.1. Effect of NAC on H₂O₂-Induced Cell Death and Cytotoxicity in SK-N-MC Cells

Human SK-N-MC neuroblastoma cells were used to evaluate the toxicity of H₂O₂ as well as the protective effects of NAC. As shown in (Fig. 1A), exposure of the cells to various concentrations of H₂O₂ (100, 200, 400, 600, and 800 μ M) resulted in a dose-dependent decrease in cell viability. Treating the cells with 200 μ M of H₂O₂ led to a significant loss of cell viability ($p < 0.001$), with approximately 60.1% of survival cells compared with the untreated control cell (Fig. 1B). However, pretreatment with NAC (1 mM) for 2 h before exposure to H₂O₂ (200 μ M) reduced the loss of cell viability induced by H₂O₂. The cell viability was increased to 82.4%, which was significantly higher than that of the cells treated with H₂O₂ alone ($p < 0.001$). Morphological changes in H₂O₂-treated SK-N-MC cells are shown in (Fig. 1C). Compared to control cells, treatment with H₂O₂ (100-400 μ M) dose-dependently resulted in morphological features of neuronal cell death, including cell shrinkage and neuritis injury. However, pretreatment with NAC (1 mM) inhibited morphological changes of SK-N-MC cells induced by H₂O₂ (200 μ M). LDH assay for cytotoxicity was also performed. The cells treated with H₂O₂ showed a remarkable increase in the LDH level compared with the untreated control cells ($p < 0.001$). However, pretreatment of the cells with NAC (1 mM) significantly reduced LDH level in H₂O₂-treated cells by 21.0% ($p < 0.001$) (Fig. 1D).

3.2. DDA MS Analysis of the Combined Proteome

In DDA MS analysis, a total of 2,806 proteins and 16,183 peptides were identified from 5 high pH PRLC fractions (Table S1 and S2). The physicochemical properties of the identified peptides and proteins were determined. From TPP, molecular weight (MW), retention time, and isoelectric point (pI) of the peptides were obtained. In addition, the grand average of hydropathy (GRAVY) values of the peptides was calculated with the GRAVY calculator (<http://gravy-calculator.de/>) [50]. Negative GRAVY values indicate hydrophilicity and vice versa. (Fig. 2A-2D) present distribution of MW, GRAVY, pI, and retention time of the identified peptides. It can be seen that the majority of the identified peptides have MW < 2,000 Da (87.6%), and particularly, more than 50% of the peptides are in the range of

MW 1,000-1,600 Da. Most of the peptides are hydrophilic (80.2%), as evidenced by GRAVY < 0. Approximately 75% of the peptides have pI in the range 4.0-7.0. The efficacy of tryptic digestion is shown in (Fig. 2E), with about 96% of the peptides having two lysine/arginine terminals. In addition, less than 7% of the peptides have missed cleavages. (Fig. 2F) presents the distribution of the identified proteins according to their length. More than 70% of the proteins have < 600 amino acids. Accordingly, various two-dimensional graphs were constructed using R (ver. 3.6.1) to reveal the relationship among different physicochemical parameters of the identified peptides (Fig. (S1)). It can be observed that acidic peptides appear throughout the RPLC running time, whereas basic peptides, particularly those with pI > 10, were mostly associated with short retention time. Highly hydrophilic peptides (GRAVY < -2) are likely eluted at an early time (< 60 min). It should also be noted that peptides with high MW (> 3,000 Da) tend to be eluted at the later time in RPLC, and have GRAVY of -2~0.5 and pI of 3-6.

3.3. DIA MS Analysis and Quantitative

After the Comet search, the whole protein list in DDA MS was used to build a spectra library for label-free quantification of DIA data with Skyline. The peptide intensities are listed in Table S3. DEPs were identified with MSstats for different comparisons ((disease vs. control, NAC vs. disease, and NAC vs. control)). The lists of DEPs are shown in Tables 1, 2, and S4. (Fig. 3A) illustrates volcano plots for each comparison with up- and down-regulated proteins being marked by blue and red colors, respectively. Details on protein name, gene name, protein description, and summary of log₂FC and p-value are listed in Table S5. Comparisons of protein abundances among the three groups are presented in Fig. (S2) for each protein. From 546 proteins that are quantified, 93 proteins are differentially expressed in at least one comparison (Table S5).

First, in the disease vs. control comparison, 39 proteins are differentially expressed (23 up and 16 down), which relate to 8 biological processes, 17 cellular components, 0 molecular functions (Table S6A), and 0 KEGG pathway. Second, in the NAC vs. disease comparison, there are 37 DEPs (22 up and 15 down). They relate to 1 biological process (protein folding), 15 cellular components, 10 molecular functions (Table S6B), and 2 KEGG pathways (protein processing in the endoplasmic reticulum (has04141) and cysteine and methionine metabolism (hsa00270)). Third, in the NAC vs. control comparison, 52 proteins are differentially expressed (32 up and 20 down), which relate to 3 biological processes, 26 cellular components, 4 molecular functions (Table S6C), and 3 KEGG pathways.

Among 93 DEPs, we filtered and selected 10, including CAAX prenyl protease 1 homolog (ZMPSTE24), 60S acidic ribosomal protein P2 (RPLP2), L-lactate dehydrogenase B chain (LDHB), guanine nucleotide-binding protein G(k) subunit alpha (GNAI3), aspartate aminotransferase, cytoplasmic (GOT1), DNA-directed RNA polymerase II subunit RPB1 (POLR2A), stress-70 protein, mitochondrial (HSPA9), tran-

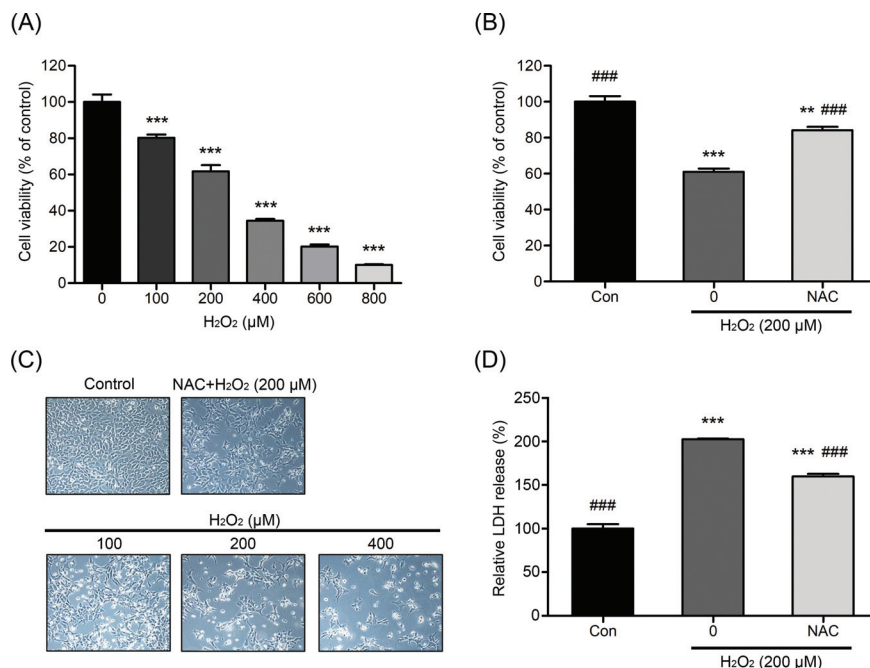


Fig. (1). Protective effects of NAC against H_2O_2 -induced cell death and cytotoxicity in human SK-N-MC neuroblastoma cells. **(A)** SK-N-MC cells were treated with H_2O_2 (100–800 μM) for 24 h. Cell viability was expressed as % compared to control. **(B)** Cells were pretreated with NAC (1 mM) for 2 h and then exposed to H_2O_2 (200 μM) for 24 h. **(C)** Morphological changes in SK-N-MC cells were observed using light microscopy ($\times 100$). **(D)** Relative LDH release in the cell supernatant was measured using LDH cytotoxicity detection kit. The results are expressed as the mean $\pm$ S.E.M from three independent experiments. ** $p < 0.01$; *** $p < 0.001$ and ### $p < 0.01$; #### $p < 0.01$ compared with untreated control cells and H_2O_2 -treated cells, respectively. Con: Control. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

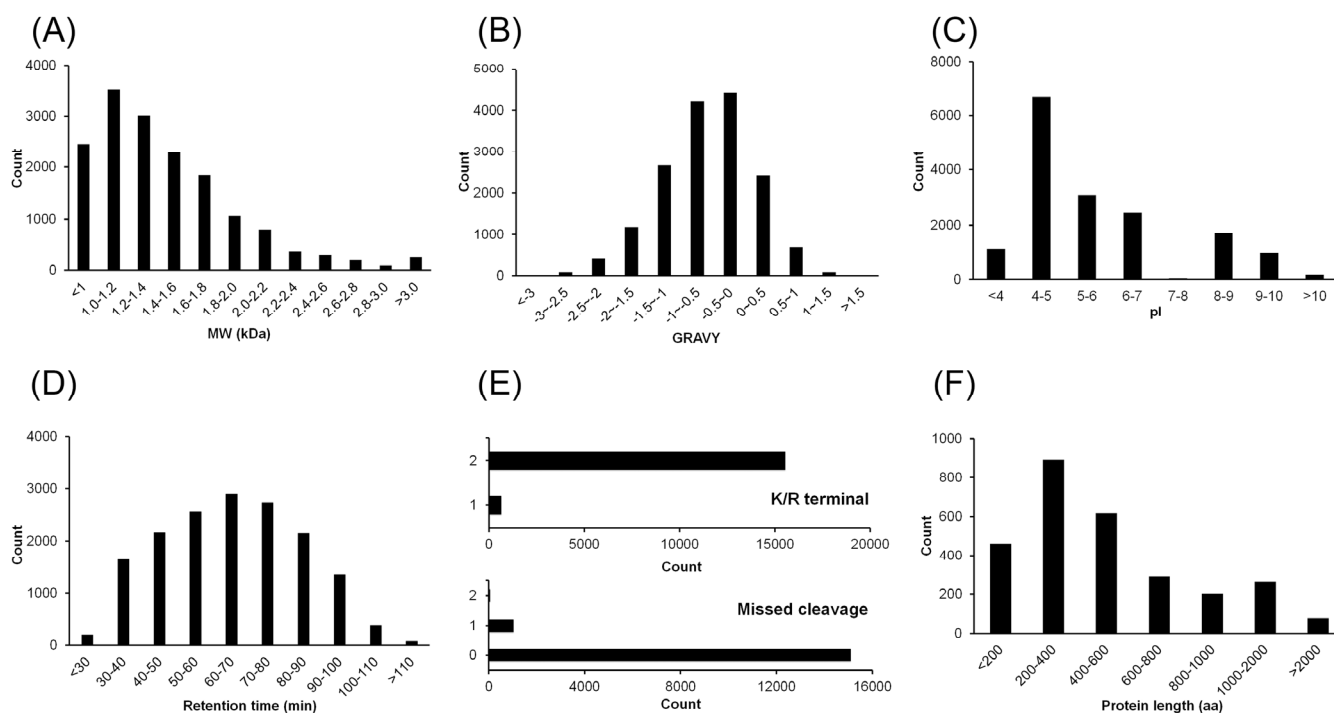


Fig. (2). Summary of results from proteomic analysis with DDA MS. **(A–D)** Distribution of identified peptide by molecular weight, GRAVY, pI, and LC retention time. **(E)** Number of K/R terminals and missed cleavages by trypsin digestion. **(F)** Distribution of identified protein by protein length (amino acid number). (A higher resolution / colour version of this figure is available in the electronic copy of the article).

Table 1. DEPs of disease *versus* control.

Up-regulation			Down-regulation		
Protein ID	log2FC	p-Value	Protein ID	log2FC	p-Value
P14927	1.02	0.00403	P05387	-3.22	0.00016
P01111	1.02	0.04024	Q9H3K6	-2.36	0.02007
Q9BUB7	1.04	0.03962	P38646	-2.25	0.00074
P54577	1.10	0.01553	P62266	-1.86	0.03154
Q10570	1.21	0.03758	P24928	-1.77	0.00005
P49356	1.22	0.00481	Q13547	-1.65	0.03701
P47755	1.23	0.00190	Q9Y217	-1.52	0.00127
Q9H773	1.25	0.00385	O75844	-1.41	0.00007
P07195	1.31	0.04974	P10606	-1.32	0.00637
Q9Y5J1	1.34	0.02639	Q9UFC0	-1.29	0.00437
P28066	1.36	0.01486	O00232	-1.25	0.01138
Q9UFW8	1.37	0.00392	Q9UBV8	-1.17	0.01164
Q96QK1	1.38	0.00891	P17174	-1.08	0.04910
P30086	1.39	0.00011	Q6PI78	-1.06	0.00468
P23284	1.40	0.01537	Q9BUF5	-1.05	0.02690
P46109	1.43	0.01775	P05556	-1.05	0.00152
P46977	1.48	0.00749	-	-	-
P13489	1.49	0.01898	-	-	-
Q8TAE8	1.54	0.04673	-	-	-
P80723	1.85	0.00073	-	-	-
P08754	2.12	0.01177	-	-	-
Q08211	2.79	0.03658	-	-	-
Q8WXX5	3.73	0.00038	-	-	-

Table 2. DEPs of NAC *versus* disease

Up-Regulation			Down-Regulation		
Protein ID	log2FC	p-Value	Protein ID	log2FC	p-Value
O75844	1.01	0.00211	P08670	-3.39	0.03918
P55072	1.04	0.03136	P80723	-2.94	0.00000
P04066	1.08	0.02600	P08238	-2.21	0.04749
P16949	1.10	0.00000	Q8WXX5	-2.19	0.02320
Q9Y3B4	1.10	0.01138	Q14692	-2.19	0.00385
P11766	1.20	0.03928	P08754	-2.05	0.01428
P17174	1.22	0.02789	P07195	-2.03	0.00386
Q15050	1.22	0.01944	P54753	-1.84	0.03670
Q6PI78	1.26	0.00108	P30040	-1.62	0.01629
Q8IY81	1.32	0.00740	Q9H1E3	-1.46	0.04440
P24928	1.33	0.00111	O43765	-1.33	0.00396
P31689	1.39	0.03755	P13639	-1.28	0.04895
P26640	1.41	0.00188	P31153	-1.23	0.01454
Q9H0Q0	1.44	0.00399	Q9UFW8	-1.04	0.02335
Q9UBK9	1.49	0.04924	Q14554	-1.01	0.04210
P51571	1.50	0.03979	-	-	-
P56537	1.53	0.00360	-	-	-

(Table 2) contd....

Up-Regulation			Down-Regulation		
Protein ID	log2FC	p-Value	Protein ID	log2FC	p-Value
Q5BKZ1	1.70	0.02976	-	-	-
P38646	1.78	0.00529	-	-	-
O43615	1.93	0.03559	-	-	-
P21281	2.05	0.00078	-	-	-
P05387	2.38	0.00224	-	-	-

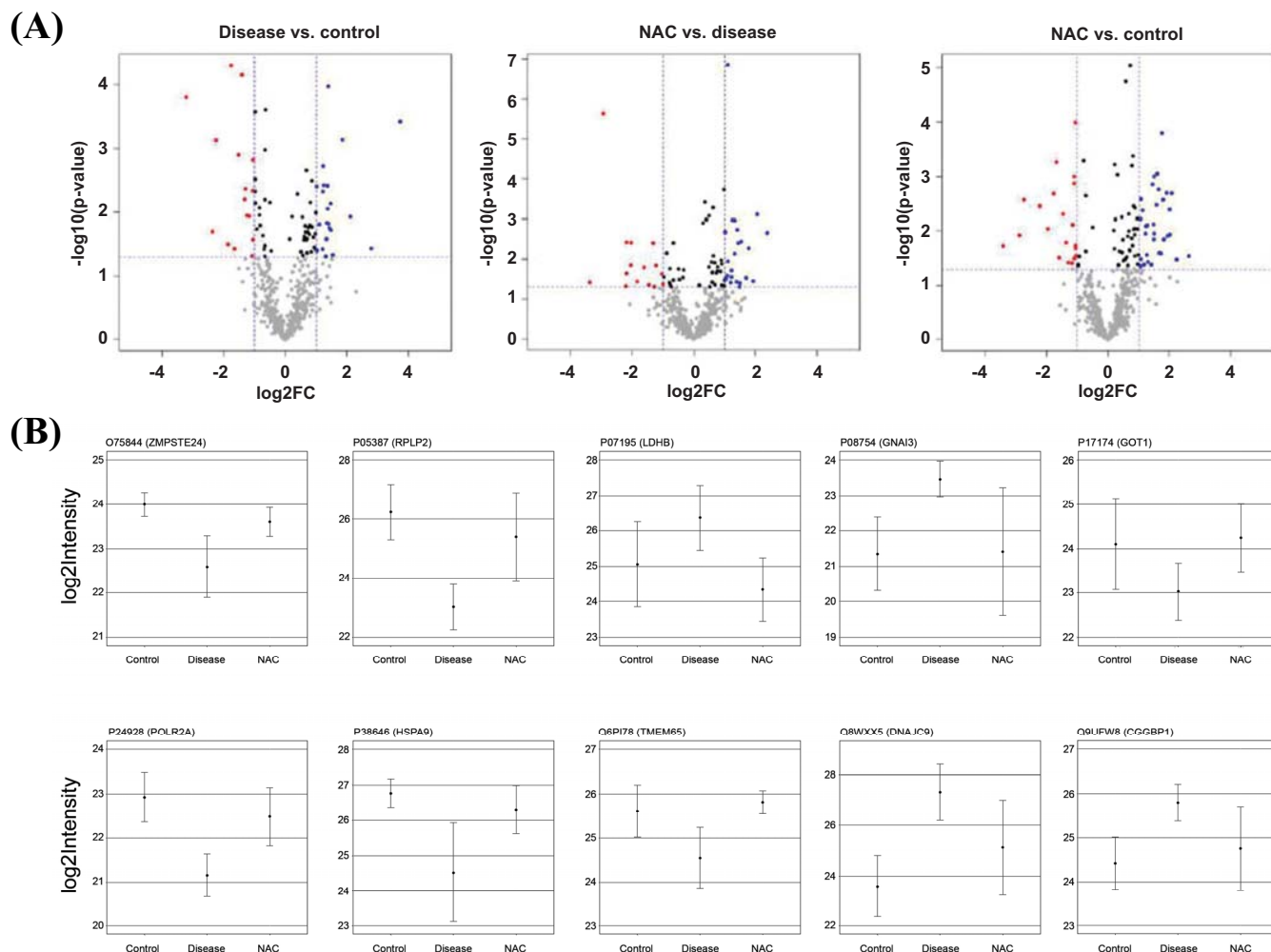


Fig. (3). Summary of results from proteomic analysis with DIA MS. **(A)** Volcano plots. **(B)** Comparison of intensity of some differentially expressed proteins. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

smembrane protein 65 (TMEM65), DNA J homolog subfamily C member 9 (DNAJC9), and CGG triplet repeat-binding protein 1 (CGGBP1). These proteins were differentially expressed when the cells were exposed to H_2O_2 but recovered to the normal state in the case of NAC-treated cells (Fig. 3B).

PPI among DEPs was defined using String and visualized via Cytoscape (Fig. 4 and Fig. (S3)). Detail interactions defined by String can be observed from Fig. (S4) and Table

S7. It can be seen that 4 of the 10 above-selected proteins, including GOT1, LDHB, HSPA9, and DNAJC9, are shown to closely interact with each other. As noted, the cysteine and methionine metabolism pathway is one KEGG pathway that resulted from DEPs of the NAC vs. disease comparison. Fig. (S5) shows the regulation of three proteins (L-lactate dehydrogenase B chain, aspartate aminotransferase, cytoplasmic, and S-adenosylmethionine synthase isoform type-2) in cysteine and methionine metabolism pathway.

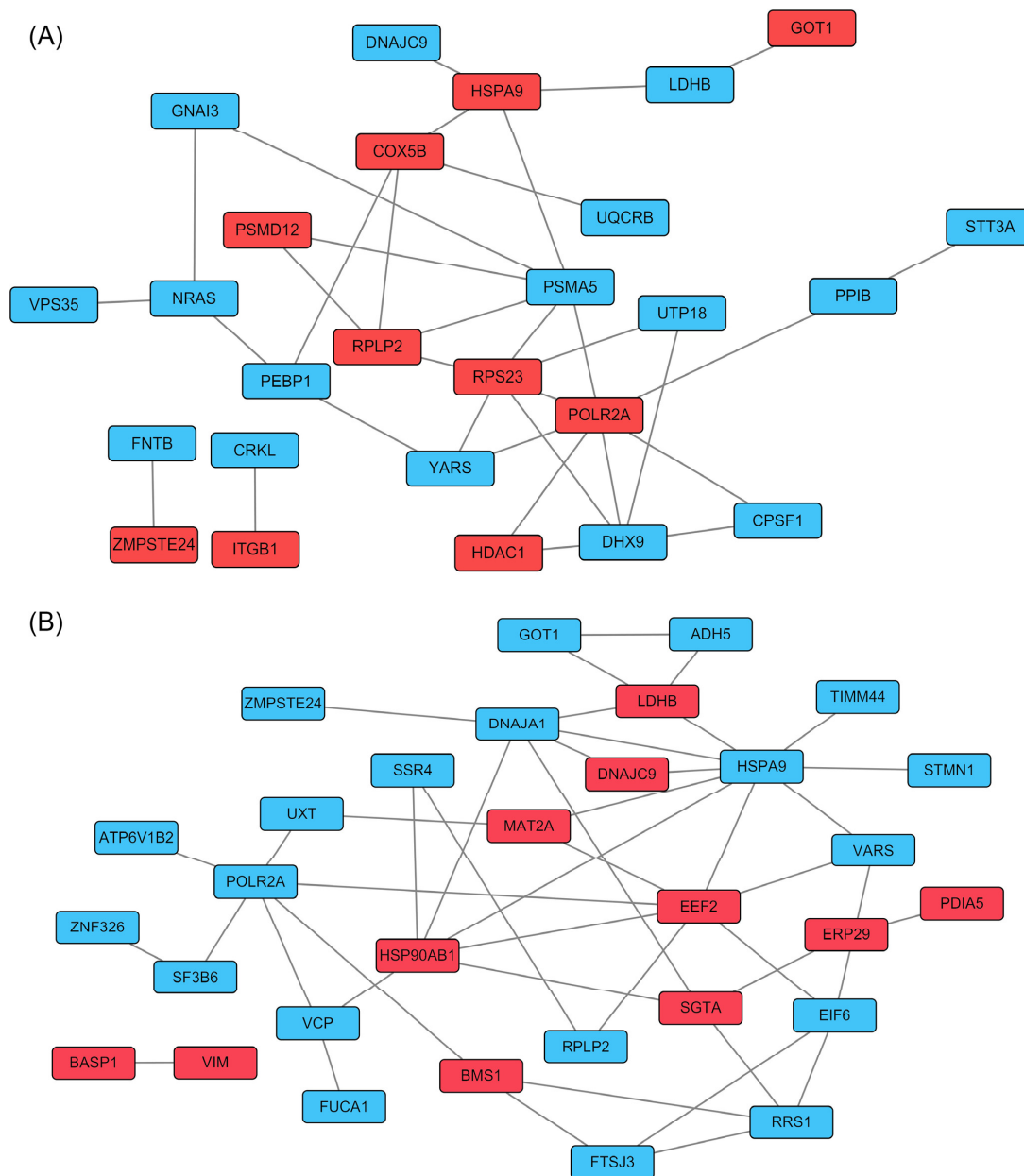


Fig. (4). Protein-protein interaction using lists of differentially expressed proteins. (A) Disease vs. control and (B) NAC vs. disease. Red: down-regulated, blue: up-regulated. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

4. DISCUSSION

In this study, quantitative proteomic analysis was performed to reveal changes in the proteome of SK-N-MC cells in 3 different conditions: control, H₂O₂ exposure (disease group), and NAC-pretreatment, followed by H₂O₂ exposure (NAC group). Proteomic analysis of cell lysates in three conditions is an effective approach to reveal changes in the proteome of the cell and partly elucidate the effects of NAC in protein levels. First, DDA MS was used to profile the combined proteome of cells in 3 conditions. A two-dimensional separation method consisting of high pH-RPLC and low pH-RPLC was used to increase peptide and protein identification [39, 51]. As a result, approximately 2,800 proteins were

identified with at least 2 unique proteins. The spectra from DDA MS were then subjected to build a library in Skyline,

which enabled the quantification of peptides and proteins from the DIA MS data.

H₂O₂ was used to induce cytotoxicity in SK-N-MC cells [52]. About 40% of cell death was observed at the concentration of 200 μ M H₂O₂. It is in agreement with the results of a previous study [17]. H₂O₂ caused changes in the expression of some proteins (Table 1) and affected some cellular biosynthetic and metabolic processes (Table S6A). However, no distinct pathway was found from those DEPs. When NAC was pretreated to cells prior to H₂O₂ exposure, it was ob-

served that NAC could considerably protect the cells from toxicity caused by H_2O_2 (Fig. 1). LDH activity is regarded as a reliable indicator of cell damage since it correlates with the disruption of the cell membrane. Thus, LDH levels are often used as an index to evaluate cell death. In this study, the relative LDH level was a measure to reflect the damage or death of the neuro cell [36]. NAC acts as an antioxidant and a free-radical scavenging agent [20]. It also increases intracellular GSH, which helps to protect the cells from oxidative stress [21]. The protective effects of NAC may be *via* inhibition of ROS or the estrogen receptor-stress-mediated apoptosis pathway [53]. Proteomics analysis revealed 37 DEPs (Table 2), which related to several binding functions in the cells (Table S6B). Notably, 10 proteins showed remarkable recovery to the normal levels in the case of NAC pretreatment (Fig. 3B), although they were differentially regulated under H_2O_2 exposure. Thus, among 39 proteins differentially expressed under H_2O_2 exposure, only 25.6% (10 proteins) was rescued with the pretreatment of NAC. Those proteins may play a critical role in the protective effect of NAC on the cells exposed to H_2O_2 . In a previous study, NAC was used to protect the brain from acute cytotoxicity induced by acrylamide. In this case, no GO or KEGG annotation enrichment was detected from 80 DEPs. Among them, 14 DEPs were rescued by NAC [54]. The effects of NAC on the protection of neural cells from oxidative stress *in vitro* in this study agreed with many previous studies [18, 25, 55–57]. The ability of NAC to cross the blood-brain barrier *in vivo* models depends on its dose and administration [20]. The poor blood-brain barrier permeability, as well as the high de-acylation of NAC during intestine absorption, may cause failure in increasing GSH levels [54].

The DEPs in NAC *vs.* disease relates to the cysteine and methionine metabolism KEGG pathway (Fig. (S5)) with 3 proteins being matched (L-lactate dehydrogenase B chain, aspartate aminotransferase, and S-adenosylmethionine synthase isoform type-2). LDH is a glycolytic enzyme that catalyzes the reversible conversion of lactate to pyruvate, with the simultaneous conversion of NAD^+ and NADH. Increases in LDH levels occur together with a wide variety of diseases. Particularly, the serum level of LDH is an indicator of cell damage since it relates to the release of LDH into the bloodstream [58]. It can be seen that L-lactate dehydrogenase B chain (LDHB) up-regulated after H_2O_2 exposure; however, NAC pretreatment helped reducing its level to normal condition (Fig. 3B). Aspartate aminotransferase (GOT1) catalyzes the biosynthesis of L-glutamate from L-cysteine or L-aspartate. It also acts as a scavenger of glutamate in brain neuroprotection. The depletion of aspartate aminotransferase relates to the increased ROS levels [59]. In addition, aspartate aminotransferase catalyzes the synthesis of 3-mercaptopyruvate, which is further converted into hydrogen sulfide (H_2S) *via* the action of 3-mercaptopyruvate sulfurtransferase. H_2S is also a crucial synaptic modulator and neuroprotectant in the brain [60]. Besides, aspartate aminotransferase is crucial to provide oxaloacetate at low glucose levels, probably to maintain the redox homeostasis [61]. It

could also reduce serum and brain glutamate levels, and the combination with oxaloacetate would be a potential approach for a successful acute-phase stroke treatment [62]. Our findings showed that level of aspartate aminotransferase was decreased when the cells were exposed to H_2O_2 . The pretreatment with NAC exhibited the significant recovery of aspartate aminotransferase to the normal level (Fig. 3B). The findings in this study, however, could not reveal the mechanisms of NAC on altering the concentrations of 10 proteins mentioned above. Thus, further studies may be conducted to uncover how NAC affects those proteins that lead to the protective actions on neuroblastoma cells. Besides, this study lacked the verification of the DEPs. Further studies should be performed to validate and confirm the expression of those proteins using orthogonal analyses, such as enzyme-linked immunosorbent assays (ELISA), western blot, and radioimmunoassay [63, 64].

CONCLUSION

In this study, the cytoprotective effects of NAC against H_2O_2 -induced cytotoxicity were confirmed using proteomics as an alternative approach. Specifically, several proteins that might be involved in the mechanism of the NAC pretreatment were identified. 10 proteins were found to be differentially expressed under H_2O_2 exposure but effectively recovered to normal levels in the NAC-pretreatment condition, including CAAX prenyl protease 1 homolog, 60S acidic ribosomal protein P2, L-lactate dehydrogenase B chain, guanine nucleotide-binding protein G(k) subunit alpha, cytoplasmic aspartate aminotransferase, DNA-directed RNA polymerase II subunit RPB1, mitochondrial stress-70 protein, transmembrane protein 65, DNA J homolog subfamily C member 9, and CGG triplet repeat-binding protein 1. L-lactate dehydrogenase B chain and aspartate aminotransferase were involved in the cysteine and methionine metabolism KEGG pathway. Further studies on these proteins may reveal mechanisms of NAC on protecting neural cells from oxidative stress.

LIST OF ABBREVIATIONS

DDA	= Data-Dependent Acquisition
DEPs	= Differentially Expressed Proteins
DIA	= Data-Independent Acquisition
FASP	= Filter-Aided Sample Preparation
GO	= Gene Ontology
GRAVY	= The Grand Average of Hydropathy
HPLC	= High-Performance Liquid Chromatography
IAA	= Iodoacetamide
KEGG	= Kyoto Encyclopedia of Genes and Genomes
LC-MS/MS	= Liquid Chromatography-Tandem Mass Spectrometry

LDH	= Lactate Dehydrogenase
MS	= Mass Spectrometry
MW	= Molecular Weight
NAC	= N-Acetylcysteine
pI	= Isoelectric Point
PPI	= Protein-Protein Interaction
TPP	= Trans-Proteomic Pipeline

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of the article will be available upon request.

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CONFLICT OF INTEREST

The authors have no conflicts of interest, financial or otherwise.

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Declared none.

SUPPORTIVE/SUPPLEMENTARY MATERIAL

The data supporting the findings of the article is available in the ProteomeXchange Consortium *via* the PRIDE [65] partner repository with the dataset identifier PXD018177. For reviewers, username is reviewer19680@ebi.ac.uk and password is JwROWCN.

REFERENCES

- [1] Dias, V.; Junn, E.; Mouradian, M.M. The role of oxidative stress in Parkinson's disease. *J. Parkinsons Dis.*, **2013**, *3*(4), 461-491. <http://dx.doi.org/10.3233/JPD-130230> PMID: 24252804
- [2] Wei, Z.; Li, X.; Li, X.; Liu, Q.; Cheng, Y. Oxidative stress in parkinson's disease: A systematic review and meta-analysis. *Front. Mol. Neurosci.*, **2018**, *11*(236)
- [3] Trist, B.G.; Hare, D.J.; Double, K.L. Oxidative stress in the aging substantia nigra and the etiology of Parkinson's disease. *Aging Cell*, **2019**, *18*(6), e13031. <http://dx.doi.org/10.1111/ace1.13031> PMID: 31432604
- [4] Lovell, M.A.W.R.; Markesbery, W.R. Oxidative DNA damage in mild cognitive impairment and late-stage Alzheimer's disease. *Nucleic Acids Res.*, **2007**, *35*(22), 7497-7504. <http://dx.doi.org/10.1093/nar/gkm821> PMID: 17947327
- [5] Butterfield, D.A.B.; Halliwell, B. Oxidative stress, dysfunctional glucose metabolism and Alzheimer disease. *Nat. Rev. Neurosci.*, **2019**, *20*(3), 148-160. <http://dx.doi.org/10.1038/s41583-019-0132-6> PMID: 30737462
- [6] Shirley, R.; Ord, E.N.J.; Work, L.M. Oxidative stress and the use of antioxidants in stroke. *Antioxidants*, **2014**, *3*(3), 472-501. <http://dx.doi.org/10.3390/antiox3030472> PMID: 26785066
- [7] Jia, Z.; Zhu, H.; Li, J.; Wang, X.; Misra, H.; Li, Y. Oxidative stress in spinal cord injury and antioxidant-based intervention. *Spinal Cord*, **2012**, *50*(4), 264-274. <http://dx.doi.org/10.1038/sc.2011.111> PMID: 21987065
- [8] Pistono, C.; Monti, M.C.; Boiocchi, C.; Berzolari, F.G.; Osera, C.; Mallucci, G.; Cuccia, M.; Pascale, A.; Montomoli, C.; Bergamaschi, R. Response to oxidative stress of peripheral blood mononuclear cells from multiple sclerosis patients and healthy controls. *Cell Stress Chaperones*, **2020**, *25*(1), 81-91. <http://dx.doi.org/10.1007/s12192-019-01049-0> PMID: 31720998
- [9] Bordini, M.; Pansarasa, O.; Dell'Orco, M.; Crippa, V.; Gagliardi, S.; Sproviero, D.; Bermuzzi, S.; Diamanti, L.; Ceroni, M.; Tedeschi, G.; Poletti, A.; Cereda, C. Nuclear phospho-sod1 protects DNA from oxidative stress damage in amyotrophic lateral sclerosis. *J. Clin. Med.*, **2019**, *8*(5), 729. <http://dx.doi.org/10.3390/jcm8050729> PMID: 31121901
- [10] Jensen, S.J.K. Oxidative stress and free radicals. *J. Mol. Struct.: Theochem*, **2003**, *666-667*, 387-392. <http://dx.doi.org/10.1016/j.theochem.2003.08.037>
- [11] Kelsey, N.A.; Wilkins, H.M.; Linseman, D.A. Nutraceutical antioxidants as novel neuroprotective agents. *Molecules*, **2010**, *15*(11), 7792-7814. <http://dx.doi.org/10.3390/molecules15117792> PMID: 21060289
- [12] Redza-Dutordoir, M.D.A. Averill-Bates. Activation of apoptosis signalling pathways by reactive oxygen species. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, **2016**, *1863*(12), 2977-2992.
- [13] Kruman, I.; Bruce-Keller, A.J.; Bredesen, D.; Waeg, G.; Mattson, M.P. Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis. *J. Neurosci.*, **1997**, *17*(13), 5089-5100. <http://dx.doi.org/10.1523/JNEUROSCI.17-13-05089.1997> PMID: 9185546
- [14] Floyd, R.A.J.M.; Carney, J.M. Free radical damage to protein and DNA: mechanisms involved and relevant observations on brain undergoing oxidative stress. *Ann. Neurol.*, **1992**, *32*(S1)(Suppl.), S22-S27. <http://dx.doi.org/10.1002/ana.410320706> PMID: 1510377
- [15] Valencia, A.; Morán, J. Role of oxidative stress in the apoptotic cell death of cultured cerebellar granule neurons. *J. Neurosci. Res.*, **2001**, *64*(3), 284-297. <http://dx.doi.org/10.1002/jnr.1077> PMID: 11319773
- [16] Choi, D.J.; Cho, S.; Seo, J.Y.; Lee, H.B.; Park, Y.I. Neuroprotective effects of the *Phellinus linteus* ethyl acetate extract against H₂O₂-induced apoptotic cell death of SK-N-MC cells. *Nutr. Res.*, **2016**, *36*(1), 31-43. <http://dx.doi.org/10.1016/j.nutres.2015.11.005> PMID: 26773779
- [17] Zhang, J-X.; Wang, R.; Xi, J.; Shen, L.; Zhu, A-Y.; Qi, Q.; Wang, Q-Y.; Zhang, L-J.; Wang, F-C.; Lü, H-Z.; Hu, J.G. Morroniside protects SK-N-SH human neuroblastoma cells against H₂O₂-induced damage. *Int. J. Mol. Med.*, **2017**, *39*(3), 603-612. <http://dx.doi.org/10.3892/ijmm.2017.2882> PMID: 28204825
- [18] Günther, M.; Davidsson, J.; Plantman, S.; Norgren, S.; Mathiesen, T.; Risling, M. Neuroprotective effects of N-acetylcysteine amide on experimental focal penetrating brain injury in rats. *J. Clin. Neurosci.*, **2015**, *22*(9), 1477-1483. <http://dx.doi.org/10.1016/j.jocn.2015.03.025> PMID: 26100161
- [19] Hicdonmez, T.; Kanter, M.; Tiryaki, M.; Parsak, T.; Cobanoglu, S. Neuroprotective effects of N-acetylcysteine on experimental closed head trauma in rats. *Neurochem. Res.*, **2006**, *31*(4), 473-481. <http://dx.doi.org/10.1007/s11064-006-9040-z> PMID: 16758355

- [20] Bavarsad Shahripour, R.; Harrigan, M.R.; Alexandrov, A.V. N-acetylcysteine (NAC) in neurological disorders: mechanisms of action and therapeutic opportunities. *Brain Behav.*, **2014**, 4(2), 108-122.
http://dx.doi.org/10.1002/brb3.208 PMID: 24683506
- [21] Arakawa, M.; Ito, Y. N-acetylcysteine and neurodegenerative diseases: basic and clinical pharmacology. *Cerebellum*, **2007**, 6(4), 308-314.
http://dx.doi.org/10.1080/14734220601142878 PMID: 17853088
- [22] Omara, F.O.; Blakley, B.R.; Bernier, J.; Fournier, M. Immunomodulatory and protective effects of N-acetylcysteine in mitogen-activated murine splenocytes *in vitro*. *Toxicology*, **1997**, 116(1-3), 219-226.
http://dx.doi.org/10.1016/S0300-483X(96)03520-2 PMID: 9020524
- [23] Tardiolo, G.; Bramanti, P.; Mazzon, E. Overview on the Effects of N-Acetylcysteine in Neurodegenerative Diseases. *Molecules*, **2018**, 23(12), 3305.
http://dx.doi.org/10.3390/molecules23123305 PMID: 30551603
- [24] Monti, D.A.; Zabrecky, G.; Kremens, D.; Liang, T-W.; Wintering, N.A.; Bazzan, A.J.; Zhong, L.; Bowens, B.K.; Chervoneva, I.; Intenzo, C.; Newberg, A.B. N-acetyl cysteine is associated with dopaminergic improvement in parkinson's disease. *Clin. Pharmacol. Ther.*, **2019**, 106(4), 884-890.
http://dx.doi.org/10.1002/cpt.1548 PMID: 31206613
- [25] Studer, R.; Baysang, G.; Brack, C. N-Acetyl-L-Cystein downregulates β -amyloid precursor protein gene transcription in human neuroblastoma cells. *Biogerontology*, **2001**, 2(1), 55-60.
http://dx.doi.org/10.1023/A:1010065103073 PMID: 11708617
- [26] Xu, Y.; Hou, X-Y.; Liu, Y.; Zong, Y.Y. Different protection of K252a and N-acetyl-L-cysteine against amyloid- β peptide-induced cortical neuron apoptosis involving inhibition of MLK3-MKK7-JNK3 signal cascades. *J. Neurosci. Res.*, **2009**, 87(4), 918-927.
http://dx.doi.org/10.1002/jnr.21909 PMID: 18951497
- [27] Zhang, Z.; Yan, J.; Taheri, S.; Liu, K.J. Hypoxia-inducible factor 1 contributes to N-acetylcysteine's protection in stroke. *Free Radic. Biol. Med.*, **2014**, 68, 8-21.
- [28] Tyers, M.; Mann, M. From genomics to proteomics. *Nature*, **2003**, 422(6928), 193-197.
http://dx.doi.org/10.1038/nature01510 PMID: 12634792
- [29] Ethel Daniela, C-R.; Víctor Manuel Torres-de la, C.; Catalina, R.-M.; Gloria María, M.-S.; María Adriana, N.-G.; María Julia, V.-S.; Catalina, L.-R. Proteomic analysis of a bioactive Aloe vera extract. *Curr. Proteomics*, **2019**, 16(3), 181-187.
- [30] Rajdeep, D.; Nisha, D.s.; Surya Kant, C.; Sethumadhavan, M.; Anura, V.K.; Amit Kumar, M. Analysis of extracellular proteome of staphylococcus aureus: a mass spectrometry based proteomics method of exotoxin characterisation. *Curr. Proteomics*, **2020**, 17(1), 3-9.
http://dx.doi.org/10.2174/1570164616666190204160627
- [31] Van-An, D.; Jeeyun, A.; Na-Young, H.; Jong-Moon, P.; Jeong-Hun, M.; Tae Wan, K. Proteomic analysis of the vitreous body in proliferative and non-proliferative diabetic retinopathy. *Curr. Proteomics*, **2020**, 17, 1-1.
- [32] Larance, M.; Lamond, A.I. Multidimensional proteomics for cell biology. *Nat. Rev. Mol. Cell Biol.*, **2015**, 16(5), 269-280.
http://dx.doi.org/10.1038/nrm3970 PMID: 25857810
- [33] Yuan, H.; Jiang, B.; Zhao, B.; Zhang, L.; Zhang, Y. Recent advances in multidimensional separation for proteome analysis. *Anal. Chem.*, **2019**, 91(1), 264-276.
http://dx.doi.org/10.1021/acs.analchem.8b04894 PMID: 30415533
- [34] Navarro, P.; Kuharev, J.; Gillet, L.C.; Bernhardt, O.M.; MacLean, B.; Röst, H.L.; Tate, S.A.; Tsou, C.-C.; Reiter, L.; Distler, U.; Rosenberger, G.; Perez-Riverol, Y.; Nesvizhskii, A.I.; Aebersold, R.; Tenzer, S. A multicenter study benchmarks software tools for label-free proteome quantification. *Nat. Biotechnol.*, **2016**, 34(11), 1130-1136.
http://dx.doi.org/10.1038/nbt.3685 PMID: 27701404
- [35] Reubsaet, L.; Sweredoski, M.J.; Moradian, A. Data-independent acquisition for the orbitrap q exactive hf: A tutorial. *J. Proteome Res.*, **2019**, 18(3), 803-813.
http://dx.doi.org/10.1021/acs.jproteome.8b00845 PMID: 30557026
- [36] Liu, Y.; Liu, K.; Wang, N.; Zhang, H. N acetylcysteine induces apoptosis via the mitochondria dependent pathway but not via endoplasmic reticulum stress in H9c2 cells. *Mol. Med. Rep.*, **2017**, 16(5), 6626-6633.
http://dx.doi.org/10.3892/mmr.2017.7442 PMID: 28901511
- [37] Arul, A-B.; Byambadorj, M.; Han, N-Y.; Park, J.M.; Lee, H. Development of an automated, high-throughput sample preparation protocol for proteomics analysis. *Bull. Korean Chem. Soc.*, **2015**, 36(7), 1791-1798.
http://dx.doi.org/10.1002/bkcs.10338
- [38] Joo, M.; Park, J-M.; Duong, V-A.; Kwon, D.; Jeon, J.; Han, M.; Cho, B-K.; Choi, H-K.; Lee, C-G.; Kang, H-G. H. Lee. An automated high-throughput sample preparation method using double-filtration for serum metabolite LC-MS analysis. *Anal. Methods*, **2019**, 11(31), 4060-4065.
http://dx.doi.org/10.1039/C9AY01137D
- [39] Yun, G.; Park, J-M.; Duong, V-A.; Mok, J-H.; Jeon, J.; Nam, O.; Lee, J.; Jin, E.; Lee, H. Proteomic profiling of *Emiliania huxleyi* using a three-dimensional separation method combined with tandem mass spectrometry. *Molecules*, **2020**, 25(13), 3028.
http://dx.doi.org/10.3390/molecules25133028 PMID: 32630776
- [40] Deutsch, E.W.; Mendoza, L.; Shteynberg, D.; Slagel, J.; Sun, Z.; Moritz, R.L. Trans-Proteomic Pipeline, a standardized data processing pipeline for large-scale reproducible proteomics informatics. *Proteomics Clin. Appl.*, **2015**, 9(7-8), 745-754.
http://dx.doi.org/10.1002/prca.201400164 PMID: 25631240
- [41] Keller, A.; Nesvizhskii, A.I.; Kolker, E.; Aebersold, R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal. Chem.*, **2002**, 74(20), 5383-5392.
http://dx.doi.org/10.1021/ac025747h PMID: 12403597
- [42] Nesvizhskii, A.I.; Keller, A.; Kolker, E.; Aebersold, R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem.*, **2003**, 75(17), 4646-4658.
http://dx.doi.org/10.1021/ac0341261 PMID: 14632076
- [43] MacLean, B.; Tomazela, D.M.; Shulman, N.; Chambers, M.; Finney, G.L.; Frewen, B.; Kern, R.; Tabb, D.L.; Liebler, D.C.; MacCoss, M.J. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics*, **2010**, 26(7), 966-968.
http://dx.doi.org/10.1093/bioinformatics/btq054 PMID: 20147306
- [44] Choi, M.; Chang, C-Y.; Clough, T.; Broudy, D.; Killeen, T.; MacLean, B.; Vitek, O. MSstats: an R package for statistical analysis of quantitative mass spectrometry-based proteomic experiments. *Bioinformatics*, **2014**, 30(17), 2524-2526.
http://dx.doi.org/10.1093/bioinformatics/btu305 PMID: 24794931
- [45] Liu, Z.; Lv, J.; Zhang, Z.; Li, H.; Yang, B.; Chen, W.; Dai, X.; Li, X.; Yang, S.; Liu, L.; Ou, L.; Ma, Y.; Zou, X. Integrative transcriptome and proteome analysis identifies major metabolic pathways involved in pepper fruit development. *J. Proteome Res.*, **2019**, 18(3), 982-994.
http://dx.doi.org/10.1021/acs.jproteome.8b00673 PMID: 30650966
- [46] Szklarczyk, D.; Gable, A.L.; Lyon, D.; Junge, A.; Wyder, S.; Huerta-Cepas, J.; Simonovic, M.; Doncheva, N.T.; Morris, J.H.; Bork, P.; Jensen, L.J.; Mering, C.V. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.*, **2019**, 47(D1), D607-D613.
http://dx.doi.org/10.1093/nar/gky1131 PMID: 30476243
- [47] Kanehisa, M.; Furumichi, M.; Tanabe, M.; Sato, Y.; Morishima, K. KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res.*, **2017**, 45(D1), D353-D361.
http://dx.doi.org/10.1093/nar/gkw1092 PMID: 27899662
- [48] Kanehisa, M.; Sato, Y.; Furumichi, M.; Morishima, K.; Tanabe, M. New approach for understanding genome variations in KEGG. *Nucleic Acids Res.*, **2019**, 47(D1), D590-D595.
http://dx.doi.org/10.1093/nar/gky962 PMID: 30321428
- [49] Kanehisa, M.; Sato, Y.; Kawashima, M.; Furumichi, M.; Tanabe, M. KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res.*, **2016**, 44(D1), D457-D462.
http://dx.doi.org/10.1093/nar/gkv1070 PMID: 26476454

- [50] Chen, D.; Shen, X. Strong cation exchange-reversed phase liquid chromatography-capillary zone electrophoresis-tandem mass spectrometry platform with high peak capacity for deep bottom-up proteomics. *Analytica Chimica Acta*, **2018**, *1012*, 1-9.
- [51] Duong, V.-A.; Park, J.-M.; Lee, H. Review of three-dimensional liquid chromatography platforms for bottom-up proteomics. *Int. J. Mol. Sci.*, **2020**, *21*(4), 1524.
<http://dx.doi.org/10.3390/ijms21041524> PMID: 32102244
- [52] Uhl, L.; Dukan, S. Hydrogen peroxide induced cell death: the major defences relative roles and consequences in *E.coli*. *PLoS One*, **2016**, *11*(8), e0159706.
<http://dx.doi.org/10.1371/journal.pone.0159706> PMID: 27494019
- [53] Sun, Y.; Pu, L.-Y.; Lu, L.; Wang, X.-H.; Zhang, F.; Rao, J.H. N-acetylcysteine attenuates reactive-oxygen-species-mediated endoplasmic reticulum stress during liver ischemia-reperfusion injury. *World J. Gastroenterol.*, **2014**, *20*(41), 15289-15298.
<http://dx.doi.org/10.3748/wjg.v20.i41.15289> PMID: 25386077
- [54] Faria, M.; Prats, E.; Gómez-Canela, C.; Hsu, C.-Y.; Arick, M.A., II; Bedrossiantz, J.; Orozco, M.; Garcia-Reyero, N.; Ziv, T.; Ben-Lulu, S.; Admon, A.; Gómez-Oliván, L.M.; Raldúa, D. Therapeutic potential of N-acetylcysteine in acrylamide acute neurotoxicity in adult zebrafish. *Sci. Rep.*, **2019**, *9*(1), 16467.
<http://dx.doi.org/10.1038/s41598-019-53154-w> PMID: 31712630
- [55] Lu, H.; Zhang, D.-M.; Chen, H.-L.; Lin, Y.-X.; Hang, C.-H.; Yin, H.-X.; Shi, J.X. N-acetylcysteine suppresses oxidative stress in experimental rats with subarachnoid hemorrhage. *J. Clin. Neurosci.*, **2009**, *16*(5), 684-688.
<http://dx.doi.org/10.1016/j.jocn.2008.04.021> PMID: 19264484
- [56] Nazıroğlu, M.; Şenol, N.; Ghazizadeh, V.; Yürüker, V. Neuroprotection induced by N-acetylcysteine and selenium against traumatic brain injury-induced apoptosis and calcium entry in hippocampus of rat. *Cell. Mol. Neurobiol.*, **2014**, *34*(6), 895-903.
<http://dx.doi.org/10.1007/s10571-014-0069-2> PMID: 24842665
- [57] Shahripour, R.B.; Maleki, A.H.Z.; Alexandrov, A.V. Application of N-Acetylcysteine in Neurological Disorders. In: *The Therapeutic Use of N-Acetylcysteine (NAC) in Medicine*; Frye, R.E.; Berk, M., Eds.; Springer Singapore, Singapore, **2019**; pp. 181-202.
http://dx.doi.org/10.1007/978-981-10-5311-5_11
- [58] Weidmann, E.; Brieger, J.; Jahn, B.; Hoelzer, D.; Bergmann, L.; Mitrou, P.S. Lactate dehydrogenase-release assay: a reliable, non-radioactive technique for analysis of cytotoxic lymphocyte-mediated lytic activity against blasts from acute myelocytic leukemia. *Ann. Hematol.*, **1995**, *70*(3), 153-158.
<http://dx.doi.org/10.1007/BF01682036> PMID: 7718644
- [59] Son, J.; Lyssiotis, C.A.; Ying, H.; Wang, X.; Hua, S.; Ligorio, M.; Perera, R.M.; Ferrone, C.R.; Mullarky, E.; Shyh-Chang, N.; Kang, Y.; Fleming, J.B.; Bardeesy, N.; Asara, J.M.; Haigis, M.C.; DePinho, R.A.; Cantley, L.C.; Kimmelman, A.C. Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. *Nature*, **2013**, *496*(7443), 101-105.
<http://dx.doi.org/10.1038/nature12040> PMID: 23535601
- [60] D'Aniello, A.; Fisher, G.; Migliaccio, N.; Cammisia, G.; D'Aniello, E.; Spinelli, P. Amino acids and transaminases activity in ventricular CSF and in brain of normal and Alzheimer patients. *Neurosci. Lett.*, **2005**, *388*(1), 49-53.
<http://dx.doi.org/10.1016/j.neulet.2005.06.030> PMID: 16039064
- [61] Zhou, X.; Curbo, S.; Li, F.; Krishnan, S.; Karlsson, A. Inhibition of glutamate oxaloacetate transaminase 1 in cancer cell lines results in altered metabolism with increased dependency of glucose. *BMC Cancer*, **2018**, *18*(1), 559-559.
<http://dx.doi.org/10.1186/s12885-018-4443-1> PMID: 29751795
- [62] Pérez-Mato, M.; Ramos-Cabrera, P.; Sobrino, T.; Blanco, M.; Ruban, A.; Mirelman, D.; Menendez, P.; Castillo, J.; Campos, F. Human recombinant glutamate oxaloacetate transaminase 1 (GOT1) supplemented with oxaloacetate induces a protective effect after cerebral ischemia. *Cell Death Dis.*, **2014**, *5*(1), e992-e992.
<http://dx.doi.org/10.1038/cddis.2013.507> PMID: 24407245
- [63] Schori, C.; Trachsel, C.; Grossmann, J.; Zygoula, I.; Barthelmes, D.; Grimm, C. The proteomic landscape in the vitreous of patients with age-related and diabetic retinal disease. *Invest. Ophthalmol. Vis. Sci.*, **2018**, *59*(4), AMD31-AMD40.
<http://dx.doi.org/10.1167/iovs.18-24122> PMID: 30025106
- [64] Cehofski, L.J.; Mandal, N.; Honoré, B.; Vorum, H. Analytical platforms in vitreoretinal proteomics. *Bioanalysis*, **2014**, *6*(22), 3051-3066.
<http://dx.doi.org/10.4155/bio.14.227> PMID: 25496257
- [65] Perez-Riverol, Y.; Csordas, A.; Bai, J.; Bernal-Llinares, M.; Hewapathirana, S.; Kundu, D.J.; Inuganti, A.; Griss, J.; Mayer, G.; Eisenacher, M.; Pérez, E.; Uszkoreit, J.; Pfeuffer, J.; Sachsenberg, T.; Yilmaz, S.; Tiwary, S.; Cox, J.; Audain, E.; Walzer, M.; Jarnuczak, A.F.; Ternent, T.; Brazma, A.; Vizcaino, J.A. The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res.*, **2019**, *47*(D1), D442-D450.
<http://dx.doi.org/10.1093/nar/gky1106> PMID: 30395289