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Original Article

Malate-aspartate shuttle mediates the intracellular ATP levels, antioxidation capacity and survival of differentiated PC12 cells

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Abstract: NAD^+ and NADH play pivotal roles in numerous redox reactions in cells. While increasing evidence has indicated important roles of NAD^+ in cell survival and cellular functions, there has been distinct deficiency in the studies regarding the biological functions of NADH. NADH shuttles mediate the transfer of the reducing equivalents of the cytosolic NADH into mitochondria. Cumulating evidence has suggested that malate-aspartate shuttle (MAS), one of the two types of NADH shuttles, plays significant roles in multiple biological processes such as glutamate synthesis in neurons. Because there has been no information regarding the roles of NADH shuttle in the energy metabolism, antioxidation capacity, and survival of any type of neural cells, in this study we used differentiated PC12 cells as a cellular model to investigate the roles of MAS in the energy metabolism, antioxidation capacity and survival of cells. We found that MAS inhibition led to a significant decrease in the levels of GSH – a major antioxidation molecule in cells, suggesting an important role of MAS in maintaining the antioxidation capacity of cells. Our study has also suggested that MAS could play critical roles in maintaining the intracellular ATP levels of the cells. Moreover, MAS inhibition was shown to significantly decrease the survival of differentiated PC12 cells. Collectively, our study has provided first evidence suggesting important roles of NADH shuttles in maintaining antioxidation capacity of cells. Our study has also suggested important roles of MAS in maintaining the intracellular ATP levels and survival of differentiated PC12 cells.

Keywords: NADH, malate-aspartate shuttle, PC12 cell, cell death, GSH, ATP

Introduction

NAD^+ and NADH (reduced form of NAD^+) are major coenzymes for the electron transfer in numerous biochemical reactions, which play fundamental roles in a number of biological functions including energy metabolism, mitochondrial functions, and calcium homeostasis [1]. Numerous studies have indicated that NAD^+ plays key roles in oxidative stress-induced cell death and such pathological processes as ischemic brain damage and traumatic brain damage [2]. While increasing evidence has indicated critical roles of NAD^+ in cell survival, there has been distinct deficiency in the studies regarding the roles of NADH in cell survival and cellular functions.

Previous studies have suggested that NADH may be used to decrease the symptoms of Parkinson's disease (PD) and Alzheimer's disease (AD) [3]. However, the mechanisms underlying the effects of NADH remain unclear. Our group has reported that NADH can be transported across the plasma membranes of astrocytes by P2X7 receptors [4], which can decrease poly(ADP-ribose) polymerase (PARP)-mediated cell death [5]. Moreover, our study has further indicated that NADH treatment can decrease the survival of C6 glioma cells by generating oxidative stress [6].

NADH shuttles mediate the transfer of the reducing equivalents of the cytosolic NADH into mitochondria [7]. There are two major types of

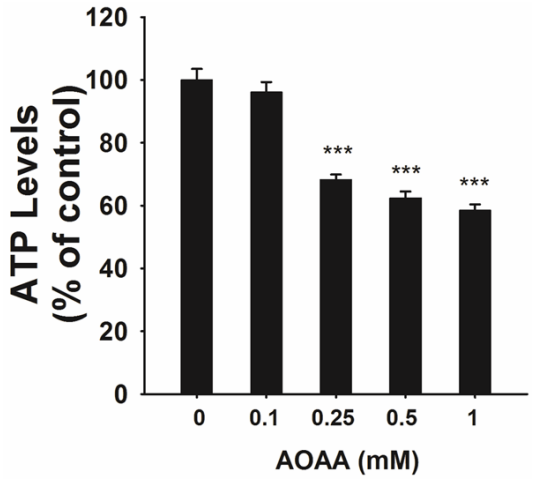

Figure 1. Aminooxyacetic acid (AOAA) treatment can significantly decrease the intracellular ATP levels of differentiated PC12 cells. AOAA can dose-dependently decrease the intracellular ATP levels of differentiated PC12 cells, assessed 3 hrs after the AOAA treatment. N = 11. Data were collected from three independent experiments. ***, $p < 0.001$.

NADH shuttles: Malate-aspartate shuttle (MAS) and glycerol-3-phosphate shuttle [1]. NADH shuttle may play critical biological roles due to the following reasons: First, cytosolic NADH levels mediate glycolysis, since increased cytosolic NADH/NAD^+ ratios can lead to inhibition of glycolysis [7]. Second, NADH is a key electron donor to mitochondrial electron transport chain, which is the major driving force for mitochondrial ATP generation [7]. Third, excessive cytosolic NADH may lead to generation of oxidative stress by providing substrate for NAD(P)H oxidase [8]. Fourth, cytosolic NADH can regulate cytosolic calcium concentrations by acting at IP_3-gated Ca^{2+} channels [9]. Cumulative evidence has suggested that MAS plays significant roles in multiple biological processes, including insulin secretion from β-islet cells, growth of breast cancer cells [10], and synthesis of glutamate and glutamine in neurons [11].

However, there has been little information regarding the roles of NADH shuttles in the antioxidation capacity, energy metabolism and survival of neural cells. In current study, we used differentiated PC12 cells as a cellular model to investigate the roles of NADH shuttles in the antioxidation capacity, energy metabolism and cell survival. Our study has suggested that MAS plays critical roles in maintaining the antioxidation capacity, intracellular ATP levels, and survival of differentiated PC12 cells.

Materials & methods

Materials

All chemicals were purchased from Sigma (St. Louis, MO, USA) except where noted.

Cell cultures

Differentiated PC12 cells were plated into 24-well cell culture plates at the initial density of 1×10^5 cells/mL in Dulbecco's Modified Eagle Medium containing 4,500 mg/L D-glucose, 584 mg/L L-glutamine, 110 mg/L sodium pyruvate (Thermo Scientific, Waltham, MA, USA), 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (PAA Laboratories, Austria). The cells were maintained at 37 °C in a 5% CO_2 incubator.

Intracellular lactate dehydrogenase (LDH) assay

Cell survival was quantified by measuring the intracellular LDH activity of the cells. In brief, cells were lysed for 20 min in lysing buffer containing 0.04% Triton X-100, 2 mM HEPES, 0.2 mM dithiothreitol, 0.01% bovine serum albumin (pH 7.5). Then 50 µl cell lysates were mixed with 150 µL 500 mM potassium phosphate buffer (pH 7.5) containing 1.5 mM NADH and 7.5 mM sodium pyruvate. The A_{340nm} change was monitored over 90 sec. Percentage of cell survival was calculated by normalizing the LDH values of samples to LDH activity measured in the lysates of control (wash only) culture wells.

ATP assay

Intracellular ATP levels were quantified using an ATP Bioluminescence Assay Kit (Roche Applied Science, Mannheim, Germany) following the standard protocol provided by the vendor. In brief, after washed once with PBS, the cells were lysed with the Cell Lysis Reagent, which was mixed with 50 µL of the Luciferase Reagent. A plate reader (Biotek Synergy 2) was used to detect the chemiluminescence of the samples. The ATP concentrations of the samples were calculated using an ATP standard, and normalized to the protein concentrations of the sam-

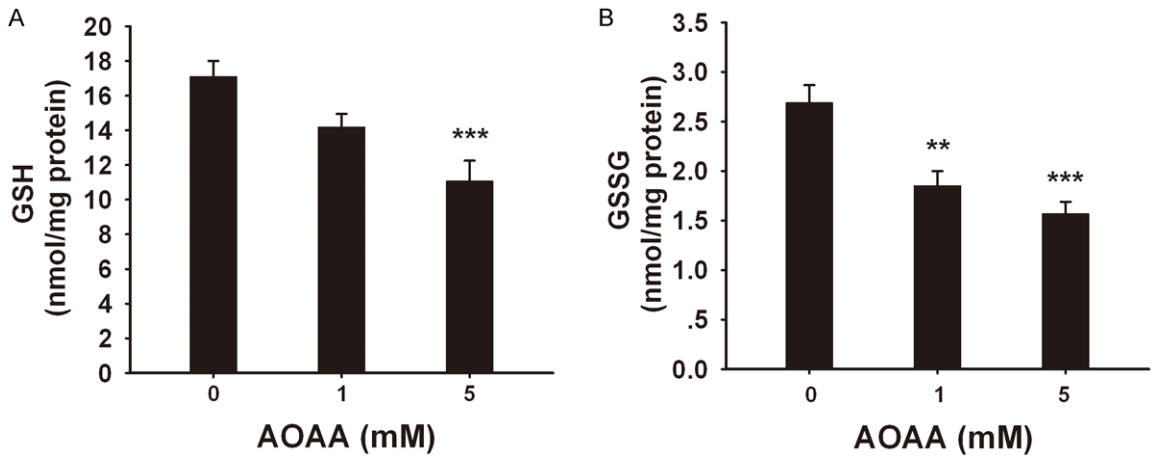

Figure 2. AOAA treatment can significantly decrease the GSH levels of differentiated PC12 cells. AOAA can dose-dependently decrease the levels of both GSH (A) and GSSG (B) of differentiated PC12 cells, assessed 6 hrs after the AOAA treatment. N = 7 - 10. Data were collected from three independent experiments. *, $p < 0.05$; **, $p < 0.05$; ***, $p < 0.001$. The comparisons were made between AOAA-treated samples and controls.

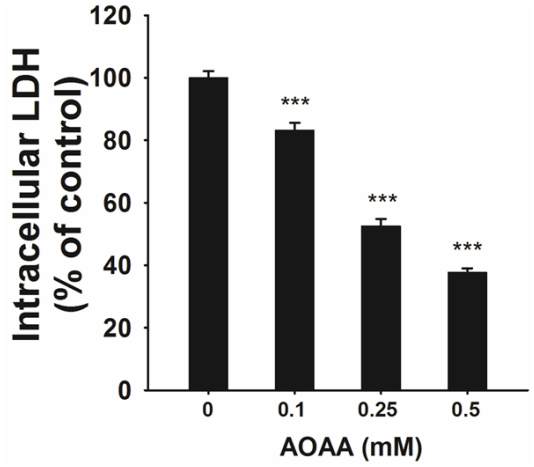

Figure 3. AOAA treatment can significantly decrease the intracellular lactate dehydrogenase (LDH) levels of differentiated PC12 cells. AOAA can dose-dependently decrease the intracellular LDH levels of differentiated PC12 cells, assessed 24 hrs after the AOAA treatment. N = 12. Data were collected from three independent experiments. ***, $p < 0.001$. The comparisons were made between AOAA-treated samples and controls.

ples, which were determined using the BCA assay.

GSH/GSSG determinations

Antioxidation capacity was assessed by measuring the intracellular GSH/GSSG levels of the cells. By using a GSH and GSSG Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China), we measured the concentrations of total gulutathione (T-GSH), reduced glutathione (GSH) and oxidized glutathione (GSSG) of the cells. The concentration of T-GSH was determined by the 5,5-dithio-bis(2-nitrobenzoic) acid-glutathione disulfide (DTNB-GSSG) reductase recycling. After GSH of a sample was removed, the GSSG of the sample was reduced to GSH by glutathione reductase, which was assessed by measuring 5-thio-2-nitrobenzoic acid (TNB) produced from the reaction of GSH with 5,5-dithio-bis(2-nitrobenzoic) acid (DTNB). The rate of TNB formation was measured by a plate reader at the wavelength of 412 nm over 15 min. The GSH concentration of the sample was determined by subtraction of GSSG from T-GSH.

Flow cytometry-based annexin V/7-AAD assay

Flow cytometry assay was performed to determine the levels of early-stage apoptosis, late-stage apoptosis, and necrosis using ApoScreen Annexin V kit (SouthernBiotech, Birmingham, AL, USA) according to the manufacturer's protocol. Briefly, differentiated PC12 cells were digested by 0.25% trypsin-EDTA, washed by cold PBS one time and resuspended in cold 1× binding buffer at concentrations between 1×10^6 and 1×10^7 cells/ml. Five μL of labeled Annexin V was added into 100 μL of the cell suspension. After incubation on ice for 15 min, 200 μL binding buffer and 5 μL 7-AAD solution

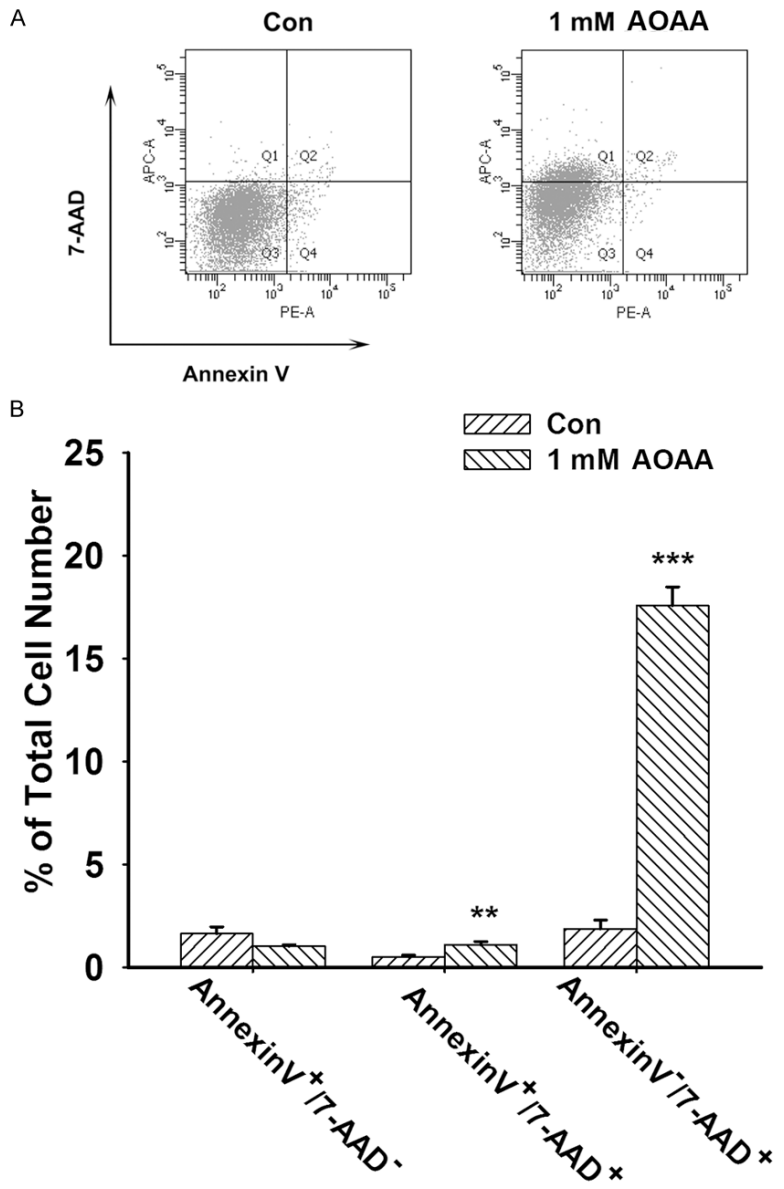

Figure 4. AOAA treatment can significantly increase necrosis of differentiated PC12 cells. A: Flow-cytometry-based assay showed that AOAA induced an increase in the number of AnnexinV$^-$/7-AAD$^+$ cells (necrotic cells). In the four fields of the original images from the flow cytometry-based study, the number of the dots indicates the number of AnnexinV$^-$/7-AAD$^-$ (the bottom-left field), AnnexinV$^+$/7-AAD$^-$ (the bottom-right field), AnnexinV$^-$/7-AAD$^+$ (the top-left field), and AnnexinV$^+$/7-AAD$^+$ cells (the top-right field), respectively. The dots in these four fields indicate the number of normal cells, cells in early-stge apoptosis, cells in late-stage apoptosis, and necrotic cells, respectively. B: Quantifications of the results from the flow-cytometry-based study showed that the AOAA treatment induced a significant increase in the number of AnnexinV$^-$/7-AAD$^+$ cells, assessed 24 hrs after the AOAA treatment. N = 15. Data were collected from three independent experiments. ***, $p < 0.001$. The comparisons were made between AOAA-treated samples and controls.

were added into the cell suspensions. The number of stained cells was assessed immediately by a flow cytometer (FACSAria II, BD Biosciences).

Statistical analyses

All data are presented as mean ± SE. Data were assessed by one-way ANOVA, followed by Student-Newman-Keuls post hoc test. *P* values less than 0.05 were considered statistically significant.

Results

Aminooxyacetic acid (AOAA) treatment can decrease the intracellular ATP levels of differentiated PC12 cells

Aminooxyacetate (AOAA) is a most widely used, specific inhibitor of MAS, which inhibits the shuttle by inhibiting aspartate aminotransferase - a key enzyme of the shuttle [12, 13]. We determined the effects of AOAA treatment on the intracellular ATP levels of differentiated PC12 cells, showing that AOAA dose-dependently decreased the intracellular ATP levels of PC12 cells, assessed 3 hrs after the AOAA treatment (**Figure 1**).

AOAA treatment can decrease the intracellular GSH and GSSG levels of differentiated PC12 cells

We also determined the effects of AOAA on the GSH/GSSG levels differentiated PC12 cells. We found that AOAA dose-dependently decreased the levels of both GSH and GSSG of differentiated PC12 cells, assessed 24 hrs after the treatment (**Figure 2**).

AOAA treatment can decrease the intracellular LDH levels of differentiated PC12 cells

We conducted intracellular LDH assay to assess the effects of AOAA on the survival of

differentiated PC12 cells. We found that AOAA dose-dependently decreased the intracellular LDH levels of differentiated PC12 cells, assessed 24 hrs after the treatment (**Figure 3**). We also determined the effects of AOAA treatment on both apoptosis and necrosis of the cells by applying flow cytometry-based annexin V/7-AAD assay. Our assay showed that AOAA induced a significant increase in the number of $AnnexinV^{-}/7\text{-}AAD^{+}$ cells, indicating that AOAA can significantly induce necrosis of the cells (**Figure 4A** and **4B**).

Discussion

The major findings of our study include: First, MAS could play an important role in maintaining the GSH levels of the cells; second, MAS also playWs significant role in maintaining the intracellular ATP levels of differentiated PC12 cells; and third, MAS is of significance for maintaining the survival of differentiated PC12 cells. To our knowledge, this is the first report suggesting an important role of NADH shuttles in the antioxidation capacity of any type of cells. Our study has also suggested important roles of MAS in maintaining both the intracellular ATP levels and survival of differentiated PC12 cells.

Previous studies have suggested that NADH may be used to decrease the symptoms of PD and AD [3], while the mechanisms remain unclear. Our group has reported that NADH can decrease PARP-mediated cell death [5]. However, there has been only insufficient information regarding the roles of NADH in cell survival and cellular functions. Cumulating evidence has suggested that MAS plays significant roles in several biological processes, including insulin secretion from β-islet cells [14], neurotransmitter synthesis [11] and survival of breast cancer cells [10]. However, there has been little information regarding the roles of NADH shuttles in the antioxidation capacity, energy metabolism and survival of neural cells.

Our study has suggested that MAS could play critical roles in maintaining the intracellular ATP levels of PC12 cells. Because ATP plays fundamental roles in cell survival and numerous biological functions such as protein phosporylation and ion transport [15, 16], our study has provided first evidence suggesting that MAS is actively involved in maintaining the energy metabolism of differentiated PC12 cells.

We found that MAS inhibition also leads to decreased level of GSH – a major antioxidation molecule in cells. This observation has provided first evidence suggesting that MAS plays a significant role in antioxidation capacity of any type of cells. Because oxidative stress plays crucial roles in the tissue injury of various neurological disorders, including brain ischemia and Parkinson's disease [8, 17], our study has suggested that MAS may become a novel target for modulating the antioxidation capacity of cells. Because excessive cytosolic NADH may lead to generation of oxidative stress by providing substrate for NAD(P)H oxidase [8], MAS inhibition might lead to decreased GSH levels by producing increased cytosolic NADH. Future studies are warranted to investigate the mechanisms underlying the effects of MAS inhibition on the GSH levels of the cells.

Our study has also suggested that MAS could play a critical role in maintaining the survival of differentiated PC12 cells. Because decreased ATP plays a significant role in cell survival [15, 16], the AOAA-induced ATP decrease (**Figure 1**) may contribute to the AOAA-induced decrease in cell survival (**Figure 3**). Because multiple mitochondrial proteins such as caspase-3, AIF, apaf-1 play important roles in cell death, MAS inhibition-produced blockage of NADH transfer from cytosol into mitochondria might affect the cell survival by producing alterations of mitochondrial functions.

Aminooxyacetate (AOAA) is a most widely used specific inhibitor of MAS, which inhibits the shuttle by inhibiting the key enzyme of the shuttle - aspartate aminotransferase [12, 13]. It has been found that AOAA is specific for aspartate aminotransferase, particularly when GABAnergic neurons are not present [13]. Since there are no GABAnergic neurons present in our study, AOAA has high specificity for MAS inhibitor in our study. In particular, in our study AOAA becomes effective in affecting the intracellular ATP levels and apoptosis at concentrations below 1 mM, which further raises the specificity of the drug on aspartate aminotransferase [13].

In summary, our study has provided first evidence suggesting an important role of MAS in maintaining antioxidation capacity of cells. Our study has also suggested important roles of MAS in maintaining both the intracellular ATP levels and survival of differentiated PC12 cells.

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References

[1] Ying W. NAD^+/NADH and $NADP^+$/NADPH in cellular functions and cell death: regulation and biological consequences. Antioxid Redox Signal 2008; 10: 179-206.

[2] Ma Y, Chen H, He X, Nie H, Hong Y, Sheng C, Wang Q, Xia W and Ying W. NAD^+ Metabolism and NAD^+-Dependent Enzymes: Promising Therapeutic Targets for Neurological Diseases. Curr Drug Targets 2012; 13: 222-229.

[3] Ying W. NAD^+ and NADH in brain functions, brain diseases and brain aging. Front Biosci 2007; 12: 1863-1888.

[4] Lu H, Burns D, Garnier P, Wei G, Zhu K and Ying W. P2X7 receptors mediate NADH transport across the plasma membranes of astrocytes. Biochem Biophys Res Commun 2007; 362: 946-950.

[5] Zhu K, Swanson RA and Ying W. NADH can enter into astrocytes and block poly(ADP-ribose) polymerase-1-mediated astrocyte death. Neuroreport 2005; 16: 1209-1212.

[6] Ma Y, Chen H, Xia W and Ying W. Oxidative stress and PARP activation mediate the NADH-induced decrease in glioma cell survival. Int J Physiol Pathophysiol Pharmacol 2011; 3: 21-28.

[7] Stryer L. Biochemistry. New York: W.H. Freeman and Company; 1995.

[8] Ying W and Xiong ZG. Oxidative stress and NAD^+ in ischemic brain injury: current advances and future perspectives. Curr Med Chem 2010; 17: 2152-2158.

[9] Bickler PE, Fahlman CS, Gray J and McKleroy W. Inositol 1,4,5-triphosphate receptors and NAD(P)H mediate Ca^{2+} signaling required for hypoxic preconditioning of hippocampal neurons. Neuroscience 2009; 160: 51-60.

[10] Thornburg JM, Nelson KK, Clem BF, Lane AN, Arumugam S, Simmons A, Eaton JW, Telang S and Chesney J. Targeting aspartate aminotransferase in breast cancer. Breast Cancer Res 2008; 10: R84.

[11] Pardo B, Contreras L and Satrustegui J. Synthesis of Glial Glutamate and Glutamine in Young Mice Requires Aspartate Provided by the Neuronal Mitochondrial Aspartate-Glutamate Carrier Aralar/AGC1. Front Endocrinol (Lausanne) 2013; 4: 149.

[12] Fitzpatrick SM, Cooper AJ and Duffy TE. Use of beta-methylene-D,L-aspartate to assess the role of aspartate aminotransferase in cerebral oxidative metabolism. J Neurochem 1983; 41: 1370-1383.

[13] McKenna MC, Waagepetersen HS, Schousboe A and Sonnewald U. Neuronal and astrocytic shuttle mechanisms for cytosolic-mitochondrial transfer of reducing equivalents: Current evidence and pharmacological tools. Biochem Pharmacol 2006; 71: 399-407.

[14] Rubi B, del Arco A, Bartley C, Satrustegui J and Maechler P. The malate-aspartate NADH shuttle member Aralar1 determines glucose metabolic fate, mitochondrial activity, and insulin secretion in beta cells. J Biol Chem 2004; 279: 55659-55666.

[15] Eguchi Y, Shimizu S and Tsujimoto Y. Intracellular ATP levels determine cell death fate by apoptosis or necrosis. Cancer Res 1997; 57: 1835-1840.

[16] Nikoletopoulou V, Markaki M, Palikaras K and Tavernarakis N. Crosstalk between apoptosis, necrosis and autophagy. Biochim Biophys Acta 2013; 1833: 3448-3459.

[17] Beal MF. Mitochondria, oxidative damage, and inflammation in Parkinson's disease. Ann N Y Acad Sci 2003; 991: 120-131.