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# Time course study of Aβ formation and neurite outgrowth disruption in differentiated human neuroblastoma cells exposed to $H_2O_2$: Protective role of autophagy

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ABSTRACT

Here, we tried to elucidate the possible role of autophagy against $H_2O_2$ and Amyloid beta (Aβ) induced neurotoxicity using retinoic acid differentiated SH-SY5Y cells. We found that $H_2O_2$ disrupted neurite outgrowth concomitant with production of Aβ. Furthermore, we showed that $H_2O_2$ could increase the apoptotic factors such as Bax/Bcl-2 ratio, caspase-3 level, and PARP activity in a time course manner. These findings were confirmed by acridine orange/ethidium bromide and Hoechst staining. In addition, we observed that $H_2O_2$ led to conversion of LC3 protein from LC3I to LC3II and an increase in autophagy flux. Autophagy factors including LC3B, Atg7, and Atg12 increased and reached their highest level after 2 h of insulting and then dropped to a lower level. Our results showed that autophagy could internalize and degrade intra- and extracellular Aβ after 3 h treatment with $H_2O_2$. However, the remaining amount of Aβ accelerated morphological atrophy and, as a result, increased neuronal death (apoptosis). Inhibition of autophagy influx, using 3-methyl-adenine, increased intra- and extracellular levels of Aβ, providing more proof for a protective role of autophagy against oxidative stress. Further studies can shed light on the important role of autophagy by finding new pathways involved in Aβ degeneration.



## 1. Introduction

The role of Amyloid beta (Aβ) peptide in neurodegeneration is extremely well studied. Aβ peptide is generated via the amyloidogenesis pathway from amyloid precursor protein. Aβ can disrupt the neuronal excitability (Mattson et al., 1993), synaptic plasticity (Turner et al., 2003), axonal growth, branching (Ikin et al., 2007; Moya et al., 1994), and dendritic growth (Mattson, 1997). Since the level of Aβ increases, in Alzheimer's disease (AD), it would be interesting to study the role of this peptide in neuronal system and determine the consequences of its synthesis and degeneration.

In addition, disruption of neurite outgrowth and neuronal degeneration occur during oxidative stress (de la Monte et al., 2000; Khodagholi et al., 2012; Raff et al., 2002). Many studies have demonstrated that neuronal changes in frontal and temporal cortex may initially lead to a mild cognitive impairment and severe memory loss (Naslund et al., 2000). So, promoting neurite outgrowth may have the potential of to be therapeutic matter for patients with neurodegenerative diseases like AD. It is widely accepted that Aβ can induce neurite degeneration followed by programmed cell death (PCD). Besides, some experiments have shown that aggregated form of Aβ had no detrimental effect on neurite outgrowth (Koo et al., 1993). In contrast, it has been demonstrated that soluble Aβ stimulates neuronal degeneration and also reduces neurite outgrowth (Koo et al., 1993; Novitskaya et al., 2006). A growing body of evidence indicates some protective pathways against degeneration in the neuronal cells such as antioxidant, inflammatory, and autophagic pathways which have crucial roles in cell survival (Alirezaei et al., 2011; Rafatian et al., 2012; Rao et al., 2010; White et al., 2010). Autophagy is one of the most interesting neuroprotective pathways which have a controversial role against stress conditions (Vellai et al., 2007). Some studies have

*Abbreviations:* AD, Alzheimer's disease; Aβ, Amyloid-β; AO/EB, acridine orange/ethidium bromide; Atg, autophagy related-gene; ECL, electrochemiluminescence; FBS, fetal bovine serum; $H_2O_2$, hydrogen peroxide; 3MA, 3-methyladenine; LC3B, microtubule-associated protein light chain 3B; MDC, Monodansylcadaverine; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium a bromide; PARP, Poly ADP-ribose polymerase; PCD, programmed cell death; RA, retinoic acid; ROS, reactive oxygen species.

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shown a neuroprotective role for autophagy in mediating the degradation of Aβ (Agholme et al., 2010; Pickford et al., 2008). Also, it is widely regarded as an intracellular hub for the removal of harmful Aβ peptides, but some studies suggested that inhibition of autophagy or knocking down *Atg* genes could protect neurons against degeneration (Tsujimoto and Shimizu, 2005). In the same line of results, some data are available showing an unfavorable function of autophagy in facilitating the production of intracellular Aβ. The two faces of autophagy in the homeostasis of Aβ place it in a very unique and intriguing position in AD pathogenesis. Moreover, there are multiple connections between apoptosis and autophagy, which together determine cells fate (Moretti et al., 2007). Although studies have pointed out that apoptosis and autophagy may have an interconnection and can be regulated by the same triggers, but recently it has been reported that autophagy can resist or suspend apoptosis.

Recently, treatment strategies have been planned to target Aβ production. For this purpose, some studies have investigated the effect of inhibitors of β- and γ-secretase pathways (Cai et al., 2001; Dovey et al., 2001). However, the ability of such inhibitors which are able to cross the blood–brain barrier is challenging. Thus, focusing on attenuating Aβ production through intrinsic molecules and pathways is one of the major interests in the recent decades. In the present study, we investigated the controversial role of the autophagy against $H_2O_2$ and Aβ toxicity in RA (retinoic acid)-differentiated SH-SY5Y cells, focusing on the role of oxidative stress in neuronal outgrowth.

We used differentiated SH-SY5Y cells by the reason that Misonou and colleagues' works revealed that exposure of cultured human neuroblastoma cells to hydrogen peroxide could result in an increase in intracellular Aβ (Misonou et al., 2000). This paradigm would closely mimic the conditions in AD brain tissue. Moreover, we demonstrated the effects of programmed cell death type I and type II pathways by detecting both intracellular and extracellular Aβ levels in a time-dependent manner.

## 2. Materials and methods

### 2.1. Materials

Antibodies directed against caspase-3, Poly (ADP-ribose) polymerase (PARP-1), Bax, Bcl-2, Atg7 (Autophagy related-gene-7), Atg12, LC3B (light chain 3), and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA). ELISA assay kit for Aβ42 detection was purchased from Wako Chemicals (Richmond, Virginia, USA). Electrochemiluminescence (ECL) kit was provided from Amersham Bioscience (Piscataway, NJ, USA). Fetal Bovine Serum (FBS) was obtained from Gibco (Big Cabin, Oklahoma, USA), and polyvinylidene fluoride membrane was purchased from Chemicon Millipor (Temecula, CA, USA). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), Retinoic acid (RA), Dulbecco's modified Eagle's medium (DMEM/F12), and 3-methyladenine (3MA) were provided from Sigma Aldrich (St. Louis, MO). Hoechst dye was obtained from Invitrogen (Carlsbad, California, US).

### 2.2. Cell culture and differentiation

SH-SY5Y neuroblastoma cells, obtained from Pasteur Institute (Tehran, Iran), were grown in DMEM/F12 (1/1) medium with L-glutamine, supplemented with 10% fetal bovine serum and 1% antibiotic mixture comprising penicillin and streptomycin, in a humidified atmosphere at 37 °C with 5% $CO_2$. For differentiation, twenty-four hours after seeding, the serum levels of the medium were reduced to 3% with RA (10 mM) for ten days prior to our treatment.

### 2.3. Drug treatment conditions

SH-SY5Y cells, planted in 75 $cm^2$ culture flasks, were incubated with $H_2O_2$ (300 μM) for different periods of time in the presence and/or absence of 3MA (10 mM). 3MA was added to culture flasks 30 min before induction of $H_2O_2$ exposure.

### 2.4. Measurement of cell viability

Conventional MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) reduction assay was conducted to measure the cell viability. The dark blue formazan crystals formed by the oxidation of MTT dye. After being solubilized in DMSO, the absorbance was measured at 550 nm, assuming the absorbance of control group cells as 100%.

### 2.5. Morphological analysis of differentiated SH-SY5Y cells

Random images were acquired from each well, taking three images per well for morphological analysis. In our study, a minimum of 50 cells per treatment were quantified in at least three independent experiments. Three parameters were quantified, including: the total number of neurites (μm), number of neurites per cell, and nodes per cell. Neurite length was calculated by summing the lengths of the primary processes with all associated branches and distinct protrusions from the cell body greater than 10 μM. The mean of neurite length was measured as a ratio of total neurite length and number of neurites. All measurements are presented as proportions using the number of cells displaying the characteristic as a sub-population of the total number of cells. To evaluate neurite networks, images were analyzed by cell counter plugin to score all branching nodes in each image. Data analysis was done by Cell^ A program.

### 2.6. Staining dye protocols for detecting apoptosis and autophagy

Apoptosis was evaluated morphologically using Hoechst 33342 and acridine orange/ethidium bromide (AO/EB) staining, followed by fluorescence microscopy inspection. In brief, differentiated SH-SY5Y cells ($1 \times 10^6$ cells/ml) were seeded in a 6-well plate and treated with $H_2O_2$ (300 μM) for different time periods (15 min, 30 min, 1 h, 2 h, 3 h, 4 h). In the next step, Hoechst 33342 (1 μg/ml) and/or AO/EB solution (1:1 v/v) were added to the cells at room temperature for 5 min. At the end, nuclear morphological changes were visualized using fluorescence microscope (Olympus IX71, Japan).

Autophagic vacuoles were detected with MDC and acridine orange staining as described previously (Biederbick et al., 1995; Kim et al., 2007). Briefly, differentiated SH-SY5Y cells were cultured in a 6-well plate and treated with $H_2O_2$ (300 μM). After different times intervals (15 min, 30 min, 1 h, 2 h, 3 h, 4 h), the cells were incubated with MDC (0.05 mM) and/or acridine orange (1 μg/ml) in PBS at 37°C for 10 min. Upon that, the cells were harvested and washed three times with PBS and were adjusted to a density of $10^6$ cells/ml of PBS. The cells were immediately analyzed by fluorescence microscope.

### 2.7. Western blot analysis

Analysis of specific proteins was performed using Western blotting. The cells were lysed in buffer comprising a complete protease inhibitor cocktail. Protein contents of cell lysate were determined according to Bradford's method (Bradford, 1976). Lysates