The effects of O-GlcNAc alteration on Alzheimer-like neurodegeneration in SK-N-SH cells

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Background. O-GlcNAcylation is a highly dynamic post-translational modification that plays a key role in regulating phosphorylation of protein and cell survival. The proteins O-GlcNAcylation level is regulated dynamically by O-GlcNAc transferase (OGT) and β-N-acetylglucosaminidase (O-GlcNAcase, OGA). Although previous studies have suggested the role of O-GlcNAcylation in neurodegenerative diseases, the mechanism of O-GlcNAcylation in Alzheimer's disease (AD) remains unclear.

Methods. The decrease of O-GlcNAcylation by alloxan, an OGT inhibitor, and increase by NAG-thiazolines (NAG-Ae), an O-GlcNAcase inhibitor were tested to investigate the effects of O-GlcNAc alteration on AD like neurodegeneration in SK-N-SH cells.

Results. The level of O-GlcNAcylation was decreased in alloxan treated cells and increased in NAG-Ae treated cells. Meanwhile, it was observed that both abnormal phosphorylation of NFs in cell bodies and apoptosis induced by alloxan treatment can be resisted by pretreatment or simultaneous treatment with appropriate NAG-Ae.

Conclusion. These results demonstrated that increasing O-GlcNAc with NAG-Ae protected AD like neurodegeneration from NFs hyperphosphorylation and the cell loss, suggesting the role of O-GlcNAc in the pathogenesis and therapy of AD.

Key words: O-GlcNAcylation; O-GlcNAcase; O-GlcNAc transferase; neurofilament phosphorylation; Alzheimer's disease

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INTRODUCTION

Alzheimer's disease (AD) is a common neurodegenerative disease. In AD brain, the aggregation of abnormally hyperphosphorylated NFs into neurofibrillary tangles and extensive neuronal loss are the two major characteristics[1-3].

Neurofilaments (NFs) are the intermediate filaments (10 nm diameter) and the most abundant cytoskeletal components, which are assembled from three NF subunits, termed light (NF-L), medium (NF-M) and heavy (NF-H) chains[4]. The hyperphosphorylation and accumulation of NFs are the AD early pathological change contributing to the retrograde degeneration of neurons. In addition to phosphorylation, NFs are also competitively modified by O-GlcNAcylation, a special type of O-glycosylation by which β-N-acetylglucosamine (GlcNAc) is linked to the serine or threonine residues via an O-linked glycosidic bond[5-8]. Our previous observations that phosphorylation of NFs was inversely regulated by O-GlcNAcylation along with NFs decreased O-GlcNAcylation in AD brain suggest that decrease of O-GlcNAcylation may contributed to the hyperphosphorylation of NFs in neurodegeneration. The O-GlcNAc transferase (OGT) transfering O-GlcNAc and the β-N-acetylglucosaminidase (O-GlcNAcase) cleaving the glycosidic bond are the two important enzymes responsible for the dynamic cycling of O-GlcNAc (ref.[9]).

However, up to now, the role of O-GlcNAcylation in nervous system and O-GlcNAcylation of NFs in SK-N-SH cells are little known and need further research. In this study, we tried to decrease the O-Glycosylation by alloxan, an OGT inhibitor[10] and increase that by NAG-thiazolines (NAG-Ae), an O-GlcNAcase inhibitor[11], to investigate the effects of O-GlcNAc alteration on AD like hyperphosphorylation and cell loss in neuroblastoma SK-N-SH cells. Our results demonstrated that increasing O-GlcNAcylation with NAG-Ae played a protective role in AD like neurodegeneration including NFs hyperphosphrylation and the cell loss. These findings provide a potential therapeutic approach for AD by inhibiting O-GlcNAc removal.

MATERIALS AND METHODS

Cell culture

Human SK-N-SH neuroblastoma cells were cultured in DMEM/F12 (1:1) Medium (Gibco, MD, USA) supplemented with 10% fetal bovine serum, 100 IU/mL Penicillin, 100 IU/mL streptomycin at 37 °C at 5% CO_2 atmosphere.

Western Blots

Protein concentrations were measured by the BCA protein Assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Proteins from cells were separated by SDS-PAGE and transferred onto a PVDF membrane. After the block with 5% milk for 30 min, the membranes were incubated with primary antibodies SMI31 (Sternberger Monoclonals, MD, USA), RL2 (abcam, Cambridge, MA, USA), R61d (Institute for Basic Research, New York, USA) against NFs and actin (Beyotime Institute of Biotechnology, Shanghai, China) overnight at 4°C, and then incubated with secondary antibodies for 2 hours at room temperature. After washing with PBS, the membrane was reacted with BeyoECL Plus reagent and was then scanned. The intensities of the bands were measured using Image J analysis software.

Immunofluorescence

The SK-N-SH cells plated on poly L-lysine-coated 8-chamberred glass slides were subjected to various treatment. One was that different concentration of alloxan (0, 4, 6 mM) was added to each chamber and incubated at 37 °C for 12 h. And for another, the cells was preincubated 20μM NAG-Ae for 12 h before 6mM alloxan was added. Then, cells were fixed for 20 min in 10% neutral formaldehyde on ice. After permeabilization with 0.5% Triton X-100 for 20 min, cells were treated with 3% hydrogen peroxide to block endogenous peroxidase and incubated with 5% BSA for 20 min. Then, cells were incubated with primary antibodies SMI31/NF160, RL2/NF160 at 4 °C overnight. After that, cells were washed with PBS and incubated with secondary antibodies, FITC-labeled goat anti- mouse IgG, Cy3-labeled goat anti-rabbit IgG (Invitrogen, NM, USA) for 30 min at 37 °C. Then the glides were covered with anti-fade mounting medium.

PI/Hoechst staining

PI/Hoechst staining was used to assess cell death. SK-N-SH cells were plated in an 8-chamberred glass slides at a density of 2×10^4/mL. When the cell quantity reached to 50%-60% per chammber, alloxan and/or NAG-Ae were added to each chamber and incubated at 37 °C. After culture, the medium was removed and 5 μL Hoechst/PI (propidium iodide) staining solution was added in proper order, incubated at 4 °C for 30 min and then observed under fluorescence microscope.

DAPI staining

The SK-N-SH cells were plated at a density of 5×10^3 cells in 8-chamberred glass slides coated with poly-D-Lysine. At 24 h after plating, alloxan and/or NAG-Ae were added to each chamber and incubated at 37 °C. After incubation, cells were fixed in 10% neutral formaldehyde on ice for 20 min and then 100 ng/mL DAPI (4', 6-di-

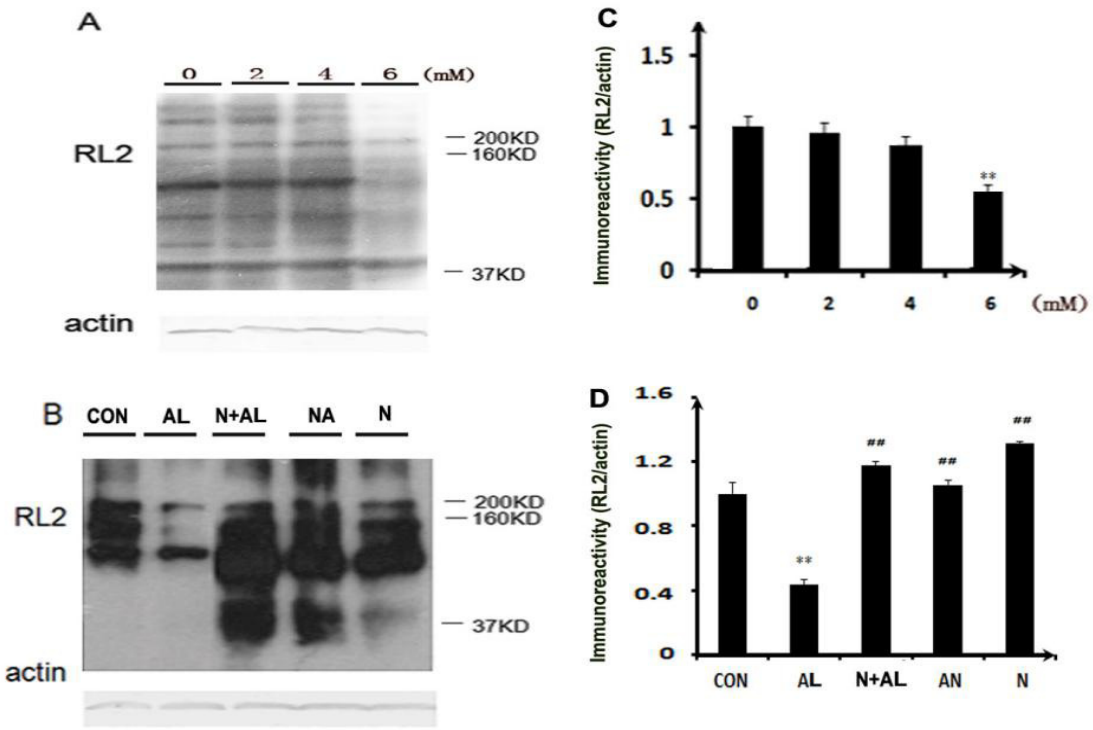

Fig. 1. The O-GlcNAc in alloxan or/and NAG-Ae treated SK-N-SH cells.
A. Representative blots of cells treated with different concentration of alloxan (AL) (0, 2, 4, 6 mM) and with specific monoclonal antibodies of RL2 and actin recognized the O-Glycosylation and β-actin.
B. Representative O-GlcNac blots of cells pretreatment with 20 μM NAG-Ae (N+AL) or treated with 20 μM NAG-Ae and 6mM alloxan (NA) at the same time with antibodies RL2 and β-actin.
C. and D. The quantities assay of blot A. and B.
**indicated $P<0.01$ compared to the control cells. $^{\#\#}P<0.01$ compared to alloxan treated cells for the same treatment time.

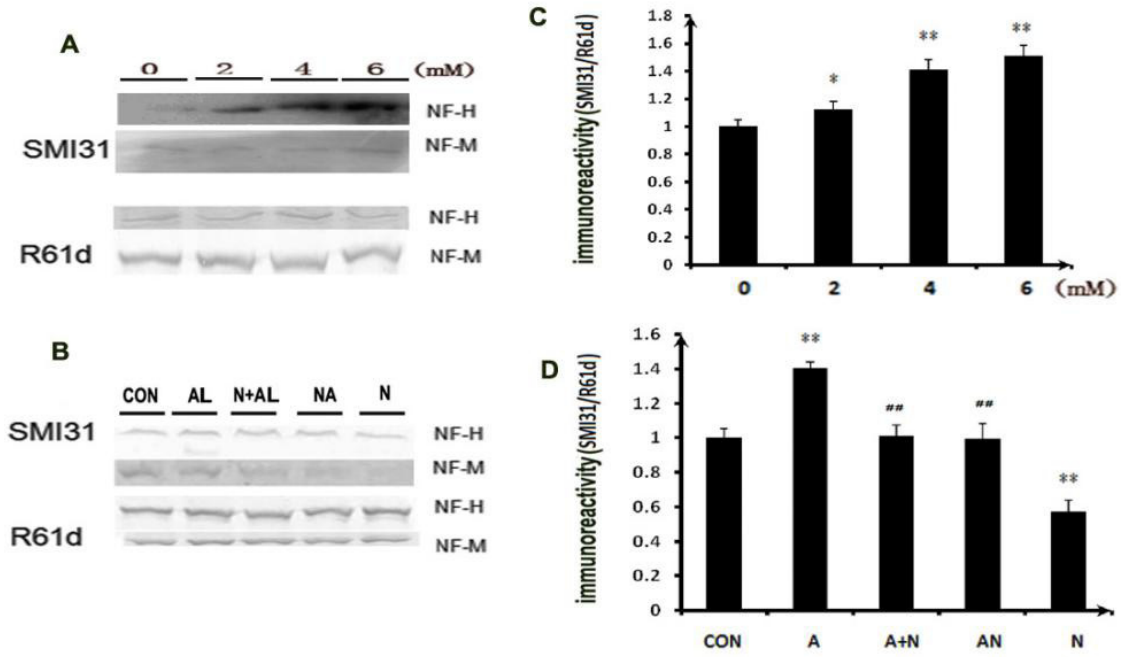

Fig. 2. The abnormal phosphorylation of NFs and accumulation in SK-N-SH cells.
A. Representative blots of cells treated with different concentration of alloxan (AL) (0, 2, 4, 6 mM) with specific monoclonal antibodies of SMI31 and R61d recognized the phorsphorylation and total of NF-M/H.
B. Representative blots of cells pretreatment with 20 μM NAG-Ae (N+AL) or treated with 20 μM NAG-Ae and 6mM alloxan at the same time (NA) with antibodies of SMI31 and R61d
C. and D. The quantities assay of blot A and B
* indicated $P<0.05$ ** $P<0.01$ compared to the control cells. ## $P<0.01$ compared to alloxan treated cells for the same treatment tim

amidino-2-phenylindole) was added and incubated for 2 min. Then the slides were covered with antifade mounting medium and observed under fluorescence microscope.

Statistical analysis

Experimental data is expressed as means ± standard deviation ($\bar{x}$ ± SD). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by unpaired t-test for data (SPSS16.0, La Jolla, CA). Significant differences between experimental groups were defined as P<0.05.

RESULTS

Alloxan and NAG-Ae causes reverse change in the level of O-GlcNAcylation in SK-N-SH cells

We detected the level of O-GlcNAcylation with antibody RL2 to recognize the O-Glycosylation of protein in cells treated with alloxan and NAG-Ae. Compared to the control, the level of O-GlcNAcylation was decreased in a dose-dependent manner as the cells exposed to alloxan (Fig. 1A, B). It was increased as cells exposed to NAG-Ae, while reduced level of O-GlcNAcylation induced by alloxan was recovered by the treatment of 20μM NAG-Ae (Fig. 1C, D).

Increased O-GlcNAcylation induces decreased phosphorylation of NFs in SK-N-SH cells

In order to observe the phosphorylation of NFs, specific antibody SMI31 and R61d were used to detect the phosphorylated tail domain of NF-M/NF-H and total NFs. The phosphorylation of NF-M/NF-H was increased by the treatment of alloxan in concentration-dependent manner, but the total of NFs that was detected by monoclonal antibody R61d had not changed (Fig. 2A, B). Furthermore, when the cells were pretreated with 20 μM NAG-Ae for 12 h, or treated with 20 μM NAG-Ae and 6 mM alloxan at the same time, the phosphorylation of NF-H/M induced by alloxan was reduced by both pretreatment and treatment of NAG-Ae. The difference is that NAG-Ae pretreatment showed a stronger protective role for abnormal NFs phosphorylation (Fig. 2C, D).

We also observed a dose-dependent decrease in O-GlcNAcylation of NF-M, as determined by monoclonal antibody NL6. Immunofluorence staining of these cells indicated that alloxan treatment markedly increased SMI31 staining, decreased NL6 staning and caused accumulation of the phosphorylated NF in the cells (Fig. 3A, B). Consistent with the results of western blot, NAG-Ae treatment protected SK-N-SH cells from the morphology change and abnormal phosphorylation (Fig. 3C, D). Taken together, these results suggested that O-GlcNAcylation and phosphorylation of NFs are regulated reciprocally in SK-N-SH cells and that increased

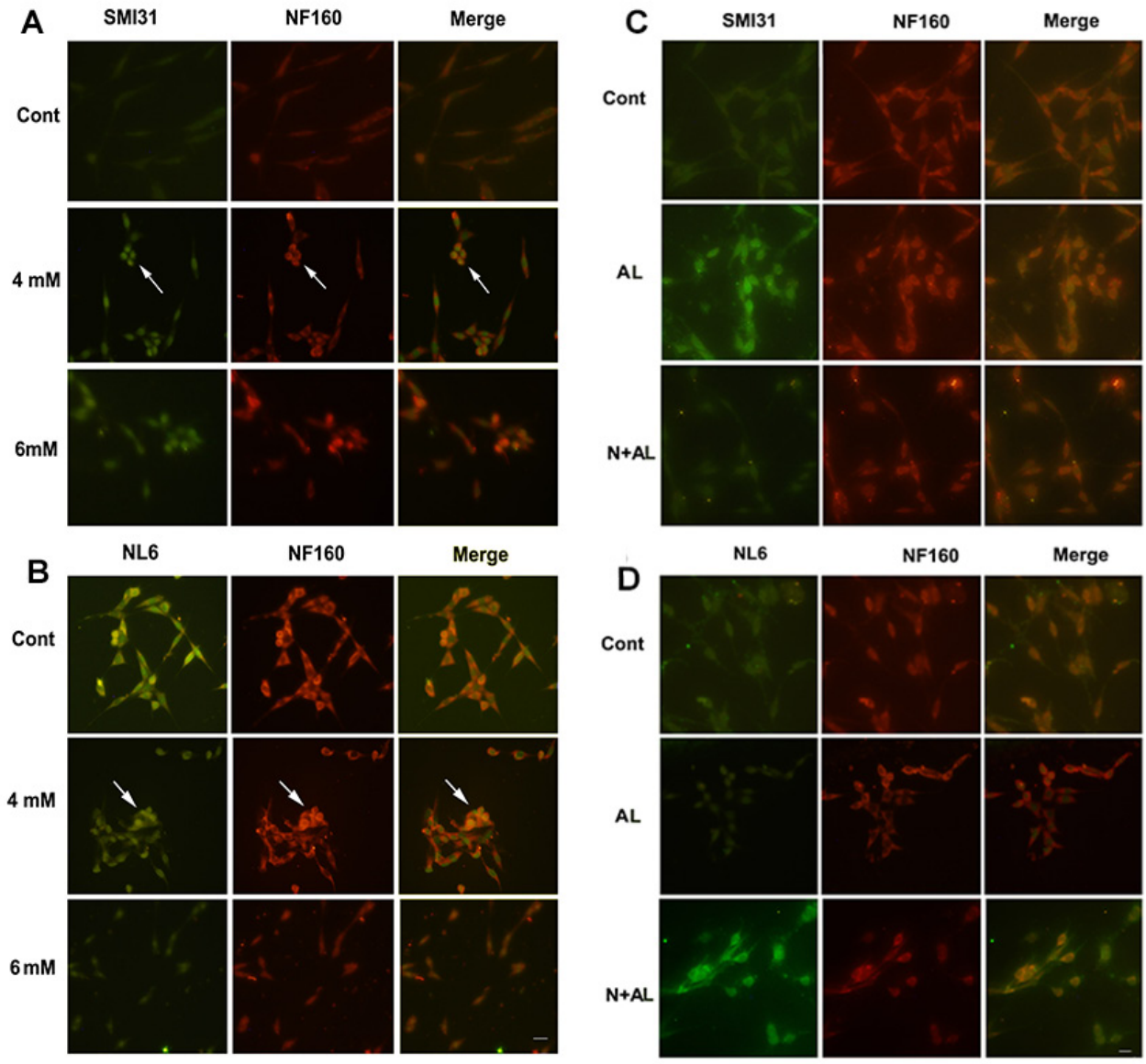

Fig. 3. The localization assay of O-GlcNAc and phosphorylation in SK-N-SH cells.
Representative pictures of Immunofluorescence in cells were treated with 4, 6 mM alloxan (A and B) or 20 μM NAG-Ae pretreatment (C and D) with antibodies SMI31/NF160 or NL6/NF160, arrow indicated cells to be round with accumulated NF.
Scale 100 μm.

O-GlcNAcylation with NAG-Ae protected NFs from abnormal phosphorylation.

The effects of phosphorylation and O-GlcNacylation on cells death

Cells death was assessed by Hoechst(Ho)/PI staining and DAPI staining since PI is excluded by viable cells and Ho can penetrate cell membranes. Apoptotic cells show a high Ho staining and a low PI staining (strong blue fluorescence and light red fluorescence), and necrotic cells are brightly stained with PI and Ho (strong blue fluorescence and strong red fluorescence). Healthy cells are dimly stained by Ho and not stained by PI, so the control cells showed light blue fluorescence without red fluorescence. We found that 4mM and 6mM alloxan treatment induced cells apoptosis and necrosis respectively (Fig. 4A). By contrast, cells exposed to NAG-Ae were similar to the control cells. Additionally, pretreatment of the cells with NAG-Ae reduce the staining of strong blue and strong red fluorescence induced by 6mM alloxan treatment (Fig. 4B).

In DAPI staining, alloxan treatment can cause cell apoptosis, while cells with treated or pretreated with NAG-Ae displayed no abnormal changes (Fig. 4C). The staining results suggest that increased phosphorylation with alloxan treatment induced cells apoptosis and necrosis. Increased O-GlcNacylation with NAG-Ae treatment or pretreatment, on the contrary, reduced cell death which was induced by alloxan in SK-N-SH cells.

DISCUSSION

Many studies have demonstrated that O-GlcNAcylation and phosphorylation competitively modify the same serine/threonine residues and are thus reciprocal to each other. Especially in the central nervous system, O-GlcNAcylation has also been shown to regulate many synaptic proteins and neuronal cytoskeleton proteins[12,13]. We also found that impaired glucose uptake/metabolism can lead to hyperphosphorylation and pathological accumulation of NFs in AD brain by decreased O-GlcNAcylation[14].

What's more, different ways of modulating the O-GlcNAcylation levels may cause conflicting results. In the present study, by using alloxan (OGT inhibitor) and NAG-Ae (OGA inhibitor) to change the O-GlcNAcylation levels in cultured neuroblastoma cells, the interrelationship similar to early studies between O-GlcNAcylation and phosphorylation of NFs was detected; This is to say, increased O-GlcNAcylation protects NFs from abnormal phosphorylation[15]. However, except OGT, alloxan has also been shown to inhibit the glucokinase and GlcNAcase of pancreatic β-cell, the effect of several protein phosphatase and kinase such as phosphatase 2A (PP2A), glycogen synthesis kinase 3 (GSK-3), cyclin-dependent kinase5 (CDK5), mitogen-activated protein kinase (MAPK), stress-activated protein kinase family (SAPK1), calcium/calmodulin-dependent protein kinase II (CaMKII) should be taken into account[10,16,17].

NAG-Ae was a more potent and selective inhibitor of O-GlcNAcase, research indicated that it has cardioprotective effects on acute ischemic myocardium by preserving the integrity of Z-line protein via increased O-GlcNAcylation[19]. Howewer, whether NAG-Ae could protect the neuronal cells is not clear. In our test, we found 20 μM NAG-Ae inhibited the abnormal phosphorylation and accumulation of NF in the bodies, but the cell viability was decreased in concentration of 80μM and 160μM NAG-Ae (Data not shown). Besides, hyper-O-GlcNAcylation leads to impaired memory and synaptic plasticity[20]. Thus, selection of proper dose and time may be the key point to develop reagents to improve the O-GlcNAc for AD therapy.

Cell loss is another one of the pathological features in AD brain. In our test, we found apoptotic morphology changes of nuclear condensation or fragmentation with alloxan treatment. In addition, the apoptosis was accompanied by hyperphosphorylation of NFs, as aggregation of phosphorylated tau is thought as the cause of apoptosis[21]. However, other investigators found that the reduced number of neurons in AD is actually neuronal apoptosis induced by increased O-GlcNAcylation via AKT signaling and NFκB (ref.[22,23]). As far as now, the fate of cells with hyperphosphorylation and early apoptosis feature is hardly understood.

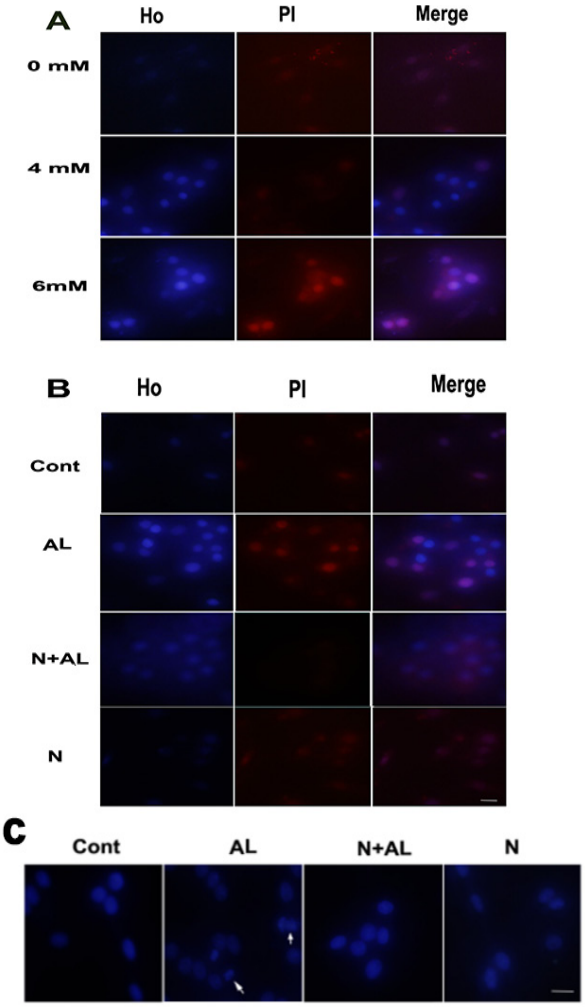

Fig. 4. Representative pictures of Hoechst (Ho)/PI staining and DAPI staining.
A. Cells exposed to different concentration of alloxan (0, 4, 6 mM) for 12 h were stained by Ho/PI.
B. Cells exposed to 6mM alloxan with or without pretreatment of NAG-Ae stained by PI/Hoechst staining.
C. Cells exposed to 6mM alloxan with or without pretreatment of NAG-Ae (N+AL) or NAG-Ae (N) only were stained by DAPI. Arrow indicated the nuclear condensation or fragmentation.
Scale 100 μm.

CONCLUSION

In summary, increasing O-GlcNAc with NAG-Ae protected Alzheimer-like neurodegeneration from NFs hyperphosphrylation and the cell loss, suggesting the role of decreased O-GlcNAc in the pathogenesis of AD. As a result, the inhibition of O-GlcNAc removal may represent a new therapeutic approach for AD.

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Author contributions: ND: performed the experiments; PP: manuscript writing; YC, JW, SC: data analysis; RA: language correction; YD: experiments design, final revision.

Conflict of interest statement: None declared.

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