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Chitooligosaccharides protect rat cortical neurons against copper induced damage by attenuating intracellular level of reactive oxygen species

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ABSTRACT

A large number of evidence has suggests that dyshomeostasis of the redox-active biometals such as copper and other metal ions can lead to oxidative stress in neurons, which plays a key role in the pathology of neurodegenerative disorders. Chitooligosaccharides (COSs) are biodegradation product of chitosan and demonstrated diverse biological activities, Here we first report that protective effects of COSs (M.W. 1500, DD. 90%) against Cu(II) induced neurotoxicity in primary cultured rat cortical neurons. The toxicity of Cu(II) to cortical neurons was obviously attenuated in a concentration-dependent manner by COSs pretreated. The data derived from lactate dehydrogenase (LDH) release and the Hoechst 33342 assay support the results from MTT assay. After DCFH assay, COSs were found to depress Cu(II) induced elevation in intracellular reactive oxygen species (ROS), These findings suggest that COSs protect against Cu(II) induced neurotoxicity in primary cortical neurons by interfering with an increase in intracellular reactive oxygen species (ROS).

Copper is an important element that plays an important role in cell physiology as a cofactor of many enzymes, such as cytochrome oxidase, Cu/Zn superoxide dismutase, dopamine hydroxylase, amine oxidases, Cu monooxygenases, and nitrite reductase/multicopper oxidase tyrosinase.[1,2] However, copper accumulation can also be toxic to neurons, the redox property of copper may catalyse the production of ROS such as hydroxyl radicals by participation in Fenton or Harber Weiss reactions.[3] Furthermore, Copper dyshomeostasis is regard as an important role in neurodegenerative diseases such as Alzheimer disease. ROS can react with biomolecules in living cells and then resulting in extensive impairment of cellular functions through membrane lipid peroxidation, protein oxidation and nucleic acid cleavage.[3] Copper accumulation can also be cytotoxic because of perturbations in copper homeostasis result in oxidative stress and increased free radical production.[4] Although the precise mechanism of copper neurotoxicity is always unclear, copper involved in many steps with the pathogenesis of AD and other neurodegenerative disorders.[5] Furthermore, Redox-active Cu(II) enhanced neurotoxicity of amyloid beta peptide with concomitant production of reactive oxygen species (ROS), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$),[6] which are believed to contribute to the neurotoxicity in primary neural cultures.

Chitooligosaccharides (COSs) are biodegradation product of chitosan. Accumulating evidence has shown an array of biological activities of COSs, including free radical scavenging activity,[7,8] antitumor activity,[9] immunostimulating effects,[10] and antimicrobial activity.[11] Moreover, COSs contains multiple amino, hydroxyl, and acetamide groups and can form complexes with many metal ions such as Cu, The resultant complex were regarded as a potential neuroprotective agent.[12–14]

As part of our ongoing investigation on COSs biological activities, Here, for the first time, we reported the copper neurotoxicity attenuated by COSs via maintaining the integrity of cell plasma membrane and decreasing intracellular ROS formation. We prepared COSs with degree of deacetylation (DD) of 90% and average molecular weight (MW) of 1500 in order to carry out an evaluation of their neuroprotective effects. This study was designed to investigate whether COSs were capable of protecting primary culture of rat cortical neurons against copper induced neurotoxicity. These results may provide an important target for medical chemistry approaches of COSs for the future treatment of AD and other neurodegenerative disorders.

In this study, Chitooligosaccharides (COSs) provided by biochemical engineering college of Beijing union university (Beijing, China). The purity of the COSs is determined by HPLC and it has achieved 90% at least. Other chemical reagents including DMSO, $CuCl_2$, $NaHCO_3$, NaCl were obtained from Beijing Chemical Industry (Beijing, China). Working solution of copper were prepared by taking a known amount of $CuCl_2$ and dissolving it in PBS buffer, the stock solution was then aliquoted and stored at −20 °C. When copper solution was required, the aliquots were diluted to the final working concentration by culture medium. COSs were dissolving in PBS buffer, and then diluted to final working concentration by

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culture medium. Unless otherwise mentioned, Prior to use all buffers and stock solutions of metal ions and COSs were filtered through a 0.22 μm filter (Millipore, Cork, Ireland) to remove any particulate matter.

Cortical neural cultures were obtained according to an existing protocol with some modification. Briefly, cortices of new born Sprague–Dawley rats were removed, dissected free of meninges and blood vessels, cut into 1–2 mm^3 tissues, and dissociated in 0.125% (W/V) trypsin, shaking every 5 min. Fifteen minutes later, 5% serum was immediately added to terminate the dissociation.[15] The dissociated cells were triturated using a filter-plugged fine pipette tip, pelleted, and resuspended in plating medium (DMEM with 10% NBS and 10% HS plus 1% penicillin streptomycin). Viable cells were counted manually using a 1 mm^2 grid (10× objective) stained with 0.4% trypan blue. Afterwards, dissected cells were plated into poly-L-lysine-coated 48-well culture plates or 25-cm^2 cell culture plate (Corning, Corning, USA) at a density of 125,000 cells/cm^2 in plating medium. All cultures were maintained in an incubator set at 37 °C with 5% CO_2. After 4 h the plating medium was replaced with fresh Neurobasal-A medium plus B27 supplements, 100 U/ml penicillin, 100 μg/ml of streptomycin, and 0.5 mM L-glutamine. This method resulted in cultures highly enriched for neurons (>95% purity) with minimal astrocyte and microglial content as determined by immunostaining of culture preparations using specific marker antibodies.

The neural cells were allowed to mature for 7 d in culture before treatment using freshly prepared serumfree Neurobasal-A medium plus B27 supplements minus antioxidants. The stock solution of COSs was prepared with Neurobasal-A medium. For cell treatment, cortical neurons were pre-incubated for 24 h in neural culture medium with or without 0.4, 0.2, or 0.1 mg/ml COSs, respectively, after removal of the medium, culture medium containing 50 μM $CuCl_2$ was added and allowing to incubate for 48 h at 37 °C in a humidified incubator, cell viability and other property were then assayed.

Cell morphology was observed by phase contrast microscopy, and cell viability was quantitated using MTT assay as described previously. Briefly, neurons were cultured in 96-well plates cells and washed three times with 250 μl of D-Hanks buffer, then placed in Neurobasal-A medium plus B27 lacking antioxidants (250 μl), and 25 μl of MTT was added to each well and incubated for 4 h at 37 °C with 5% CO_2. After removal of the medium, the absorbance of the samples was measured at a wavelength of 570 with 630 nm as a reference wavelength. Unless otherwise indicated, the extent of MTT conversion in cells is expressed as a percentage of the control.

LDH is a stable cytoplasmic enzyme presented in all cells including neurons. It is rapidly released into the cell culture supernatant when the cell plasma membrane was damaged. Therefore, the LDH level in the culture medium is a dependable biochemical index for neural plasma membrane damage. In this study, LDH release from cytosol of damaged neurons into the medium following copper exposure was detected using Lactate dehydrogenase assay kit, which determined the LDH activity in the medium to enzymatically convert the lactate and NAD^+ to pyruvate and NADH. The dinitrophenylhydrazine salt produced in the enzymatic reaction was then reduced to red formazan in the presence of pyruvate, thus allowing a colorimetric detection for neural membrane integrity.

The cultured cortical Chromatin condensation was detected by nucleus staining with Hoechst 33342 assay, neurons grown in a 48-well plates were washed with 4 °C phosphate buffered saline (PBS) and fixed with 4% formaldehyde in PBS. Cells were then stained with Hoechst 33342 (5 μg/mL) for 10 min at 4 °C. Nuclei were visualized using a fluorescence microscope (Nikon, Japan) at 400× magnification.

Measurement of intracellular ROS was based on ROS-mediated conversion of nonfluorescent 2′,7′-DCFH-DA into DCFH. The intensity of fluorescence reflects enhanced intracellular oxidative stress. After the incubation studies as before, cortical neurons in black 96-well plates were washed with PBS (pH 7.4) and then incubated with DCFH-DA (20 μM) in PBS at 37 °C for 2 h. At the end of incubation, the DCFH fluorescence of the cells from each well was evaluated using a confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany).The background was deduct from cell-free conditions. Results were expressed as percentage of control (non-stimulated cortical neurons) fluorescence intensity.

Data were analyzed as means ± S.D. Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) with subsequent Turkey's tests. Differences were considered statistically significant at $p < 0.05$.

The effects of COSs on primary cortical neurons were investigated, cells were exposed to different concentrations of COSs (0.01–2 mg/mL). MTT assay indicated that cells exposed to 2 mg/mL COSs induced neural death in 48 h (data not shown), the neural axon and dendrite turned straight and slight. These results were not according with previous research which suggest that COSs working concentration was achieved at 4 mg/mL.[16,17] These difference may arise from the discrepancy of deacetylation degree and average molecular weight. In our study, COSs below concentration of 1 mg/mL has no side-effects on cell viability (Fig. 1). These results indicated that the effects of COSs on primary cortical neurons was a non-linear relation.

Primary cortical neurons had the appearance of axon and dendrite emerged from the cell bodies while medium was replaced by serum free Neurobasal-A Medium 12 h later, the neurons achieved maturity after being cultured for 7 d and their morphology could be clearly seen under inverted microscope, neurons of control group had big and round cell bodies, the neural axon turned obvious and communicated with each other, halo surround cell bodies imply that these cells grows in good condition (Fig. 2A). However, many cell bodies emerged shrinkage shape and their axon turned thin when copper incubated 48 h at 50 μM concentration (Fig. 2B). Groups pretreated with 0.4 mg/mL COSs performed much slighter cell bodies damage than groups treated with copper only, moreover, the recovery of neural bodies is much more significant than neural axions. (Fig. 2C), and the profound mechanism requires further research.

Hoechst 33342 staining assay displayed that chromatin condensation and nuclear fragmentation appeared in rat cortical neurons following copper stimulation. As shown in Figure 3A, chromatin of control group cells were even and pale, in contrast, neurons

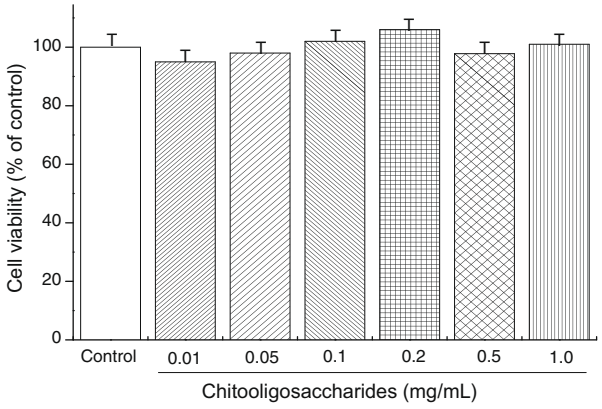

Figure 1. Effects of COSs on cultured cortical neurons indicated by MTT assays, respectively. Cortical neurons were incubated with0, 0.01, 0.05, 0.1, 0.2, 0.5 and 1.0 mg/ml COSs for 48 h, Both $^*p < 0.05$ and $^{**}p < 0.01$ versus control group.

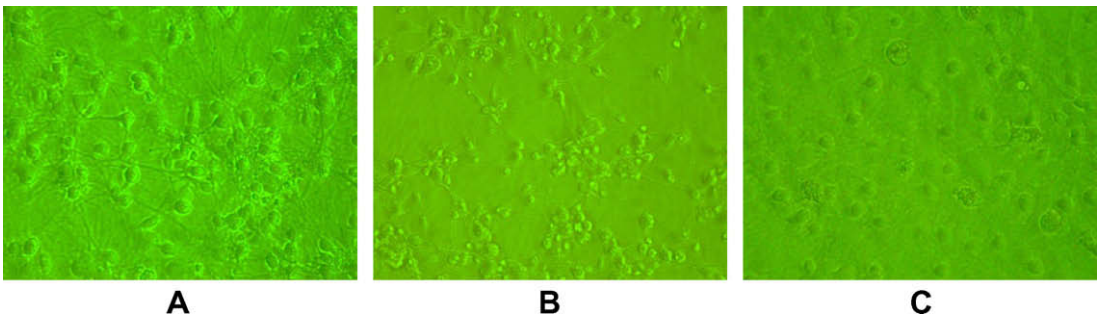

Figure 2. Light microscopic photomicrographs show the morphology of cortical neurons. Control (A); incubated with 50 μM copper (B); pretreated with 0.4 mg/mL COSs and then incubated with 50 μM copper (C), Each sample has been cultured for 7 d. Original magnification 400×.

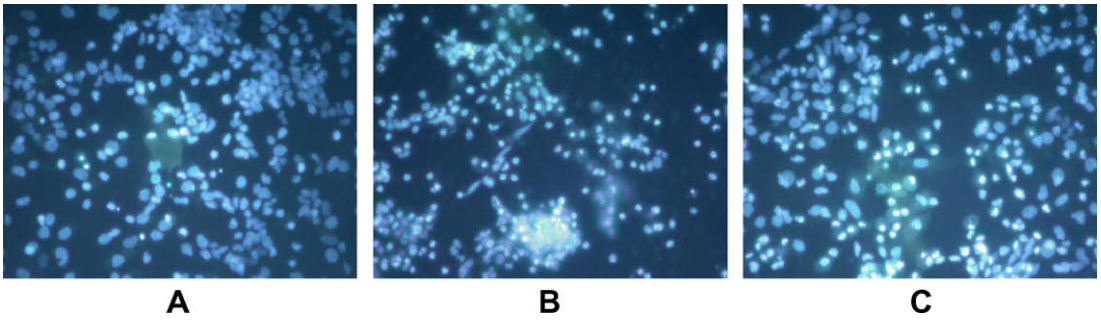

Figure 3. COSs pretreatment attenuated copper induced nuclear condensation in cultured cortical neurons as indicated by Hoechst 33342 staining. Control (A); incubated with 50 μM copper (B), pretreated with 0.4 mg/mL COSs and then incubated with 50 μM copper (C), Each sample has been cultured for 7 d. Original magnification 400×.

exposed to copper stimulation displayed intense chromatin condensation (Fig. 3B). However, the condensation was obviously attenuated by COSs pretreatment under fluorescence microscope (Fig. 3C).

MTT assay showed that the cell viability of rat cortical neurons decreased on exposure to 50 μM copper after 48 h, groups pretreatment with 0.4 mg/ml COSs significantly attenuated the copper induced decrease in the cell viability of cortical neurons. These protective effects showing a concentration-dependent pattern (Fig. 4A). On the other hand, the release of LDH into medium in cortical neurons increased after exposure to copper intensely, but pretreatment with 0.4 mg/ml COSs significantly decreased the LDH release (Fig. 4B). It is imply that copper induced neurotoxicity is associated with collapse integrity of plasmalemma.

To explore the underlying mechanism associated with copper neurotoxicity, we measured DCFH-DA assay to investigate whether increase of intracellular ROS level is associated with copper neurotoxicity. Our results suggest that intracellular ROS level obviously elevated after copper stimulation 48 h later (Fig. 5B), meanwhile, the DCFH fluorescence intensity was obviously declined in a dose-dependent manner by COSs pretreatment (Fig. 5C–E). These results confirmed the previous studies which demonstrated that copper induced reactive oxygen species (ROS) generation in vitro, the neuroprotective effects of COSs was associated with its antioxidant actions. Excess copper is extremely toxic by leading to many pathological conditions that are associate with oxidative damage to membranes and molecules,[18,19] The precise mechanism of neurotoxicity induced by high concentration copper ions is also unclear, it is widely accepted that the neurotoxic mechanism of copper in the brain is associate with the Fenton reaction catalyzed by reduced forms of copper and then accelerated formation of intracellular Reactive Oxygen Species.[20] A large body of evidence suggests that oxidative stress in brain is a predominant pathological feature of AD and other neurodegenerative disorders. Metabolic signs of oxidative stress in the neocortex of AD patients, such as free radical-mediated damage of brain proteins, lipids, and DNA; systemic signs of oxidative stress; and the response of antioxidant systems have all been observed in AD tissues.[21–23]

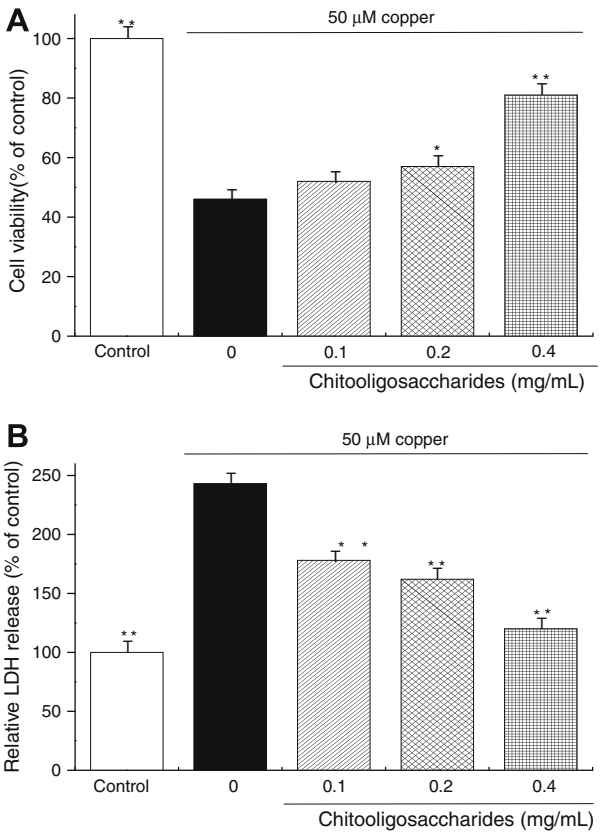

Figure 4. COSs pretreatment attenuated copper induced decrease in the cell viability of cultured cortical neurons indicated by MTT (A) and LDH release (B) assays, respectively. COSs pretreatment groups: cortical neurons were pre-incubated with 0.1, 0.2, or 0.4 mg/ml COSs for 24 h, and then replaced medium with 50 μM copper for 48 h. Copper group: without COSs pretreatment (0 mg/mlCOSs) and with copper only. Control group: neither COSs pretreatment nor copper. Both $^{*}p$ <0.05 and $^{**}p$ <0.01 versus copper group.

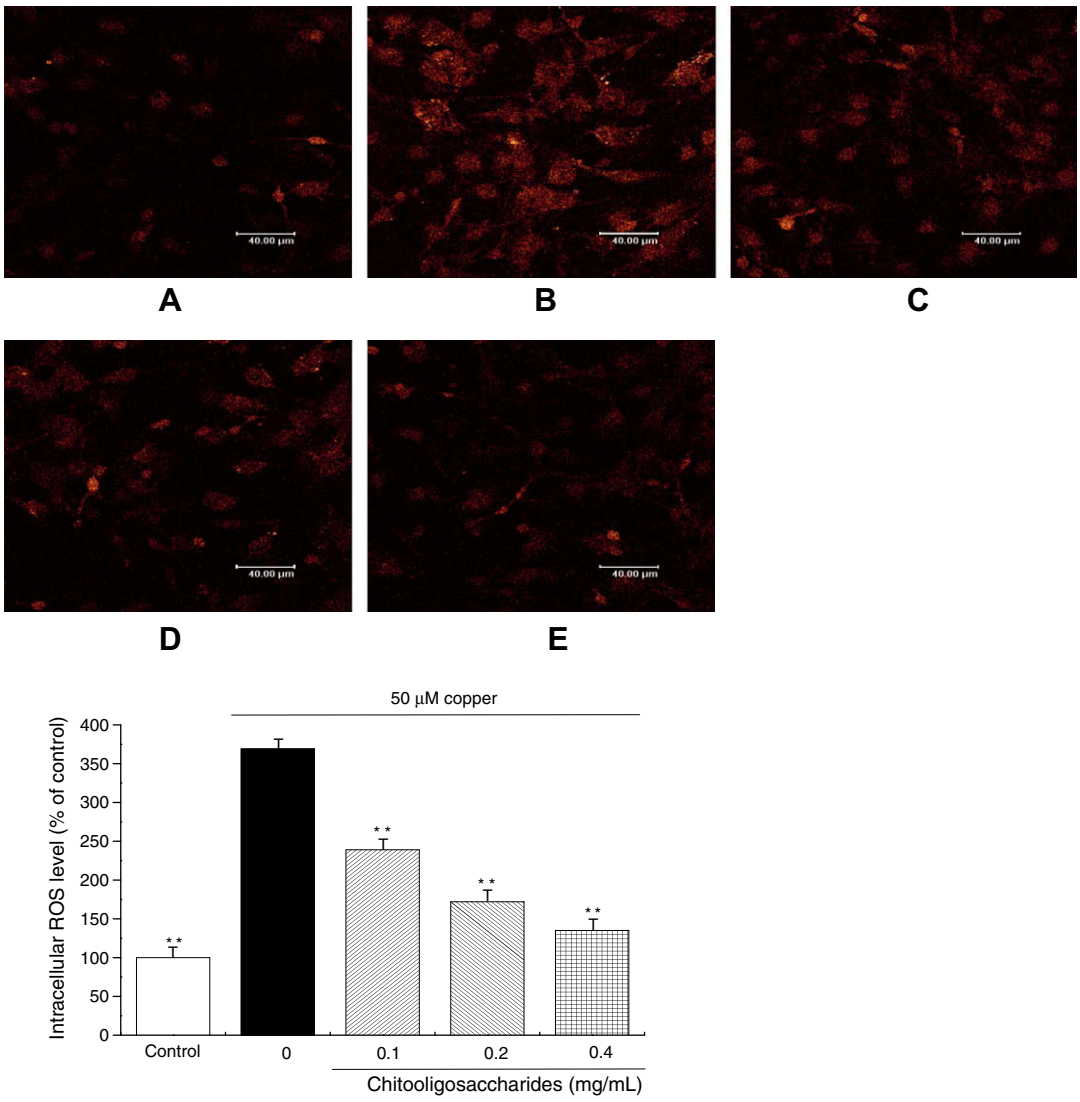

Figure 5. COSs pretreatment attenuated intracellular Reactive Oxygen Species level of cultured cortical neurons indicated by DCFH assay, respectively. Control (A), incubated with 50 μM copper (B), pretreated with 0.1 mg/mL COSs and then incubated with 50 μM copper (C), pretreated with 0.2 mg/mL COSs and then incubated with 50 μM copper (D), pretreated with 0.4 mg/mL COSs and then incubated with 50 μM copper (E), Each sample has been cultured for 7 d prior to Laser scanning confocal microscope. Both $^{*}p$ <0.05 and $^{**}p$ <0.01 versus control group.

Wherefore, in this study, we studied copper induced neurotoxicity and its relevant mechanisms in primary rat cortical neurons for the first time. We demonstrated that moderate toxic levels (50 μM) of copper, while beneficial at subtoxic levels,[24] induce obvious cell death in primary cortical neurons (Fig. 2B). We also found that higher concentration (100 μM) copper stimulation will induce more intense necrosis (data not shown). Copper induced cell death was markly decreased when pretreated with COSs indicated by MTT assay, showing a concentration-dependent pattern. Copper stimulation may destroy cell plasmalemma, LDH release and then cell death, the relation between copper stimulation and poor plasmalemma integration may associate with intracellular ROS production, The ROS-induced membrane damage induces further calcium influx, and resultant accentuated calcium influx will in turn induce the further generation of ROS,[25] consequently, membrane damage bring on cell death.

Therefore, the inhibitory actions of COSs on copper stimulated increase cell death are likely associated with ROS-scavenging abilities of COSs. COSs are biodegradation of chitosan, many research indicates that COSs possess favorable ROS-scavenging abilities in vitro.[26,27] On the other hand, the underlying mechanisms of neuroprotective effect of COSs is probably associate with copper chelation action of chitosan, previous studies demonstrates that chitosan could chelating copper efficiently, COSs molecular units contains innumerable amidocyanogen and hydroxy, these units could bind with copper,[28,29] chitosan-copper complex may interdict copper induce Fenton reaction catalyzed by reduced forms of copper and then inhibit formation of intracellular Reactive Oxygen Species.

In conclusion, our present studies clearly show that COSs can attenuate copper induced oxidative stress injury in primary neurons. The potential mechanism of protective effects of COSs are probably partly because of the inhibiting intracellular ROS production, along with the capacity of suppressing primary cultured neurons death subsequent to the amelioration of ROS elevation. The antioxidant properties of COSs hold great potential for the treatment of oxidative diseases. Our findings suggest a potentia application for COSs in the treatment of Alzheimer disease and other neurodegenerative disorders.

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