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The effect of Cu^{2+} and Zn^{2+} on the $A\beta_{42}$ peptide aggregation and cellular toxicity

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Abstract

The coordination chemistry of Cu and Zn metal ions with the amyloid β (Aβ) peptides has attracted a lot of attention in recent years due to its implications in Alzheimer's disease. A number of reports indicate that Cu and Zn have profound effects on Aβ aggregation. However, the impact of these metal ions on Aβ oligomerization and fibrillization is still not well understood, especially for the more rapidly aggregating and more neurotoxic $A\beta_{42}$ peptide. Here we report the effect of Cu^{2+} and Zn^{2+} on $A\beta_{42}$ oligomerization and aggregation using a series of methods such as Thioflavin T (ThT) fluorescence, native gel and Western blotting, transmission electron microscopy (TEM), and cellular toxicity studies. Our studies suggest that both Cu^{2+} and Zn^{2+} ions inhibit $A\beta_{42}$ fibrillization. While presence of Cu^{2+} stabilizes $A\beta_{42}$ oligomers, Zn^{2+} leads to formation of amorphous, non-fibrillar aggregates. The effects of temperature, buffer, and metal ion concentration and stoichiometry were also studied. Interestingly, while Cu^{2+} increases the $A\beta_{42}$-induced cell toxicity, Zn^{2+} causes a significant decrease in $A\beta_{42}$ neurotoxicity. While previous reports have indicated that Cu^{2+} can disrupt β-sheets and lead to nonfibrillar Aβ aggregates, the neurotoxic consequences were not investigated in detail. The data presented herein including cellular toxicity studies strongly suggest that Cu^{2+} increases the neurotoxicity of $A\beta_{42}$ due to stabilization of soluble $A\beta_{42}$ oligomers.

Introduction

Alzheimer's disease (AD) is an irreversible, progressive neurodegenerative disease that slowly destroys memory and thinking skills, and eventually causes senile dementia. More than 5 million in the US and 24 million people worldwide suffer from this disease.[1,2] The pathogenesis of AD is far from being understood, and 42- and 40-amino acids long amyloid β peptides ($A\beta_{42}$ and $A\beta_{40}$, respectively) are proposed to play a central role in the onset of AD.[3] Although $A\beta_{40}$ is present in larger amounts in the brain, $A\beta_{42}$ was found to be more neurotoxic and have a higher tendency to aggregate.[4,5] The amyloid cascade hypothesis

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suggests the ultimate products of Aβ aggregation, the amyloid plaques, are responsible for neurodegeneration.[6] However, recent *in vivo* studies have shown that soluble Aβ oligomers are more neurotoxic than amyloid plaques and most likely responsible for synaptic dysfunction and memory loss in AD.[7–11]

Post-mortem examination of the brain suggested that bio-available metals (*i.e.*, copper, zinc, and iron) are found in high concentration in the amyloid plaques. These metal ions are believed to play a key role in the peptide aggregation processes.[12–15] For example, it was shown that that metal ions promote Aβ aggregation,[16,17] as well as formation of reactive oxygen species (ROS) and lead to oxidative stress.[18–20] While several studies have investigated the metal coordination environment of Aβ-M^{n+} complexes,[14,21–24] only few have employed the full-length Aβ peptides, shorter non-aggregating forms like $Aβ_{1–16}$ or $Aβ_{1–28}$ being used instead.[14,22–25]

Although these studies suggest that metal ions alter the Aβ aggregation pathways and toxicity,[26] the molecular mechanisms of metal–Aβ species interactions are not completely understood, especially for the more neurotoxic $Aβ_{42}$ peptide.[13,15,27,28] Several studies showed that Zn^{2+} promotes formation of nonfibrillar aggregates,[16,29–31] yet conflicting results have been reported for the Cu^{2+}-mediated Aβ aggregation.[21,28,32,33] In some studies Cu^{2+} appears to be involved in disrupting or reducing $Aβ_{42}$ aggregation,[28,32,34–37] while other studies report that Cu^{2+} enhances aggregation.[17,33,38] Moreover, in the cell toxicity studies the presence of transition metal ions showed both increased[39–42] or decreased[34,43] toxicity of Aβ. The likely reason for these conflicting results is the use of different experimental conditions and methods to measure Aβ aggregation. There are reports using Thioflavin T (ThT) fluorescence,[28,32,33] atomic force microscopy (AFM)[32,35] or TEM,[28,36,44] and various cellular toxicity[33] assays to evaluate the Cu^{2+}-mediated Aβ aggregation and neurotoxicity. However, no report has employed all these techniques under similar experimental conditions to provide a detailed picture of the neurotoxicity implications of the Cu^{2+}- and Zn^{2+}-mediated formation of soluble and/or insoluble $Aβ_{42}$ aggregates. Hence, more comprehensive studies on the effect of metal ions on $Aβ_{42}$ aggregation and neurotoxicity are needed to better understand their role in AD progression.

Herein we have employed for the first time a wide range of techniques to study the effect of metal ions on the oligomerization and aggregation of the more neurotoxic $Aβ_{42}$ peptide. ThT fluorescence, native gel electrophoresis and Western blotting, transmission electron microscopy (TEM), and cellular toxicity studies were used to determine the formation in presence and absence of Cu^{2+} and Zn^{2+} of both soluble and insoluble $Aβ_{42}$ aggregates. Most importantly, we have correlated these *in vitro* results with neurotoxicity studies, in order to address to potential *in vivo* role of metal–$Aβ_{42}$ interactions in AD. We observed a significantly low ThT fluorescence for Cu^{2+}- and Zn^{2+}-containing Aβ aggregates, and TEM and native gel/Western blotting studies suggest that Cu^{2+} and Zn^{2+} both inhibit fibrillization. While Zn^{2+} leads to formation of amorphous aggregates, Cu^{2+} is shown to stabilize soluble Aβ species. Cellular toxicity studies suggest that while Zn^{2+} reduces the $Aβ_{42}$ toxicity, Cu^{2+} significantly increases $Aβ_{42}$ neurotoxicity. Based on these results, we propose that Cu^{2+} stabilizes the neurotoxic soluble $Aβ_{42}$ oligomers, with direct implications into the physiological role of Cu^{2+} in increasing $Aβ_{42}$ neurotoxicity in AD.

Experimental details

Materials

All reagents were purchased from commercial sources and used as received unless stated otherwise. All solutions and buffers were prepared using metal-free Millipore water that was treated with Chelex overnight and filtered through a 0.22 μm nylon filter.

Aβ samples preparation

Aβ monomeric films were prepared by dissolving synthetic $A\beta_{42}$ or $A\beta_{40}$ peptide (Keck Biotechnology Resource Laboratory, Yale University) in hexafluoroisopropanol (HFIP) (1 mM) and incubating for 1 h at room temperature. The solution was then aliquoted out and evaporated overnight. The aliquots were vacuum centrifuged and the resulting monomeric films stored at −80 °C. Aβ fibrils were generated by dissolving monomeric Aβ films in DMSO, diluting into the appropriate buffer, and incubating for 24 h at 37 °C with continuous agitation at 150 rpm (final DMSO concentration was <2%). For metal-containing fibrils, the corresponding metal ions were added in various stoichiometric ratios (0.25 to 2 equiv. *vs.* Aβ) before initiation of fibrillization. For preparation of soluble $A\beta_{42}$ oligomers, a literature protocol was followed:[45] a monomeric film of $A\beta_{42}$ was dissolved in anhydrous DMSO and diluted in DMEM : F12 media (1 : 1 v : v, without phenol red, Invitrogen). The $A\beta_{42}$ solution (50–100 μM) was incubated at 4 °C for 24 h and then centrifuged at 10 000g for 10 min. The supernatant was used as a solution of soluble $A\beta_{42}$ oligomers.

Native gel electrophoresis and Western blotting

All gels, buffers, membranes, and other reagents were purchased from Invitrogen and used as directed except where otherwise noted. Samples were separated on 10–20% gradient tris-tricine mini gels. The gels were transferred to a nitrocellulose membrane on an ice bath and the protocol was followed as directed except that the membrane was blocked overnight at 4 °C. After blocking, the membrane was incubated in a solution (1 : 2000 dilution) of 6E10 anti-Aβ primary antibody (Covance) for 3 h. Invitrogen's Western Breeze Chemiluminescent kit was used to visualize the Aβ species. An alkaline phosphatase antimouse secondary antibody was used, and the protein bands were imaged using a FUJIFILM Luminescent Image Analyzer LAS-1000CH.

Cytotoxicity studies

Cell viability studies were performed using the Alamar Blue assay (Invitrogen). Mouse neuroblastoma Neuro2A (N2A) cell lines were purchased from the American Type Culture Collection (ATCC). Cells were grown in DMEM/10% FBS, which is the regular growth media for N2A cells. N2A cells were plated in each well of a 96 well plate (2.5×10^4/well) with DMEM/10% FBS. The media was changed to DMEM/N2 after 24 h. After 1 h, the reagents (20 μM $A\beta_{42}$ species and metal ions) were added. After an additional incubation of 40 h, the Alamar blue solution was added in each well, the cells were incubated for 90 min at 37 °C, and the absorbance was measured at 570 nm (control OD = 600 nm).

Fluorescence measurements

All fluorescence measurements were performed using a SpectraMax M2e plate reader (Molecular Devices). For ThT fluorescence studies, samples were diluted to a final concentration of 2.5 μM Aβ in PBS containing 10 μM ThT and the emission measured at 485 nm (λ_{ex} = 435 nm).

Transmission electron microscopy (TEM)

Glow-discharged grids (Formvar/Carbon 300-mesh, Electron Microscopy Sciences) were treated with Aβ samples (25 μM, 5 μL) for 2–3 min at room temperature. The excess solution was removed using filter paper and the grids were rinsed twice with H_2O (5 μL). Grids were stained with uranyl acetate (1% w/v, H_2O, 5 μL) for 1 min, blotted with filter paper, and dried for 15 min at room temperature. Images were captured using a FEI G2 Spirit Twin microscope (60–80 kV, 6500–97 000 magnification).

Results and discussion

$A\beta_{42}$ aggregation monitored by ThT fluorescence

Thioflavin T (ThT) is a fluorescent dye widely used for detection of amyloid peptide aggregation.[46] However, ThT does not interact with unstructured Aβ monomer or oligomers, nor with amorphous aggregates.[46] The fluorescence intensity of ThT increases by 20 fold in presence of pre-formed Aβ fibrils (Fig. 1), as observed previously for similar $A\beta_{42}$ peptide concentrations.[32] Interestingly, we observe a significantly low ThT fluorescence with the $A\beta_{42}$ aggregates prepared in presence of various amounts of Cu^{2+} and Zn^{2+} (Fig. 1). A decreased ThT fluorescence[32,36] in presence of Cu^{2+} ions can be accounted for by less fibril formation, although a quenching of fluorescence by the paramagnetic Cu^{2+} ions could also be involved.[47] Previous conflicting results suggested an increased or decreased ThT fluorescence intensity for Cu-Aβ aggregates. Exley *et al.*[28,36] and Sugimoto *et al.*[32] reported a decreased ThT fluorescence and suggested that Cu^{2+} prevents amyloid fibril formation, while Viles *et al.*[33] suggested an increased ThT fluorescence and thus an increased extent of fibrillization for the Aβ-Cu^{2+} species. Overall, our results are in line with the former reports, with a decreased ThT fluorescence observed for the Cu-$A\beta_{42}$ aggregates. In the presence of diamagnetic Zn^{2+} ions, although the effect is slightly lower than for Cu^{2+}, the decrease in ThT fluorescence suggests the formation of non-fibrilar aggregates (see below),[48] suggesting that the nature of Aβ aggregates determines the intensity of ThT fluorescence.

As described above, the use of ThT fluorescence to probe the aggregation of Aβ peptide has limitations as the results can be affected by metal complex formation, paramagnetic quenching effect, pH of media, and formation of amorphous aggregates. Thus, other methods of analysis such as TEM and native gel electrophoresis/Western blotting are needed to accurately evaluate the effect of metal ions on $A\beta_{42}$ aggregation.

Aβ aggregation monitored by TEM and native gel/Western blotting

Using both native gel electrophoresis/Western blotting and TEM techniques, a more detailed picture of the extent and pathways of Aβ aggregation can be obtained. While the former type of analysis reveals the presence of smaller, soluble Aβ species, the latter method reveals the

morphology of the larger, insoluble Aβ aggregates that cannot be characterized by gel electrophoresis.

We have focused mainly on studying the effect of Cu^{2+} and Zn^{2+} on the $A\beta_{42}$ peptide aggregation, since $A\beta_{42}$ was shown to be more neurotoxic and form soluble $A\beta_{42}$ oligomers.[7,8,11] To confirm this observation, we studied the aggregation of $A\beta_{40}$ and $A\beta_{42}$ in presence and absence of Cu^{2+} and Zn^{2+}. TEM images and the corresponding native gel/ Western blots are shown in Fig. 2. Panels a–c show the TEM images of $A\beta_{40}$ aggregation in absence and presence of Cu^{2+} and Zn^{2+}, respectively. $A\beta_{40}$ and $A\beta_{42}$ both form fibrils after incubation for 24 h at 37 °C (Fig. 2a and d). In the Western blot, although $A\beta_{40}$ does not show formation of any oligomeric species, it shows a dark band at the top of gel (lane a). This band likely corresponds to Aβ fibrils or large aggregates which cannot enter the gel. The Western blot of $A\beta_{42}$ shows formation of various size oligomers (trimer, tetramer, and higher MW oligomers) as well as a small band at gel entrance (lane d). These results strongly support the fact that $A\beta_{42}$ is the oligomer-forming peptide rather than $A\beta_{40}$.

In presence of Cu^{2+}, $A\beta_{40}$ forms fibrils although in a lower amount as observed by TEM, yet the nature of aggregates formed remains fibrillar (Fig. 2, panel b). Notably, the $A\beta_{40}$ peptide does not form oligomers either in presence or in absence of metal ions (Fig. 2, lanes a–c), a similar behavior being observed for $A\beta_{40}$ by SDS-PAGE.[49] In contrast to $A\beta_{40}$, $A\beta_{42}$ did not form amyloid fibrils in presence of Cu^{2+}, small $A\beta_{42}$ aggregates being formed instead as seen by TEM (panel e). Western blot confirms the formation of small soluble $A\beta_{42}$ oligomers (lane e), the formation of high MW oligomers being inhibited. Thus, Cu^{2+} seems to reduce the aggregation of $A\beta_{42}$ quite significantly. In presence of Zn^{2+}, both $A\beta_{40}$ and $A\beta_{42}$ form amorphous aggregates, as observed by TEM (panels c and f). Western blotting of Zn^{2+}-$A\beta_{42}$ aggregates shows formation of small soluble $A\beta_{42}$ oligomers, while high MW oligomers were not observed. Overall, the TEM and Western blotting results suggest that metal ions inhibit the $A\beta_{42}$ fibrillization process by stabilizing soluble $A\beta_{42}$ aggregates or formation of amorphous aggregates. Since the $A\beta_{42}$ aggregation was significantly inhibited by Cu^{2+} and thus could influence the etiology of AD, we have probed the effect of buffer, temperature, incubation time, and Cu^{2+} (and Zn^{2+}) stoichiometry on the observed metal-mediated formation of various $A\beta_{42}$ species and their neurotoxicity.

Effect of buffer on Cu^{2+}-mediated $A\beta_{42}$ aggregation

The commomly used buffers for Aβ aggregation studies are HEPES and PBS. In addition, we tested two concentrations of HEPES, 20 μM (pH 6.6) and 20 mM (pH 6.6 and pH 7.4). The former concentration has been used in previous reports for studying Aβ aggregation in presence of Cu^{2+},[50–52] although we believe this concentration may not be enough to maintain a bufferred solution of 25 μM Aβ. We did not observe any significant change between the two different pHs of HEPES. Under all conditions tested, TEM analysis shows a drasticaly reduced amount of $A\beta_{42}$ fibrils formed in presence of Cu^{2+} (Fig. 3). While some large $A\beta_{42}$ aggregates were observed in 20 μM HEPES, overall the inhibition of $A\beta_{42}$ aggregation by Cu^{2+} does not seem to be dependent on the buffer used.

Effect of temperature on Cu^{2+}-mediated $A\beta_{42}$ aggregation

As temperature is expected to dramatically affect the aggregation process, we performed experiments at both 25 °C[30,53] and 37 °C (physiological temperature),[32,50–52] the most commonly used temperatures to study Aβ aggregation *in vitro*. $A\beta_{42}$ forms amorphous aggregates after incubation for 72 h at room temperature and forms a range of soluble oligomers (Fig. 4, panel a and lane a). However, the presence of Cu^{2+} limits the formation of insoluble aggregates and high MW oligomers and seems to stabilize small MW oligomers (Fig. 4, panel b and lane b). At 37 °C the $A\beta_{42}$ peptide forms amyloid fibrils within 24 h as observed in TEM, as well as large MW oligomers as seen in the Western blot (Fig. 4, panel c and lane c). By contrast, the presence of Cu^{2+} leads to formation of small MW oligomers, which suggests that Cu^{2+} stabilizes $A\beta_{42}$ oligomers even at 37 °C (Fig. 4, lane d). Overall, these experiments suggest that Cu^{2+} stabilizes soluble $A\beta_{42}$ species at both temperatures and use of RT allows for a detailed study of the Cu^{2+}-mediated oligomerization process.

Dose-dependent Cu^{2+}-$A\beta_{42}$ aggregation

The presence of a trace amount of Cu^{2+} was found to affect the Aβ aggregation behavior in AFM studies.[35] Thus, various concentrations of Cu^{2+} were added to a fixed $A\beta_{42}$ concentration (25 μM) and the aggregation behavior was investigated by TEM and Western blotting. Five different concentrations of Cu^{2+} ranging from 0.25 to 2 equiv. relative to $A\beta_{42}$ were tested (Fig. 5). All concentrations of Cu^{2+} were effective in reducing $A\beta_{42}$ fibrillization, as monitored by ThT fluorescence (Fig. 1) and TEM (Fig. 5). While the presence of 0.25 equiv. Cu^{2+} (6.25 μM) does not completely inhibit $A\beta_{42}$ aggregation, Cu^{2+} amounts larger than 0.5 equiv. lead to formation of soluble $A\beta_{42}$ species. Importantly, the TEM images in Fig. 5 are representative of the entire surface of TEM grids that show a homogeneous distribution of the observed Aβ species. In addition, the Western blots of these dose-dependent experiments suggest that all Cu^{2+} concentrations inhibit the $A\beta_{42}$ aggregation. The formation of insoluble aggregate and high MW oligomers (80–160 kDa) was inhibited by any amount of Cu^{2+} present (Fig. 5), and higher concentrations of Cu^{2+} lead to an increased formation of smaller soluble $A\beta_{42}$ oligomers (Fig. 5, lanes d–f).

Overall, both TEM and native gel/Western blotting studies strongly suggest that Cu^{2+} has an inhibitory effect on $A\beta_{42}$ fibrillization and leads to the formation of soluble $A\beta_{42}$ oligomers. Our results are in line with the recent report by Exley *et al.*[28] that employs ThT fluorescence and TEM to study the role of Cu^{2+} in preventing fibril formation. However, we have also employed native gel/Western blotting to reveal the formation of soluble $A\beta_{42}$ species in presence of Cu^{2+}, strongly supporting our hypothesis that Cu^{2+} stabilizes such soluble $A\beta_{42}$ species. In addition, the Cu^{2+}-mediated stabilization of soluble $A\beta_{42}$ species also occurs in presence of Zn^{2+} ions – as observed by TEM, and thus is expected to be physiologically relevant. Importantly, these results have direct implications into the neurotoxicity of $A\beta_{42}$ (see below).

Dose-dependent Zn^{2+}-$A\beta_{42}$ aggregation

A similar experiment was carried out to study the effect of Zn^{2+} concentration on $A\beta_{42}$ aggregation, and five different concentrations of Zn^{2+} ranging from 0.25 to 2 equiv. relative to $A\beta_{42}$ were tested. Interestingly, while $A\beta_{42}$ fibrils were observed after incubation in

absence of Zn^{2+} at room temperature for 1 day, presence of as low as 0.25 equiv. Zn^{2+} causes formation of amorphous aggregates as observed by TEM (Fig. 6), a similar behavior being observed in the presence of higher concentrations of Zn^{2+}. The experiment suggests that even a substoichiometric amount of Zn^{2+} dramatically affects $A\beta_{42}$ fibrillization and leads to formtion of amorphous $A\beta_{42}$ aggregates instead of soluble oligomers.

Time-dependent $A\beta_{42}$ oligomerization–aggregation with Cu^{2+} and Zn^{2+}

We then investigated the time dependency of the $A\beta_{42}$ oligomerization–aggregation process in absence and presence of metal ions. Equivalent amounts of $A\beta_{42}$ and M^{2+} were incubated at room temperature for four days and the aggregation was monitored by native gel/Western blotting. $A\beta_{42}$ by itself forms large MW oligomers at room temperature (Fig. 7); this is in contrast with the incubation at 37 °C in which $A\beta_{42}$ fibrils are formed instead (Fig. 4). Interestingly, the presence of Cu^{2+} seems to inhibit formation of the large MW oligomers and selectively stabilizes small MW oligomers, even after four days (Fig. 7A). On the other hand, Zn^{2+} leads to disappearance of high MW oligomers, only small $A\beta_{42}$ oligomers being observed (Fig. 7B) that form likely along with an appreciable amount of insoluble amorphous aggregates (Fig. 6).

Cell toxicity studies

The role of metal ions in the etiology of AD is still unclear, and conflicting results on the effect of metal ions on Aβ toxicity have been reported. An increased[39–42] or decreased[34,43] toxicity of Aβ has been observed in the presence of transition metal ions, and these *in vitro* studies have employed either $A\beta_{40}$ or $A\beta_{42}$ peptides. It was reported recently that an increased ratio of $A\beta_{42}/A\beta_{40}$ in amyloid plaques increases their toxicity,[5] therefore we have investigated the effect of Cu^{2+} and Zn^{2+} on the cell toxicity of the more neurotoxic $A\beta_{42}$ peptide.[54–56] The viability of Neuro-2A (N2A) cells[57] was probed using the Alamar Blue cell assay, which was shown to be more appropriate for Aβ toxicity studies *vs.* the MTT assay.[58,59]

The cell toxicity results suggest that monomeric $A\beta_{42}$ leads to 58 ± 2% cell survival, likely due to formation of soluble $A\beta_{42}$ oligomers during the 40 h incubation with the cells (Fig. 8, lane 1). In addition, the cell survival with preformed $A\beta_{42}$ oligomers (OAβ) and $A\beta_{42}$ fibrils (FAβ) is 45 ± 6% and 86 ± 8%, respectively (Fig. 8, lanes 2 and 3), supporting the more neurotoxic nature of $A\beta_{42}$ oligomers.[60,61] While Cu^{2+} was not toxic to cells in the absence of $A\beta_{42}$ – a 90 × 5% cell survival being observed in presence of Cu^{2+} (Fig. 8, lane 4), a markedly reduced 48 ± 2% cell survival was observed in the presence of $A\beta_{42}$ and Cu^{2+} (Fig. 8, lane 5). Thus, the presence of Cu^{2+} leads to a statistically significant decrease in cell viability, which is similar to that observed for preformed $A\beta_{42}$ oligomers (Fig. 8, lane 5 *vs.* lane 2). It is important to mention here that earlier it was suggested that substoichiometric amounts of Cu^{2+} promote Aβ fibrillization and thus enhance Aβ-induced toxicity, yet an equimolar or excess amount of Cu^{2+} did not have a similar effect.[33] However, our results suggest that Cu^{2+} in an equimolar ratio to $A\beta_{42}$ also leads to an appreciable cell toxicity, strongly supporting our hypothesis that Cu^{2+} stabilizes $A\beta_{42}$ oligomers and thus increases the neurotoxicity of $A\beta_{42}$ aggregates. By contrast, Zn^{2+} reduces the toxicity of monomeric $A\beta_{42}$, (Fig. 8, lane 6 *vs.* lane 1), while a similar cell survival (80 ± 5%) was observed for

Zn^{2+} in both presence or absence of $A\beta_{42}$ (Fig. 8, lanes 6 and 7). Zn^{2+} reduces the toxicity of $A\beta_{42}$ most likely by formation of non-toxic amorphous aggregates. The results are in line with previous reports suggesting a neuroprotective role of Zn^{2+}.[30,62] Overall, these cell toxicity studies are supported by ThT fluorescence, native gel/Western blotting, and TEM, showing the formation of neurotoxic soluble $A\beta_{42}$ species in presence of Cu^{2+}, while non-toxic amorphous aggregates were observed in presence of Zn^{2+} (see above).

Conclusion

In summary, we have studied herein the effect of Cu^{2+} and Zn^{2+} on $A\beta_{42}$ oligomerization and aggregation. ThT fluorescence, native gel electrophoresis/Western blotting, and TEM studies suggest that Cu^{2+} and Zn^{2+} inhibit $A\beta_{42}$ fibrillization: while Zn^{2+} forms insoluble amorphous aggregates, Cu^{2+} leads to formation of soluble $A\beta_{42}$ oligomers. Importantly, the cell toxicity assays show that Cu^{2+} leads to an enhanced toxicity due to formation of neurotoxic soluble $A\beta_{42}$ oligomers, while Zn^{2+} decreases $A\beta_{42}$ toxicity by formation of non-toxic insoluble amorphous aggregates. Previous studies have reported conflicting results, especially regarding the effect of Cu^{2+} on $A\beta_{42}$ aggregation. Our cellular toxicity studies confirm that $A\beta_{42}$ oligomers are more neurotoxic than insoluble $A\beta_{42}$ aggregates and the increased toxicity observed in presence of both $A\beta_{42}$ and Cu^{2+} strongly supports our hypothesis that Cu^{2+} stabilizes soluble $A\beta_{42}$ oligomers. These studies rule out a preventive role of Cu^{2+} and suggest that Cu^{2+} may lead to an increased $A\beta_{42}$ neurotoxicity *in vivo* due to formation of $A\beta_{42}$ oligomers. Thus, this Cu^{2+}-promoted increased neurotoxicity of soluble $A\beta_{42}$ species may play an important role in the etiology of AD.

It has been considered that modulating metal ion homeostasis *via* metal chelation therapy may be a valid method to control the onset of AD.[63] However, we have previously shown that for the $A\beta_{42}$ peptide, in contrast to the $A\beta_{40}$ peptide, the previously employed strategy of inhibiting Aβ aggregation and promoting amyloid fibril disaggregation may not be optimal for the development of potential AD therapeutics, due to formation of neurotoxic soluble $A\beta_{42}$ oligomers.[29] Thus, we consider that the development of metal chelating compounds which do not lead to formation of toxic $A\beta_{42}$ oligomers should be promoted. Research efforts in this direction are currently ongoing in our laboratory.

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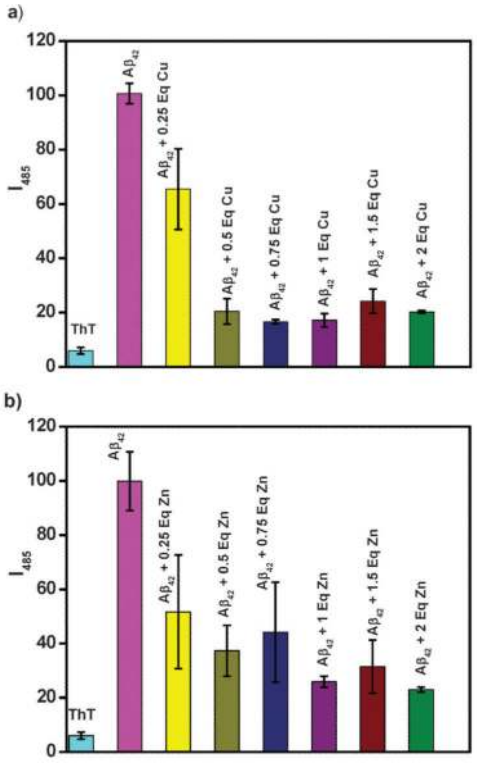

Fig. 1.

ThT fluorescence for $A\beta_{42}$ incubated with various stoichiometric ratios of (a) Cu^{2+} and (b) Zn^{2+} for 24 h at 25 °C in PBS. Conditions: [$A\beta_{42}$] = 25 μM, [M^{2+}] = 0–2 equiv., [ThT] = 10 μM.

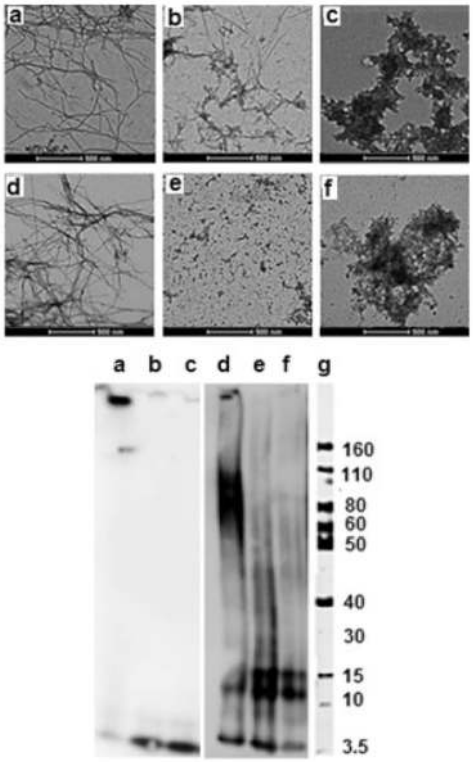

Fig. 2.

TEM images and native gel/Western blots of $A\beta_{40}$ and $A\beta_{42}$ fibrils in absence and presence of Cu and Zn. Top: (a) $A\beta_{40}$; (b) $A\beta_{40}$ + Cu^{2+}; (c) $A\beta_{40}$ + Zn^{2+}; (d) $A\beta_{42}$; (e) $A\beta_{42}$ + Cu^{2+}; (f) $A\beta_{42}$ + Zn^{2+}; bottom: Western analysis of same samples. Conditions: 24 hours with agitation in PBS at 37 °C, [Aβ] = [M] = 25 μM. The scale bar represents 500 nm.

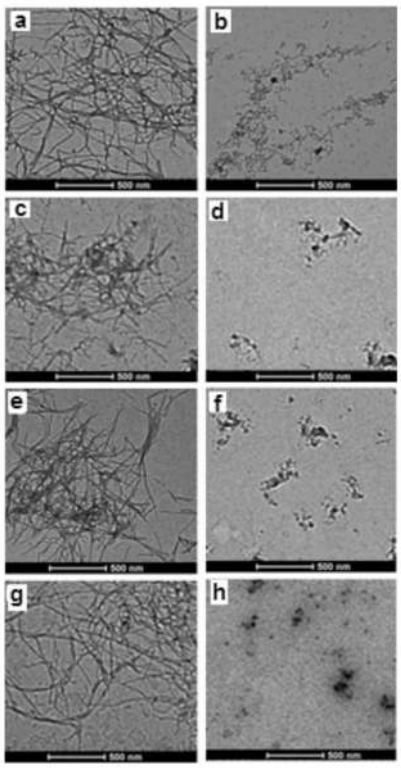

Fig. 3.

TEM images of samples containing (a) $A\beta_{42}$ (20 μM HEPES, 150 μM NaCl, pH 6.6); (b) $A\beta_{42}$ + Cu^{2+} (20 μM HEPES, 150 μM NaCl, pH 6.6); (c) $A\beta_{42}$ (20 mM HEPES, pH 6.6); (d) $A\beta_{42}$ + Cu^{2+} (20 mM HEPES, pH 6.6); (e) $A\beta_{42}$ (20 mM HEPES, pH 7.4); (f) $A\beta_{42}$ + Cu^{2+} (20 mM HEPES, pH 7.4); (g) $A\beta_{42}$ (PBS, pH 7.4); (h) $A\beta_{42}$ + Cu^{2+} (PBS, pH 7.4). Conditions: 24 hours with agitation at 37 °C, [Aβ] = [Cu^{2+}] = 25 μM. The scale bar represents 500 nm.

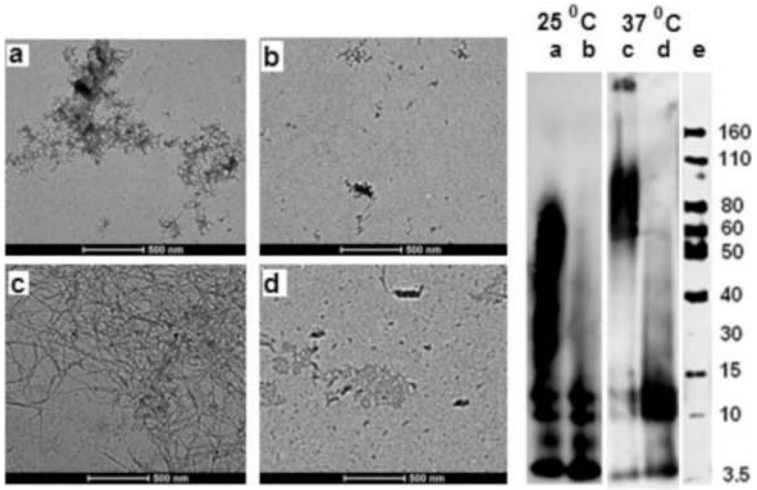

Fig. 4.

TEM images and native gel/Western blot of $A\beta_{42}$ aggregation in absence and presence of Cu^{2+}. Left: TEM images for (a) $A\beta_{42}$ aggregation for 72 h at 25 °C; (b) $A\beta_{42}$ + Cu^{2+} aggregation for 72 h at 25 °C; (c) $A\beta_{42}$ aggregation for 24 h at 37 °C; (d) $A\beta_{42}$ + Cu^{2+} aggregation for 24 h at 37 °C. Right: the corresponding Western blots, (e) molecular weight ladder. Conditions: [Aβ] = [Cu^{2+}] = 25 μM, PBS. The scale bar represents 500 nm.

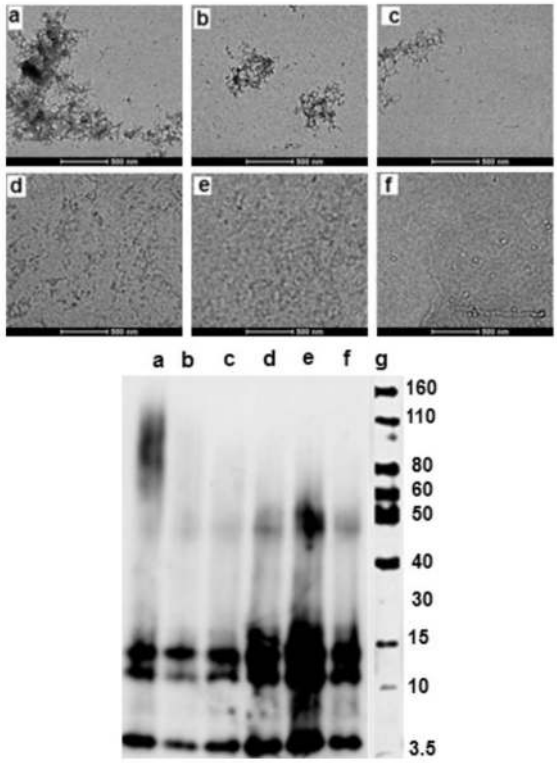

Fig. 5.

Top: TEM images and for samples containing 25 μM $A\beta_{42}$ with: (a) 0 equiv. Cu^{2+}; (b) 0.25 equiv. Cu^{2+} (6.25 μM); (c) 0.5 equiv. Cu^{2+} (12.5 μM); (d) 1.0 equiv. Cu^{2+} (25 μM); (e) 1.5 equiv. Cu^{2+} (37.5 μM); (f) 2 equiv. Cu^{2+} (50 μM). Bottom: the corresponding native gel/ Western blots, (g) molecular weight ladder. Conditions: 24 h, 25 °C, PBS. The scale bar represents 500 nm.

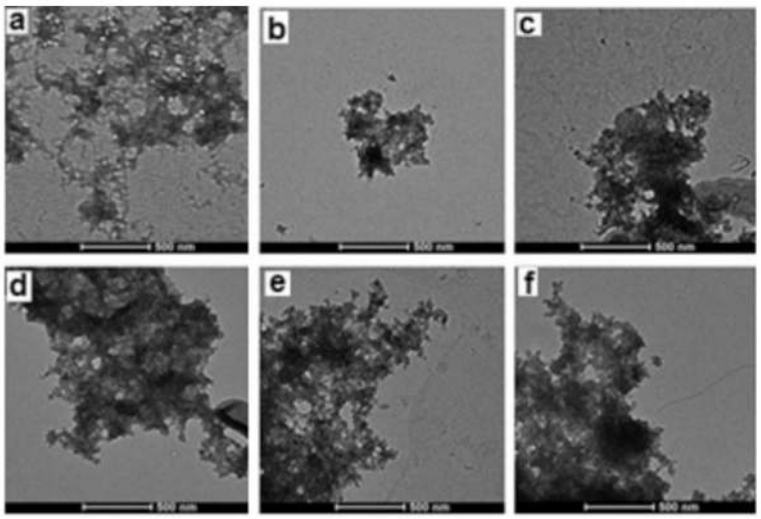

Fig. 6.

TEM images of samples containing 25 μM $A\beta_{42}$ with: (a) 0 equiv. Zn^{2+}; (b) 0.25 equiv. Zn^{2+} (6.25 μM); (c) 0.5 equiv. Zn^{2+} (12.5 μM); (d) 1 equiv. Zn^{2+} (25 μM); (e) 1.5 equiv. Zn^{2+} (37.5 μM); (f) 2 equiv. Zn^{2+} (50 μM). Conditions: 24 h, 25 °C, PBS. The scale bar represents 500 nm.

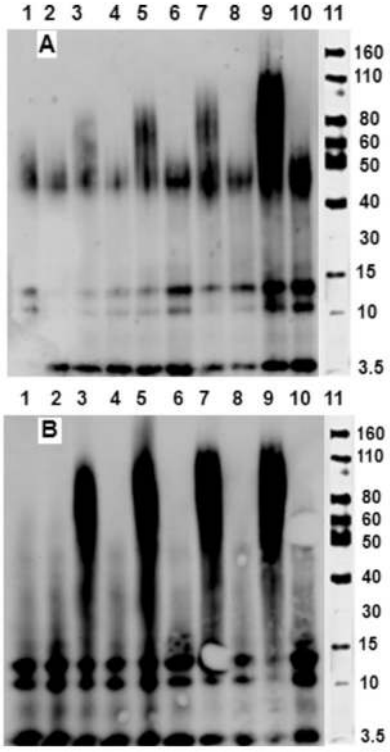

Fig. 7.

Native gel/Western blots for time-dependent aggregation of $A\beta_{42}$ with and without Cu^{2+} or Zn^{2+}. Panel A: lane 1, $A\beta_{42}$, day 0; lane 2, $A\beta_{42} + Cu^{2+}$, day 0; lane 3, $A\beta_{42}$, day 1; lane 4, $A\beta_{42} + Cu^{2+}$, day 1; lane 5, $A\beta_{42}$, day 2; lane 6, $A\beta_{42} + Cu^{2+}$, day 2; lane 7, $A\beta_{42}$, day 3; lane 8, $A\beta_{42} + Cu^{2+}$, day 3; lane 9, $A\beta_{42}$, day 4; lane 10, $A\beta_{42} + Cu^{2+}$, day 4. Panel B: lane 1, $A\beta_{42}$, day 0; lane 2, $A\beta_{42} + Zn^{2+}$, day 0; lane 3, $A\beta_{42}$, day 1; lane 4, $A\beta_{42} + Zn^{2+}$, day 1; lane 5, $A\beta_{42}$, day 2; lane 6, $A\beta_{42} + Zn^{2+}$, day 2; lane 7, $A\beta_{42}$, day 3; lane 8, $A\beta_{42} + Zn^{2+}$, day 3; lane 9, $A\beta_{42}$, day 4; lane 10, $A\beta_{42} + Zn^{2+}$, day 4; lanes 11, molecular weight ladder. Conditions: 24 h, 25 °C, PBS.

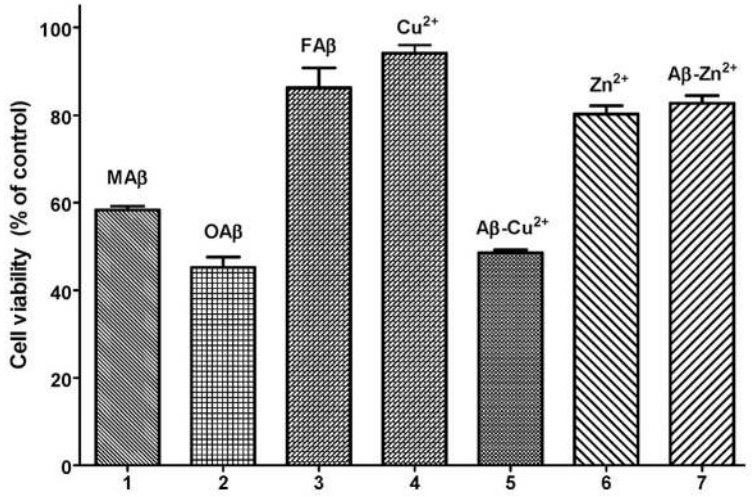

Fig. 8.

Cell viability (% of control) upon incubation of Neuro-2A cells with (1) monomeric $A\beta_{42}$ (MAβ); (2) $A\beta_{42}$ oligomers (OAβ); (3) $A\beta_{42}$ fibrils (FAβ); (4) Cu^{2+}; (5) $MA\beta_{42} + Cu^{2+}$; (6) Zn^{2+}; (7) $MA\beta_{42} + Zn^{2+}$. Conditions: $[A\beta_{42}] = [M^{2+}] = 20\ \mu M$. The *t*-test analysis of experimental data reveals values of $p < 0.001$ for any two of the above lanes, except for lanes 2 and 5 that are not statistically different ($p = 0.206$).