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Physiological cholesterol concentration is a neuroprotective factor against β-amyloid and β-amyloid–metal complexes toxicity

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ABSTRACT

Alzheimer's disease is one of the most common forms of dementia in the elderly. One of its hallmarks is the abnormal aggregation and deposition of β-amyloid (Aβ). Endogenous and exogenous metal ions seem to influence β-amyloid folding process, aggregation and deposition. Besides these variables other elements appear to affect β-amyloid behavior, such as cholesterol. The physiological concentration of cholesterol in the cerebrospinal fluid (CSF) was used in order to determine the extent in which Aβ and Aβ–metal complexes *in vitro* aggregation and their toxicity on human neuroblastoma cell cultures is affected. Cholesterol did not appear to influence Aβ and Aβ–metal complexes aggregation, but it was effective in protecting neuroblastoma cells against Aβ complexes' toxicity. The Aβ–Al complex seemed to be the most effective in disrupting and damaging membrane external layer, and simultaneously it appears to increase its toxicity on cell cultures; both of these effects are preventable by cholesterol. The presence in physiological concentrations of cholesterol seemed to compensate membrane damage that occurred to neuroblastoma cells. These findings appear to contradict some data reported in literature. We believe that our results might shed some light on the role played by cholesterol at physiological concentrations in both cellular balance and membrane protection.

1. Introduction

Alzheimer's disease (AD) is one of the most common forms of dementia in the elderly. It has been estimated that it affects 17 million people worldwide [1]. The incidence increases with age: it is 1% in people between the ages of 60 and 70 years, while is dramatically higher in mid-80s population [2]. AD is characterized by two macro events: senile plaques (SPs) and neurofibrillary tangles (NFTs). The latter consists in hyperphosphorilated Tau proteins (τ), while SPs contain insoluble deposits of β-amyloid (Aβ). Although SPs are the macroscopic hallmark, recently, researchers' interest shifted to the Aβ soluble oligomers, considered the most toxic Aβ species [3–5]. Aβ is released from a precursor protein called APP (Amyloid Precursor Protein). APP metabolism is due to three enzymes α-, β- and γ-secretase, but only when APP is cleaved by the last two, Aβ is formed. This is the first step of the so called "*amyloid cascade hypothesis*" [6]. When Aβ is released it follows a well-ordered and encoded aggregation pathway: first, monomers turn into soluble, low molecular weight (LMW) oligomeric structures, then into paranuclei (higher ordered structure), then into protofibrils (flexible structure consisting in a finite number of paranuclei) and at last into mature fibrils [7–9]. While these studies have been performed in a controlled environment, Aβ could have a different ongoing in the presence of other endogenous or exogenous elements, such as metal ions. It has been widely demonstrated that a disrupted homeostasis of metals in the brain could play a key role in the aggregation and neurotoxicity of Aβ [10–12]. The most prevalent metal ions involved in Aβ aggregation are Fe, Cu and Zn, also known to be fundamental for brain function (*e.g.* in hippocampus) and development [13,14], but a central role is also played by an exogenous metal, Al. High concentrations of Al were found indeed in AD brain [15–17] and several evidences highlight their role in worsening Aβ neurotoxicity when they are complexed with it [18–20]. This model is further complicated by other variables as well as other physiological molecules including cholesterol, a ubiquitous steroid metabolite essential for life. Meanwhile, growing evidence support the relevance of cholesterol in AD development [21–23]; however, its role in Aβ and Aβ–metal complexes aggregation has not been well researched and data in literature seem contradictory and inconsistent [24–26]. This paper aims to shed light on the role played by cholesterol, used at physiological concentrations [22,27], in influencing Aβ and Aβ–metal complexes aggregation and toxicity on neuroblastoma cell cultures. In order to better understand the interaction of Aβ and Aβ–metal complexes with cell membranes, we utilized molecular models consisting of bilayers of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), representative of phospholipid classes located in the outer and inner monolayers of many cell membranes, respectively [28,29]. The capacity of Aβ and of its complexes with Al, Zn, Cu and Fe in the absence and presence of

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cholesterol to perturb the multibilayer structures of DMPC and DMPE was evaluated by X-ray diffraction.

2. Materials and methods

2.1. Materials

Human β-amyloid 1–42 from Invitrogen, 8-anilino-1-naphtalene-sulfonic acid (ANS), L-lactic acid aluminum salt, $FeCl_3$, $CuCl_2$, $ZnCl_2$, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and cholesterol from Sigma-Aldrich (St. Louis, Mo.), and Congo Red from Merck & Co., Inc (Whitehouse Station, N.J.) were used. Experiments were carried out in 0.1 M Tris/HCl pH 7.4 buffer plus 0.15 M NaCl (standard medium).

2.2. Preparation of Aβ–metal complexes

Human $A\beta_{1-42}$ was dissolved in hexafluorisopropanol (HFIP) for 40 min at room temperature and then separated into aliquots. HFIP was removed under vacuum in a Speed Vac (Sc110 Savant Instruments). This treatment was repeated three times (modified protocol from Ref. [30]). The $A\beta_{1-42}$ metal complexes were prepared by 24 h dialysis against 10 mM metal solutions ($[CH_3CH(OH)COO]_3Al$, $FeCl_3$, $CuCl_2$, $ZnCl_2$) at 4 °C using Spectra/Por® Float-A-Lyser® tubes (Spectrum Labs) with 1000 Molecular Weight Cut Offs (MWCO). Then, $A\beta_{1-42}$ metal complexes were dialyzed against distilled water (three water changes) for 24 h to remove the excess of metals. The same treatment was also performed with Aβ alone. Aliquots of $A\beta_{1-42}$ and $A\beta_{1-42}$ metal complexes were stored at −20 °C until used.

2.3. Congo Red spectroscopy assay

Congo Red (CR) spectroscopic assay was performed according to Nilsson's protocol [31] using a 300 µL 96-well plate with U-bottom. Kinetic was followed for 24 h by monitoring the changes in absorbance at 498 nm using a Microplate SPECTRAmax® reader. Spectral differences at 498 nm are indicative of amyloid fibrils formation. The final protein concentration in each well was 5 µM, while cholesterol concentration was 50 µM (1:10 M ratio). Cholesterol was dissolved in absolute ethanol and the final ethanol concentration in wells was 2% (v/v). Signals due to the buffer alone and the buffer plus cholesterol were subtracted.

2.4. Fluorescence measurements

Fluorescence measurements were performed in a Perkin Elmer LS 50 spectrofluorimeter equipped with a thermostatic cell holder and magnetic stirring. The experiments were carried out at 25 °C. Fluorescence tests with ANS (25 µM) were developed in solutions containing 5 µM human $A\beta_{1-42}$, alone or in a complex with Al^{3+}, Fe^{3+}, Cu^{2+} and Zn^{2+}, and 50 µM cholesterol previously dissolved in absolute ethanol (2% v/v concentration in the final solution). $A\beta_{1-42}$ and Aβ–metal complexes' spectra were recorded after 24 h of incubation with cholesterol at room temperature.

2.5. X-Ray diffraction studies of phospholipid multilayers

We determined by X-ray diffraction the capacity of cholesterol, Aβ, the four Aβ–metal complexes alone and in the presence of cholesterol to modify the bilayer structures of DMPC and DMPE. About 2 mg of each phospholipid was mixed in Eppendorf tubes with 160 µL of double distilled water, and aqueous solutions of Aβ, Aβ–Al, Aβ–Fe, Aβ–Cu and Aβ–Zn in the absence and presence of cholesterol (previously dissolved in absolute ethanol). The effect of cholesterol alone on DMPC and DMPE multilayers was tested in a range of concentrations (5 to 50 µM). The final concentrations used consisted in 5 µM for the Aβ–metal complexes, 50 µM for cholesterol (1:10 M ratio) and 0.2% v/v for the ethanol as cosolvent. Each sample was incubated for 15 min at 30 °C and 60 °C with DMPC and DMPE, respectively. After that, samples were centrifuged for 10 min at 2200 rpm, the specimens were then transferred into 1.6 mm dia special glass capillaries (Glas Technik and Konstruktion, Berlin, Germany) and X-ray diffracted with Ni-filtered CuKα from a Bruker Kristalloflex 760 (Karlsruhe, Germany). Sample distances from the film were 8 and 14 cm. The relative reflections intensities were obtained in a MBraun PSD-50 M linear position-sensitive detector system (Garching, Germany); all the experiments were performed at 17 ± 2 °C, which is below the main phase transition temperature of both DMPC and DMPE. Each experiment was repeated twice, and further measurements were performed if there was uncertainty in the data.

2.6. Transmission electron microscopy (TEM)

All samples at 10 µM protein concentration, after an incubation period of 24 h, were absorbed onto glow-discharged carbon-coated butwar films on 400-mesh copper grids. Grids were negatively stained with 1% uranyl acetate and observed at 40,000x by transmission electron microscopy (TEM) (Tecnai G2, FEI). The samples contained $A\beta_{1-42}$ and its metal complexes with cholesterol 100 µM (1:10 M ratio) in 2% v/v of absolute ethanol.

2.7. Neuroblastoma cells

SH-SY5Y human neuroblastoma cells were from ECACC (European Collection of Cell Culture, Salisbury, UK). The medium in which they were cultured contained DMEM/F12 (Gibco, Carlsbad, CA, USA) with 15% (v/v) fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO), 100 units/mL penicillin and 100 µg/mL streptomycin (Gibco, Carlsbad, CA USA) and 1% (v/v) MEM (Minimum Essential Media) non essential amino acid (NEAA) (Sigma-Aldrich, St. Louis, MO). Cells were stored at 37 °C with 5% CO_2 in a humidified atmosphere (90% humidity). Cells were used until passage 25. The culture medium was replaced every two days.

2.8. Cell viability assay

Cell viability was determined by means of MTT reduction assay. SH-SY5Y cells were seeded into 24-well plates at a density of 15×10^4 cells per well in 1 mL culture medium. 15% FBS-culture medium containing $A\beta_{1-42}$, and $A\beta_{1-42}$ metal complexes with or without cholesterol was added to the cells for 24 h. Cholesterol was dissolved in absolute ethanol; the final ethanol concentration in the medium was 0.2% (v/v) that resulted largely non-toxic (data not shown). 100 µL of MTT (5 mg/mL) was added to each well and incubated in the dark at 37 °C for 3 h. After that, cells were lysed with 1 mL of acidic isopropanol (0.04 M HCl in absolute isopropanol) [32]. Color intensity was measured with a 96-well ELISA plate reader at 550 nm (Microplate SPECTRAmax®). All MTT essays were performed three times in triplicate. Viability was defined as the relative absorbance of treated *vs.* untreated, expressed as a percentage.

2.9. Statistical analysis

Congo Red spectroscopy and MTT essays were statistically analyzed by Student–Newman–Keuls *t*-test. Results were reported as highly statistically significant if $P<0.01$ and statistically significant if $P<0.05$. Results are presented as mean ± standard deviation.

3. Results

To understand Aβ complexes aggregation pathway in the presence of cholesterol, three biophysical techniques were used: Congo Red spectroscopic assay to detect fibril formation, ANS fluorescence assay to detect the exposure of hydrophobic clusters and TEM micrographs to visualize the morphology of the aggregates. In order to understand Aβ complexes interactions with cell membranes, X-ray diffraction of DMPC and DMPE was used.

4. Congo Red spectroscopy assay

Congo Red (CR) is a secondary diazo dye used in histology to detect Aβ fibrils; it is also used to detect fibrils formation *in vitro* through a spectrophotometric assay [31]. An increase in absorbance at 498 nm indicates fibril formation. Data herein reported are expressed as percentage increase of absorbance compared to the controls. As shown in Fig. 1 cholesterol seemed to have a modest anti-aggregative role. In fact, every Aβ complex showed a reduction of fibrillogenesis after 24 h of incubation with cholesterol (50 μM) at room temperature. The most notable decrease was observed for Aβ–Al, Aβ–Cu and Aβ–Zn, but only the last one was statistically significant. It is notable that cholesterol did not stop the aggregation process but only slowed it, except for Aβ–Zn complex; in this case an increase in CR absorbance was not observed. It is worth noting that Al did not promote amyloid fibrillization; on the contrary, aluminum seemed to "freeze" Aβ in an oligomeric state as previously demonstrated by Drago et al. [33], while the presence of cholesterol promoted the not aggregative role played by aluminum. In addition, absorbance due to cholesterol, CR and metal ions (Al, Fe, Cu and Zn) was measured to exclude an interaction between the three compounds; in this case we did not observe differences compared to control (CR alone) (data not shown). Thioflavine T (ThT) fluorescence assay, commonly used to detect fibril formation *in vitro*, was not performed to show Aβ–metal complexes aggregation, because preliminary analysis suggested that cholesterol could bias the results through non spectroscopic interferences (data not shown).

5. ANS fluorescence

According to Uversky et al. [34], changes in ANS fluorescence are characteristic hallmarks of the interaction of this dye with the solvent-exposed hydrophobic clusters of partially folded peptides. Each Aβ–metal complex was tested for surface hydrophobicity after 24 h incubation with cholesterol (50 μM). Aβ and Aβ–metal complexes concentration was 5 μM in each cuvette. Fig. 2 showed that aluminum is the metal that promoted the greatest increase in ANS fluorescence compared with the other metals and Aβ alone. This implied that Aβ–Al is the complex that exposed most of its hydrophobic clusters. The exposition was enhanced by the presence of cholesterol for both Aβ–Al and Aβ–Zn, and Aβ alone. As for Aβ–Cu we observed a negligible effect of cholesterol in promoting exposition of hydrophobic clusters. Aβ–Fe metal complex showed the lowest capability of exposing lipophilic clusters; the presence in solution of cholesterol did not change ANS fluorescence intensity, but unlike the other complexes the signal due to Aβ–Fe + cholesterol was lower than the signal due to Aβ–Fe alone.

6. Toxicity on cell culture

To evaluate the viability of cell cultures the capability to convert MTT to formazan crystals by mitochondrial respiratory chain reactions was measured. This cell line cannot be considered a primary choice of brain cell, but could be considered a paradigmatic model to fix the basis for further and more detailed studies. Cells were treated with Aβ and Aβ–metal complexes with or without cholesterol. The final Aβ–metal complexes concentration in the medium was 0.5 μM, while the final cholesterol concentration was 5 μM. As shown in Fig. 3, cholesterol seemed to reduce the toxicity of the two most toxic species of amyloid: Aβ and Aβ–Al. In fact it was previously demonstrated by this laboratory that Aβ–Al is more effective in

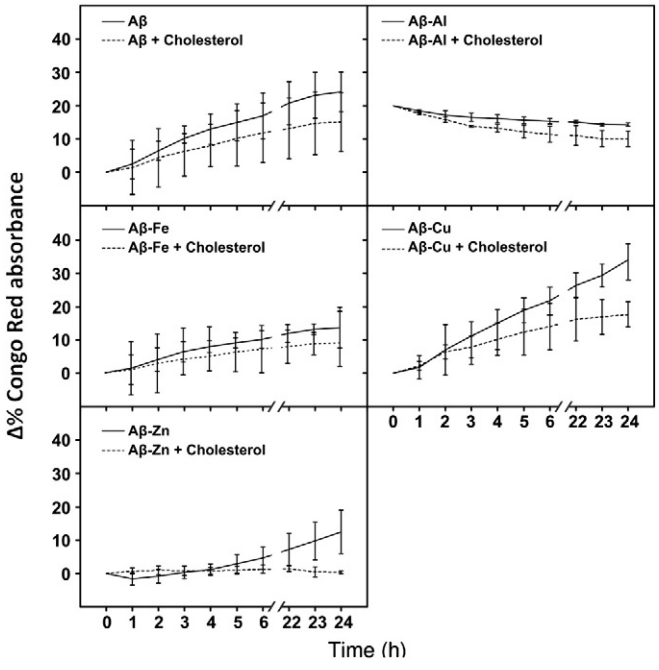

Fig. 1. Congo Red spectroscopic assay. Congo Red percentage increase of absorbance *vs.* time is indicative of amyloid fibril formation. Protein concentration in each well was 5 μM, while cholesterol concentration was 50 μM (1:10 M ratio). Ethanol concentration in the medium was 2% v/v. Solutions containing CR and metal ions (Al, Fe, Cu and Zn) were also tested to avoid interactions between the dye and metals; no significant differences were observed (data not shown).

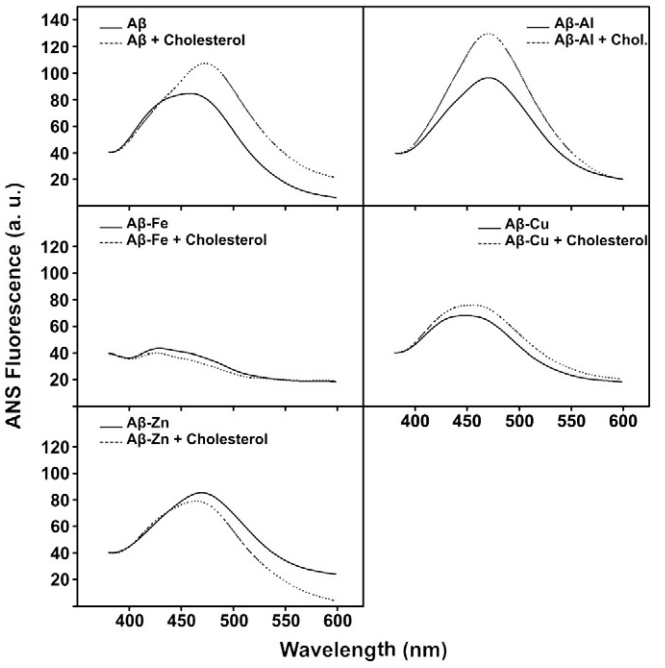

Fig. 2. ANS fluorescence assay. Increase in ANS fluorescence is indicative of solvent-exposed hydrophobic clusters. ANS spectra were recorded after 24 h incubation at RT of Aβ or Aβ–metal complexes (5 μM) in the presence or in the absence of cholesterol (50 μM). Emission spectra were recorded from 400 to 700 nm with excitation at 360 nm. The signals due to the free dye and buffer were subtracted.

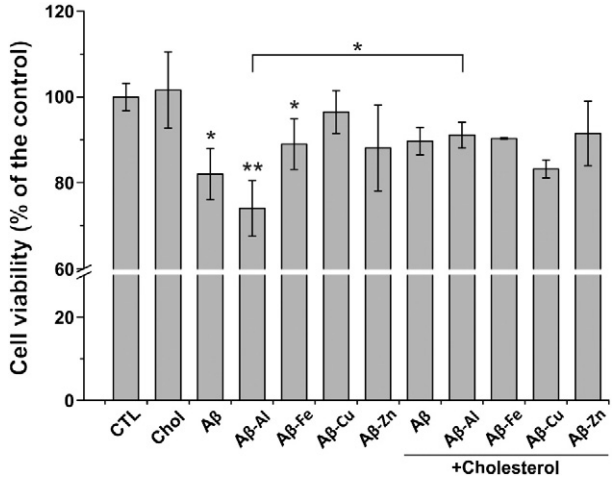

Fig. 3. Cell viability assay. SH-SY5Y neuroblastoma cells were incubated with Aβ and Aβ–metal complexes (0.5 μM) for 24 h in the presence or in the absence of cholesterol (5 μM). Data presented are expressed as % cell death as compared with control. Results are mean ± SD of three individual experiments, each done in triplicate. (* $P<0.05$, ** $P<0.01$).

decreasing cell viability when compared with the other Aβ–metal complexes [19]. Simultaneously cholesterol showed a modest and not significant capability to increase Aβ–Cu toxicity, while no effects were observed for both Aβ–Fe and Aβ–Zn in the presence of cholesterol.

7. TEM

Electron micrographs were recorded to assess the morphology of aggregates in the presence of cholesterol. Aβ and Aβ–metal complexes were incubated with cholesterol at the same concentrations used during Congo Red spectroscopic assay. Aliquots were removed at time zero and after 24 h of incubation at room temperature. As shown in Fig. 4, we observed in each micrograph the presence of cholesterol microcrystals. Aβ oligomers observed at time zero reassembled in mature fibrils after 24 h of incubation, while Aβ–Al retained its oligomeric state accordingly to the capability of aluminum to hold fibril formation [11]. Aβ–Cu in the presence of cholesterol appeared to form unstructured aggregates already at time zero; then, after 24 h fibrils became the dominant specie intercalated within cholesterol micro crystals. The aliquot containing Aβ–Fe + cholesterol showed the typical aggregation pathway of Aβ–Fe complex. In fact the micrograph recorded at time zero shows the presence of small oligomers (≈15 nm of diameter); after 24 h of incubation a large amount of amorphous aggregates was detected. Aβ–Zn, as confirmed by CR spectroscopic assay, forms at the beginning small and unstructured aggregates that after 24 h did not increase in dimension nor assumed a well defined fibrillary structure, as already reported by our laboratory [19] and confirmed by other studies [35]. Furthermore, Aβ–Zn micrographs confirmed after 24 h the anti-aggregative role of cholesterol to the amyloid–zinc complex.

8. X-ray diffraction studies of phospholipids multilayers

Fig. 5 exhibits the results obtained by incubating DMPC with water, metal-free Aβ, and Aβ–metal complexes. As expected, water altered the DMPC structure: its bilayer repeat (bilayer width plus the width of the water layer between bilayers) increased from about 55 Å in its dry crystalline form [36] to 64.5 Å when immersed in water, and its low-angle reflections (indicated as (LA) in the figure), which correspond to DMPC polar terminal groups, were reduced to only the first two orders of the bilayer repeat. On the other hand, only one strong reflection of 4.2 Å (indicated as (WA) showed up in the wide-angle region, which corresponds to the average distance between fully extended acyl chains organized with rotational disorder in hexagonal packing. These results were indicative of the fluid state reached by DMPC bilayers, which showed the typical characteristics of a Pβ′ phase [37]. Fig. 5A shows that after its exposure to metal-free Aβ and Aβ complexes with Fe, Cu and Zn in the 5 μM concentration the DMPC diffraction pattern remained practically unchanged. However, 5 μM Aβ–Al induced a considerable weakening of the low- and wide-angle lipid reflection intensities, indicative of a strong interaction of the Al complex with DMPC bilayers. Fig. 5B shows that cholesterol in

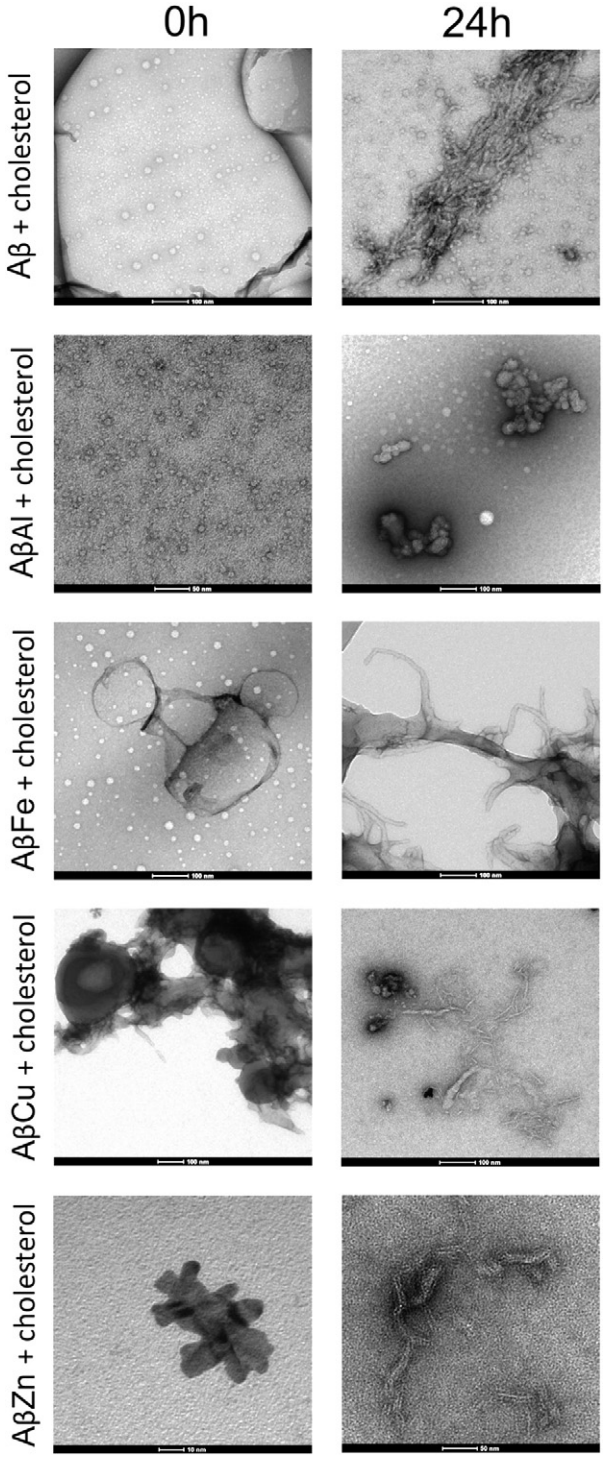

Fig. 4. TEM micrographs. TEM micrographs of Aβ and Aβ–metal complexes in the presence of cholesterol at time 0 and after 24 h incubation at room temperature.

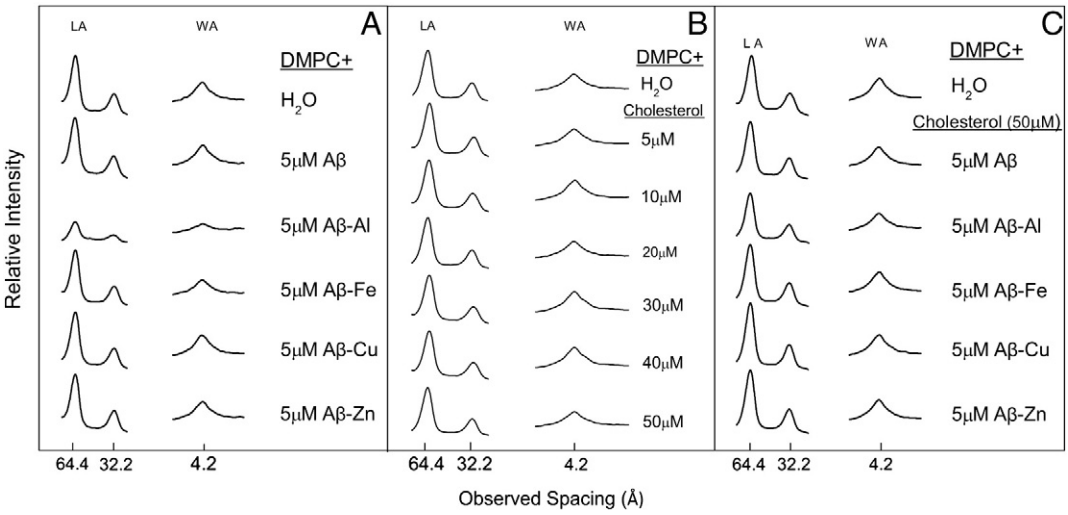

Fig. 5. X-ray diffraction of cellular membrane model. Microdensitograms from X-ray diffraction diagrams of DMPC in the presence of (A) Aβ and Aβ–metal complexes, (B) cholesterol, and (C) cholesterol and Aβ complexes; (LA) low-angle and (WA) wide-angle reflections.

the 5–50 μM concentration range did not induced changes to DMPC bilayers. Results from incubating DMPC with 5 μM metal-free Aβ and Aβ–metal complexes including that of Al in the presence of 50 μM cholesterol showed no perturbation effects of any of these compounds on the lipid structure (Fig. 5C). Similar experiments were performed on DMPE bilayers. Fig. 6 shows that the DMPE bilayer repeat expanded from about 51 Å when dry [36] to 56.4 Å when subjected to maximum hydration. On the other hand, only two reflections were observed, one more pronounced of 56.4 Å, corresponding to the first order of the bilayer repeat, and the other of 4.2 Å, indicating the fluid state reached by DMPE. Fig. 6(A, B, C) also shows that 5 μM metal-free Aβ and the four Aβ–metal complexes in the absence and presence of cholesterol did not induce changes to DMPE bilayers. From these results it can be concluded that neither metal-free Aβ, cholesterol nor the Aβ–metal complexes with Fe, Cu and Zn produced any significant structural perturbation to DMPC or to DMPE bilayers, and only that of Al significantly affected DMPC. On the other hand, the results presented in Fig. 5A demonstrate that the strong interaction of the Aβ–Al complex with DMPC bilayers was not due to the metal-free peptide, as a similar concentration of Aβ did not induce any structural perturbation to this lipid. Therefore, it can be concluded that only the association of Al with Aβ is able to interact and disturb the structure of DMPC, which is preferentially located in the outer monolayer of many cell membranes [28,29].

9. Discussion

One of the toughest challenges in the study of AD is to establish the pathological *primum movens* that sets the tone to the progression of the disease. The difficulty is due to the multifactorial character of this pathology; a recent review underlines the complexity of this pathology caused by the huge amount of variables that could interact with its development [37]. Within all the possible variables influencing the course of AD, metal ions seemed to play a critical role on Aβ aggregation and consequently its toxicity. It has been demonstrated by this laboratory that aluminum is the most effective metal ion influencing Aβ toxicity on neuroblastoma cells, while other metals tested (*e.g.*: Fe, Cu and Zn) showed minor or negligible effects [11,17,19,38]. The aim of this study was to investigate the role of cholesterol on Aβ and Aβ–metal complexes aggregation and in influencing their interaction with lipid model of cellular membrane. The role of high levels of cholesterol as AD aggravating factor has been demonstrated largely [39–41] and its ability to influence Aβ aggregation has received considerable attention despite some inconsistencies in the literature (see Table 1) [4,25,48,49]. We

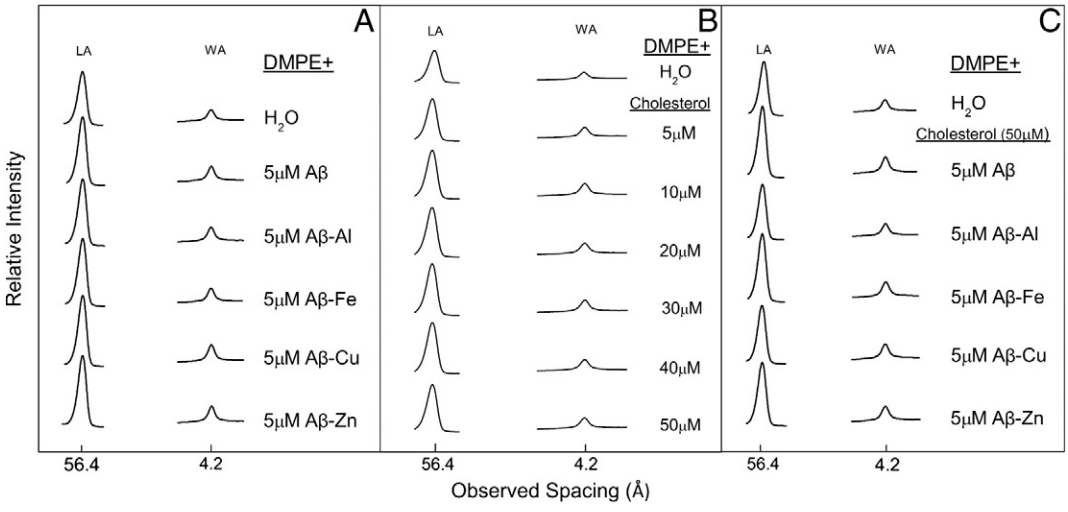

Fig. 6. X-ray diffraction of cellular membrane model. Microdensitograms from X-ray diffraction diagrams of DMPE in the presence of (A) Aβ and Aβ–metal complexes, (B) cholesterol, and (C) cholesterol and Aβ complexes; (LA) low-angle and (WA) wide-angle reflections.

Table 1
Comparison between this and other studies on Aβ aggregation and toxicity in the presence of cholesterol. Concentrations and solvent used are expressed when known. (↑: effect increased by cholesterol; ↓: effect decreased by cholesterol; = : no differences observed).

Author(s)	Reference	[Cholesterol]	Solvent	Toxicity	Aβ aggregation
Frears et al.	[49]	517 μM	Water	–	–
Ferreira et al.	[4]	50 μM	–	↑	–
Harris JR	[25]	2.58 mM	Water	–	↑
Mizuno et al.	[50]	–	–	–	↑
This paper		5 μM	Ethanol	↓	=

have previously hypothesized that the toxicity of Aβ–metal complexes is related to their ability in perturbing cellular membranes [20]. In this connection there is a huge body of evidences supporting Aβ role in disrupting membrane structure [42–45]. The ability of Aβ–metal complexes to alter membrane structures is linked to their ability to retain the oligomeric state and to expose hydrophobic clusters that interact with the lipid bilayer [46]. Aβ–Al was the most effective Aβ–metal complex to combine these features also in the presence of cholesterol (Figs. 1 and 2). In agreement, we demonstrated that Aβ–Al was the most effective in perturbing cell membrane models as well as in causing neuronal death [19,20]. In our experimental conditions, cholesterol (at the same molar ratio used on cell cultures, 1:10) seemed to balance the increased membrane disorder caused by the presence of Aβ–Al. As shown in Fig. 5B, cholesterol alone did not induce changes on DMPC bilayer structure. On the contrary, the presence of cholesterol in solution with Aβ–Al drastically reduced alterations on DMPC bilayer caused by this metal complex. This result could be explained on the basis of the role played by cholesterol in membrane structure. It has been amply demonstrated that cholesterol modulates the properties of lipid bilayers, making them less deformable and thereby less permeable to small water-soluble molecules [47]. These results appeared consistent with data obtained from cell viability assay (Fig. 3). We observed a significant decrease in Aβ–Al toxicity in the presence of cholesterol. Also Aβ alone resulted less toxic even not significantly in the presence of cholesterol. Only Aβ–Cu in the presence of cholesterol resulted more toxic if compared with Aβ–Cu alone. We could speculate that this result is most likely due to oxidative stress properties of Cu emphasized by the presence of cholesterol, however further and more specific investigations are required. Meanwhile, treatment with Aβ–Fe and Aβ–Zn in the presence or in the absence of cholesterol exerted only negligible effects, both in cell viability and X-ray diffraction essays. As for cell viability, Aβ–Fe showed a higher reproducibility in cells treated with Aβ–Fe in the presence of cholesterol (SD = ±0,14%) unlike those treated with Aβ–Fe alone (SD = ±5,9%); we can hypothesize that this result is related to an increased stabilization of the Aβ–Fe complex in the presence of cholesterol; further investigations are required to confirm this speculation and clarify the interaction between Aβ–Fe and cholesterol.

One aim of our study was to investigate the effect of Aβ and Aβ–metal complexes on a lipid model of cellular membrane. We found that Aβ–Al complex was the most effective in perturbing DMPC bilayer (Fig. 5A) compared to the other metal complexes. Considerably less pronounced was the effect of Aβ–Al complex on DMPE bilayer (Fig. 6A). This result can be explained on the basis of their different structures. DMPC and DMPE differ only in their terminal amino groups, these being $^{+}N(CH_3)_3$ in DMPC and $^{+}NH_3$ in DMPE. Moreover, both molecular conformations are very similar in their dry crystalline phases [36] with the hydrocarbon chains mostly parallel and extended, and the polar head groups lying perpendicularly to them. However, the gradual hydration of DMPC results in water filling the highly polar interbilayer spaces with the resulting increase of their width. This phenomenon allows for the incorporation of the Aβ–Al complex into DMPC bilayers disrupting their arrangement and consequently the whole of the bilayer structure. Moreover, DMPE molecules pack tighter than those of DMPC due to their smaller polar groups and higher effective charge, resulting in a very stable bilayer system that is not significantly affected by water [36]. On the other hand, the Al complexed with Aβ may induce a change in the net charge of the peptide which can promote abnormal lipid–peptide interactions, thus promoting pathological oligomerization of Aβ. Recently, our group has demonstrated that when Al was bound to Aβ, forming a stable metallorganic complex, the surface hydrophobicity of the peptide dramatically increased as a consequence of metal-induced conformational changes, favoring misfolding/aggregation phenomena [50]. Aβ–Al, thanks to its higher lypophilicity compared to the other Aβ–metal complexes, could intercalate with the acyl chain region altering the bilayer arrangement. In accordance, we showed that Aβ–Al was able to promote a greater increase in membrane fluidity mostly in the lipid tail/polar heads border areas of cell membrane with respect to the other Aβ–metal complexes [20].

Collectively, our results indicate that cholesterol seems to slow down Aβ and Aβ–metal complexes aggregation pathway without blocking it, with the exception of Aβ–Al (see above). In addition, our results indicate that physiological concentrations of cholesterol avoid external lipid bilayer leaflet disruption caused by Aβ–Al. In agreement we also observed a decrease in Aβ and Aβ–metal complexes toxicity, with the not significant exception discussed above. These data could suggest a protective role played by cholesterol at concentrations closed to those physiological, highlighting once more the thin limit between positive or negative effects caused by cholesterol. Nevertheless future studies will be required to fully understand the mechanisms that underlie these results.

Acknowledgments

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