Researches about important factors for endocytic transfer of amyloid beta (Aβ-42) through biomimetic and biological membranes

Neha Sharma

Japan Advanced Institute of Science and Technology

Doctoral Dissertation

Researches about important factors for endocytic transfer of amyloid beta (Aβ-42) through biomimetic and biological membranes

Neha Sharma

Supervisor: Professor Masahiro Takagi

School of Materials Science

Japan Advanced Institute of Science and Technology

June 2017

Acknowledgements

I would like to sincerely thank my Ph.D. supervisor, Professor Masahiro Takagi, for his embracing guidance and support. He provided me a well-equipped research environment that helped me to finish this valuable work. I am very thankful for his constant support. I am very thankful that he understood my situation and gave me precious chances of visiting my family in India. I am extremely grateful to him for providing me financial support in every aspect especially for my stay and study in Kagoshima for minor research training.

I would like to thank my Assistant Professor Naofumi Shimokawa, for giving me important comments which helped to improve and advance my study. He provided valuable guidance and support throughout my research.

I would extent my gratitude towards my sub-supervisor, Associate Professor Toshiaki Taniike, for providing the valuable comments and appreciation in my study.

I highly appreciate Japan Advanced Institute of Science and Technology for proving me with the scholarship and great environment to study and live a good life. I am highly grateful to the administrative staff of JAIST to support and help to make my stay convenient here.

I am deeply thankful to Associate Professor Mun'delanji Catherine Vestergaard, (Kagoshima University) for her guidance and support in my minor research training. She helped to broaden my knowledge and improve many skills such as problem-solving and critical thinking. I am highly thankful to the Tamaki laboratory members and administrative staff members of Kagoshima University to make my stay comfortable.

I am thankful to Professor Yuzuru Takamura, for his guidance and support to conduct remaining part of minor research in JAIST and to his lab members for helping me to use the equipment and for maintaining a friendly atmosphere in the laboratory.

I also extend my appreciation to Takagi laboratory members KeangOk Baek, Shoji, Ko Sugahara, Satomi Yabuuchi, Shinji Shimokawa, Ryota Yamamoto and all the other members for providing a sincere and friendly environment to study for my research. I am highly thankful to them for their constant support and help which is very precious to me.

I want to express my kind regards towards my teachers and administrative department of University of Delhi (DU) to introduce and to provide a chance to join JAIST in my master degree course from there. It was a great opportunity to develop my understanding in research and to develop my personality from an international exposure.

I sincerely express my love and thankfulness to my parents, siblings and friends to encourage me, care for me. Their love strongly motivated me to finish the fruitful study here.

JAIST, June 2017

Neha Sharma

Abstract

Alzheimer's disease is a worldwide leading dementia affecting millions of life globally. Deposition of amyloid beta (Aβ) peptides into extracellular plaques and formation of intracellular neurofibrillary tangles (NFT) are the pathological hallmarks in Alzheimer's disease. Aggregation, accumulation, misfolding and cytotoxicity of the peptide are the key events in the pathogenesis of the neurological illness. Accumulation of misfolded proteins induces stress to endoplasmic reticulum (ER) which is responsible for the correct folding of newly synthesized proteins. This phenomenon is generally known as ER stress which is linked with other cellular responses such as release of calcium ions, inflammation and apoptosis. It has been reported that ER stress may occur by externally added Aβ which would possibly cause similar effect on the cellular processes. I have speculated that exogenous Aβ may be internalizes into the cell via endocytic pathway, then causes stress to the organelle. In this dissertation, I aimed to investigate the important factors mediating the endocytic transport of Aβ-42. These factors are associated with the risk factors for Alzheimer's disease such as aging, oxidative stress, advanced glycation end products (AGEs), diabetes, genetic or hereditary factors. There is a critical link between Alzheimer's disease and diabetes where oxidative stress, AGEs etc. are the common features between these two ailments. Oxidative stress causes lipid oxidation which generates oxygenated derivatives of cholesterol, known as oxysterols. Recently, oxysterols have been recognized as risk factors for Alzheimer's disease which can be generated by enzymatic oxidation of cholesterol. Thus, in this study role of oxysterols in Alzheimer's disease was emphasized with other factors involved in the endocytic transport of the peptide.

Accumulating evidence have suggested that interaction of Aβ with cell membranes has important role in these processes. Biomimetic and biological membranes possessing different levels of complexities were employed to achieve the aim of this dissertation. 7-ketocholesterol (7-KC) and 25-hydroxycholesterol (25-OHC) were the two oxysterols used to substitute the membrane cholesterol level among which latter is produced by reactive oxygen species (ROS) and former gets generated on the enzymatic oxidation to make the transport of cholesterol from brain in form of its derivatives. Notably, toxicity of Aβ depends upon its isoforms and their state of aggregation, thus based on the current understanding, protofibrillar Aβ-42 was used throughout the study.

In the heterogeneous biomimetic membranes, after substituting the cholesterol level with 25-OHC, I have found that the localization of protofibrillar Aβ-42 was influenced in comparison to the membrane without substitution. Predominantly, it occurred in the liquid-disordered phase of lateral compartments of the membrane. Next, I assess the aggregation kinetics of the peptide in the presence and absence of oxysterol. As oxysterols are cytotoxic in nature, thus presumably, they would enhance the toxicity induced by Aβ peptide. So as happened after the addition of 25-OHC, more toxic species were formed sine the fibrillation of Aβ-42 was restricted to form oligomers and protofibrils as compared to less toxic monomers and mature fibrils.

Studies on biological membranes, using Jurkat T cells, a leukemic cell line were performed to assess the effect of oxysterols on the interaction of the peptide with cell membranes. With previous understanding about the oxysterols which suggested induction of peptide insertion into the membrane, increase in excess surface area, enhance surface interaction of Aβ with the membranes. This dissertation have clearly shown that 25-OHC mediates the endocytic transport of protofibrillar Aβ-42 in the cell in correspondence of negative curvature induction by cholera toxin B subunit and GM1 interaction. After insertion of Aβ-42, they were transported to ER via microtubules within the cell. As a result, intracellular calcium ions release was observed which induced after disruption of calcium homeostasis by the action of Aβ-42 and oxysterols in a concentration dependent manner. Although Aβ-42 and oxysterols are cytotoxic in nature but they could not cause severe damage to the Jurkat cells under used conditions.

Undifferentiated human neuroblastoma SH-SY5Y cell line which is an analogue of neuronal cells was used to investigate the Aβ-induced toxicity in the progression of Alzheimer's disease. To assess the effect of concentration of peptide, I used different concentrations of protofibrillar Aβ-42 and observed that toxicity of the peptide was dependent upon the concentration of Aβ-42 protofibrils. This was in accordance with a previous study which proposed that the aggregation of peptide varies with its density. At higher concentrations, Aβ-42 was lethal to cells whose effect was increased in the presence of both oxysterols in a time-dependent experiment. Lower concentration of the peptide

was used to analyze the internalization affected by oxysterols at different incubation times. Higher amount of protofibrillar Aβ-42 was internalized into the cells in the presence of oxysterols. After internalizing into the cell, protofibrillar Aβ-42 was localized into the ER which indicates the induction of ER stress after accumulation of misfolded proteins in the lumen of ER which was the inspiration and speculation of this study.

In conclusion, use of biomimetic and biological membranes provided an advantage to further our understanding about the mechanism behind changes induced by Aβ in Alzheimer's disease. Through the findings of this dissertation, a clear view about the risk factors for the disease was represented. Oxysterols, glycosyl chains in the membrane, nano-structures of the peptide were the risk factors studied here. Thus, prevention of oxidation of cholesterol, fibrillation of Aβ-42 and avoiding glycation may be a substantial approach in the treatment of Alzheimer's disease.

Keywords: Amyloid-beta (Aβ-42), oxysterols, toxicity, endoplasmic reticulum, biomimetic membranes, biological membranes.

Table of Contents

Chapter 1:

General Introduction

Abstract

Background of the study, with close review of related literature was introduced in this chapter. Importance of amyloid beta (Aβ), its toxic forms and their role in the pathology of Alzheimer's disease (AD) which is known as an age-related dementia, was discussed. Additionally, risk factors of AD were explained. Role of oxygenated derivatives of cholesterol (oxysterols) in neurodegenerative disorders was explained with a general overview of their structural and functional aspects. Presence of oxysterols has been reported at significant and consistent levels in AD brains makes them relevant to the study. Integral function of cholesterol and its derivatives in endocytic transport of Aβ through biological membranes were studied. Based on the literature background and factual details, objectives of the study were proposed.

Keywords: Oxysterols, amyloid beta, endocytic transport, membrane, Alzheimer's disease.

1.1. Pathology in Alzheimer's disease- cellular toxicity induced by Aβ

1.1.1. *Alzheimer's disease (AD)*

Alzheimer's disease (AD) is the worldwide old age dementia increasing at an alarming rate. Accumulation, aggregation, misfolding of amyloid beta (Aβ) is the pathological hallmark in AD. This illness was introduced in 1906 by Dr. Alois Alzheimer (1). It is a chronic neurological disorder which accompanies with memory loss, cognitive decline etc. It is characterized with intracellular neurofibrillary tangles of phosphorylated tau protein, extracellular amyloid plaques, and vascular damage resulting from extensive plaque deposition, neuronal cells and synapse loss. Foremost damage occurs in the hippocampus area of the brain which is responsible for memory functioning. It has been divided into three stages such as mild, moderate and severe stages of the ailment. Mild AD involves short term memory loss. This will progress into long memory loss, cognitive disabilities known as moderate AD. It is followed by severe AD where difficulties in communication and loss of body functions apparently leads to death (2). Furthermore, AD can be characterized into familial AD and late-onset AD. Familial AD is also known as early-onset AD which affects people in their 40s to 50s, it is possible due to genetic abnormalities. Late-onset AD often called as sporadic AD, affecting people after the age of 65. It is associated with aging and contributes majorly with the disease (3).

Now, AD has become a global life threat to human health, specially influencing health of aged people. It is the leading dementia, accounts for around 60 to 80 percent of the total dementia cases. Only in United States, roughly 5.4 million people are already fighting with AD. It is among six leading causes of death in the continent. On the gender basis, women has been found to be affected more than men (4). According to World Health Organization, there is a sharp increase in the number of people suffering from the dementia, in 2006, it was around 35.6 million in the world. This number is predicted to rise to 75.6 million in coming 13 years and 135.5 million by 2050 (5). Due to its wide-spreading approach, AD has come out to be an imminent threat to human health and healthcare system over the globe (6).

1.1.2. *Different approaches in Alzheimer's disease*

There are multiple hypothesis about the development of AD in the brain, plenty of researches are going on, but the exact mechanism behind the stimulation of disease has not been cleared yet. Among those multiple approaches amyloid cascade (7), cholinergic (8), oxidative stress (9), tau protein hypotheses (10) proposed till date, hypothesis suggested by Hardy and Higgins (1995) is believed to be the most significant proposal (11). It has demonstrated the formation of plaques through the deposition of amyloid beta (Aβ) peptides in brain tissues. Relation of Down syndrome which has trisomy of APP gene and some of these gene mutations having high chances of AD (12) has given rise to 'amyloid cascade hypothesis'. This proposal have shown the Aβ-induced cytotoxicity and accumulation of peptides is an integral part in the pathology of AD. This was accepted and supported by numerous studies across the world for more than two decades, also demonstrated the involvement of Aβ-42 isoform to be more serious than 40-residue. Aβ is considered to be the causative agent in AD which amplifies the formation of neurofibrillary tangles of tau protein, neural cells loss and vascular damages. There are two pathological events in AD; firstly, production of Aβ from its precursor protein which is known as amyloid precursor protein (APP), and next is the toxicity induced by Aβ to neuronal cells and the formation of neurofibrillary tangles of tau. In the proposal, a number of causes have mentioned for the production, deposition and toxicity of Aβ peptides, which constitutes many important biological processes such as genomic, metabolic, histopathologic, inflammatory, cholinergic conditions and oxidative stress. After all these studies and proposal, it is still a challenge to unravel the exact mechanism behind the activation or appearance of AD in humans.

1.1.3. *Generation of Aβ from amyloid precursor protein (APP)*

Amyloid precursor protein abbreviated as APP, generates Aβ peptides on enzymatic cleavage. APP is a transmembrane protein (~100-130 kDa) possessing large extracellular domains (13, 14). Its gene; APP gene gives instructions for the processing of APP. APP gene locates in chromosome 21 (12) which undergoes alternative slicing to produce 8 different isoforms (15). There are three common isoforms; 695-, 751- and 770-residue which resides in central nervous system (CNS), among them the first one is expressed superiorly while the other two are expressed pervasively.

APP processing takes place using two different pathways; amyloidogenic and non-amyloidogenic. Amyloidogenic pathway, first uses beta-site APP cleaving enzyme (BACE) and then γ-secretase to produce soluble N-terminal ectodomains (sAPPβ), the identical intracellular C-terminal (AICD) and Aβ peptides. Non-amyloidogenic pathway uses α-secretase for protein cleavage in the extracellular domain and then γ-secretase in the transmembrane domain. Products obtained in this pathway are: sAPPα, P3 peptides and AICD as shown in figure 1.1 (16). Among the two pathways, non-amyloidogenic is more common type of processing for proteolysis of APP which takes place in cell membranes. Amyloidogenic pathway occurs primarily in the trans-Golgi network and endosomes due to the presence of BACE in the intracellular compartments (14, 15, 17). After the formation of Aβ, it is then released into extracellular spaces by the process of vesicle recycling (15).

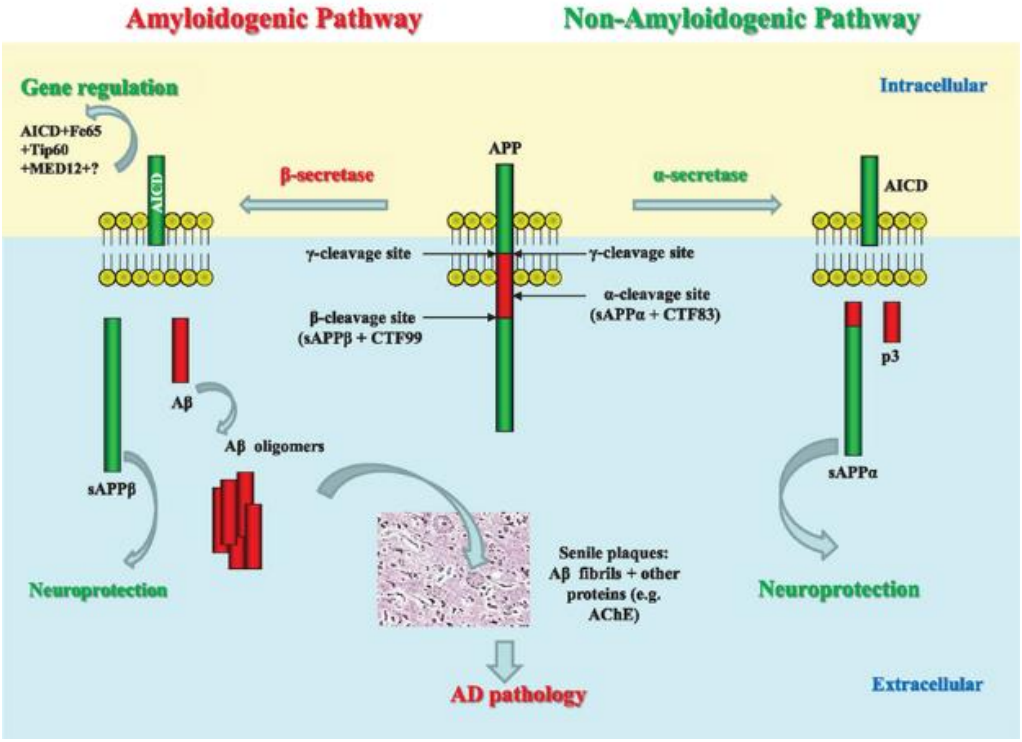


Figure 1.1. Processing of Aβ by amyloidogenic and non-amyloidogenic pathways (15).

When there are genetic and/or metabolic abnormalities in the body, it causes modifications in APP processing which concomitantly produce Aβ in excess amount or alters the ratio of its isoforms. In metabolic abnormalities, alterations in lipid composition of membranes changes

normal activities of cleaving enzymes in processing of APP since they are membrane-linked proteins, which influences the amyloidogenic pathway for APP processing (14, 15). On the other hand, in genetic mutations, genes associated with AD are affected. In Down syndrome, APP gene number increases to three times as compare to normal physiological conditions, thus these mutant APP genes changes the residue next to cleavage site of BACE and promotes formation of Aβ (18). Presenilin (PS-1) and PS-2 are other genes which are components of enzyme, γ secretase, mutations in these genes enhances the production of more toxic residue (-42 isoform) than Aβ-40 (19).

Large amount of APP is synthesized in neurons. APP is considered significant in cell signaling, cognitive and synaptic functions, cell and synaptic adhesion, synapotropic and neuroprotection (20). Nascent proteins swiftly internalizes into the cell membranes after they are transported with the help of endosomes. Endosomes takes proteins from Golgi apparatus to cell membranes. They can also take back the proteins to the cell surface or lysosomes, for degradation (17). In general physiological conditions, cells can get rid of excess of Aβ using three different mechanisms. Pathways for clearance of Aβ in the brain are as follows, i) insulin-degrading enzymes-induced extracellular degradation, ii) Aβ peptide release from CNS to blood, and iii) intracellular proteolytic degradation in microglia and astrocytes. If these clearance pathways are hindered, production of Aβ peptides significantly increases, resulting in accumulation of peptides in the brain (21).

1.1.4. *Structure and aggregation of Aβ peptides*

APP processing produces Aβ peptides, comprising of 39-43 amino acids, among which 40 and 42 residues are the superior isoforms. Estimated weight of Aβ is around 4.5 kiloDalton (kDa) with hydrodynamic radius of 0.9±0.1 nm (22). Under normal physiological conditions, ratio of two isoforms of Aβ is 9:1 (14, 23). Aβ peptide comprises a hydrophobic C-terminal (residues 28-43) and hydrophilic N-terminal (residues 1-27) molecule, thus it is an amphiphilic molecule. Central region of the peptide also constitutes a hydrophobic core (residues 16-22). In its N-terminal, there are acidic and basic amino acids. Thus, Aβ is negatively and electrically neutral at physiological and weakly acidic pH, respectively (24). The peptide is present in a helix-turn-helix-like conformation in its native state in APP (23) which gets converted into soluble form after amyloidogenic APP processing. Previous studies have shown that Aβ monomers exhibited random

coil structure in aqueous environment (25). On the contrary, some other studies proposed about forming a partly α-helical (26) or a β-sheet form (23). Hence, changes in temperature, pH and concentration influences the conformation of Aβ monomers which plays phenomenal impact on amyloidogenicity (23, 27).

All amyloid peptides are prone to aggregate and form fibrils. *In vitro* studies reported concentration to be at μM range for impetuous aggregation (28). Commonly, Aβ aggregation considered as a nucleation-dependent polymerization. There are two crucial steps in this polymerization process which are i) nucleation and ii) fibril elongation (29, 30). Formation of nuclei from soluble monomers is the first step where unfolded/α-helix-enriched structure changes to β-sheet-enriched structure. This conformational transition is an important step in nuclei formation, occurring at high peptide concentration or acidic pH. Hydrophobic C-terminal and central region are also thought to be important for the transition (23). It also depends upon the ionic strength, presence of some ions and cell membranes (23–27). β-sheet enriched structure tends to aggregates on its own (31) and produces dimers, trimmers and finally nuclei (30–32). Intermediate species of Aβ, including oligomers and protofibrils were reported to appear during Aβ fibrillation. According to few researchers, oligomeric species serves as a nuclei of fibril growth (33, 34). These oligomeric species are found to present as prefibrillar oligomers comprising beta-sheet structure (35, 36) globulomers having globular shape, or pentamers (disc-shaped) composed of loosely aggregated strands (34). Thus oligomers are present in different size (37) and structure. Small elongated oligomers are known as protofibrils (38) which are considered as the precursor of fibrils. Fibrils are generally longer and straight than protofibrils (33). Oligomers and protofibrils have been demonstrated as the primary toxic species of Aβ peptides (36). Aβ oligomers exhibits a great degree of polymorphism with various biophysical, structural, and cytotoxic properties. Specifically, oligomeric species of the peptide have been categorized as A+ and A- oligomers, stating A- to be less toxic than A+ oligomers, probably as a culmination of exposure of hydrophobic residues which would be important in their interaction with the plasma membrane (39, 40).

In the process of fibril elongation, nuclei of Aβ peptides polymerize to form fibrils which exhibits β-sheet conformation (32, 34). In residue-40 isoform (Aβ-40), residues 1-10 is unstructured while conformation of residues 11-40 is β-strand-turn-β-strand, with side chain

packing between Phe 19 and Ile32, Leu34 and Val36, and between Gln15 and Val36. On the other hand, in isoform Aβ-42, residues 18-42, β-turn-β structure is observed from the bindings between Phe19 and Gly38 and between Met35 and Ala42 (41). It was demonstrated that Phe19 and Leu34, Gln35 and Gly37, and His13 and Gln15 can create the side chain packing in Aβ-42 (34). Moreover, when β-strand bends at Glu22, Asp23, and Lys28, the conformation acquires stability by salt bridge among residues in both isoforms and hydrophobic interactions (see figure 1.2) (34). Two additional hydrophobic amino acids in Aβ-42 cause faster aggregation as compare to Aβ-40 (33). Different states of aggregated Aβ peptides induces varied neurotoxicity, thus aggregation is crucial aspect in Aβ (30).

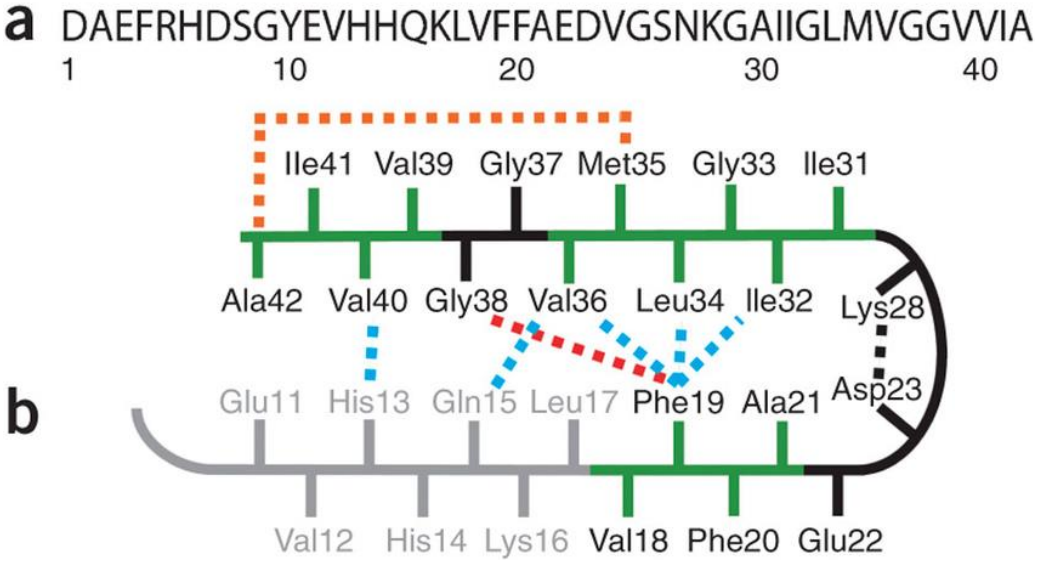


Figure 1.2. Sequence and structure of the monomer of Aβ-40 and Aβ-42. (a) Sequence of Aβ-42, derived from human APP. (b) Structural constraints in Aβ-40 and Aβ-42. NMR measurements of Aβ40 fibrils have shown that residues 1–10 are unstructured and residues 11–40 adopt a β-turn-β fold19, 20. Side chain packing is observed between Phe19 and Ile32, Leu34 andVal36 and between Gln15 and Val36 as well as between His13 and Val40 (blue dashed lines). In Aβ-42, residues 1–17 may be unstructured (in gray), with residues 18–42 forming a β-turn-β fold14. Molecular contacts have been reported within the monomer unit of Aβ-42 fibrils between Phe19 and Gly38 (red dashed line) 14 and between Met35 and Ala42 (orange dashed line) 18. In both Aβ-40 and Aβ-42, the turn conformation is stabilized by hydrophobic interactions (green residues) and by a salt bridge between Asp23 and Lys28 (black dashed line) (33).

1.1.5. *Toxicity of Aβ peptide to living cells*

In the brain, Aβ accumulation activates a cascade of toxic processes including synapse dysfunction, neuron loss, cognitive impairment and dementia (12, 18). Aβ shows many harmful effects on synapse and neurons. Among different aggregates species of Aβ, oligomers and protofibrils are reported as dominantly toxic species where Aβ-42 exhibit adverse nature than Aβ-40 (33, 36).

It was observed that when Aβ was incubated with glucose or fructose, elevation in oligomeric species was observed at the expense of fibrillar species (42). Since mechanism behind Aβ-induced toxicity is direct correlated with the conformation of Aβ, it may be leading to increase the cell viability due to the formation of stable oligomers whereas the fibrillar species ae reported to be more toxic than the soluble oligomers (43). On the other hand, in vitro and in vivo studies have suggested the toxicity of soluble oligomers (44, 45). Cellular toxicity induced by Aβ oligomers can be explained by the fact that same peptide may produce structurally different oligomers depending on the hydrophobic residues, growth conditions, compactness, possessing different stability and cytotoxicity (46).

Accumulation of Aβ in endoplasmic reticulum (ER) triggers ER stress, which enhances calcium efflux from ER to cytosol (47). In addition, Aβ induces dyshomeostasis of Ca^{2+} which is considerable in learning, memory, neuron survival and death. Level of Ca^{2+} in cytosol is increased by the changes induced by Aβ in calcium pumps and ion channels in cell membranes (48). Another effect of peptide is disruption of ionotropic glutamate receptors (membrane receptors) (49) that are important to synaptic activities (50). Alteration in synaptic activities triggers neuron apoptosis. The peptide is also able to induce dysfunction of mitochondrial which promotes ROS production (48). Moreover, Aβ peptide impairs the activity of GTP-binding proteins, ion-motive ATPases, (18, 51) glucose and glutamate transporters which increase cellular oxidative stress and thus, cause oxidation of lipids (49). Combination of Aβ with Fe^{2+} and Cu^{2+} (redox-active metals) potentially oxidizes lipids in the membrane (52).

Neuroinflammation is another major consequence of Aβ accumulation. Enough evidences have shown that Aβ activates microglia that are important to initial responses of CNS to injuries, and astrocytes, another glia cells. Astrocytes are responsible for multiple functions such as injury response, organization of brain, and blood-brain-barrier formation. Under normal physiological

conditions, microglia and astrocytes are capable of these cells, which leads to production of inflammatory factors (cytokines). Neurotoxic effects such as synaptic damages and neuron apoptosis are induced by these cytokines (53).

As mentioned earlier, appearance of Aβ extracellular plaques and intracellularly tangles are characterizes as pathological hallmarks of AD. Accumulation of Aβ peptides has been demonstrated to induce formation of tangles of hyperphosphorylated tau proteins, present as paired helical filaments. Activity of several kinases and phosphatases changes in Aβ accumulation, which facilitates the phosphorylation of tau and neurofilaments that eventually leads to the formation of neurofibrillary tangles. Tau protein is mainly found in the cytosol and occurs unfolded in its native state (50). Tangles of tau protein induces cytoskeletal disruption, activate apoptosis proteins such as caspase-3 and finally cause apoptosis of neuronal cells (51, 54).

1.2. Risk factors of Alzheimer's disease

1.2.1. *Leading risk factors for Alzheimer's disease*

AD is a worldwide leading dementia which can be advanced with the help of some factors. They are known as risk factors of AD, including age, genetics and family history. The biggest known risk factor for AD is aging, mostly affected people are above 85 years of age. Aging leads to accumulation of reactive oxygen species which cause lipid peroxidation.

There are mainly two types of genes associated with AD that are *risk genes* and *deterministic genes*. Risk genes increase the chances to attain AD, apolipoprotein E-e4 (ApoE-e4) is the most influential risk gene which holds substantial effect in the disease. Basically in APOE there are three common genes; APOE-e2, APOE-e3, APOE-e4. Among these forms, APOE-e4 is the most vulnerable form which have tendency to prepone the possibility of AD in younger age. Other than risk genes, deterministic genes are the risk factors for AD; APP gene, PS-1 and PS-2 falls under the category of deterministic genes. APP gene was the first gene known to involve in AD, investigated first in 1987. As mentioned before, mutations in PS-1 and PS-2 have relevance with the early-onset of AD.

1.2.2. *Diabetes: manageable risk factor for Alzheimer's disease*

The above mentioned risk factors are uncontrollable but there are others factors related to AD which occurs after the damage in blood and heart vessels. It includes blood pressure, high cholesterol, cardiac diseases and diabetes. Diabetes results from the impairment in the regulation of insulin protein. Emerging evidence have demonstrated that signaling by impaired insulin probably contribute to the pathogenesis of AD. It was reported that diabetes (Type 2) increase the risk by two times of a patient having dementia and patients on insulin had four times the risk. When insulin attains remarkably high level, Aβ peptides that are accumulated in senile plaques, varies. When elevation in insulin levels in plasma is observed exaggeratedly, it induces an increase in Aβ peptide in the cerebrospinal fluid, leading to memory loss. Diabetes and AD have signs of increased oxidative stress and advanced glycation end products (AGEs) (discussed later in detail). AGEs accumulation is associated with the pathological features of AD such as formation of neurofibrillary tangles and amyloid plaques in AD brains. Thus, diabetic patients have a higher risk for AD.

Researchers at Northwestern University found the Aβ-derived diffusible ligands (ADDLs) have been implicated as a cause of impaired insulin signaling. ADDLs may contribute to decrease the insulin resistance and levels in AD brains. They are small oligomers that are more diffusible and thus considered as more harmful than Aβ. Under normal physiological conditions, insulin binds to a receptor at a synapse that leads to memory formation. It was demonstrated that ADDLs disrupt the mechanism and itself binds and alters the shape of the synapse. Since the shape is changed, insulin can no longer binds to the synapse. This dysfunction the synapse and disrupts signal transduction, resulting in insulin resistance. It has been proposed that insulin resistance in the brain of AD's patients, is a response to ADDLs due to the fact that they appear to influence the insulin receptor signaling in neurons. It induces a neurological form of diabetes. Neurons without ADDLs exhibit sufficient number of insulin receptors. These ligands have been linked to AD since they induce synapse loss, produce oxidative damage, and reduce the plasticity of the synapse, leading in AD-like tau hyperphosphorylation.

Another hypothesis which establishes a bio-chemical link between diabetes and AD is cholinergic hypothesis. It suggests that AD is the result of insufficient production of acetylcholine which may also links to insulin resistance and blood sugar abnormalities. Acetylcholine is an

important neurotransmitter for cognition, present at synapses, at neuromuscular junctions, and at a variety of sites within the CNS. Additionally, it has been shown that the enzyme responsible for acetylcholine synthesis, choline acetyltransferase (CAT), gets activated by insulin, thus insulin contributes in neurological function. Hence, alteration in insulin levels and impairment in its receptor sensitivity can cause a decrease in acetylcholine, which bridges the possibility of diabetes and AD.

1.2.3. *AGEs and Oxidation – Common link between Diabetes and AD*

Another key to link diabetes with AD is that both diseases shares common characteristics that are increased production of AGEs and oxidative stress. AGEs are the products first identified in 1912, obtained after consequent steps resulting in the modification of the protein by a process known as glycation. Reducing sugars such as glucose, fructose, and ribose etc. react with the amino groups of proteins to produce cross-linked complexes and unstable compounds, thus modifies the structure and functions of the proteins. This is a non-enzymatic process and also known as Maillard reaction (55). Since it occurs after their translation of new proteins, called as post-translation modifications. The association between vascular dementia and AGEs is already established, whereas novel evidence proposed a link between AGEs and AD. AGEs enhances the accumulation and aggregation of Aβ peptides and are also known to modify plaques and neurofibrillary tangles, both implicated in AD. These products have been recognized in Aβ containing senile plaques and neurofibrillary tangles composed of phosphorylated tau.

Type 2 diabetes found to accelerate the production of AGEs, thus it is another influential factor in the development of AD. Analysis of toxic AGEs in the serum or cerebrospinal fluid have been proposed as an inherent bio-marker for early detection of AD. AGEs have been detected in different organs including retinal vessels, kidneys, peripheral nerves, and the CNS of diabetic patients. Combination of AGEs with free radicals tend to induce oxidative damage, which in turn leads to cellular injury. Thus, diabetic patients acquires a higher risk of AD than normal individuals. AGEs also produce by oxidative stress, creating a vicious cycle. More extensive and detailed researches are needed to be done to implicate these risk factors and their acquired knowledge in the treatment strategies. The information that there is a depression in the sensitivity to insulin in AD patients who are not ApoE4-positive indicates that regulation of blood sugar

levels may produce medicinal benefits. For example, use of insulin-sensitizing agents in the setting of early AD.

1.3. Cholesterol, its oxygenated derivatives (oxysterols) and AGEs in Alzheimer's disease

1.3.1. Cholesterol: structure, metabolism and function

Cholesterol is a sterol, a type of lipid, first identified in 1769 which is composed of four fused and nonplanar rings (56). It is structured by the fusion of a tetracyclic ring known as the common nucleus in all sterols, hydroxyl group attached to the tetracyclic ring at the carbon positioned at 3rd place, and a branched iso-octyl side chain at C17 of the ring (see figure 1.3.). Cholesterol is a weak amphiphilic molecule possessing hydrophobic rings and hydrocarbon tail along with hydrophilic head group. It is synthesized from acetate, involving a series of various enzymatic reactions taking place primarily in the liver and rest in reproductive organs, intestines and adrenal glands. By receptor mediated endocytosis, liver cells can intake cholesterol form our diet.

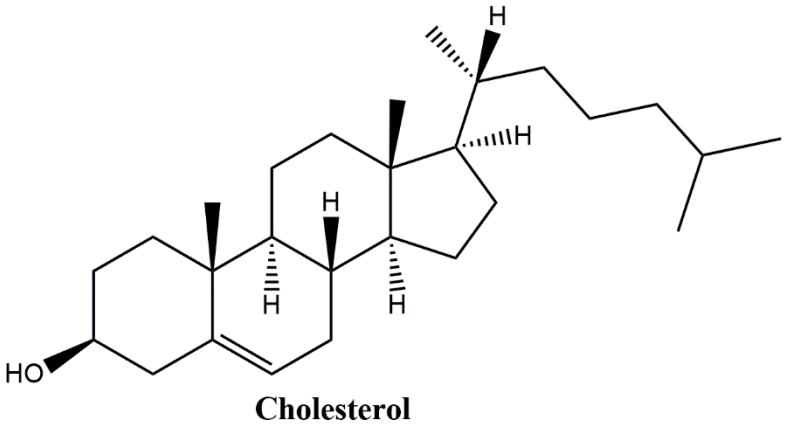


Figure 1.3. Structure of Cholesterol

Cholesterol synthesized in liver and other organs are used for regular cellular processes and digestion in all cells. But brain has its own biological synthesis for cholesterol. Brain is the most abundant cholesterol containing organ (57). Cholesterol in CNS is separated from all the other tissues by blood-brain-barrier block the efflux of lipoproteins in plasma to CNS and to impede the lipoproteins of CNS to the blood streams of peripheral system (58) . On the other hand,

recently it was reported that there is a small transfer of cholesterol to CNS from peripheral system (59). Moreover, excess cholesterol in CNS oxidizes at 24-C and converts to 24-hydroxycholesterol (24-OHC) which has the potential to move across the blood-brain-barrier (60).

All animal and plant cell synthesize cholesterol as it is the key component of the cell membranes, used in various cellular processes. Solely in plasma membrane, cholesterol comprises 30-40% of total lipid composition of the membrane (61). In lipid bilayers, cholesterol is aligned in perpendicular direction to the bilayers, the sterol nucleus interact with the hydrocarbon chains of other lipids present in the bilayers and reduces their ability to move, leading to a strong lipid packing. It has been shown that cholesterol modulates the fluidity and permeability of membranes (62).

In addition, cholesterol has an integral role in the development and metabolism of the body. Steroid hormones which controls various biological processes and bile acids, which are essential for the fat-absorption and digestion in the intestines (63), are formed from cholesterol as the starting moiety. Even in the embryonic development, cholesterol has major significance in neuronal functions such as generation and plasticity of synapse and neurotransmission (64). Thus, hindrance in the proper regulation of cholesterol has been related to the impairment of cellular functions leading to the development of several disease such as cardiac diseases, stroke and neurodegenerative disorders.

1.3.2. Oxygenated products of cholesterol: oxysterols

Cholesterol oxidation causes the formation of compounds, known as oxysterols. Lipid peroxidation, caused by aging which is one of the leading risk factor for AD. Cholesterol may oxidizes enzymatically or non-enzymatically to produce oxygenated derivatives of cholesterol (65). In enzymatic oxidation, side chain of the cholesterol oxidizes to produce 24-hydroxycholesterol (24-OHC), 25-hydroxycholesterol (25-OHC) and 27-hydroxycholesterol (27-OHC), compounds bearing an extra polar hydroxyl group. In the case of non-enzymatic reactions, reactive oxygen species (ROS) cause oxidation of cholesterol to produce 7-ketocholesterol (7-KC) baring an extra oxygen. It occurs as the result of oxidative stress which is primarily affected by aging and other exogenous factors. These oxysterols have been found to have notable impact in the pathogenesis of AD where they were detected in the plasma at a considerable concentration (66). Since oxysterols contains extra oxygen in their structure, they exhibit different hydrophilicity and

distinguished orientation than cholesterol. They have a tendency to expose both –OH group to the polar interface of membrane leading to a horizontal alignment of the side chain and tilted conformation of the oxidized sterol nucleus (67, 68). Since oxysterols are more permeable than cholesterol, they can cross the plasma membrane relatively easier than cholesterol (69). Unlike cholesterol, oxysterols do not stiffens the lipid packing and alters properties of membrane more profoundly.

Kandutsch and Chen have demonstrated that some oxysterols inhibits the biological synthesis of cholesterol *in vitro* and suppresses the activity of controlling enzyme, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in the synthesis (70, 71). Oxysterols shows different characteristics and impact in multiple biological processes, they serves as intermediate products in the transport of cholesterol and thus, homeostasis and metabolism of cholesterol. Oxysterols are inherently involved in modulation of gene expression, as an example, generation of sterol regulatory element binding protein (SREBP) was restrained by 25-OHC, SREBP is the transcription factor of genes in the synthesis of cholesterol. In addition, oxysterols exhibit cytotoxic effects and at elevated concentrations ultimately leads to cell death (66, 72). Oxysterols have been indicated as pathological factors in various illnesses such as cancer, atherosclerosis, cataract, sickle cell disease and neurological disorders. Their detrimental outcomes may be ascribed to their potential to alter membrane properties (73) which has evolved oxysterols in the pathogenesis of human disease.

1.3.3. Role of cholesterol and its derivatives in the toxicity induced by Aβ

Cholesterol has been contemplated as an essential factor in AD, although it was found to have significant effects in the production and aggregation of Aβ and also influences the cytotoxicity, but effect of cholesterol as a promoter or inhibitor is still a matter of discussion among the researchers (74, 75). ApoE, genes present in the cholesterol, reported to induce accumulation of Aβ peptide in the brain (76). On the contrary, it was suggested that depletion of cholesterol induces interaction of Aβ with the neuronal cell membranes (77), increases Aβ production (78). Various reports on the interaction of Aβ with the membranes have been proposed in the recent years. It was indicated that cholesterol cause conformation change in monosialoganglioside (GM1) to make it able to bind with Aβ, thus increasing the interaction of Aβ with the membrane (79, 80). Another report suggested that formation of GM1 cluster induces by cholesterol and thus it

facilitates the binding of Aβ with GM1 (81, 82). Elevated levels of cholesterol were found to change the membrane properties thus enhancing the interaction of Aβ with lipid bilayers (83). On the other hand, a group of researchers have demonstrated that Aβ will find and interacts with cholesterol after inserting into membranes and not essential for the binding with membranes (84).

Additionally, researchers are aiming to study the role of oxysterols in the generation, aggregation and cytotoxicity of Aβ in the brain. 24-OHC and 27-OHC serves as modulators in cholesterol biosynthesis in the brain and mediators in traversing of cholesterol between peripheral and central nervous system. It was shown that these two oxysterols adverts Aβ generation by reducing APP processing in the brain (85). Another oxysterol, 22(R)-OHC was proposed to decrease Aβ production by destabilizing C-terminal of APP (86). Moreover, effect of oxysterols in the clearance of Aβ has been studied. ApoE gene is related with the release of cholesterol and Aβ from the brain (21) and oxysterols such as 22(R)-OHC, 24-OHC, 25-OHC and 27-OHC are known as crucial modulators of ApoE gene and ligands of Liver X activated receptors (LXR) (87). Among all oxysterols, 22(R)-OHC and its derivatives taken as therapeutic candidate in AD (88). As it was mentioned that oxysterols exhibit dual behavior, they tend to produce detrimental effects in AD. Potential of 27-OHC was shown to induce Aβ generation (89), its elevation was observed at the expense of 24-OHC in AD brains (90).

1.3.4. *Advanced glycation end products (AGEs)*

AGEs are the oxidant compounds with significant role in diabetes, AD and other chronic diseases, commonly called as glycotoxins (91–96) . Although generation of AGEs is a regular metabolic activity, but they are threatening when produced in excess amount and release in tissues and blood streams (92). Since AGEs binds with proteins and cell surface receptors, they are able to stimulate oxidative stress and inflammation in the body (97–99). Since, proteins in deposits of amyloid commonly found in glycated forms, it indicates that there is a relation between formation of amyloid and glycation of protein (100–106). Moreover, glycation may interferes in the process of protein degradation (107), advancing aggregation of proteins (108–110) and identified to cause misfolding of the proteins. Derivatives of methyl-glyoxal and N6-carboxymethyllysine are the most common AGEs detected and they can be derived from lipid glycoxidation and protein (111, 112).

In addition, the AGE-modified proteins plays major role in physiological and pathological mechanisms. Usually, AGEs binds with particular cellular receptors resulting in the stimulation of various signaling pathways. RAGE (receptor for advanced glycation end products), possessed by the immunoglobulin superfamily is a multiple ligand receptor and is the most popular AGE receptor (113–115). Stimulation of RAGE modulates crucial cellular processes such as autophagy, inflammation, proliferation and apoptosis. According to current researches, RAGE has been found to co-relate with the pathology of amyloidosis (116, 117). RAGE is the binding site for Aβ on the surface in different cells such as endothelial, glial and neuronal cells and intervenes the pathogenesis of Alzheimer's disease (116, 118, 119). Upregulation of RAGE has been found to arbitrate oxidative stress caused by Aβ, stimulate transcription factor Nf-κB, and finally led to apoptosis (120, 121). According to a recent report, this upregulation and stimulation of death-signaling pathways are aggravated by incubation of Aβ with methylglyoxal (122).

It was found that senile plaques of amyloid in AD brains contains AGEs three times more than in normal brains (108). Furthermore, it was observed in immunohistochemical studies that AGEs have been detected as primary constituents in those senile plaques of amyloid in the brain (123). As previously mentioned, aggregation of Aβ is a two-step process; formation of the nuclei followed by its growth phase. It was demonstrated that AGE-modified-Aβ peptide nuclei accelerates the aggregation of the soluble Aβ peptide in comparison with unchanged Aβ nuclei (108). Under normal conditions, nucleus formation occurs in a reversible manner which depends on the number of Aβ monomers, therefore, it is possible that at an inadequate concentration, process may be initiated in an irreversible manner which involves cross-linking of AGE-modified monomers (108). Moreover, AGEs, promoting nucleation were indicated as primary glycated species that are formed after the interaction with glucose. In addition, fructose too induces aggregation of Aβ peptide in *in vitro* studies. Thus, both steps involved in the aggregation process are being influenced by AGE-modified peptide (42, 124).

Additionally, glycation can also influence the physiological, chemical and structural aspects of Aβ oligomers, thus interaction of Aβ with the cell membrane may be affected and as a consequence, caused different cellular toxicities. AGEs produced by methylglyoxal induces the synthesis of β-sheets, oligomers, and protofibrils in peptide. Enlargement of oligomers was also observed indicating towards increase in inter and intramolecular interactions that cause stability in

aggregated species (125). Apparently, contradictive results about cellular toxicities induced by glycated Aβ can be related to the information that process of glycation is associated with two different kinds of mechanisms: sudden cyto-protective effect due to different structural properties of the oligomers, and cytotoxic effect at longer incubation times due to formation of AGEs.

Although formation of AGEs takes place inside the body, they also exist in food products. Animal studies have shown that intake of food products containing AGEs cause elevation of AGEs in tissues, blood and increase chances of kidney diseases (126) and atherosclerosis (127). And constraining the AGEs form the diet helps to promote insulin sensitivity (128, 129), avert the possibilities of kidney and vascular dysfunction (126, 127), enhance wound healing (130) and diabetes type 1 or type 2 (131). AGEs in diet have been shown to have linked with AGEs in the blood stream, such as CML and MG, with oxidative stress (132). In addition, reduction in the intake of AGEs comprising diet reduces oxidative stress in diabetics (133), patients of kidney disease (134, 135) and in the healthy individuals (136). Thus these findings are beneficial to help in circumventing the food products containing AGEs from our diet to defer aging and chronic diseases in living beings (94).

1.4. Previous studies with biomimetic and biological membranes

1.4.1. Cell membranes: structure and function

Cell membranes possesses highly intricate structure, most popular model of cell membrane was given by Singer and Nicolson in 1972, known a fluid mosaic model. In this model, membranes are composed of two-dimensional lipid bilayers and floating proteins within these bilayer (see figure 1.4.) (62). Usually, cell membranes constitutes plasma membrane and other cell organelles membranes. Membranes are made up of lipids stated in decreasing order in their availability in the membranes: glycerophospholipids, sphingolipids and cholesterol. Glycerophospholipids acquires glycerol, 1 phosphate group and two unsaturated or saturated hydrocarbon tails. Glycerol is a 3-carbon backbone which acts a polar moiety in these lipids.

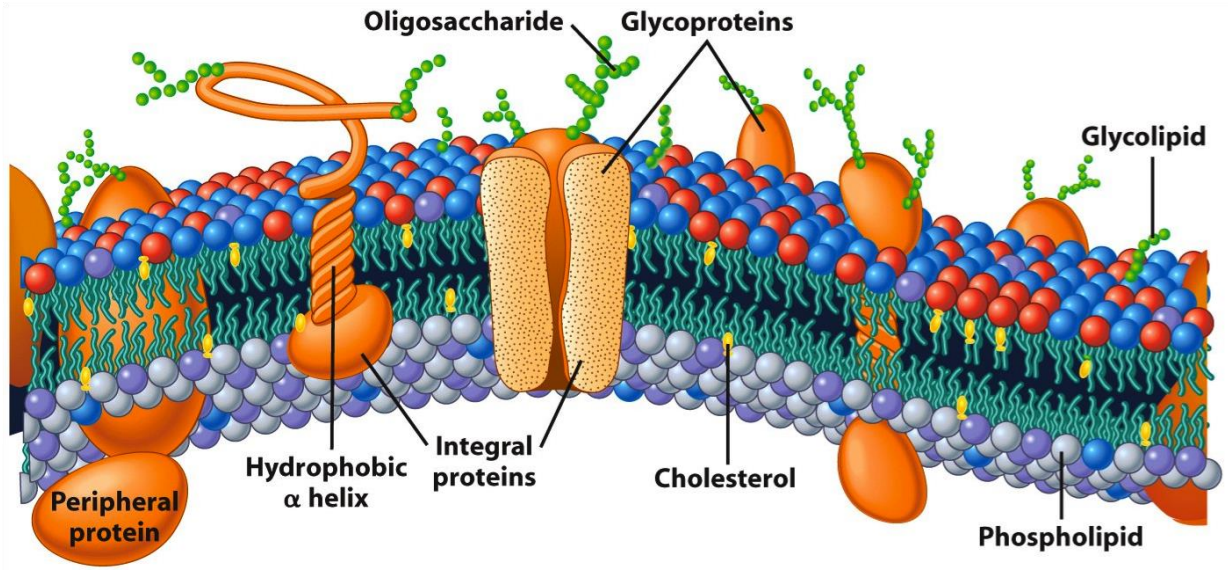


Figure 1.4. Structure of plasma membrane proposed by fluid mosaic model (61).

Although like other lipids, glycerophospholipids are insoluble in water, but due to their distinctive geometry, they aggregate into bilayers without any external energy. Sphingolipids are made up of sphingosine molecules and fatty acid chains. Mostly, these fatty acid chains are saturated and their head-groups are longer than those of glycerophospholipids (62). In the lipid bilayers, head-groups of the lipids are exposed to the interface of water and hydrophilic part of lipid and side chains which are hydrophobic, hide inside. Three types of interactions are present among the molecules of lipid which are a) hydrophilic interactions at the interface of water and hydrocarbon, b) hydrophobic interaction of hydrocarbon chains and repulsion of hydrophilic head-groups (see figure 1.5.). There is an asymmetric orientation of lipid molecules among the two layers. Altogether, lipids ascribes for around 50% of total mass of cell membranes. Cholesterol is about 20% of the total lipid content in the plasma membranes. But it is not present in other membranes such as bacterial or mitochondrial membranes. Composition of lipid, primarily cholesterol, regulates different membrane properties, fluidity and phase behavior of the membrane. Hence alterations in lipid composition induces notable effects on properties and functions of membrane.

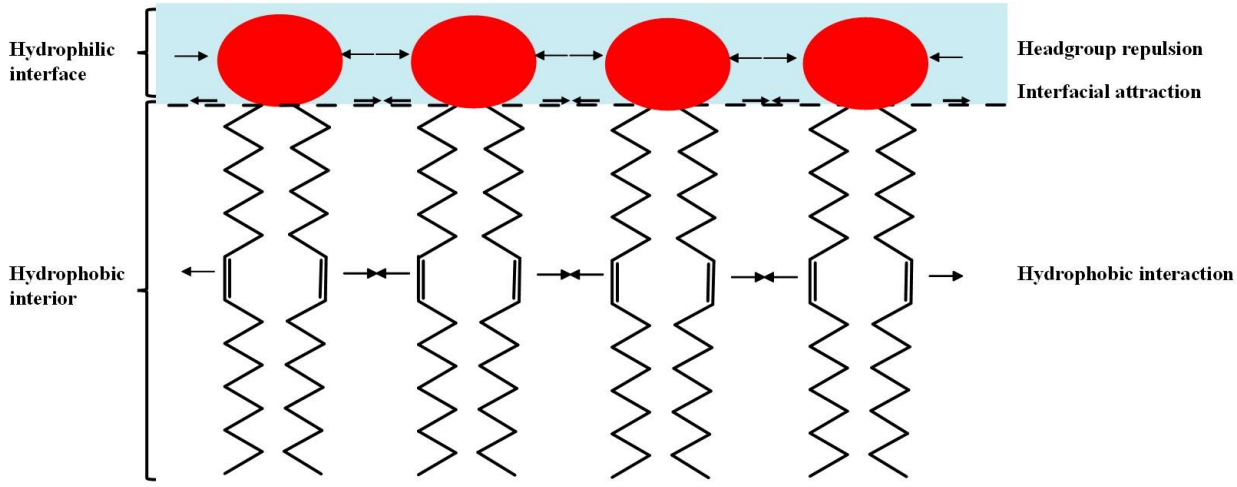


Figure 1.5. The hydrophobic and hydrophilic interaction between phospholipids of bilayer.

As previously indicated in fluid mosaic model, in addition to lipids, membranes composed of freely floating proteins. Approximately, 50% mass of most cellular membranes is accounted for proteins. Some of the proteins are emerging from both sides of the membrane and rest are embedded into the membrane, they are known as transmembrane proteins. They traverse across the membrane several times, attributed to the interaction between hydrocarbon chains of lipids and hydrophobic moiety of proteins (see figure 1.6.). Thereby, this combination of lipids and proteins that forms a cellular membrane depends on biophysical properties to form and function. Many proteins cannot move on their own in living cells and generally stabilized in a place within the cell membrane.

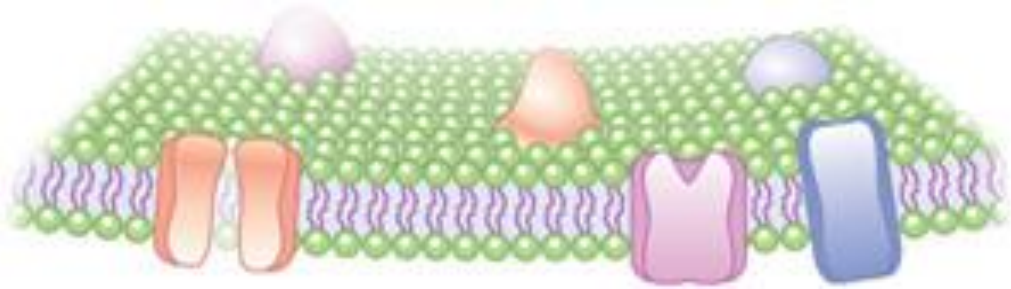


Figure 1.6. The glycerophospholipid bilayer embedded with transmembrane proteins (137).

In 1997, Simons and Ikonen suggested another model of membrane structure, called as 'Lipid raft model' (see figure 1.7.) (137). They had proposed that lipids are laterally organized in the membrane and forms microdomains of lipids, named lipid rafts. Lipid rafts are cholesterol and sphingolipids-rich area in the membrane. Thickness of lipid rafts is more than other parts of bilayer and these domains act as binding sites for the proteins. Integral role of lipid rafts has been demonstrated in signal transduction and membrane trafficking (137).

Cell membranes perform essential roles in different biological processes. Membranes separates the cell from near surroundings and thus protect them. Cell membranes are semi-permeable, so it allows selective molecules, which are necessary for cell survival, to diffuse across the lipid bilayer. Small hydrophobic molecules and gases may cross membranes swiftly. However, transport of highly charged molecules, such as ions, and molecules such as sugars and amino acids is restricted. Their movement across the membranes depends on particular transport proteins resides in the membrane.

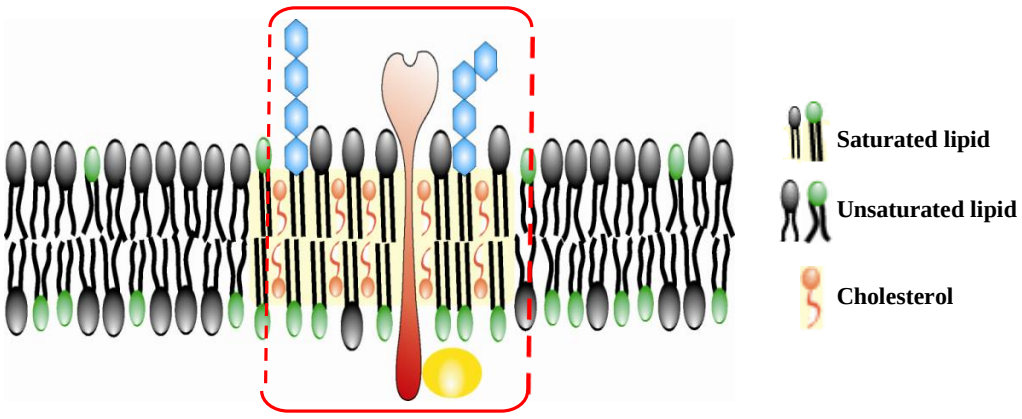


Figure 1.7. Lipid raft model of cell membrane (137).

Membranes play important part in intercellular communication and communication of cell with extracellular environment. Most of the membrane proteins are signaling receptors. Additionally, cell membranes are involved in ion conductivity, cell adhesion and act as foundation for cytoskeleton in the cell (62). Previous studies have demonstrated the importance of membrane in the pathology of several disease. Thus, finding the mechanism behind the membrane response

is of major interest, knowledge about specific interaction of membrane with proteins would be implicated to find out treatment of some protein-related illnesses.

1.4.2. Interaction of Aβ with cell membranes through different pathways

AD was introduced in 1906, since then a lot of researchers are trying to find out its cause, effects and mechanism. Apart from many remarkable achievements and progress in the area, still lots of unanswered questions are remained yet. So far, it is known that Aβ interacts with cell membranes through different pathways. These pathways involves interaction of Aβ with lipid bilayer (138), membrane receptors, including glutamate receptors, prion protein (139) and ganglioside GM1. GM1 is a kind of sphingolipid in the membrane and Aβ preferentially binds with GM1 (140). Since most of GM1 is distributed in the lipid raft of cell membranes, thus lipid rafts are known as binding sites of Aβ. Aβ undergoes conformational change when binds with GM1, ultimately leads to fibrillation of peptide (141).

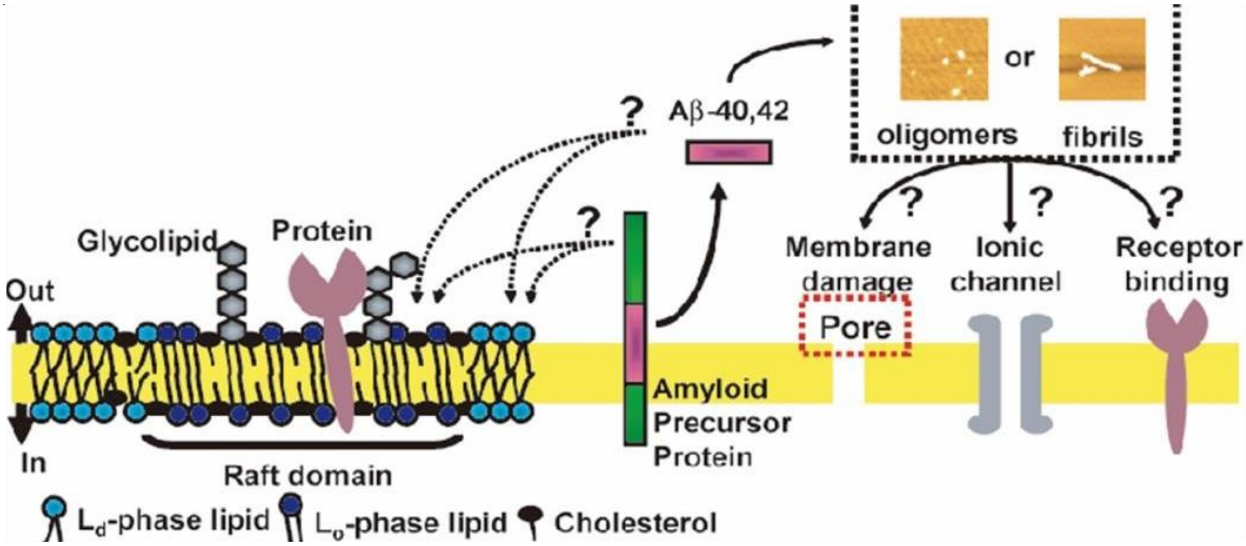


Figure 1.8. Proposed membrane-mediated cytotoxicity of Aβ (73).

Although in interaction with lipid bilayers, Aβ adsorbed on the surface of membranes but insertion of Aβ is also possible. Hydrophobic interaction between hydrocarbon tail of lipid and hydrophobic C-terminal of Aβ can induce the internalization of Aβ (142). Previous studies have proposed involvement of Aβ-cell membrane interaction in aggregation and cytotoxicity of the peptide (see figure 1.8.).

Additionally, Aβ is able to make pores to internalize into the lipid bilayer which is accompanied with dyshomeostasis of calcium ions. In the interaction of Aβ with cell, internalization or endocytosis involves absorption of peptide which induces toxicity to other cell organelles (28, 143). Since composition and properties of membrane influence the interaction of membrane with Aβ peptides (139), it is crucial to understand role of different components present in the membrane.

1.4.3. Use of biomimetic (model) membranes

Biomimetic membranes are synthetic or model membranes, have been used as model for the biological membranes. They are similar in size, composition, structure with bio-membranes. Since they are composed of only lipids, are simpler than their biological counterpart. Therefore, it is a good option to understand the mechanism relatively at easier platform by neglecting the intricate structure of biological membranes. At the same time, use of biomimetic membranes broadens the understanding about cellular processes with high precision.

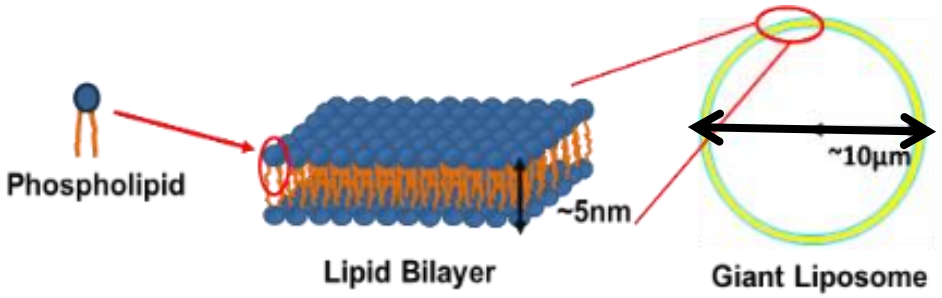


Figure 1.9. Representation of Lipid vesicle (spherical lipid bilayer) with diameter of 10 µm (13).

These membranes have been categorized into four types: monolayers (144), Supported lipid bilayer (SLB) (145), hybrid membranes (146, 147) and cell-sized lipid vesicles. Lipid vesicles are spherical membranes, also known as liposomes contains bilayers, and similar in size with a cell, having diameter ≥10 µm (figure 1.9.) (14). They can be heterogeneous (more than one lipid phase) or homogenous (single lipid phase), where in heterogeneous vesicles, lipids distributes themselves into lateral compartments of the membrane (148). Other than size, composition and

bilayer structure. Lipid vesicles exhibit features such as transformation and curvature. Due to their properties, lipid vesicles have been widely used in the studies of membrane response and dynamics, to confine large molecules inside the membranes. In the recent times, model membranes have been used to understand the mechanism behind AD. My group have investigated the localization of Aβ peptide using heterogeneous lipid membranes (149). Incorporation of cholesterol and oxysterols was demonstrated to alter the composition of membrane and observed membrane response to Aβ exposure (150) .

1.5. Research objectives and thesis overview

1.5.1. Research objectives

According to the previous knowledge, it was shown that there are multiple factors in the pathology of AD. Interaction of Aβ with the cell membrane, importance of cholesterol, involvement of oxygenated derivatives of cholesterol, change in membrane composition, importance of glycosyl chains, aggregated species of Aβ and other factors associated with endocytic transport of the peptide hold their individual importance. Moreover, combination of above mentioned factors is equally important. All these factors are related to the risk factors for AD. Based on this understanding, this thesis was an advancement to the past study and it was performed with toxic species of Aβ peptide, oxysterols, biomimetic and biological membranes.

This dissertation aimed to investigate the essential factors for endocytic transport of Aβ which was conducted into following steps:

1. Investigation of influence of oxysterols on the localization of Aβ-42 using biomimetic membranes and impact of oxidized derivatives on the aggregation kinetics of the peptide.
2. Elucidation of the important factors involved in the endocytic transport of protofibrillar Aβ-42 in Jurkat cells, which were used as a mimic to neuronal cells. Using biomimetic membranes and Jurkat cells provided an advantage to find out the components necessary for the internalization of peptide before the use of undifferentiated human neuroblastoma SH-SY5Y cells. Introduction of oxysterols in the membranes allows the physiological changes in the membrane which could facilitate higher interaction of Aβ-42 with the cells.

3. Estimation of toxicity induced by the peptides on the undifferentiated human neuroblastoma SH-SY5Y cells.
4. Investigation of the pathway chosen by Aβ-42 to internalize into the membrane and it could affect in the presence of oxysterols such as 7-KC and 25-OHC at different concentrations at varied time intervals.

1.5.2. Outline of thesis

This thesis has been divided into five chapters:

Chapter 1 gives the overview of the complete research. It is the background of the study, explaining about the pathologies of AD, importance of *amyloid cascade hypothesis,* production of Aβ, risk factors involved in AD. Further detail about different risk factors such as aging, advanced glycation end products, related vascular and metabolic conditions. In the latter part, I have discussed about the use of biomimetic and biological membranes and their interaction with Aβ peptides we have known so far.

Chapter 2 explains the localization of Aβ-42 in the lateral compartments of the heterogeneous model membranes and tells about the toxicity induced by 25-OHC which facilitates the aggregation of the peptide in toxic species.

Chapter 3 includes the factors important to induce endocytic transport of Aβ peptides. Here, Jurkat cell line was used as a model before using neural cells. It helped to understand the possible mechanism behind the internalization of peptides in cells. It was shown that combination of oxysterols (25-OHC), GM1 and protofibrillar species of Aβ-42 creates an appropriate system which caused Aβ peptides to internalize into through the cell membrane of Jurkat cells. This transfer was demonstrated to be cytoskeleton dependent where Aβ peptides swayed through microtubules and reaches to ER. These factors were closely related to the risk components of AD.

Chapter 4 shows the use of undifferentiated human neuroblastoma SH-SY5Y cell line to determine the toxicity induced by protofibrillar Aβ-42 at different concentrations and with multiple incubation periods. It was also compared in the presence of oxysterols (7-KC and 25-OHC) in the cell membrane. In addition, internalization of the peptides was observed at basal cholesterol level

and after altering the composition of the membrane by changing the time duration of treatment. Moreover, localization of protofibrillar Aβ-42 was observed in the ER which support the hypothesis in this dissertation.

Chapter 5 comprises all the conclusions obtained throughout the study. These findings were helpful to unravel the mechanism behind AD involving endocytic transport of Aβ peptides which is possible using primary toxic species of peptides and to deepen our understanding about the risk factor for AD. For the advancement of study, it will be interesting to investigate the effect of 7-KC and 25-OHC together in a single system.

References

1. Berchtold, N. C., and Cotman, C. W. (1998) Evolution in the Conceptualization of Dementia and Alzheimer's Disease: Greco-Roman Period to the 1960s. *Neurobiol. Aging* **19**, 173–189 [online] http://www.sciencedirect.com/science/article/pii/S0197458098000529 (Accessed May 1998).
2. ZS, K. (1985) Diagnosis of alzheimer's disease. *Arch. Neurol.* **42**, 1097–1105 [online] http://dx.doi.org/10.1001/archneur.1985.04060100083029 (Accessed November 1, 1985).
3. Blennow, K., de Leon, M. J., and Zetterberg, H. Alzheimer's disease. *Lancet* **368**, 387–403 [online] http://dx.doi.org/10.1016/S0140-6736(06)69113-7.
4. https://www.alz.org/documents_custom/2016-facts-and-figures.pdf
5. http://www.who.int/mediacentre/factsheets/fs362/en/
6. Mount, C., and Downton, C. Alzheimer disease: progress or profit? [online] http://www.nature.com/nm/journal/v12/n7/full/nm0706-780.html.
7. Karran, E., Mercken, M., and De Strooper, B. (2011) The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics. *Nat. Rev. Drug Discov.* **10**, 698–712
8. Francis, P. T., Palmer, A. M., Snape, M., and Wilcock, G. K. (1999) The cholinergic hypothesis of Alzheimer's disease: a review of progress. *J. Neurol. Neurosurg. &*

Psychiatry **66**, 137–147 [online] http://jnnp.bmj.com/content/66/2/137 (Accessed February 1, 1999).

9. Markesbery, W. R. (1997) Oxidative Stress Hypothesis in Alzheimer's Disease. *Free. Radic. Biol. Med.* **23**, 134–147 [online] http://www.sciencedirect.com/science/article/pii/S0891584996006296 (Accessed 1997).
10. Mudher, A., and Lovestone, S. (2002) Alzheimer's disease – do tauists and baptists finally shake hands? *Trends Neurosci.* **25**, 22–26 [online] http://www.sciencedirect.com/science/article/pii/S0166223600020312 (Accessed January 1, 2002).
11. Hardy, J. A., and Higgins, G. A. (1992) Alzheimer's disease: the amyloid cascade hypothesis. *Science* **256**
12. Hardy, J. A., and Higgins, G. A. Alzheimer's disease: the amyloid cascade hypothesis. [online] http://search.proquest.com/openview/e965729ff54db4d8a61fdb184525be25/1?pq-origsite=gscholar.
13. Vestergaard, M., Kerman, K., and Tamiya, E. (2006) The study of Alzheimer's disease biomarkers. *NanoBiotechnology* **2**, 5–16 [online] http://dx.doi.org/10.1007/s12030-006-0002-y (Accessed 2006).
14. Vestergaard, M., Hamada, T., and Takagi, M. (2008) Using model membranes for the study of amyloid beta: lipid interactions and neurotoxicity. *Biotechnol. Bioeng.* **99**, 753–763 [online] https://doi.org/10.1002%2Fbit.21731 (Accessed 2008).
15. O'Brien, R. J., and Wong, P. C. (2011) Amyloid Precursor Protein Processing and Alzheimer's Disease. *Annu. Rev Neurosci* **34**, 185–204 [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3174086/ (Accessed 2011).
16. Hicks, D., Nalivaeva, N., and Turner, A. (2012) Lipid Rafts and Alzheimer's Disease: Protein-Lipid Interactions and Perturbation of Signaling. *Front. Physiol.* **3** [online] http://journal.frontiersin.org/article/10.3389/fphys.2012.00189 (Accessed 2012).
17. Thinakaran, G., and Koo, E. H. (2008) Amyloid Precursor Protein Trafficking, Processing, and Function. *J. Biol. Chem.* **283**, 29615–29619 [online] https://doi.org/10.1074%2Fjbc.r800019200 (Accessed July 2008).
18. Hardy, J., and Selkoe, D. J. (2002) The Amyloid Hypothesis of Alzheimer's Disease:

Progress and Problems on the Road to Therapeutics. *Science* **297**, 353–356 [online] http://science.sciencemag.org/content/297/5580/353 (Accessed 2002).

19. Citron, M., Westaway, D., Xia, W., Carlson, G., Diehl, T., Levesque, G., Johnson-wood, K., Lee, M., Seubert, P., Davis, A., Kholodenko, D., Motter, R., Sherrington, R., Perry, B., Yao, H., Strome, R., Lieberburg, I., Rommens, J., Kim, S., Schenk, D., Fraser, P., St George Hyslop, P., and Selkoe, D. J. (1997) Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid [beta]-protein in both transfected cells and transgenic mice. *Nat Med* **3**, 67–72 [online] http://dx.doi.org/10.1038/nm0197-67 (Accessed January 1997).
20. Zheng, H., and Koo, E. H. (2011) Biology and pathophysiology of the amyloid precursor protein. *Mol. Neurodegener.* **6** [online] http://dx.doi.org/10.1186/1750-1326-6-27 (Accessed 2011).
21. Wildsmith, K. R., Holley, M., Savage, J. C., Skerrett, R., and Landreth, G. E. (2013) Evidence for impaired amyloid β clearance in Alzheimer's disease. *Alzheimer's Res. & Ther.* **5** [online] http://dx.doi.org/10.1186/alzrt187 (Accessed 2013).
22. Nag, S., Sarkar, B., Bandyopadhyay, A., Sahoo, B., Sreenivasan, V. K. A., Kombrabail, M., Muralidharan, C., and Maiti, S. (2011) Nature of the Amyloid- Monomer and the Monomer-Oligomer Equilibrium. *J. Biol. Chem.* **286**, 13827–13833 [online] https://doi.org/10.1074%2Fjbc.m110.199885 (Accessed February 2011).
23. Takano, K. (2008) Amyloid beta conformation in aqueous environment. *Curr. Alzheimer Res.* **5**, 540–547 [online] https://www.ncbi.nlm.nih.gov/pubmed/19075580 (Accessed NaN, 2008).
24. Matsuzaki, K. (2007) Physicochemical interactions of amyloid β-peptide with lipid bilayers. *Biochim. Biophys. Acta* **1768**, 1935–1942 [online] //www.sciencedirect.com/science/article/pii/S0005273607000570 (Accessed August 2007).
25. McLaurin, J., Franklin, T., Fraser, P. E., and Chakrabartty, A. (1998) Structural transitions associated with the interaction of Alzheimer β-amyloid peptides with gangliosides. *J. Biol. Chem.* **273**, 4506–4515
26. Bossis, F., and Palese, L. L. (2013) Amyloid beta(1–42) in aqueous environments: Effects of ionic strength and E22Q (Dutch) mutation. *Biochim. et Biophys. Acta* **1834**, 2486–2493

[online] //www.sciencedirect.com/science/article/pii/S1570963913003087 (Accessed December 2013).

27. Serpell, L. C. (2000) Alzheimer's amyloid fibrils: structure and assembly. *Biochim. Biophys. Acta* **1502**, 16–30 [online] //www.sciencedirect.com/science/article/pii/S0925443900000296 (Accessed July 26, 2000).
28. Lee, J.-M., Crick, S., Pappu, R., Frieden, C., and Hu, X. Amyloid seeds formed by cellular uptake, concentration, and aggergation of the $Abeta_{1-42}$ peptide. *Alzheimer's & Dementia: J. Alzheimer's Assoc.* **5** [online] http://dx.doi.org/10.1016/j.jalz.2009.04.007.
29. Lomakin, A., Chung, D. S., Benedek, G. B., Kirschner, D. A., and Teplow, D. B. (1996) On the nucleation and growth of amyloid beta-protein fibrils: detection of nuclei and quantitation of rate constants. *Proc Natl Acad Sci U S* **93**, 1125–1129 [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC40042/ (Accessed February 6, 1996).
30. Sabaté, R., Gallardo, M., and Estelrich, J. (2003) An autocatalytic reaction as a model for the kinetics of the aggregation of β-amyloid. *Pept. Sci.* **71**, 190–195 [online] http://dx.doi.org/10.1002/bip.10441 (Accessed 2003).
31. Lansbury, P. T. (1999) Evolution of amyloid: What normal protein folding may tell us about fibrillogenesis and disease. *Proc Natl Acad Sci U S* **96**, 3342–3344 [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC34271/ (Accessed March 30, 1999).
32. Mousseau, N., and Derreumaux, P. (2005) Exploring the Early Steps of Amyloid Peptide Aggregation by Computers. *Accounts Chem. Res.* **38**, 885–891 [online] http://dx.doi.org/10.1021/ar050045a (Accessed November 1, 2005).
33. Harper, J. D., Wong, S. S., Lieber, C. M., and Lansbury, P. T. (1997) Observation of metastable Aβ amyloid protofibrils by atomic force microscopy. *Chem. & Biol.* **4**, 119–125 [online] http://www.sciencedirect.com/science/article/pii/S1074552197902556 (Accessed 1997).
34. Ahmed, M., Davis, J., Aucoin, D., Sato, T., Ahuja, S., Aimoto, S., Elliott, J. I., Van Nostrand, W. E., and Smith, S. O. (2010) Structural conversion of neurotoxic amyloid-[beta]1-42 oligomers to fibrils. *Nat Struct Mol Biol* **17**, 561–567 [online] http://dx.doi.org/10.1038/nsmb.1799 (Accessed May 2010).
35. Glabe, C. G. (2008) Structural Classification of Toxic Amyloid Oligomers. *J. Biol. Chem.*

283, 29639–29643 [online] https://doi.org/10.1074%2Fjbc.r800016200 (Accessed August 2008).

36. Kayed, R., and E Head, J. T., TM McIntire… Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. [online] http://science.sciencemag.org/content/300/5618/486.short.

37. Cizas, P., Budvytyte, R., Morkuniene, R., Moldovan, R., Broccio, M., Lösche, M., Niaura, G., Valincius, G., and Borutaite, V. (2010) Size-dependent neurotoxicity of β-amyloid oligomers. *Arch Biochem. Biophys* **496**, 84–92 [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2853175/ (Accessed April 15, 2010).

38. Hane, F., Drolle, E., Gaikwad, R., Faught, E., and Leonenko, Z. (2011) Amyloid-β aggregation on model lipid membranes: An atomic force microscopy study. *J. Alzheimer's Dis.* **26**, 485–494

39. Jusakul, A., Yongvanit, P., Loilome, W., Namwat, N., and Kuver, R. (2011) Mechanisms of oxysterol-induced carcinogenesis. *Lipids Health Dis* **10**, 44–44 [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3061933/ (Accessed March 9, 2011).

40. Linseisen, J., Wolfram, G., and Miller, A. B. (2002) Plasma 7β-Hydroxycholesterol as a Possible Predictor of Lung Cancer Risk. *Cancer Epidemiol. Biomarkers & Prev.* **11** [online] http://cebp.aacrjournals.org/content/11/12/1630.abstract (Accessed December 1, 2002).

41. Masuda, Y., Uemura, S., Nakanishi, A., Ohashi, R., Takegoshi, K., Shimizu, T., Shirasawa, T., and Irie, K. (2008) Verification of the C-terminal intramolecular β-sheet in Aβ42 aggregates using solid-state NMR: Implications for potent neurotoxicity through the formation of radicals. *Bioorganic & Med. Chem. Lett.* **18**, 3206–3210 [online] //www.sciencedirect.com/science/article/pii/S0960894X08004642 (Accessed June 1, 2008).

42. Fernandez-Busquets, X., Ponce, J., Bravo, R., Arimon, M., Martianez, T., Gella, A., Cladera, J., and Durany, N. (2010) Modulation of amyloid β peptide1-42 cytotoxicity and aggregation in vitro by glucose and chondroitin sulfate. *Curr. Alzheimer Res.* **7**, 428–438

43. Pike, C. J., Burdick, D., Walencewicz, A. J., Glabe, C. G., and Cotman, C. W. (1993) Neurodegeneration induced by beta-amyloid peptides in vitro: the role of peptide assembly state. *J. Neurosci.* **13** [online] http://www.jneurosci.org/content/13/4/1676.abstract

(Accessed April 1, 1993).

44. Dahlgren, K. N. (2002) Oligomeric and Fibrillar Species of Amyloid-beta Peptides Differentially Affect Neuronal Viability. *J. Biol. Chem.* **277**, 32046–32053 [online] https://doi.org/10.1074%2Fjbc.m201750200 (Accessed June 2002).

45. Chromy, B. A., Nowak, R. J., Lambert, M. P., Viola, K. L., Chang, L., Velasco, P. T., Jones, B. W., Fernandez, S. J., Lacor, P. N., Horowitz, P., Finch, C. E., Krafft, G. A., and Klein, W. L. (2003) Self-Assembly of Aβ1-42 into Globular Neurotoxins. *Biochemistry* **42**, 12749–12760 [online] http://dx.doi.org/10.1021/bi030029q (Accessed November 1, 2003).

46. Sakono, M., and Zako, T. (2010) Amyloid oligomers: formation and toxicity of Abeta oligomers. *FEBS J.* **277**, 1348–1358 [online] http://www.ncbi.nlm.nih.gov/pubmed/?term=20148964 (Accessed NaN, 2010).

47. Fonseca, A. C. R. G., Ferreiro, E., Oliveira, C. R., Cardoso, S. M., and Pereira, C. F. (2013) Activation of the endoplasmic reticulum stress response by the amyloid-beta 1–40 peptide in brain endothelial cells. *Biochim. et Biophys. Acta* **1832**, 2191–2203 [online] //www.sciencedirect.com/science/article/pii/S092544391300272X (Accessed December 2013).

48. Pagani, L., and Eckert, A. (2011) Amyloid-Beta Interaction with Mitochondria. *Int J Alzheimers Dis* **2011** [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3065051/ (Accessed March 15, 2011).

49. Mattson, M. P. (2004) Pathways towards and away from Alzheimer's disease. *Nature* **430**, 631–639 [online] http://dx.doi.org/10.1038/nature02621 (Accessed August 5, 2004).

50. Petroff, O. A. C. Book review: GABA and glutamate in the human brain. [online] http://journals.sagepub.com/doi/abs/10.1177/1073858402238515.

51. Dickson, D. W. (2004) Apoptotic mechanisms in Alzheimer neurofibrillary degeneration: cause or effect? *J Clin Invest* **114**, 23–27 [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC437977/ (Accessed July 1, 2004).

52. Butterfield, D. A., Drake, J., Pocernich, C., and Castegna, A. (2001) Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid β-peptide. *Trends Mol. Med.* **7**, 548–554 [online] //www.sciencedirect.com/science/article/pii/S1471491401021736 (Accessed December 1, 2001).

53. Meraz-Ríos, M. A., Toral-Rios, D., Franco-Bocanegra, D., Villeda-Hernández, J., and

Campos-Peña, V. (2013) Inflammatory process in Alzheimer's Disease. *Front. Integr Neurosci* **7** [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3741576/ (Accessed August 13, 2013).

54. LaFerla, F. M. (2010) Pathways linking Aβ and tau pathologies. *Biochem. Soc. Trans.* **38** [online] http://www.biochemsoctrans.org/content/38/4/993.abstract (Accessed July 26, 2010).
55. O'Brien, J., Morrissey, P. A., and Ames, J. M. (1989) Nutritional and toxicological aspects of the Maillard browning reaction in foods. *Crit. Rev. Food Sci. Nutr.* **28**, 211–248 [online] http://dx.doi.org/10.1080/10408398909527499 (Accessed January 1, 1989).
56. Voet, D., Voet, J. G., and Pratt, C. W. *Fundamentals of biochemistry*, Wiley New York
57. Jurevics, H., and Morell, P. (1995) Cholesterol for Synthesis of Myelin Is Made Locally, Not Imported into Brain. *J. Neurochem.* **64**, 895–901 [online] http://dx.doi.org/10.1046/j.1471-4159.1995.64020895.x (Accessed 1995).
58. Vance, J. E., Hayashi, H., and Karten, B. (2005) Cholesterol homeostasis in neurons and glial cells. *Semin. Cell & Dev. Biol.* **16**, 193–212 [online] //www.sciencedirect.com/science/article/pii/S1084952105000212 (Accessed April 2005).
59. Di Paolo, G., and Kim, T.-W. (2011) Linking Lipids to Alzheimer's Disease: Cholesterol and Beyond. *Nat Rev Neurosci* **12**, 284–296 [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3321383/ (Accessed May 30, 2011).
60. Journal of Lipid Research Volume 39, 1998 Cholesterol homeostasis in human brain: turnover of 24S-hydroxycholesterol and evidence for a cerebral origin of most of this oxysterol in the
61. McMullen, T. P., Lewis, R. N., and McElhaney, R. N. (2004) Cholesterol–phospholipid interactions, the liquid-ordered phase and lipid rafts in model and biological membranes. *Curr. Opin. Colloid & Interface Sci.* **8**, 459–468 [online] http://dx.doi.org/10.1016/j.cocis.2004.01.007 (Accessed 2004).
62. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002) Molecular biology of the cell. new york: Garland science; 2002. *Class. Textb. now its 5th Ed.*
63. Agellon, L. B. (2002) in *New Comprehensive Biochemistry*, Elsevier [online] //www.sciencedirect.com/science/article/pii/S0167730602360186 (Accessed 2002).
64. Orth, M., and Bellosta, S. (2012) Cholesterol: Its Regulation and Role in Central Nervous

System Disorders. *Cholesterol* **2012** [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3483652/ (Accessed October 17, 2012).

65. Brown, A. J., and Jessup, W. (2009) Oxysterols: Sources, cellular storage and metabolism, and new insights into their roles in cholesterol homeostasis. *Mol. Asp. Med.* **30**, 111–122 [online] //www.sciencedirect.com/science/article/pii/S0098299709000041 (Accessed June 2009).
66. Schroepfer, G. J. (2000) Oxysterols: Modulators of Cholesterol Metabolism and Other Processes. *Physiol. Rev.* **80** [online] http://physrev.physiology.org/content/80/1/361.abstract (Accessed January 1, 2000).
67. Olkkonen, V. M., and Hynynen, R. (2009) Interactions of oxysterols with membranes and proteins. *Mol. Asp. Med.* **30**, 123–133 [online] //www.sciencedirect.com/science/article/pii/S0098299709000053 (Accessed June 2009).
68. Mintzer, E., Charles, G., and Gordon, S. (2010) Interaction of two oxysterols, 7-ketocholesterol and 25-hydroxycholesterol, with phosphatidylcholine and sphingomyelin in model membranes. *Chem. Phys. Lipids* **163**, 586–593 [online] //www.sciencedirect.com/science/article/pii/S0009308410000782 (Accessed June 2010).
69. DeBose-Boyd, R. A. (2008) Feedback Regulation of Cholesterol Synthesis: Sterol-Accelerated Ubiquitination and Degradation of HMG CoA Reductase. *Cell Res* **18**, 609–621 [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2742364/ (Accessed June 2008).
70. Kandutsch, A. A., and Chen, H. W. (1974) Inhibition of sterol synthesis in cultured mouse cells by cholesterol derivatives oxygenated in the side chain. *J. Biol. Chem.* **249**, 6057–6061
71. Kandutsch, A. A., and Chen, H. W. (1973) Inhibition of sterol synthesis in cultured mouse cells by 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol. *J. Biol. Chem.* **248**, 8408–8417
72. Yamanaka, K., Saito, Y., Yamamori, T., Urano, Y., and Noguchi, N. (2011) 24 (S)-hydroxycholesterol induces neuronal cell death through necroptosis, a form of programmed necrosis. *J. Biol. Chem.* **286**, 24666–24673
73. Iuliano, L. (2011) Pathways of cholesterol oxidation via non-enzymatic mechanisms. *Chem. Phys. Lipids* **164**, 457–468 [online]

//www.sciencedirect.com/science/article/pii/S000930841100212X (Accessed September 2011).

74. Vestergaard, M., Hamada, T., and Takagi, M. M. and M. (2010) Cholesterol, Lipids, Amyloid Beta, and Alzheimers. *Curr. Alzheimer Res.* **7**, 262–270 [online] http://www.eurekaselect.com/node/86087/article (Accessed 2010).

75. Liguri, M. S. and G. (2009) Cholesterol in Alzheimers Disease: Unresolved Questions. *Curr. Alzheimer Res.* **6**, 15–29 [online] http://www.eurekaselect.com/node/83854/article (Accessed 2009).

76. Donahue, J. E., and Johanson, C. E. (2008) Apolipoprotein E, Amyloid-β, and Blood-Brain Barrier Permeability in Alzheimer Disease. *J. Neuropathol. & Exp. Neurol.* **67**, 261–270 [online] http://dx.doi.org/10.1097/NEN.0b013e31816a0dc8 (Accessed April 1, 2008).

77. Evangelisti, E., and C Cecchi, R. C., C. Sgromo… Membrane lipid composition and its physicochemical properties define cell vulnerability to aberrant protein oligomers. [online] http://jcs.biologists.org/content/125/10/2416.short.

78. Arispe, N., and Doh, M. (2002) Plasma membrane cholesterol controls the cytotoxicity of Alzheimer's disease AβP (1–40) and (1–42) peptides. *FASEB J.* **16**, 1526–1536

79. Yahi, N., Aulas, A., and Fantini, J. (2010) How Cholesterol Constrains Glycolipid Conformation for Optimal Recognition of Alzheimer's β Amyloid Peptide (Aβ1-40). *PLOS ONE* **5** [online] http://dx.doi.org/10.1371%2Fjournal.pone.0009079 (Accessed February 5, 2010).

80. Fantini, J., Yahi, N., and Garmy, N. (2013) Cholesterol accelerates the binding of Alzheimer's β-amyloid peptide to ganglioside GM1 through a universal hydrogen-bond-dependent sterol tuning of glycolipid conformation. *Front. Physiol* **4** [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3677124/ (Accessed June 10, 2013).

81. Kakio, A., Nishimoto, S., Yanagisawa, K., Kozutsumi, Y., and Matsuzaki, K. (2001) Cholesterol-dependent formation of GM1 ganglioside-bound amyloid β-protein, an endogenous seed for Alzheimer amyloid. *J. Biol. Chem.* **276**, 24985–24990

82. Kakio, A., Nishimoto, S., Kozutsumi, Y., and Matsuzaki, K. (2003) Formation of a membrane-active form of amyloid β-protein in raft-like model membranes. *Biochem. Biophys. Res. Commun.* **303**, 514–518

83. Yu, X., and Zheng, J. (2012) Cholesterol promotes the interaction of Alzheimer β-amyloid

monomer with lipid bilayer. *J. Mol. Biol.* **421**, 561–571

84. Devanathan, S., Salamon, Z., Lindblom, G., Gröbner, G., and Tollin, G. (2006) Effects of sphingomyelin, cholesterol and zinc ions on the binding, insertion and aggregation of the amyloid Aβ1- 40 peptide in solid-supported lipid bilayers. *Febs J.* **273**, 1389–1402
85. Brown, J., Theisler, C., Silberman, S., Magnuson, D., Gottardi-Littell, N., Lee, J. M., Yager, D., Crowley, J., Sambamurti, K., and Rahman, M. M. (2004) Differential expression of cholesterol hydroxylases in Alzheimer's disease. *J. Biol. Chem.* **279**, 34674–34681
86. Koldamova, R. P., Lefterov, I. M., Ikonomovic, M. D., Skoko, J., Lefterov, P. I., Isanski, B. A., DeKosky, S. T., and Lazo, J. S. (2003) 22R-Hydroxycholesterol and 9-cis-Retinoic Acid Induce ATP-binding Cassette Transporter A1 Expression and Cholesterol Efflux in Brain Cells and Decrease Amyloid Secretion. *J. Biol. Chem.* **278**, 13244–13256 [online] https://doi.org/10.1074%2Fjbc.m300044200 (Accessed January 2003).
87. Vaya, J., and Schipper, H. M. (2007) Oxysterols, cholesterol homeostasis, and Alzheimer disease. *J. Neurochem.* **102**, 1727–1737
88. Papadopoulos, V., and Lecanu, L. (2012) Caprospinol: Discovery of a Steroid Drug Candidate to Treat Alzheimer's Disease Based on 22R-Hydroxycholesterol Structure and Properties. *J. Neuroendocrinol.* **24**, 93–101 [online] http://dx.doi.org/10.1111/j.1365-2826.2011.02167.x (Accessed 2012).
89. Dasari, B., Prasanthi, J. R., Marwarha, G., Singh, B. B., and Ghribi, O. (2010) The oxysterol 27-hydroxycholesterol increases β-amyloid and oxidative stress in retinal pigment epithelial cells. *BMC Ophthalmol.* **10**
90. Björkhem, I., Heverin, M., Leoni, V., Meaney, S., and Diczfalusy, U. (2006) Oxysterols and Alzheimer's disease. *Acta Neurol. Scand.* **114**, 43–49
91. Brownlee, M. (2001) Biochemistry and molecular cell biology of diabetic complications. *Nature* **414**, 813–820 [online] http://dx.doi.org/10.1038/414813a (Accessed December 13, 2001).
92. Ulrich, P., and Cerami, A. (2001) Protein glycation, diabetes, and aging. *Recent Prog. Horm. Res.* **56**, 1–22
93. Huebschmann, A. G., Regensteiner, J. G., Vlassara, H., and Reusch, J. E. B. (2006) Diabetes and Advanced Glycoxidation End Products. *Diabetes Care* **29** [online] http://care.diabetesjournals.org/content/29/6/1420.abstract (Accessed May 26, 2006).

94. Vlassara, H., and Uribarri, J. (2004) Glycoxidation and Diabetic Complications: Modern Lessons and a Warning? *Rev. Endocr. Metab. Disord.* **5**, 181–188 [online] http://dx.doi.org/10.1023/B:REMD.0000032406.84813.f6 (Accessed 2004).

95. Goldin, A., Beckman, J. A., Schmidt, A. M., and Creager, M. A. (2006) Advanced Glycation End Products. *Circulation* **114** [online] http://circ.ahajournals.org/content/114/6/597.abstract (Accessed August 7, 2006).

96. Bohlender, J. M., Franke, S., Stein, G., and Wolf, G. (2005) Advanced glycation end products and the kidney. *Am. J. Physiol. - Ren. Physiol.* **289** [online] http://ajprenal.physiology.org/content/289/4/F645.abstract (Accessed September 13, 2005).

97. Schmidt, A. M., Yan, S. D., Wautier, J.-L., and Stern, D. (1999) Activation of Receptor for Advanced Glycation End Products. *Circ. Res.* **84** [online] http://circres.ahajournals.org/content/84/5/489.abstract (Accessed March 19, 1999).

98. Vlassara, H. (2001) The AGE-receptor in the pathogenesis of diabetic complications. *Diabetes/Metabolism Res. Rev.* **17**, 436–443 [online] http://dx.doi.org/10.1002/dmrr.233 (Accessed 2001).

99. Eble, A. S., Thorpe, S. R., and Baynes, J. W. (1983) Nonenzymatic glucosylation and glucose-dependent cross-linking of protein. *J. Biol. Chem.* **258**, 9406–9412

100. Miyata, T., Oda, O., Inagi, R., Iida, Y., Araki, N., Yamada, N., Horiuchi, S., Taniguchi, N., Maeda, K., and Kinoshita, T. (1993) beta 2-Microglobulin modified with advanced glycation end products is a major component of hemodialysis-associated amyloidosis. *J Clin Invest* **92**, 1243–1252 [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC288264/ (Accessed September 1993).

101. Sasaki, N., Fukatsu, R., Tsuzuki, K., Hayashi, Y., Yoshida, T., Fujii, N., Koike, T., Wakayama, I., Yanagihara, R., Garruto, R., Amano, N., and Makita, Z. (1998) Advanced Glycation End Products in Alzheimer's Disease and Other Neurodegenerative Diseases. *Am J Pathol* **153**, 1149–1155 [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1853056/ (Accessed October 18, 1998).

102. Kikuchi, S., Ogata, A., Shinpo, K., Moriwaka, F., Fujii, J., Taniguchi, N., and Tashiro, K. (2000) Detection of an Amadori product, 1-hexitol-lysine, in the anterior horn of the amyotrophic lateral sclerosis and spinobulbar muscular atrophy spinal cord: evidence for

early involvement of glycation in motoneuron diseases. *Acta Neuropathol.* **99**, 63–66

103. Ledesma, M. D., Bonay, P., Colaco, C., and Avila, J. (1994) Analysis of microtubule-associated protein tau glycation in paired helical filaments. *J. Biol. Chem.* **269**, 21614–21619

104. Gomes, R., Sousa Silva, M., Quintas, A., Cordeiro, C., Freire, A., Pereira, P., Martins, A., Monteiro, E., Barroso, E., and Ponces Freire, A. (2005) Argpyrimidine, a methylglyoxal-derived advanced glycation end-product in familial amyloidotic polyneuropathy. *Biochem. J* **385**, 339–345 [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1134703/ (Accessed January 15, 2005).

105. Dukic-Stefanovic, S., Schinzel, R., Riederer, P., and Münch*, G. (2001) AGES in brain ageing: AGE-inhibitors as neuroprotective and anti-dementia drugs? *Biogerontology* **2**, 19–34

106. Münch, G., Lüth, H. J., Wong, A., Arendt, T., Hirsch, E., Ravid, R., and Riederer, P. (2000) Crosslinking of α-synuclein by advanced glycation endproducts—an early pathophysiological step in Lewy body formation? *J. Chem. Neuroanat.* **20**, 253–257

107. Höhn, A., Jung, T., and Grune, T. (2014) Pathophysiological importance of aggregated damaged proteins. *Free. Radic. Biol. Med.* **71**, 70–89

108. Vitek, M. P., Bhattacharya, K., Glendening, J. M., Stopa, E., Vlassara, H., Bucala, R., Manogue, K., and Cerami, A. (1994) Advanced glycation end products contribute to amyloidosis in Alzheimer disease. *Proc. Natl. Acad. Sci.* **91**, 4766–4770

109. Bouma, B., Kroon-Batenburg, L. M., Wu, Y.-P., Brünjes, B., Posthuma, G., Kranenburg, O., de Groot, P. G., Voest, E. E., and Gebbink, M. F. (2003) Glycation induces formation of amyloid cross-β structure in albumin. *J. Biol. Chem.* **278**, 41810–41819

110. Chellan, P., and Nagaraj, R. H. (1999) Protein crosslinking by the Maillard reaction: dicarbonyl-derived imidazolium crosslinks in aging and diabetes. *Arch. Biochem. Biophys.* **368**, 98–104

111. Abordo, E. A., Minhas, H. S., and Thornalley, P. J. (1999) Accumulation of α-oxoaldehydes during oxidative stress: a role in cytotoxicity. *Biochem. Pharmacol.* **58**, 641–648 [online] //www.sciencedirect.com/science/article/pii/S000629529900132X (Accessed August 15, 1999).

112. Fu, M.-X., Requena, J. R., Jenkins, A. J., Lyons, T. J., Baynes, J. W., and Thorpe, S. R.

(1996) The advanced glycation end product, N-(carboxymethyl) lysine, is a product of both lipid peroxidation and glycoxidation reactions. *J. Biol. Chem.* **271**, 9982–9986 [online] http://www.jbc.org/content/271/17/9982.short (Accessed 1996).

113. Du Yan, S., Chen, X., Fu, J., and Chen, M. (1996) RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease. *Nature* **382**

114. Xie, J., Méndez, J. D., Méndez-Valenzuela, V., and Aguilar-Hernández, M. M. (2013) Cellular signalling of the receptor for advanced glycation end products (RAGE). *Cell. Signal.* **25**, 2185–2197

115. Ott, C., Jacobs, K., Haucke, E., Navarrete Santos, A., Grune, T., and Simm, A. (2014) Role of advanced glycation end products in cellular signaling. *Redox Biol* **2**, 411–429 [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3949097/ (Accessed January 9, 2014).

116. Lue, L.-F., Yan, S. D., Stern, D. M., and Walker, D. G. (2005) Preventing activation of receptor for advanced glycation endproducts in Alzheimer's disease. *Curr. Drug Targets-CNS & Neurol. Disord.* **4**, 249–266

117. Miranda, H. V., and Outeiro, T. F. (2010) The sour side of neurodegenerative disorders: the effects of protein glycation. *J. Pathol.* **221**, 13–25 [online] http://dx.doi.org/10.1002/path.2682 (Accessed 2010).

118. Wan, W., Chen, H., and Li, Y. (2014) The potential mechanisms of Aβ-receptor for advanced glycation end-products interaction disrupting tight junctions of the blood-brain barrier in Alzheimer's disease. *Int. J. Neurosci.* **124**, 75–81

119. Galasko, D., Bell, J., Mancuso, J. Y., Kupiec, J. W., Sabbagh, M. N., van Dyck, C., Thomas, R. G., and Aisen, P. S. (2014) Clinical trial of an inhibitor of RAGE-Aβ interactions in Alzheimer disease. *Neurology* **82**, 1536–1542

120. Takuma, K., Fang, F., Zhang, W., Yan, S., Fukuzaki, E., Du, H., Sosunov, A., McKhann, G., Funatsu, Y., and Nakamichi, N. (2009) RAGE-mediated signaling contributes to intraneuronal transport of amyloid-β and neuronal dysfunction. *Proc. Natl. Acad. Sci.* **106**, 20021–20026

121. Hadding, A., Kaltschmidt, B., and Kaltschmidt, C. (2004) Overexpression of receptor of advanced glycation end products hypersensitizes cells for amyloid beta peptide-induced cell death. *Biochim. et Biophys. Acta* **1691**, 67–72

122. Li, X. H., Du, L. L., Cheng, X. S., Jiang, X., Zhang, Y., Lv, B. L., Liu, R., Wang, J. Z., and

Zhou, X. W. (2013) Glycation exacerbates the neuronal toxicity of β-amyloid. *Cell death & Dis.* **4**

123. Smith, M. A., Taneda, S., Richey, P. L., Miyata, S., Yan, S. D., Stern, D., Sayre, L. M., Monnier, V. M., and Perry, G. (1994) Advanced Maillard reaction end products are associated with Alzheimer disease pathology. *Proc Natl Acad Sci U S* **91**, 5710–5714 [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC44066/ (Accessed June 7, 1994).

124. Münch, G., Mayer, S., Michaelis, J., Hipkiss, A. R., Riederer, P., Müller, R., Neumann, A., Schinzel, R., and Cunningham, A. M. (1997) Influence of advanced glycation end-products and AGE-inhibitors on nucleation-dependent polymerization of β-amyloid peptide. *Biochim. et Biophys. Acta* **1360**, 17–29 [online] //www.sciencedirect.com/science/article/pii/S0925443996000622 (Accessed February 27, 1997).

125. Chen, K., Maley, J., and Yu, P. H. (2006) Potential implications of endogenous aldehydes in β-amyloid misfolding, oligomerization and fibrillogenesis. *J. Neurochem.* **99**, 1413–1424

126. Zheng, F., He, C., Cai, W., Hattori, M., Steffes, M., and Vlassara, H. (2002) Prevention of diabetic nephropathy in mice by a diet low in glycoxidation products. *Diabetes/Metabolism Res. Rev.* **18**, 224–237 [online] http://dx.doi.org/10.1002/dmrr.283 (Accessed 2002).

127. Lin, R.-Y., Reis, E. D., Dore, A. T., Lu, M., Ghodsi, N., Fallon, J. T., Fisher, E. A., and Vlassara, H. (2002) Lowering of dietary advanced glycation endproducts (AGE) reduces neointimal formation after arterial injury in genetically hypercholesterolemic mice. *Atherosclerosis* **163**, 303–311

128. Hofmann, S. M., Dong, H.-J., Li, Z., Cai, W., Altomonte, J., Thung, S. N., Zeng, F., Fisher, E. A., and Vlassara, H. (2002) Improved Insulin Sensitivity Is Associated With Restricted Intake of Dietary Glycoxidation Products in the db/db Mouse. *Diabetes* **51** [online] http://diabetes.diabetesjournals.org/content/51/7/2082.abstract (Accessed July 1, 2002).

129. Sandu, O., Song, K., Cai, W., Zheng, F., Uribarri, J., and Vlassara, H. (2005) Insulin Resistance and Type 2 Diabetes in High-Fat–Fed Mice Are Linked to High Glycotoxin Intake. *Diabetes* **54** [online] http://diabetes.diabetesjournals.org/content/54/8/2314.abstract (Accessed July 25, 2005).

130. Peppa, M., Brem, H., Ehrlich, P., Zhang, J.-G., Cai, W., Li, Z., Croitoru, A., Thung, S., and Vlassara, H. (2003) Adverse Effects of Dietary Glycotoxins on Wound Healing in Genetically Diabetic Mice. *Diabetes* **52**, 2805–2813 [online] http://diabetes.diabetesjournals.org/content/52/11/2805 (Accessed 2003).

131. Peppa, M., He, C., Hattori, M., McEvoy, R., Zheng, F., and Vlassara, H. (2003) Fetal or Neonatal Low-Glycotoxin Environment Prevents Autoimmune Diabetes in NOD Mice. *Diabetes* **52** [online] http://diabetes.diabetesjournals.org/content/52/6/1441.abstract (Accessed June 1, 2003).

132. Uribarri, J., Cai, W., Peppa, M., Goodman, S., Ferrucci, L., Striker, G., and Vlassara, H. (2007) Circulating Glycotoxins and Dietary Advanced Glycation Endproducts: Two Links to Inflammatory Response, Oxidative Stress, and Aging. *J Gerontol Biol Sci Med Sci* **62**, 427–433 [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2645629/ (Accessed April 2007).

133. Vlassara, H., Cai, W., Crandall, J., Goldberg, T., Oberstein, R., Dardaine, V., Peppa, M., and Rayfield, E. J. (2002) Inflammatory mediators are induced by dietary glycotoxins, a major risk factor for diabetic angiopathy. *Proc Natl Acad Sci U S* **99**, 15596–15601 [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC137762/ (Accessed November 26, 2002).

134. Uribarri, J. (2003) Restriction of Dietary Glycotoxins Reduces Excessive Advanced Glycation End Products in Renal Failure Patients. *J. Am. Soc. Nephrol.* **14**, 728–731 [online] https://doi.org/10.1097%2F01.asn.0000051593.41395.b9 (Accessed March 2003).

135. Uribarri, J., Peppa, M., Cai, W., Goldberg, T., Lu, M., Baliga, S., Vassalotti, J. A., and Vlassara, H. (2003) Dietary glycotoxins correlate with circulating advanced glycation end product levels in renal failure patients. *Am. J. Kidney Dis.* **42**, 532–538 [online] //www.sciencedirect.com/science/article/pii/S0272638603007790 (Accessed September 2003).

136. Vlassara, H., Cai, W., Goodman, S., Pyzik, R., Yong, A., Chen, X., Zhu, L., Neade, T., Beeri, M., Silverman, J. M., Ferrucci, L., Tansman, L., Striker, G. E., and Uribarri, J. (2009) Protection against Loss of Innate Defenses in Adulthood by Low Advanced Glycation End Products (AGE) Intake: Role of the Antiinflammatory AGE Receptor-1. *J Clin Endocrinol Metab* **94**, 4483–4491 [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2775660/ (Accessed November 9, 2009).

137. Simons, K., and Ikonen, E. (1997) Functional rafts in cell membranes. *Nature* **387**

138. Giacomelli, C. E., and Norde, W. (2005) Conformational Changes of the Amyloid β-Peptide (1–40) Adsorbed on Solid Surfaces. *Macromol. Biosci.* **5**, 401–407 [online] http://dx.doi.org/10.1002/mabi.200400189 (Accessed 2005).

139. Williams, T. L., and Serpell, L. C. (2011) Membrane and surface interactions of Alzheimer's Aβ peptide--insights into the mechanism of cytotoxicity. *FEBS J.* **278**, 3905–3917 [online] http://www.ncbi.nlm.nih.gov/pubmed/21722314 (Accessed NaN, 2011).

140. Valdes-Gonzalez, T., Inagawa, J., and Ido, T. (2001) Neuropeptides interact with glycolipid receptors A surface plasmon resonance study. *Peptides* **22**, 1099–1106 [online] //www.sciencedirect.com/science/article/pii/S0196978101004326 (Accessed July 2001).

141. Matsuzaki, K., Kato, K., and Yanagisawa, K. (2010) Aβ polymerization through interaction with membrane gangliosides. *Biochim. et Biophys. Acta* **1801**, 868–877 [online] //www.sciencedirect.com/science/article/pii/S1388198110000211 (Accessed August 2010).

142. D'Errico, G., Vitiello, G., Ortona, O., Tedeschi, A., Ramunno, A., and D'Ursi, A. M. (2008) Interaction between Alzheimer's Aβ(25–35) peptide and phospholipid bilayers: The role of cholesterol. *Biochim. et Biophys. Acta* **1778**, 2710–2716 [online] //www.sciencedirect.com/science/article/pii/S0005273608002216 (Accessed December 2008).

143. Lai, A. Y., and McLaurin, J. (2011) Mechanisms of Amyloid-Beta Peptide Uptake by Neurons: The Role of Lipid Rafts and Lipid Raft-Associated Proteins. *Int J Alzheimers Dis* **2011** [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3010653/ (Accessed December 20, 2011).

144. Brezesinski, G., and Möhwald, H. (2003) Langmuir monolayers to study interactions at model membrane surfaces. *Adv. Colloid Interface Sci.* **100**, 563–584

145. Chan, Y.-H. M., and Boxer, S. G. (2007) Model membrane systems and their applications. *Curr. Opin. Chem. Biol.* **11**, 581–587

146. Plant, A. L. (1999) Supported hybrid bilayer membranes as rugged cell membrane mimics. *Langmuir* **15**, 5128–5135

147. Madhusudhana Rao, N., Silin, V., Ridge, K. D., Woodward, J. T., and Plant, A. L. (2002) Cell membrane hybrid bilayers containing the G-protein-coupled receptor CCR5. *Anal.*

Biochem. **307**, 117–130 [online] //www.sciencedirect.com/science/article/pii/S0003269702000039 (Accessed August 1, 2002).

148. Veatch, S. L., and Keller, S. L. (2003) Separation of liquid phases in giant vesicles of ternary mixtures of phospholipids and cholesterol. *Biophys. J.* **85**, 3074–3083

149. Phan, H. T. T., and MC Vestergaard, K. B. Localization of amyloid beta (Aβ1-42) protofibrils in membrane lateral compartments: Effect of cholesterol and 7-Ketocholesterol. [online] http://onlinelibrary.wiley.com/doi/10.1016/j.febslet.2014.08.007/full.

150. Phan, H. T. T., and T Hata, M. M., T. Yoda, T. Hamada… The effect of oxysterols on the interaction of Alzheimer's amyloid beta with model membranes. [online] http://www.sciencedirect.com/science/article/pii/S0005273613002101.

Chapter 2:

Localization and aggregation of amyloid-beta (Aβ-42) under the influence of 25-hydroxycholesterol in biomimetic membranes

Abstract

Lipid organization is an integral feature in the structure and functions of membranes, where alteration in the assembly of lipids may cause disturbance in their homeostasis. Dyshomeostasis of lipids is observed in the pathology of Alzheimer's disease (AD). I have shown that the alteration of organization of lipids in heterogeneous membranes influences the association of amyloid beta (Aβ) with the membrane. Specifically, the peptides are localized in the liquid-disordered region of the membrane in the presence of 25-hydroxycholesterol (25-OHC). In addition, aggregation of Aβ is closely related to the toxicity induced by peptide. This property of Aβ is affected by the incorporation of oxysterols in the lipid membrane which I have shown by introduction of 25-OHC. Fibrillation of Aβ-42 hindered by 25-OHC to cause the production of more toxic species. Thus, oxidation of cholesterol enhances the association and cellular toxicity induction by Alzheimer's amyloid protein.

Keywords: Amyloid beta-42, 25-hydroxycholesterol, localization, liquid-disordered phase, liquid-ordered phase, aggregation, biomimetic membranes.

2.1. Introduction

Interaction between Aβ and the cell membrane has been proposed as a key event in the pathogenesis of AD (1, 2). Cell membrane possesses different structural and functional properties; one of the important membrane structural property is lateral heterogeneity. Different lipid species exhibit different phase behavior, thus it has been proposed that lipid domains present in membranes exhibit difference in composition, function and physical properties (3). Simons and Ikonen hypothesized in their 'lipid raft model' that cholesterol preferentially binds to sphingolipids and forms cholesterol-sphingolipid clusters. These clusters are termed as lipid raft domains which are more ordered than other parts of the membrane. In addition to cholesterol and sphingolipids, unsaturated-phosphatidylcholines (PC) are also present which corresponds to liquid phase of lipid bilayers (4).

Cholesterol is an integral component of lipid rafts, has implicated in the pathology of AD (5–7). The importance of cholesterol in AD is ascribed to the potential of cholesterol to change physiochemical properties of cell membranes which modulates the production (8–10), aggregation, and cytotoxicity of Aβ-42 peptide, the neurotoxic species involved in AD (8, 9, 11). Cholesterol constitutes 30-40% of the total lipid composition of the membrane (12). Hence, modulates the packing of membrane lipids, affects the fluidity, permeability, and phase separation of membrane (13, 14). Oxidation of cholesterol influences the cytotoxicity induced by Aβ peptide. Cholesterol produces different derivatives (oxysterols) depending on the type of oxidation which may be enzymatic oxidation which gives 24-hydroxycholesterol (24-OHC), 25-hydroxycholesterol (25-OHC), 27-hydroxycholesterol (27-OHC) etc. Oxysterols are more hydrophilic than cholesterol due to an extra oxygen group in the structure, which makes them less capable in the packing (15, 16). Thereby, can influence the fluidity and induces peptide insertion into the biomimetic (model) membranes (16–18).

Previous reports have shown that lipid rafts serves as the sites for binding and aggregation of Aβ peptides (19). Aβ preferentially binds to glycosphingolipid GM1 of lipid rafts (20), which is facilitated by cholesterol (21) where it promotes the formation of GM1 clusters which are highly recognized by the peptides (22). In addition, lateral compartments of model membranes have been reported to interact with Aβ depending on their structure and properties (23, 24). Since cholesterol

modulates the structure and features of membrane domains, it may affect the association of the peptide with the domains present in the bilayer of lipid membrane.

Aggregation of Aβ is a crucial event in the neuronal damage in AD. Peptide aggregation is a two-step nucleation-dependent polymerization process: (i) formation of nucleus and (ii) fibril elongation (25). Firstly, soluble monomeric Aβ, generated from the amyloidogenic processing of amyloid precursor protein (APP), changes its conformation from random coil to β-sheet structure (26, 27) and/or interchanges between α-helical to β-sheet conformation depending upon the conditions (28). Particularly, β-sheet self-aggregates to produce dimers, trimmers, and finally nuclei (n-mers) (25). Subsequent step involves the formation of intermediate species of Aβ that are oligomers or protofibrils (29) which concomitantly joins together end-to-end or laterally, finally leads to the formation of insoluble fibrils (25, 30). These fibrils accumulates as extracellular amyloid plaques that are characterized as pathological hallmarks in AD (31). Aggregated species of Aβ indicates towards the cellular toxicity produced by the peptide. Monomers and insoluble fibrils are less toxic than oligomeric and protofibrillar species of Aβ (30, 32, 33). It is attributed to the fact that oligomers and protofibrils can modulate integrity of the membrane by interacting with cell membranes (34). In addition, these intermediate species may cause dysfunction of mitochondria and disturb synaptic function (35) by internalizing into cells (36). Thereby, regulation of aggregation of Aβ and the formation of neurotoxic species has emerged as a treatment in the progression of AD (37, 38).

There is a phenomenal association between cell membranes and Aβ aggregation. Membranes serve as an aggregation matrix which seeds and promotes formation of Aβ-42 into fibrils (39, 40). Subsequent changes in the conformation of the peptide into β-sheet structure, induce association and fibrillation of Aβ (41–43). Aggregation mediated by membrane depends upon different factors such as peptide density (42) and composition of lipid membrane (42, 44). In addition to GM1, other lipids also have been suggested to affect the rate of Aβ assembly. The peptide accumulates preferentially in positively charged (DOTAP) lipid bilayers than negatively charged (DOPG) or neutral (DOPC, DPPC) systems (8). Furthermore, rate of accumulation of peptide depends upon the oxidation of phospholipids where oxidized phospholipids induced faster accumulation than non-oxidized ones (12). Aβ fibrillation was attempted by few researchers but the results were contradicting (45, 46). Elucidation of influence of oxysterols on Aβ aggregation

important to understand the effect of oxidation of cholesterol in Aβ-induced toxicity. 25-OHC is an important derivative of cholesterol which is produced by enzymatic oxidation, whereas 7-ketocholesterol (7-KC) is a dominantly produced by oxidation of cholesterol by reactive oxygen species (ROS). In addition, protofibrillar Aβ-42 showed higher reactivity towards membrane in comparison to Aβ-40 (18). Thus, I used 7-KC, 25-OHC and protofibrillar Aβ-42 for my further studies.

This chapter aims to investigate the effect of cholesterol derivative, 25-OHC on the localization of protofibrillar Aβ-42 in phase-separated heterogeneous membranes. Previously, my group has suggested that 25-OHC and 7-KC prominently influenced the interaction between Aβ peptide and homogeneous biomimetic membranes. Moreover, I have investigated the kinetics of Aβ-42 aggregation in the presence of 25-OHC. My results have revealed that 25-OHC significantly influences the localization of Aβ-42 in Ld phase of the membrane and it promotes the appearance of toxic species of Aβ-42.

2.2. Experimental procedures

2.2.1. Materials-

Membrane lipids such as 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3phosphocholine (DPPC) and cholesterol (Chol) were purchased from Avanti Polar Lipids (USA). 25-hydroxycholesterol (25-OHC) and 7-ketocholesterol (7-KC) were obtained from Sigma-Aldrich (USA). Amyloid beta protein (Human, 1-42) (Aβ-42) was from Peptide Institute Inc. (Japan). Hilyte Fluor^{TM} 488-labelled (λex=503 nm, λem=528 nm) Aβ-42 (fluorescent Aβ-42) and Thioflavin T were from Anaspec, Inc. (USA) and Tokyo chemical industry co. (Japan), respectively. Rhodamine b 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (Rho-DHPE) (λex=560 nm, λem=580 nm) was from Invitrogen (USA). Chloroform, methanol, and Tris(hydroxymethyl)aminomethane (Tris) were purchased from Takara Bio Inc., Kanto-Chemical, and Nacalai Tesque (Japan).

2.2.2. Preparation of biomimetic membranes-

Heterogeneous and homogeneous biomimetic membranes or liposomes were prepared by the natural swelling method (21). Lipid mixture was dissolved in chloroform/methanol (2:1, v/v) at the final concentration of 0.1 mM. A gentle N_2 stream was used to evaporate the solvent. The tube was subsequently dried in a desiccator for a period of 3 h, resulting in a thin lipid film at the bottom of tube. The film was swollen with Tris buffer-HCl (1.25 mM, pH = 7.4) overnight at 37 ^{o}C to form the liposomes. Lo/Ld liposomes was prepared with Chol 30% (DOPC/DPPC/Cholesterol = 35/35/30, molar ratio) and to study the effect of oxysterols, 25-OHC and 7-KC, cholesterol was partially replaced with oxysterols at 75/25 (Chol/25-OHC or 7-KC) molar ratio. Concentration of cholesterol was kept at 30% in Lo/Ld liposomes because this cholesterol concentration is nearly similar to that in biological membranes (47). To visualize membrane lateral compartments, Rho-DHPE, a specific probe of DOPC, was added into lipid mixture at 0.05% (mol/mol) concentration. Homogeneous membranes were formed at DOPC (100), DOPC/Chol (50/50) and DOPC/Chol/Oxy (50/40/10) where oxy= 7-KC or 25-OHC.

2.2.3. Preparation of protofibrillar Aβ-42-

Aβ-42 (200 μM) solutions were prepared by dissolving Aβ-42 powder in 0.02% (v/v) ammonia solution and stored the vials at -80 °C. Peptide solution was diluted and later incubated with biomimetic membranes in the ratio of 5:4 (membranes/peptide) at 80 μM for different incubation periods (0, 6, 12, 24, 36, and 48 h). For localization experiments, fluorescence-labelled Aβ-42 and Aβ-42 were mixed at 1:2 (fluorescence-labelled Aβ42/Aβ-42) molar ratio. The mixture was diluted in Tris buffer (20 mM, pH 7.4) at 80 μM concentration, subsequently incubated at 37 ºC for 12 h. The morphology of Aβ-42 was demonstrated by atomic force microscopic observation (48).

2.2.4. Observation of protofibrillar Aβ-42 localization in heterogeneous model membranes-

Fluorescence-labelled Aβ-42 were added to suspension of lipid vesicles at final concentration of 5 μM. The resultant mixture was immediately poured into a silicon well (0.01 mm) placed on a glass slide and was observed using confocal microscopy (Olympus FV-1000, Japan) at room temperature (~ 21.5 ºC) within 2 min. This observation period is short enough to avoid the effect of fluorescence quenching as reported previously (39). Lateral compartments of

the membrane and protofibrillar Aβ-42 were labeled with Rho-DHPE and fluorescence-labelled Aβ-42, respectively. The intensity of fluorescent Aβ-42 was subsequently analyzed and used to evaluate localization of Aβ-42 peptide in membranes. The data were expressed as means ± SE of three independent sets of experiments.

2.2.5. Observation of aggregation of Aβ-42 in the presence of 25-hydroxycholesterol-

To assess the aggregation of Aβ-42, ThT assay was used. 5 µM ThT solution was used as working concentration, diluted in the Tris buffer (20 mM). 100 µL solution containing Aβ-42, ThT solution, buffer was added to the 96-well microtiter plate and fluorescence intensity was measured using Varioskan flash spectral scanning multimode reader at excitation at 440 nm and emission wavelength at 484 nm at temperature 37°C for a period of 24 h.

2.3. Results and discussion

2.3.1. Localization of protofibrillar amyloid–beta (Aβ-42) in 25-OHC added biomimetic membranes

Oxidative stress plays important role in the pathology of AD (49). Previously, it was reported that 7-KC enhances the association of Aβ with homogeneous and heterogeneous membranes (18, 50). Thus, I assessed the effect of 25-OHC in the localization of protofibrillar Aβ-42 in membrane lateral compartments. Here, I used heterogeneous biomimetic membranes, composed of lipids (cholesterol, unsaturated and saturated phospholipid). There are three phases in these membranes, which are, a liquid-disordered (Ld) phase, solid-ordered (So) domains and liquid-ordered (Lo) domains, co-exists depending on the concentration fraction of cholesterol (51). It has been reported that Lo domains are lipid raft-like domains while Ld and So are considered as liquid and gel states of biological membranes (52). Lo/Ld heterogeneous membranes were used with 30% cholesterol concentration because this is closer to the concentration in biological membranes (47). 25-OHC was introduced to the above stated membranes at 7.5 mM concentrations which is similar to the one used for 7-KC (50), corresponding to the level detected in rat hippocampus after kainite excitotoxicity (53). Confocal microscopy was used to observe chol-containing and 25-OHC containing membranes (figure 2.1A), the amount of protofibrillar

Aβ-42 localizing in Lo/Ld membrane systems was remarkably increased by the substitution of membrane cholesterol with 25-OHC (figure 2.1B). The elevation in the localization of the peptides was much higher in the Ld phase of 25-OHC-containing membranes.

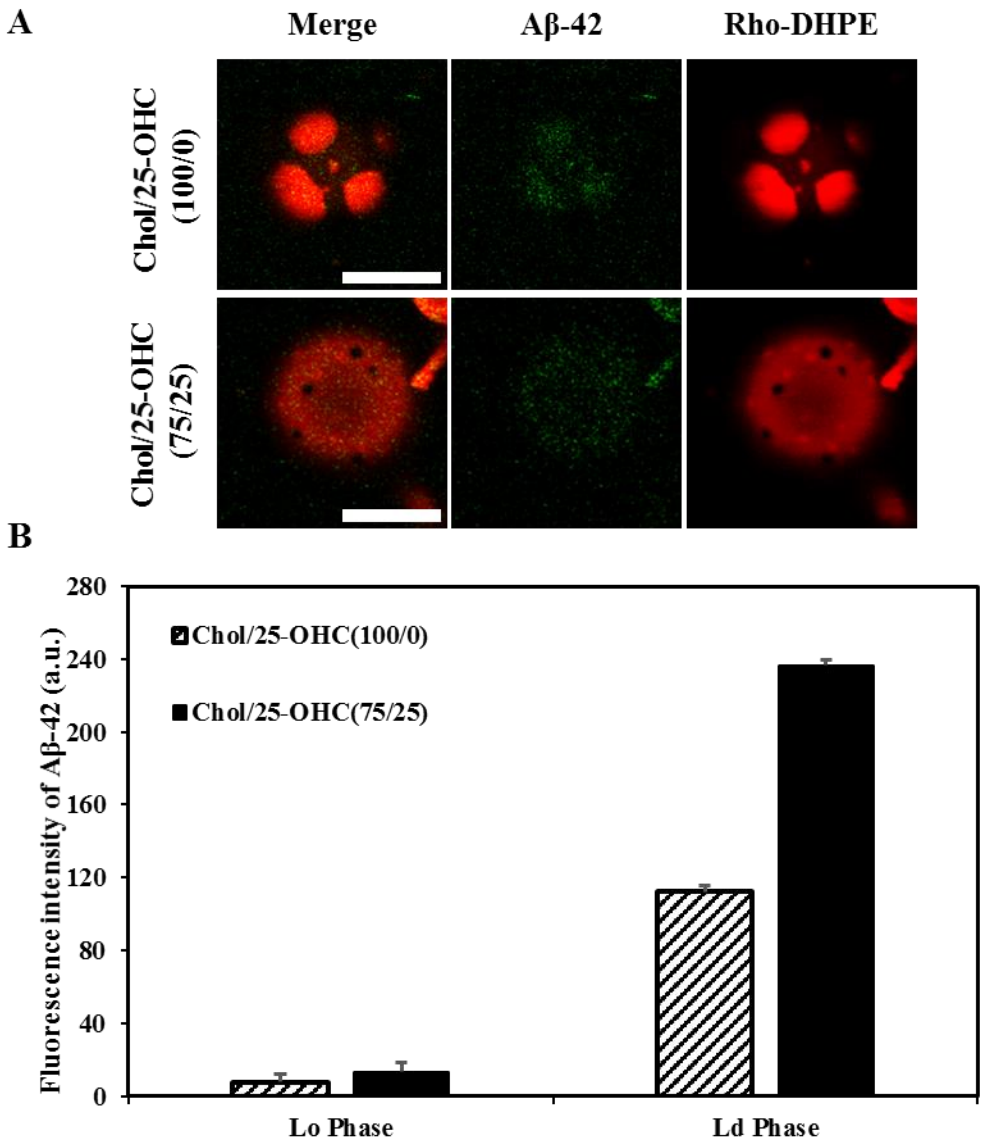


Figure 2.1. Effect of 25-hydroxycholesterol on localization of amyloid beta (1-42) protofibrils in membranes. **A)** Confocal microscopy images of lipid membranes, and **B)** Fluorescence intensity values reflecting the amount of Aβ-42 in Lo/Ld phase-separated membranes. Red and black regions indicate Ld and Lo domains, respectively. The values are mean ± SE of three independent experiments. Scale bars are 5 μm.

The interaction of protofibrillar Aβ-42 with Lo domains was slightly changed in the presence of 25-OHC in comparison with cholesterol-containing membrane. This indicated that the oxysterol exert influence in the association of the peptide predominantly with Ld phase of heterogeneous membranes. To draw a comparison between 25-OHC and 7-KC, I have shown figure 2.2., where it can be seen that the above results are in good agreement with the previous report that 7-KC influences the localization of Aβ-42 in heterogeneous biomimetic membranes, that too significantly in Ld phase of the heterogeneous membranes (50). Both oxysterols showed prominent effect in the association of Aβ-42 with the membranes in Ld phase, whereas impact produced by 25-OHC was more than that of 7-KC. Nevertheless, 25-OHC slightly affected the localization of the peptide in Lo region too unlike 7-KC which showed almost similar effect to that of cholesterol.

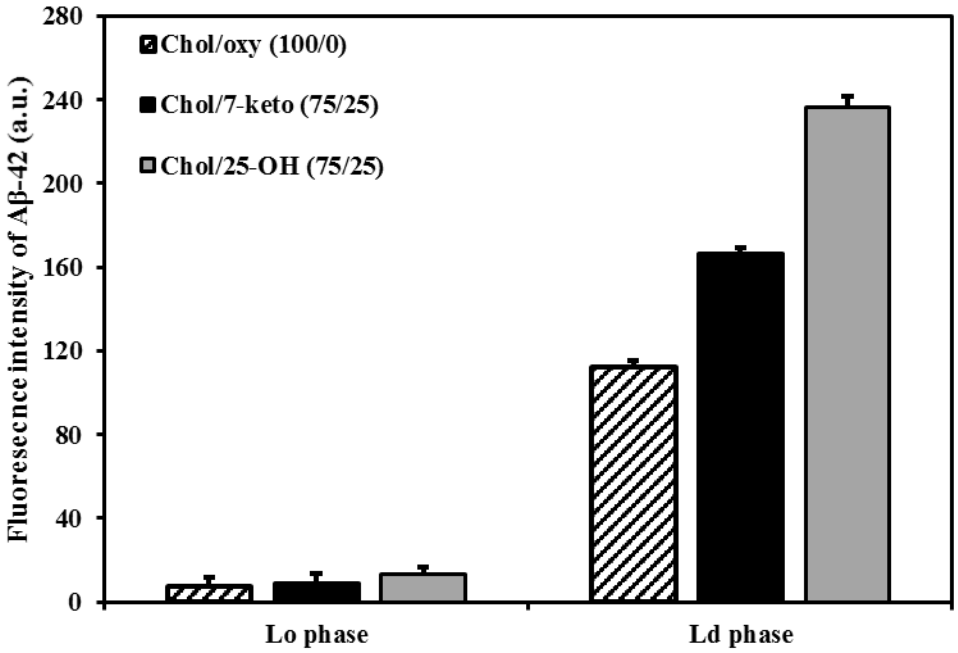


Figure 2.2. Effect of cholesterol, oxysterols (25-hydroxycholesterol and 7-ketocholestrol) on localization of amyloid beta (Aβ-42) protofibrils in membranes. Fluorescence intensity values reflecting the amount of Aβ-42 in Lo/Ld phase-separated membranes. The values are mean ± SE of three independent experiments.

2.3.2. Aggregation of amyloid beta (Aβ-42) in the presence of 25-hydroxycholesterol

To assess the aggregation kinetics of Aβ in the presence of 25-OHC, ThT assay was used. It is a popular method, where ThT exhibit fluorescence on binding with the β-sheet of amyloid peptide at 483 nm while free ThT is observed at 445 nm (54, 55). ThT assay shows the degree of fibrillation as a function of time. Fibrillation of Aβ occurs in a two-step process where nucleus formation takes place which is followed by the elongation of fibrils. Previously, my group have observed that presence of lipid vesicles influences fibrillation of Aβ-42. DOPC systems facilitates amyloid nucleus formation and inhibits fibril elongation whereas, cholesterol-containing vesicles strongly inhibited the kinetics of nucleus formation and promotes Aβ-42 to grow in toxic species (unpublished data). Although, partial substitution in the composition of membrane with 7-KC influences the aggregation kinetics of Aβ-42 in an identical manner as in DOPC systems, 7-KC-containing lipid bilayers remarkably impede elongation of the fibrils as compared to DOPC bilayers.

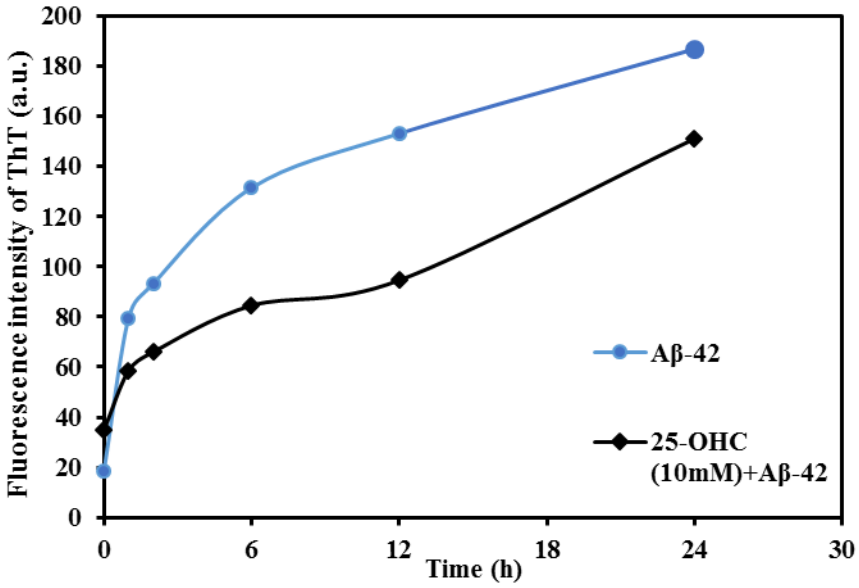


Figure 2.3. Fibrillation curve showing the aggregation kinetics of Aβ-42 in the absence and presence of 25-hydroxycholesterol. Fluorescence intensity values of ThT reflecting the degree of the peptide over 24 h and monitored using varioskan flash spectral scanning multimode reader at Ex/Em=440/484 nm.

Fibrillation of Aβ-42 from monomers in the absence and presence of the oxysterol as a function of time as shown in figure 2.3. Aggregation of the peptide exhibited a typical profile as shown previously (25, 56). In general, nucleus formation is reflected in the initial part of curve, known as lag phase, subsequently proceeds by the elongation phase which corresponds to the fibril growth, and finally saturation phase appears when most of the peptide in solution has aggregated into fibrils (57). Concentration of the peptide is an important variable in the process of nucleation (25). After addition of 25-OHC, the lag phase of the curve could not be observed very clearly, while the speed of elongation was lowered remarkably (figure 2.3.).

According to previous reports, cholesterol accelerates the fibrillation of aggregated species of Aβ-42 on sphingomyelin (SM)/cholesterol bilayers (58) and 1,2-dipalmitoyl-snglycero-3-phosphocholine (DPPC)/cholesterol (59). Oxysterols (7-KC) induced an increase in nucleation rate constant whereas lowered the elongation rate constant. These results indicated that 7-KC in the membrane could maintain the existence of Aβ-42 in intermediate toxic species (oligomers and protofibrils) by impeding fibril growth and promoting nucleus formation. A number of studies have shown the aggregation of Aβ-42 on the surface of membrane (41, 43), where a research group have demonstrated that incubation of Aβ for a day with DOPC bilayers produces less fibrils in comparison with incubation in buffer for the same time (8).

Molecular mechanism of aggregation of the peptide has been proposed in the absence of GM1 in the lipid bilayers (8, 58). They suggested that binding of hydrophilic terminal of the peptide to hydrophilic head group of the lipid results into the adsorption of peptide onto the bilayer of lipids, Hydrophilic residues may forms strong hydrogen bonds with carbonyl and phosphate oxygen groups of the phospholipids. And hydrophobic carbon residues would place into the hydrophobic interior of lipid bilayers by hydrophobic interaction. Subsequent changes of the peptide will result into a conformational transition from α-helix-rich to β-sheet-rich structures which then initiate the nucleus formation and fibril growth (8). Aggregation of Aβ-42 is influenced by its insertion into the lipid bilayer where DOPC is in fluid phase (58).

Three lipid vesicles systems were studied in the previous study and Aβ-42 without any vehicle was used as the control. DOPC and 7-KC lipid vesicles impeded the fibrillation of Aβ-42, on the other hand cholesterol-containing membranes promoted the process. Effect of oxysterol was more than DOPC whereas, cholesterol facilitated possibly due to its ability to lower the association

of the peptide with the membrane (50). On the contrary, after nuclei formation, speed of elongation increases (45) ascribed to the fact that after the incorporation of cholesterol in the membranes, peptide may not be able to insert deeply into the lipid bilayer and Aβ-42 remains adsorbed on to the surface of the membrane.

As demonstrated in a previous study, cholesterol promotes interaction between Aβ and GM1 which seeds the nucleus for aggregation of the peptide (41), then cholesterol is also able accelerate the fibrillation process by influencing the interaction of Aβ with membrane lipid bilayer. This was in accordance with the induction of high fluidity and higher association with the membrane (18). In addition, previous studies on the morphology and association of Aβ-42 with cell membranes have shown that cholesterol-containing membranes promoted the formation of fibrillar Aβ-42, while oxysterols containing membranes enhanced the appearance of more toxic protofibrils. I have observed the similar effect of oxysterols, inhibiting the growth of fibrils and inducing the formation of more harmful species of Aβ-42. Hence, these findings proposed that the oxidation of membrane cholesterol induced by oxidative stress may enhance Aβ/membranes interaction, thus being a risk factor of Aβ-induced toxicity in AD's pathology.

2.4. Conclusions

In conclusion, biomimetic membranes were useful for the observation of localization of Aβ-42 in different compartments of membrane. I have shown that the oxysterols influences the association of the peptide with the membrane. Particularly, 25-OHC showed higher recruitment of protofibrillar Aβ-42 in the Ld region of the membrane which indicates towards changes in the fluidity of the membrane. In accordance with the previous results about homogeneous and heterogeneous membranes, it is certain the oxidation of cholesterol induced by enzymatic or non-enzymatic processes is harmful for the membrane and affects its physiochemical properties. Moreover, oxysterols facilitates the cytotoxicity induced by Aβ-42 as 25-OHC hindered the fibrillation and promote the formation of toxic species. Hence, oxidized derivatives of cholesterol are potential risk factor in peptide-induced toxicity ascribed to their potential to impede the aggregation from more toxic protofibrils to fibrils.

References

1. Karran, E., Mercken, M., and Strooper, B. D. (2011) The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics. *Nat Rev Drug Discov* **10**, 698–712 [online] http://dx.doi.org/10.1038/nrd3505 (Accessed September 2011).
2. Swerdlow, R. H. (2007) Pathogenesis of Alzheimer's disease. *Clin. Interv. Aging* **2.**
3. Owen, D. M., and Gaus, K. (2013) Imaging lipid domains in cell membranes: the advent of super-resolution fluorescence microscopy.
4. Simons, K., and Ikonen, E. (1997) Functional rafts in cell membranes. *Nature* **387.**
5. Puglielli, L., Tanzi, R. E., and Kovacs, D. M. (2003) Alzheimer's disease: the cholesterol connection. *Nat. Neurosci.* **6**, 345–351.
6. Liguri, M. S. and G. (2009) Cholesterol in Alzheimers Disease: Unresolved Questions. *Curr. Alzheimer Res.* **6**, 15–29 [online] http://www.eurekaselect.com/node/83854/article (Accessed 2009).
7. Vestergaard, M., Hamada, T., and Takagi, M. M. and M. (2010) Cholesterol, Lipids, Amyloid Beta, and Alzheimers. *Curr. Alzheimer Res.* **7**, 262–270 [online] http://www.eurekaselect.com/node/86087/article (Accessed 2010).
8. Hane, F., Drolle, E., Gaikwad, R., Faught, E., and Leonenko, Z. (2011) Amyloid-β aggregation on model lipid membranes: An atomic force microscopy study. *J. Alzheimer's Dis.* **26**, 485–494.
9. Eckert, G. P., Kirsch, C., Leutz, S., Wood, W. G., and Müller, W. E. (2003) Cholesterol modulates amyloid beta-peptide's membrane interactions. *Pharmacopsychiatry* **36**, 136–143.
10. Kojro, E., Gimpl, G., Lammich, S., März, W., and Fahrenholz, F. (2001) Low cholesterol stimulates the nonamyloidogenic pathway by its effect on the α-secretase ADAM 10. *Proc. Natl. Acad. Sci.* **98**, 5815–5820.
11. LaFerla, F. M., Green, K. N., and Oddo, S. (2007) Intracellular amyloid-β in Alzheimer's disease. *Nat. Rev. Neurosci.* **8**, 499–509.
12. Van Meer, G. (1989) Lipid traffic in animal cells. *Annu. Rev. cell Biol.* **5**, 247–275.
13. McMullen, T. P., Lewis, R. N., and McElhaney, R. N. (2004) Cholesterol–phospholipid interactions, the liquid-ordered phase and lipid rafts in model and biological membranes. *Curr. Opin. Colloid & Interface Sci.* **8**, 459–468 [online] http://dx.doi.org/10.1016/j.cocis.2004.01.007 (Accessed 2004).

14. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002) Molecular biology of the cell. new york: Garland science; 2002. *Class. Textb. now its 5th Ed.*
15. Brown, A. J., and Jessup, W. (1999) Oxysterols and atherosclerosis. *Atherosclerosis* **142**, 1–28.
16. Mintzer, E., Charles, G., and Gordon, S. (2010) Interaction of two oxysterols, 7-ketocholesterol and 25-hydroxycholesterol, with phosphatidylcholine and sphingomyelin in model membranes. *Chem. Phys. Lipids* **163**, 586–593 [online] //www.sciencedirect.com/science/article/pii/S0009308410000782 (Accessed June 2010).
17. Kim, D. H., and Frangos, J. A. (2008) Effects of amyloid β-peptides on the lysis tension of lipid bilayer vesicles containing oxysterols. *Biophys. J.* **95**, 620–628.
18. Phan, H. T., Hata, T., Morita, M., Yoda, T., Hamada, T., Mun'delanji, C. V., and Takagi, M. (2013) The effect of oxysterols on the interaction of Alzheimer's amyloid beta with model membranes. *Biochim. et Biophys. Acta* **1828**, 2487–2495.
19. Rushworth, J. V., and Hooper, N. M. (2010) Lipid rafts: linking Alzheimer's amyloid-β production, aggregation, and toxicity at neuronal membranes. *Int. J. Alzheimer's Dis.* **2011.**
20. Kakio, A., Nishimoto, S., Kozutsumi, Y., and Matsuzaki, K. (2003) Formation of a membrane-active form of amyloid β-protein in raft-like model membranes. *Biochem. Biophys. Res. Commun.* **303**, 514–518.
21. Yahi, N., Aulas, A., and Fantini, J. (2010) How Cholesterol Constrains Glycolipid Conformation for Optimal Recognition of Alzheimer's β Amyloid Peptide (Aβ 1-40). *PLoS One* **5.**
22. Kakio, A., Nishimoto, S., Yanagisawa, K., Kozutsumi, Y., and Matsuzaki, K. (2001) Cholesterol-dependent formation of GM1 ganglioside-bound amyloid β-protein, an endogenous seed for Alzheimer amyloid. *J. Biol. Chem.* **276**, 24985–24990.
23. Choucair, A., Chakrapani, M., Chakravarthy, B., Katsaras, J., and Johnston, L. J. (2007) Preferential accumulation of Aβ (1- 42) on gel phase domains of lipid bilayers: An AFM and fluorescence study. *Biochim. et Biophys. Acta* **1768**, 146–154.
24. Morita, M., Hamada, T., Tendo, Y., Hata, T., Vestergaard, M. C., and Takagi, M. (2012) Selective localization of Alzheimer\textquotesingles amyloid beta in membrane lateral compartments. *Soft Matter* **8** [online] http://dx.doi.org/10.1039/c2sm07185a (Accessed 2012).
25. Sabaté, R., Gallardo, M., and Estelrich, J. (2003) An autocatalytic reaction as a model for the

kinetics of the aggregation of β-amyloid. *Pept. Sci.* **71**, 190–195.

26. Lovas, S., Zhang, Y., Yu, J., and Lyubchenko, Y. L. (2013) Molecular mechanism of misfolding and aggregation of Aβ (13–23). *J. Phys. Chem. B* **117**, 6175–6186.
27. Petkova, A. T., Buntkowsky, G., Dyda, F., Leapman, R. D., Yau, W.-M., and Tycko, R. (2004) Solid state NMR reveals a pH-dependent antiparallel β-sheet registry in fibrils formed by a β-amyloid peptide. *J. Mol. Biol.* **335**, 247–260.
28. Zagorski, M. G., and Barrow, C. J. (1992) NMR studies of amyloid. beta.-peptides: proton assignments, secondary structure, and mechanism of an. alpha.-helix. fwdarw.. beta.-sheet conversion for a homologous, 28-residue, N-terminal fragment. *Biochemistry* **31**, 5621–5631.
29. Harper, J. D., Wong, S. S., Lieber, C. M., and Lansbury, P. T. (1997) Observation of metastable Aβ amyloid protofibrils by atomic force microscopy. *Chem. & Biol.* **4**, 119–125.
30. Harper, J. D., Lieber, C. M., and Lansbury, P. T. (1997) Atomic force microscopic imaging of seeded fibril formation and fibril branching by the Alzheimer's disease amyloid-β protein. *Chem. & Biol.* **4**, 951–959.
31. Hardy, J. A., and Higgins, G. A. (1992) Alzheimer's disease: the amyloid cascade hypothesis. *Science* **256.**
32. Walsh, D. M., Hartley, D. M., Kusumoto, Y., Fezoui, Y., Condron, M. M., Lomakin, A., Benedek, G. B., Selkoe, D. J., and Teplow, D. B. (1999) Amyloid β-protein fibrillogenesis Structure and biological activity of protofibrillar intermediates. *J. Biol. Chem.* **274**, 25945–25952.
33. Zhao, L. N., Long, H. W., Mu, Y., and Chew, L. Y. (2012) The toxicity of amyloid ß oligomers. *Int. J. Mol. Sci.* **13**, 7303–7327.
34. Demuro, A., Mina, E., Kayed, R., Milton, S. C., Parker, I., and Glabe, C. G. (2005) Calcium Dysregulation and Membrane Disruption as a Ubiquitous Neurotoxic Mechanism of Soluble Amyloid Oligomers. *J. Biol. Chem.* **280**, 17294–17300 [online] https://doi.org/10.1074%2Fjbc.m500997200 (Accessed February 2005).
35. Selkoe, D. J. (2004) Cell biology of protein misfolding: the examples of Alzheimer's and Parkinson's diseases. *Nat. cell Biol.* **6**, 1054–1061.
36. Cha, M.-Y., Han, S.-H., Son, S. M., Hong, H.-S., Choi, Y.-J., Byun, J., and Mook-Jung, I. (2012) Mitochondria-specific accumulation of amyloid β induces mitochondrial dysfunction leading to apoptotic cell death. *PLoS One* **7.**

37. Hyung, S.-J., DeToma, A. S., Brender, J. R., Lee, S., Vivekanandan, S., Kochi, A., Choi, J.-S., Ramamoorthy, A., Ruotolo, B. T., and Lim, M. H. (2013) Insights into antiamyloidogenic properties of the green tea extract (-)-epigallocatechin-3-gallate toward metal-associated amyloid-β species. *Proc. Natl. Acad. Sci.* **110**, 3743–3748.
38. Re, F., Airoldi, C., Zona, C., Masserini, M., Ferla, B. L., Quattrocchi, N., and Nicotra, F. (2010) Beta amyloid aggregation inhibitors: small molecules as candidate drugs for therapy of Alzheimer's disease. *Curr. Med. Chem.* **17**, 2990–3006.
39. Kotler, S. A., Walsh, P., Brender, J. R., and Ramamoorthy, A. (2014) Differences between amyloid-β aggregation in solution and on the membrane: insights into elucidation of the mechanistic details of Alzheimer's disease. *Chem. Soc. Rev.* **43**, 6692–6700.
40. Bokvist, M., and Gröbner, G. (2007) Misfolding of amyloidogenic proteins at membrane surfaces: the impact of macromolecular crowding. *J. Am. Chem. Soc.* **129**, 14848–14849.
41. Ikeda, K., Yamaguchi, T., Fukunaga, S., Hoshino, M., and Matsuzaki, K. (2011) Mechanism of amyloid β-protein aggregation mediated by GM1 ganglioside clusters. *Biochemistry* **50**, 6433–6440.
42. Kakio, A., Nishimoto, S., Yanagisawa, K., Kozutsumi, Y., and Matsuzaki, K. (2002) Interactions of amyloid β-protein with various gangliosides in raft-like membranes: importance of GM1 ganglioside-bound form as an endogenous seed for Alzheimer amyloid. *Biochemistry* **41**, 7385–7390.
43. Lemkul, J. A., and Bevan, D. R. (2013) Aggregation of Alzheimer's amyloid β-peptide in biological membranes: a molecular dynamics study. *Biochemistry* **52**, 4971–4980.
44. Sani, M.-A., Gehman, J. D., and Separovic, F. (2011) Lipid matrix plays a role in Abeta fibril kinetics and morphology. *FEBS Lett.* **585**, 749–754.
45. Tashima, Y., Oe, R., Lee, S., Sugihara, G., Chambers, E. J., Takahashi, M., and Yamada, T. (2004) The effect of cholesterol and monosialoganglioside (GM1) on the release and aggregation of amyloid β-peptide from liposomes prepared from brain membrane-like lipids. *J. Biol. Chem.* **279**, 17587–17595
46. Yip, C. M., Elton, E. A., Darabie, A. A., Morrison, M. R., and McLaurin, J. (2001) Cholesterol, a modulator of membrane-associated Aβ-fibrillogenesis and neurotoxicity. *J. Mol. Biol.* **311**, 723–734.
47. Bucciantini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J., Taddei, N.,

Ramponi, G., Dobson, C. M., and Stefani, M. (2002) Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature* **416**, 507–511.

48. Morita, M., Vestergaard, M., Hamada, T., and Takagi, M. (2010) Real-time observation of model membrane dynamics induced by Alzheimer's amyloid beta. *Biophys. Chem.* **147**, 81–86.
49. Zhao, Y., and Zhao, B. (2013) Oxidative stress and the pathogenesis of Alzheimer's disease. *Oxidative Med. Cell. Longev.* **2013.**
50. Phan, H. T., Vestergaard, M. C., Baek, K., Shimokawa, N., and Takagi, M. (2014) Localization of amyloid beta (Aβ1-42) protofibrils in membrane lateral compartments: Effect of cholesterol and 7-Ketocholesterol. *FEBS Lett.* **588**, 3483–3490.
51. Veatch, S. L., and Keller, S. L. (2003) Separation of liquid phases in giant vesicles of ternary mixtures of phospholipids and cholesterol. *Biophys. J.* **85**, 3074–3083.
52. Barenholz, Y., and Cevc, G. (2000) Structure and properties of membranes. *Phys. Chem. Biol. surfaces*, 171–241.
53. Kim, J.-H., Jittiwat, J., Ong, W.-Y., Farooqui, A. A., and Jenner, A. M. (2010) Changes in cholesterol biosynthetic and transport pathways after excitotoxicity. *J. Neurochem.* **112**, 34–41.
54. Durairajan, S. S. K., Yuan, Q., Xie, L., Chan, W.-S., Kum, W.-F., Koo, I., Liu, C., Song, Y., Huang, J.-D., and Klein, W. L. (2008) Salvianolic acid B inhibits Aβ fibril formation and disaggregates preformed fibrils and protects against Aβ-induced cytotoxicty. *Neurochem. Int.* **52**, 741–750.
55. Vestergaard, M., and Kerman, K. (2009) Analytical Tools for Detecting Amyloid Beta Oligomerisation and Assembly. *Curr. Pharm. Anal.* **5**, 229–245.
56. Miura, Y., and Mizuno, H. (2010) Interaction analyses of amyloid β peptide (1–40) with glycosaminoglycan model polymers. *Bull. Chem. Soc. Jpn.* **83**, 1004–1009.
57. Padrick, S. B., and Miranker, A. D. (2002) Islet amyloid: phase partitioning and secondary nucleation are central to the mechanism of fibrillogenesis. *Biochemistry* **41**, 4694–4703.
58. Devanathan, S., Salamon, Z., Lindblom, G., Gröbner, G., and Tollin, G. (2006) Effects of sphingomyelin, cholesterol and zinc ions on the binding, insertion and aggregation of the amyloid Aβ1- 40 peptide in solid-supported lipid bilayers. *Febs J.* **273**, 1389–1402.
59. Zhao, L. N., Chiu, S.-W., Benoit, J., Chew, L. Y., and Mu, Y. (2011) Amyloid β peptides

aggregation in a mixed membrane bilayer: a molecular dynamics study. *J. Phys. Chem. B* **115**, 12247–12256.

Chapter 3:

Intracellular transport of amyloid beta (Aβ-42) mediated by cholera toxin B subunit and 25-hydroxycholesterol in Jurkat T cells

Abstract

Amyloid beta (Aβ) peptide is responsible for the Alzheimer's disease. Misfolding and accumulation of the peptide lead to neural cell apoptosis through Endoplasmic reticulum (ER) stress. This stress could also be the result of exogenous Aβ which eventually leads to same neurotoxicity and apoptosis to those by endogenous peptide. I have proposed endocytic transport of Aβ to be the cause of ER stress. Previously it was shown that oxysterols, in particular, 7-ketocholesterol (7-KC) induced higher surface interaction than cholesterol between Aβ and Jurkat T cells. Although, it was not sufficient to induce intracellular transfer of the peptide. Here in, primary interest was to investigate the effect of another oxysterol, 25-hydroxycholesterol (25-OHC) in the membrane raft dependent transport of Aβ-42 in Jurkat T cells. Interestingly, intracellular transfer of Aβ-42 was observed in the presence of 25-OHC only after inclusion of cholera toxin B subunit (CT-B), used for the detection of raft domain. Furthermore, CT-B together with GM1 provided negative curvature which positively results into endocytic transport of Aβ-42. Notably, I have used protofibrillar species of Aβ-42 in the study. I have demonstrated that the transport was microtubule dependent since it could not be observed in depolymerized state of microtubule. I have demonstrated important factors for internalization such as oxysterols, glycosyl chains of membranes and nano-structures of the peptide. These components are associated with aging and advanced glycation which are the risk factors involved in AD. Thus, this study will be beneficial to improve the understanding of the pathology of the disease.

Keywords: Amyloid beta-42, 25-hydroxycholesterol, Cholera toxin subunit B, endocytic transport, T cells, GM1.

3.1. Introduction

Aggregation and accumulation of Amyloid beta (Aβ) is the pathological pathway of neurological disorder commonly known as Alzheimer's disease (AD). Amyloid precursor protein (APP) is a transmembrane protein (Type 1) which undergoes different catabolic pathways at three main cleavage sites. Though APP is present in every tissue but highly expressed in brain. It is believed to be the precursor protein for Aβ, produced on cleavage of APP through BACE (beta site APP cleaving enzyme) and γ-secretase. Endoplasmic reticulum (ER) is responsible for proper folding or processing of newly synthesized proteins. When misfolded form of proteins accumulate in endoplasmic reticulum (ER) they cause stress to the organelle. This phenomenon is called as ER stress. Usually ER homeostasis can be altered by physiological changes which results in accumulation of misfolded proteins in the lumen of ER. These physiological changes could be high protein demand, viral infections, inflammatory cytokines etc. According to the previous reports, externally added amyloid in primary neurons could cause ER stress (1–3). Oligomeric species of Aβ-42 found to induce ER stress. I speculated that this could be also result of exogenously added amyloid beta. Inflammation and apoptosis are eventual outcomes of this stress. ER stress also occurs when ER homeostasis is disturbed, which is related to other neurodegenerative diseases and diabetes. Diabetes is linked with AD and known as a risk factor for AD's patients. There are some risk factors which increases the chances of AD, including aging, advanced glycation end products (AGEs), genetic and metabolic abnormalities. For example, aging, higher the prospects of development of AD through production of reactive oxygen species (ROS) which cause oxidation of lipids. In this study, I have taken these factors into consideration and explored their role in endocytic movement of Aβ peptides. Latest studies suggested about different mechanisms for the internalization of Aβ; such as endocytic mechanisms, pore forming protein perforin and receptor mediated pathways (4). Endocytic transport or endocytosis is a membrane phenomenon and basic structure of membrane is lipid bilayer which is majorly composed of cholesterol, sphingolipids and glycerophospholipids (5) .

Major cholesterol [25%] of our body is present in the brain (6) which gets oxidized enzymatically and non-enzymatically to produce, either, side chain oxygenated derivatives with an additional hydroxyl group such as 24-hydroxycholesterol (24-OHC), 25-hydroxycholesterol (25-OHC) and 27-hydroxycholesterol (27-OHC) or with an extra oxygen group resulted after

ROS-induced oxidation such as 7-ketocholesterol (7-KC), 7β-hydroxycholesterol (7β-OHC) etc. Recent reports demonstrates the importance of oxysterols in AD progression due to altered cholesterol metabolism and oxidative stress (7). 24-OHC and 25-OHC control cholesterol metabolism through binding to Liver X receptor α (LXR α). Yamanaka et al. found that 24S-OHC caused necroptosis of neural cells in a concentration dependent manner (8). Accumulation of Aβ was suggested to be higher in cholesterol-poor domains in biological membranes (9, 10).

25-OHC was reported to induce higher thermosensitivity than cholesterol and 7-KC in lipid membranes (10). 25-OHC was shown to be most cytotoxic in comparison with other oxysterols such as 7β-OHC, 7-KC, 19-OHC, 22(R)-OHC and 22(S)-OHC (11) and reported to induce cytotoxicity to various cell types (12–15) . With other oxysterols, consistent levels of 25-OHC was found in mouse brains (16). It was also reported that 25-OHC opened mitochondrial permeability transition pore and increased the production of ROS, which resulted in release of cytochrome c and further activation of caspase-3 and -9 (15).

I have performed a comparative study about interaction of the peptide with membranes. Inspired from studies about peptide Aβ using its two isoforms Aβ-40 and Aβ-42 in biomimetic membranes (9, 10, 17, 18). Among the two isoforms of protein, I had used Aβ-42 which is more hydrophobic and toxic than Aβ-40. Considerably, I have used protofibrillar (elongated oligomers) species, which is considered to be primary toxic species of Aβ-42 along with oligomers (19).
In the current study, I have used Jurkat T cells (leukemic cells). They have been reported as a target of Aβ (20) and also express a certain level of caspase-8, a key mediator of the extrinsic apoptotic pathway (21). Jurkat cell line is a potential candidate in study of AD since their plasma membrane is rich in sphingomyelin (SM) possessing functional and structural diversity of neural cells having GM1 rich domains (22). Using Jurkat T cells provides an opportunity to deepen the knowledge about essential factors involved in endocytic mechanism.

It was reported that inclusion of 7-KC resulted in greater surface interaction of protofibrillar Aβ-42 with Jurkat T cells (18). Although, transport of peptides was not observed. Thus, for the present study, I choose 25-OHC to investigate the role of oxysterols in the endocytic movement of Aβ-42 in Jurkat T cells and compared its effect from cholesterol and 7-KC. To achieve the target, I had studied cellular toxicity induced by peptide, membrane-peptide interaction, changes in intracellular calcium level and mechanistic pathway of protein in 25-OHC added cells. I have found that 25-OHC alone was also not good enough to induce the endocytic transport of the

peptide, thus I have added CT-B which binds with GM1 and suggested to provide negative curvature to the membrane (23). GM1 clusters are reported to binds and accelerate the formation of toxic Aβ fibrils (24).

In this chapter, I sought to investigate that 25-OHC together with CT-B was important for endocytic vesicular transport of Aβ-42 in Jurkat T cells. It will be the basis to understand the mechanism involved in AD. In best of my knowledge, this is the first study to investigate mechanism involving in the endocytic transport of Aβ-42 which could be a cause of ER stress.

3.2. Experimental procedures

3.2.1. Materials-

25-hydroxycholesterol (25-OHC), 7-ketocholesterol (7-KC) bovine serum albumin (BSA), and trypan blue were obtained from Sigma-Aldrich (USA). Amyloid beta protein (Human, 1-42) (Aβ-42), Hilyte FluorTM 488-labeled (λex=503 nm, λex=528 nm) Aβ-42 and HiLyte Fluor-555-labeled (λex= 551 nm, λex= 567 nm) Aβ-42 were from Peptide Institute Inc. (Japan) and Anaspec, Inc. (USA), respectively. ER-Tracker Blue-White DPX, Oregon Green 488 taxol, Roswell Park Memorial Institute 1640 (RPMI1640) medium, fetal bovine serum and Alexa Fluor 555 conjugated cholera toxin subunit B (CT-B) (λex= 555 nm, λem= 565 nm) were from Invitrogen (USA). Phosphate buffer salts (PBS), and Tris (hydroxymethyl) aminomethane (Tris) were purchased from Takara Bio Inc. and Kanto-Chemical (Japan), respectively.

3.2.2. Cell Culture-

Jurkat human leukemic T cells (Riken Cell bank, Japan) were cultured in medium containing RPMI 1640 supplemented with 10 % fetal bovine serum in a humidified incubator at 37°C in the presence of 5% CO_2.

3.2.3. Preparation of protofibrillar Aβ-42 –

200μM Aβ-42 solutions were prepared by dissolving Aβ-42 powder in 0.02% (v/v) ammonia solution and stored at -80°C. Before experiments, Aβ-42 solution was diluted to 80 μM concentration with Tris buffer (20mM, pH 7.4), and subsequently incubated at 37°C for 12 h (10). To prepare the protofibrillar species, fluorescence labeled Aβ-42 and normal Aβ-42 was mixed at 1:2 molar ratio before the incubation (9).

3.2.4. Measurement of cell viability-

Trypan blue exclusion assay was used to estimate Jurkat cell viability upon the effect of Aβ-42 protofibrils (25, 26). Untreated (Control) and 25-OHC added cells cultured in 48-well plate were exposed to 10 μM protofibrillar Aβ-42 for 24 h. Thioflavin T assay showed that the confirmation of protofibrillar Aβ-42 did not significantly change in this condition (data was not shown). The cells were then washed with PBS and treated with trypan blue at the final concentration of 0.01% (v/v) in PBS for 10 min at room temperature. The number of dead cells and viable cells were counted using a hemocytometer. The cell having a clear cytoplasm defined as a viable cell, while a dead cell is the one with a blue cytoplasm (27). Cell viability was calculated following the formation: cell viability=total viable cells/total cells. Data is expressed as mean ± standard deviation of at least three independent experiments.

3.2.5. Lipid raft staining and observation of Aβ-42-

Membrane cholesterol level was altered by treating the cells with 25-OHC in PBS at two different concentrations such as 5 μM and 10 μM for 10 min at 37°C. Lipid rafts were labelled by treating cells with 1 μg/ml CT-B, which specifically binds to GM1 in lipid rafts (28), and 0.02% (v/v) BSA in PBS at 0°C for 30 min, followed by an incubation at 37°C for 10 min. Then cells were washed with PBS buffer.

3.2.6. Staining and depolymerization of microtubule-

To stain microtubule, Oregon Green® 488 Taxol was used. For depolymerization, cells were treated with 20 μg/ml nocodazole for 10 min at 37°C in a humidified incubator in the presence of 5% CO_2. Then cells were washed twice with PBS.

3.2.7. Staining of Endoplasmic Reticulum-

ER organelle was labelled by treating cells with 1 μg/ml ER-Tracker (Blue-White DPX dye) in 0.02% BSA, followed by an incubation at 37 °C for 30 min. Then cells were washed with PBS buffer.

3.2.8. Microscopy-

Fluorescence labelled Aβ-42 was added to T cell suspension at the final concentration of 5 μM. The resultant mixture was placed in a glass bottom dish and observed using Confocal Laser Scanning Microscope (Olympus FV-1000, Japan) with 60X oil immersion lens at room temperature within 2 min. The observation period is short enough to avoid the effect of fluorescent quenching (10).

3.2.9. Measurement of intracellular calcium level-

The intracellular Calcium level was measured using a visible Ca^{2+} fluorescent probe, Fluo-3-AM (29). Untreated (Control), 25-OHC added cells were cultured in 48-well plates, subsequently exposed to 10 μM protofibrillar Aβ-42 for 60 min (30). Then washed with RPMI 1640 (non-serum) and load with 4 μM Fluo-3-AM with 20% (v/v) plorunic F127 in addition with PBS at 37°C for 20 min. 0.01% (w/v) BSA in PBS was added to dilute the cells five times and incubated further for 40 min. Probe loaded cells were washed and re-suspended in HEPES buffer. Calcium level was measured at an excitation of 488 nm (31) using confocal laser scanning microscopy.

3.2.10. Statistical analysis-

Data is expressed as means ± standard deviations of three independent experiments.

3.3. Results and discussion

3.3.1. Incorporation of 25-hydroxycholesterol in the membranes of Jurkat T Cells

The oxysterols are derivatives of cholesterol and considered to have substantial biological effects. Role of different oxysterols such as 7-KC, 24-OHC, 22-OHC, 7β-OHC in AD has been studied in the past. They are attribute to modulate the cell permeability (32, 33), regulate the cholesterol homeostasis (34–36), gene expression (37, 38) and act as receptors in cell signaling (39–41). 27-OHC and 24-OHC are reported to involve in AD (8, 42) and AMD (43) and 25-OHC which possesses similar structure, was suggested to interpose the transcription factors related to synthesis of cholesterol and fatty acid (44). Previously, it was shown that interaction of Aβ-42 with

cells was enhanced after inclusion of 7-KC in the cellular membrane (18). Last study, was a comparison among basal level cholesterol, excessively added cholesterol and 7-KC added cells. In either of the cases, endocytic transport of peptides could not be observed. In the present study, role of another oxysterol i.e. 25-OHC was studied. Herein, I had focused on the requirement of 25-OHC in the endocytic transport of Aβ-42 in Jurkat cells.

I have investigated the interaction of Aβ-42 with the Jurkat T cells in presence of 25-OHC (see figure 3.1.). Observation was done with cells having basal cholesterol level and 25-OHC was added in cells at two different concentrations such as 5 µM and 10 µM. Confocal laser scanning microscope was used to observe the cells. Even this system was not good enough to induce endocytic transport of Aβ peptide.

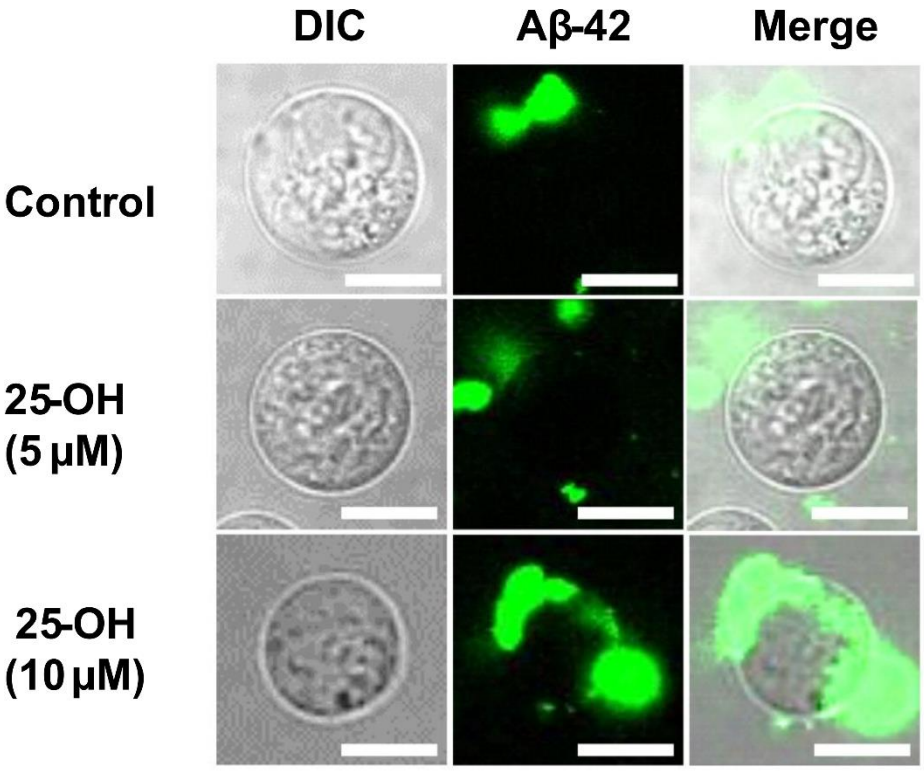


Figure 3.1. Microscopic images of control (basal cholesterol level) and 25-hydroxycholesterol (5 µM and 10 µM) added Jurkat T cells after the exposure of protofibrillar Aβ-42. Green fluorescence represents HiLyte fluor labeled Aβ-42 (488 nm). Gray images are differential interference contrast image, fluorescent images are from confocal laser scanning and merge is overlapping of two. Scale bars=10 µm.

3.3.2. Involvement of GM1 in endocytic transfer of Aβ-42 in Jurkat T Cells

Dhingra et al. have demonstrated that interaction of GM1 and CT-B facilitates the endocytic movement and transformation in the membrane (23). Thus, CT-B which binds with GM1 was used to highlight the lipid raft. This interaction was efficient for the endocytic movement of Aβ-42. After the inclusion of CT-B in the 25-OHC added cells, endocytic vesicular transport of Aβ-42 was observed (see figure 3.2). Involvement of CT-B appeared to be necessary for the transport of protein as in the absence of CT-B, Aβ-42 was not internalized into the membrane. GM1 and CT-B induces negative curvature which resulted in the endocytic transport of Aβ-42. At 5 μM 25-OHC, Aβ-42 was internalized and co-localized with the raft which could be called as raft-dependent endocytosis. But at 10 μM, Aβ-42 appeared without raft, called as raft-independent endocytosis. It might be possible that at higher concentration of 25-OHC, raft is de-stabilized but I still need to clarify the reasons more precisely. Clustering of Aβ-42 inside the cellular spaces was shown with the help of stacking images. Cross-sectional images of a cell from top to middle portion are shown in (see figure 3.3.). It ensured the presence of Aβ-42 inside the cells.

I had changed the composition of the membrane by adding 25-OHC and compared the data with results obtained with the inclusion of excess cholesterol and 7-KC in Jurkat cells. Excessive cholesterol caused higher accumulation of Aβ-42 on the cell surface (18). In comparison with oxysterols, cholesterol addition showed weaker interaction of Aβ-42 and cells. Alteration with 7-KC showed stronger surface interaction of Aβ-42 with Jurkat cells. Internalization of protofibrillar Aβ-42 was observed in the presence of 25-OHC in Jurkat cells.

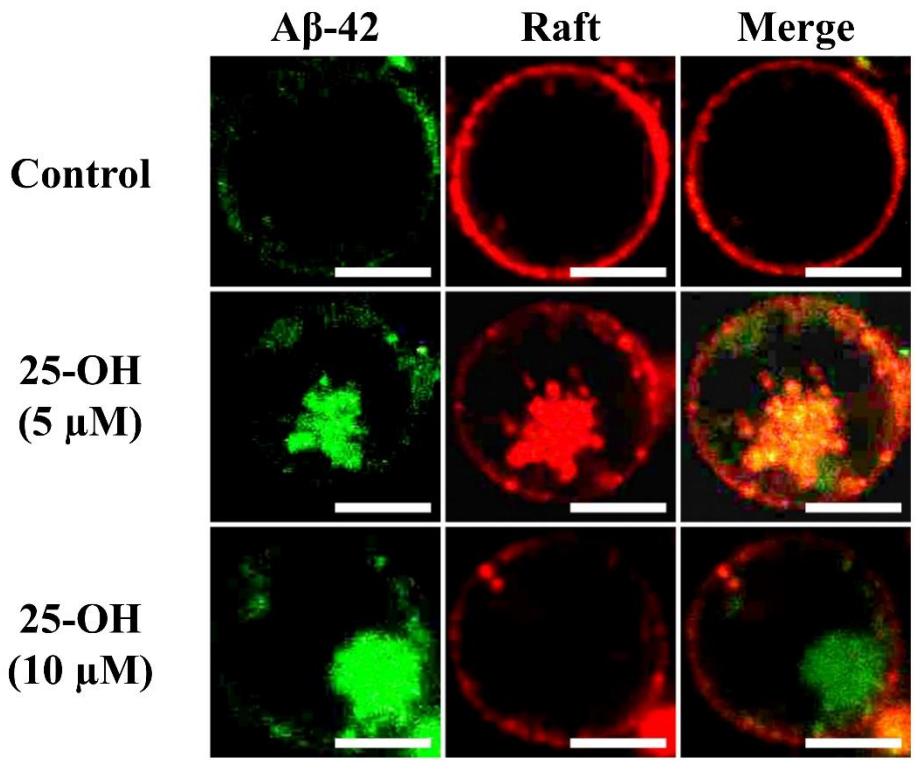


Figure 3.2. Microscopic images of control and 25-hydroxycholesterol (5 µM and 10 µM) added Jurkat T cells. Green and red fluorescence represents HiLyte fluor labeled Aβ-42 (488 nm) and CT-B (555nm), respectively. Scale bars=10 µm.

In addition to 25-OHC, CT-B was also needed in the internalization of Aβ peptides. CT-B binds with GM1, GM1 is a widely distributed sphingolipid present in all types of tissue. Highest concentration of GM1 is present in central nervous system (CNS). Interaction of GM1 and CT-B suggested to affect membrane curvature and surface, changes the processes of surface interaction (23), as a result of negative curvature produced by CT-B and GM1, endocytic movement of Aβ occurs. Incorporation of GM1 and CT-B causes membrane deformations and endocytic formations in model membranes. Enough evidence had suggested integral role of membrane lipids in the accumulation and formation of Aβ fibrils (45). They are reported to accumulate in GM1 rich regions which itself is highly concentrated in CNS. Proteins associated with lipid rafts play crucial part in the internalization of the peptide, trafficking and signal transduction (46). Originally, association of Aβ with lipid rafts was first discussed in cholesterol studies (46). It was found that oligomeric species of Aβ co-localized with CT-B in cervical sympathetic neurons, thus it was

proposed that internalization of Aβ takes place at lipid rafts, probably after binding with GM1 (47). These facts strengthen the theories involved in the onset of AD which is a neurological disorder. According to previous reports, 25-OHC has potential to induce profound morphological changes with Aβ-42 in model membranes (10). Aβ-42 peptides are reported to internalize via raft-dependent endocytosis in the neuroblastoma cells (48).

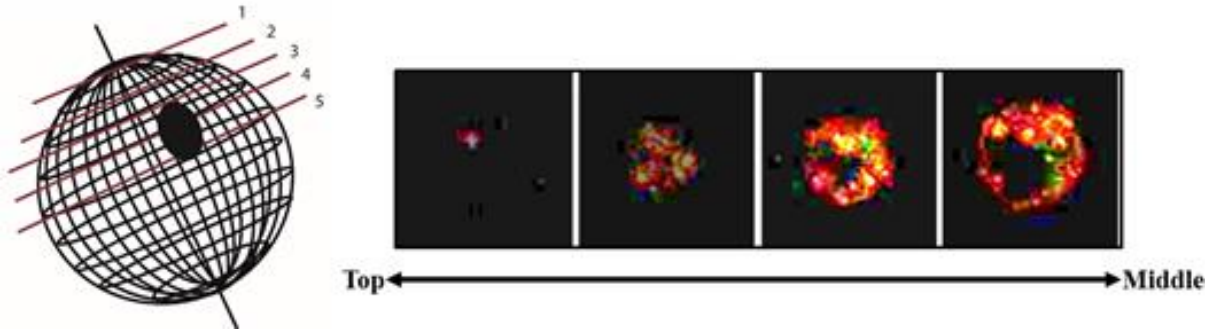


Figure 3.3. Sliced images of 25-hydroxycholesterol added cells showing internalized Aβ-42. Green and red fluorescence represents HiLyte fluor labeled Aβ-42 (488 nm) and CTB (555nm), respectively. Scale bars=10 µm.

3.3.3. Effect of oxysterols with Aβ-42 on cell viability

To measure effect of toxicity induced by Aβ-42 in oxysterol-added cells, cell viability was measured using Trypan blue exclusion test. Cells were treated with protofibrillar Aβ-42 [10 µM] for 24 h which cause a mild decrease in cell viability. Alteration of membrane composition with 25-OHC at 5 µM and 10 µM cause further decrease in the viability of cells. Effect of 25-OHC was higher than 7-KC which was used at similar concentrations and incubation time (see figure 3.4.). Though inclusion of oxysterol and exposure to Aβ-42 was harmful for the cells but it was not so much significant at the specified conditions for Jurkat cell line. Similar concentrations of 25-OHC was lethal for neural cells (12, 49) which had resulted into death of ~35-40% of cells. Cytotoxic effects of 7-KC and 25-OHC was demonstrated at 5 µM in EL4 lymphoma and K36 leukemic cells after 48-72 h of incubation with the oxysterols (50). In my case, incubation time was 24 h after the

addition of Aβ-42 in the system, so it could be possible that cells survived and viability was more at the time of observation.

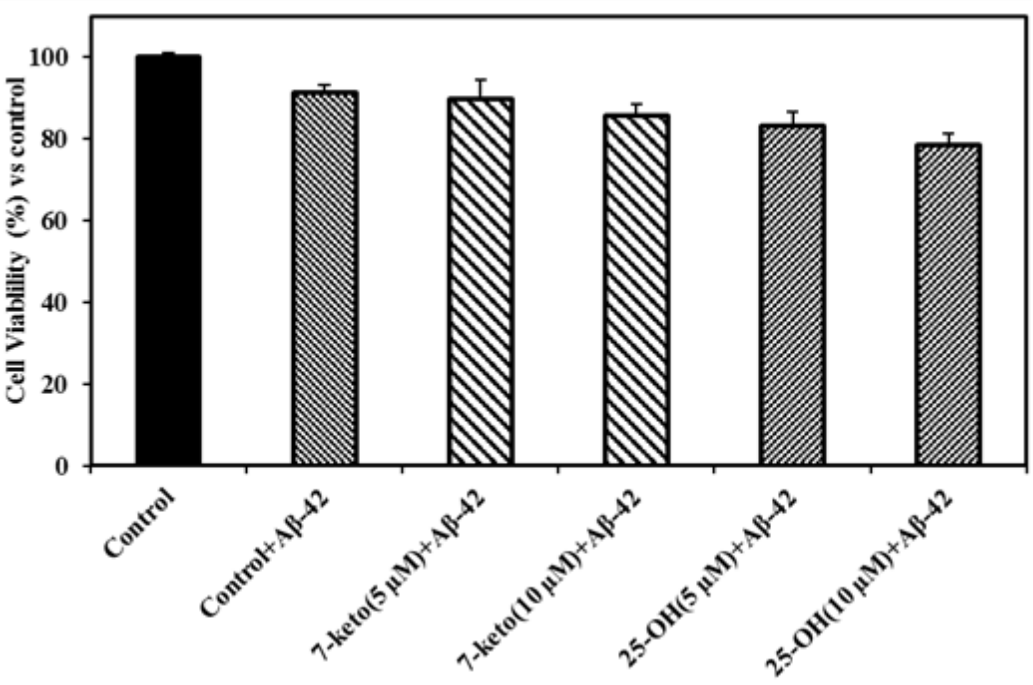


Figure 3.4. Cell viability of control (basal level cholesterol), 7-ketocholesterol and 25-hydroxycholesterol (5 μM and 10 μM) added cells after exposure to protofibrillar Aβ-42 for 24 h. Viability was measured using Trypan blue exclusion assay as described in "Experimental procedures". The values are mean of three independent set of identical experiments.

I have checked the viability of cells after exposure to Aβ-42 and oxysterols. 25-OHC decreased the cell viability to 61% after 3 days treatment on PC 12 cells (12). Though the changes induced by 7-KC and 25-OHC are not highly significant in Jurkat cells, 25-OHC showed larger decrease in cell viability than 7-KC (see figure 3.4.). Previously, 25-OHC stimulates apoptosis of oligodendrocyte and arouse the expression of $sPLA_2$-IIA (51) . Possibly, in 24 h, 25-OHC together with Aβ-42 could not produce prominent effect on cell viability as it is reported to induce apoptosis in lymphoma cells (RDM4) at 5 and 10 μM in 48 h treatment (52).

3.3.4. Role of Microtubules in the transfer of Aβ-42

Cytoskeleton is very important for cell and it is comprised into three parts, one of them is known as microtubule which is the longest filament. After endocytic transport of Aβ-42, it must follow some pathway to move up to endoplasmic reticulum. I have used Oregon green to stain microtubule and to depolymerize them, nocodazole which is a depolymerizing agent, was used. In the Figure 3.5., it is shown that in untreated cells, Aβ-42 could not be observed inside the cells. Though in the presence of 25-OHC, Aβ-42 appeared inside the cells along with microtubules. Afterwards, in depolymerized state, Aβ-42 could not be observed. Hence, it is possibly true that Aβ-42 internalized and reaches to the ER via microtubules. It was suggested that the delivery of proteins to their specified destination is ensured by microtubules, sorting signals and motor proteins in neural cells.

After observation of internalization of Aβ-42, it was interesting to know about the pathway used by peptides to transport into the cell. Thus, analysis of cytoskeleton was chosen to determine the mode of transport mechanism. To evaluate whether internalization is cytoskeleton dependent or independent, nocodazole was used. Nocodazole is a chemical used to depolymerize microtubules, internalization of Aβ-42 could occur in polymerized state and not in the depolymerized one. Internalization of Aβ-42 could occur in polymerized state, not in the depolymerize one. Microtubule stabilizes the structure of cell and they are organized by tau protein, the other protein involved in AD. Disruption of microtubules is an integral feature in AD's patients. Primary mechanism implicated in these changes is still unknown.

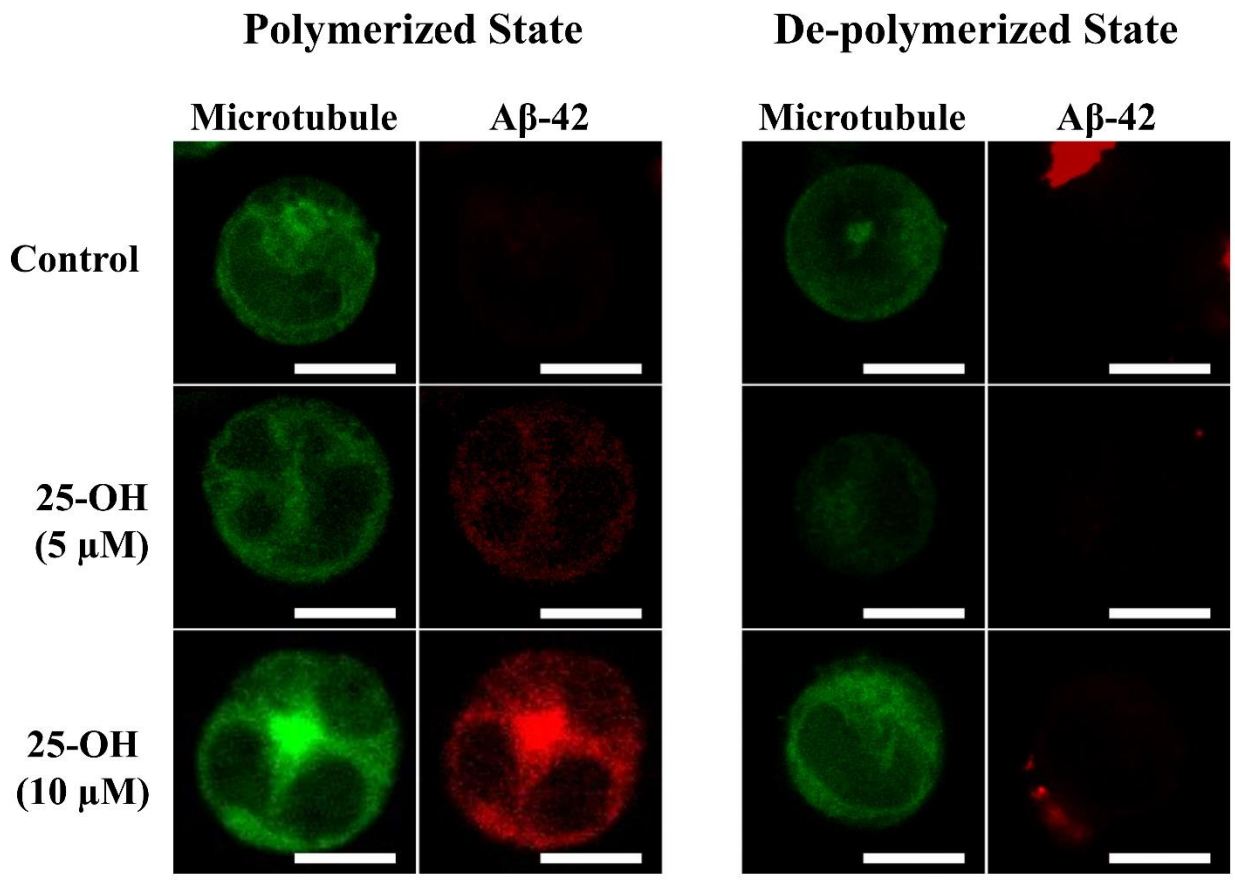


Figure 3.5. Microscopic images of untreated (used as control) and 25-hydroxycholesterol (5 µM and 10 µM) added Jurkat T cells after the exposure of protofibrillar Aβ-42. Polymerized state of microtubule (top) and de-polymerized state of microtubule (bottom). Red and green fluorescence represents Hilyte fluor labeled Amyloid beta-42 (555nm) and Oregon green (488nm), respectively. Scale bars=10 µm.

3.3.5. Transfer of Aβ-42 to Endoplasmic reticulum

ER is responsible for the synthesis of one-third of total body proteins. Excess accumulation of proteins or externally misfolded proteins may cause ER stress. This stress cause cell death and accompanied by release of calcium ions as ER is known to be a calcium reservoir. Although, this increase in intracellular calcium ions could be result of release of calcium ions from other reservoir such as mitochondria. Here, I assess the localization of Aβ-42 when it gets internalized into the membrane. Using confocal scanning microscopy, single-cell observation was accomplished when

green and blue fluorescence indicating towards the peptide and ER, respectively as shown in figure 3.6. Aβ-42 was co-localized with ER in Jurkat T cells. Co-localization of Aβ-42 and ER indicates that after internalizing into the cell, Aβ-42 reaches to ER. In comparison to cholesterol, 25-OHC swiftly transports from cell membrane to the ER (53).

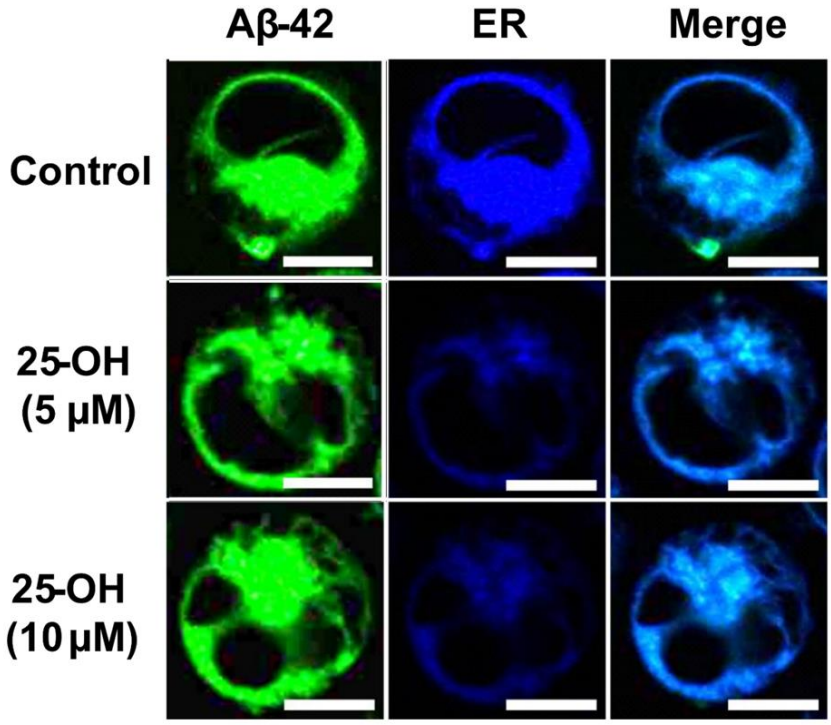


Figure 3.6. Microscopic images of control (basal level cholesterol) and 25-hydroxycholesterol (5 μM and 10 μM) added Jurkat T cells after the exposure of protofibrillar Aβ-42. Blue and green fluorescence represents ER (DAPI filter) and Hilyte flour labeled (488nm) Amyloid beta-42. Scale bars=10 μm.

Illustration of the endocytic transport of Aβ-42 in the 25-OHC substituted membrane in the presence of CT-B binding with GM1 is shown in figure 3.7. After internalizing into the membrane peptide has reached to ER where it may cause stress to the organelle. In general, it is known that microtubules are the carrier of the protein and finally delivers the protein to its specified destination which could be ER. Thus, Aβ-42 was carried form the vicinity of the plasma membrane by microtubules and delivered to the ER as depicted in the illustration.

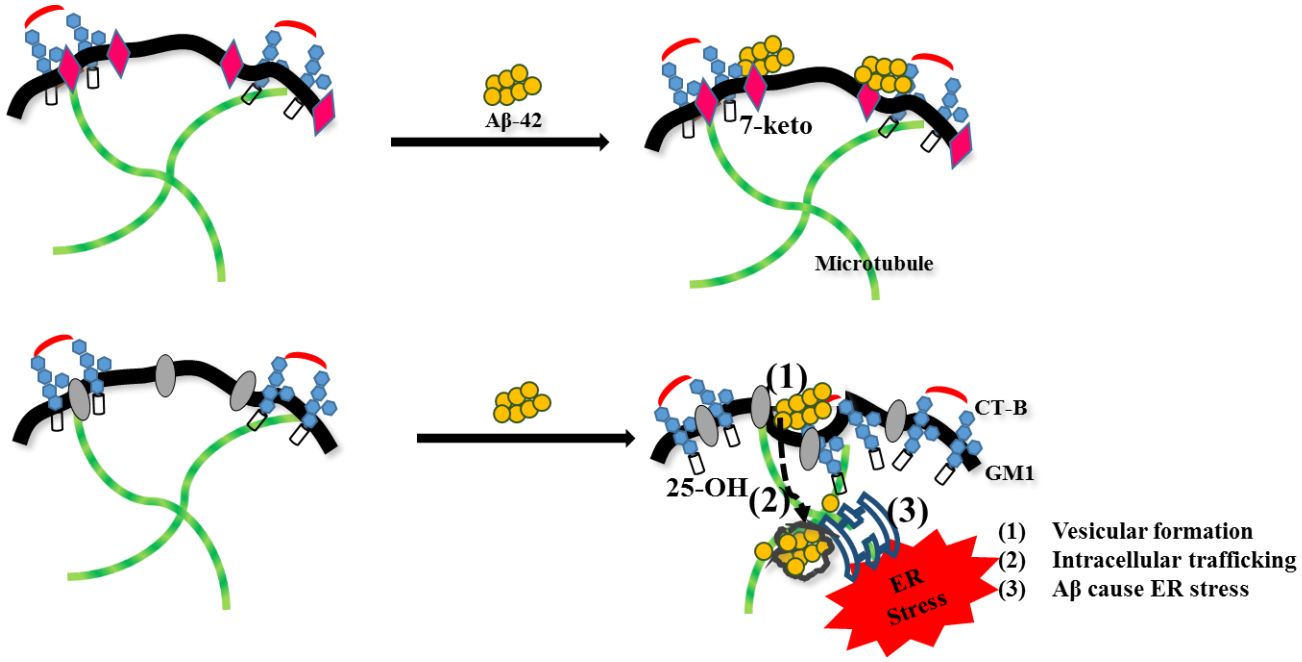


Figure 3.7. Illustration of the intracellular transport pathway of protofibrillar Aβ-42 in 25-hydroxycholesterol added cells and not in 7-ketocholeserol added cells in the presence of CT-B which binds with GM1.

3.3.6. Intracellular calcium release in presence of oxysterols and Aβ-42

Toxicity produced by Aβ peptides have been linked with different processes and mechanisms, changes induced in the intracellular calcium ions is one of them and have been closely related with the pathology of AD (54–56). Commonly, calcium dyshomeostasis is interrelated with cellular apoptosis and excess phosphorylation of major proteins. As mentioned in the previous section, ER has storage for the intracellular calcium ions. Since the concentration of free calcium ions in cytosol (~ 10-4 mM) differs from that of in extracellular spaces (~ 1-2 mM), a calcium gradient establishes across cell membranes (57). This gradient has major function in a number of different biological processes, including inflammatory responses (57), neuron survival and apoptosis (55). Calcium ions acts as a signaling molecule in human body. Aβ, protein responsible for AD, has been reported to accelerate influx of calcium ions (58, 59), thereby disrupting the calcium homeostasis of the cells.

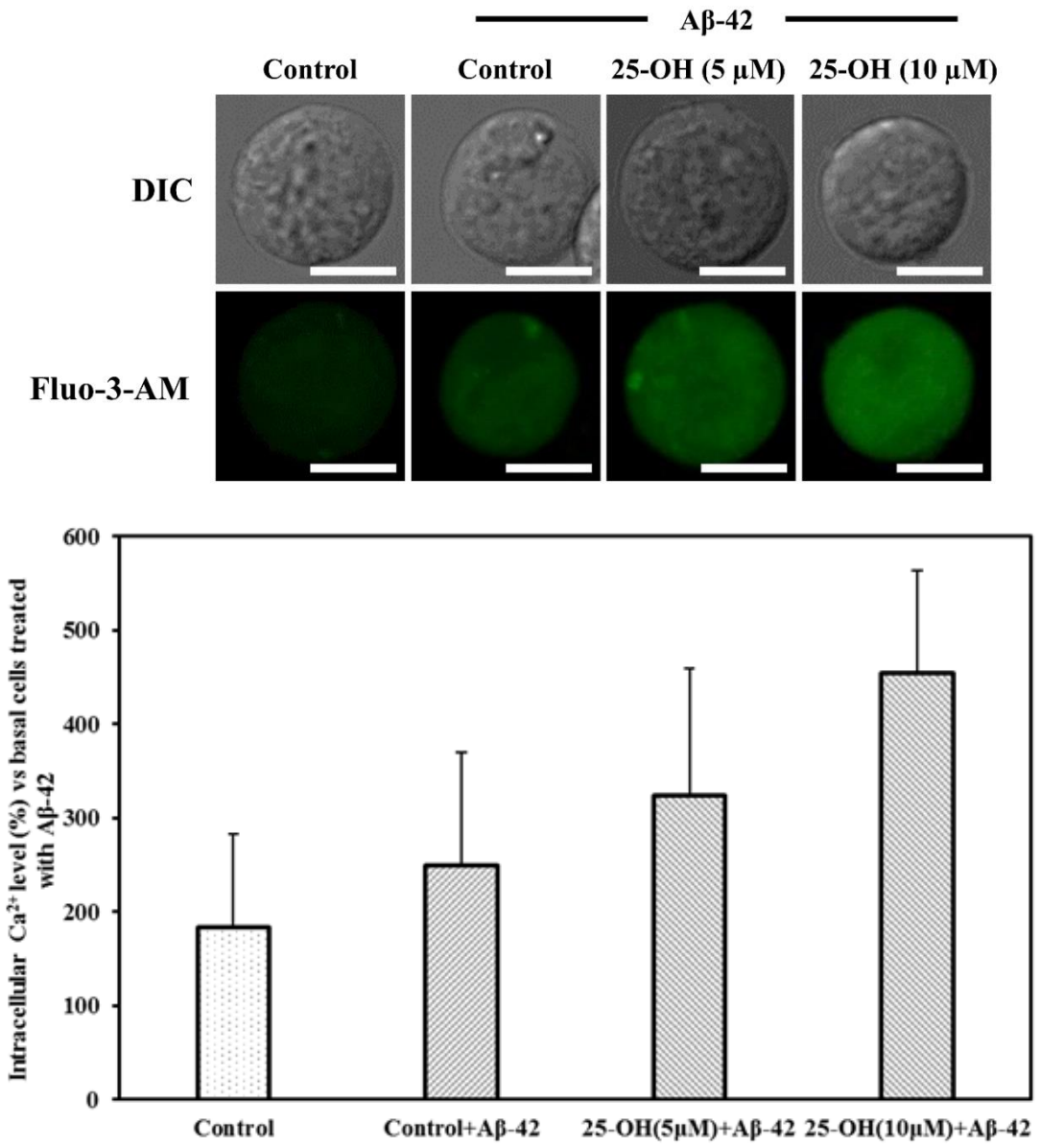


Figure 3.8. A) Microscopic images of untreated (used as control) and 25-hydroxycholesterol added cells with and without exposure to protofibrillar Aβ-42 for 1 h and then loaded with Fluo-3-AM to measure the calcium release. B) Graphical representation of effect of 25-hydroxycholesterol (5 µM and 10 µM) in Intracellular calcium release. Values are represented as means ± SE. Scale bars=10 µm.

Thereby, I assessed the possible outcomes after treating the Jurkat cells with Aβ-42 protofibrils. In addition, to understand whether oxysterols (7-KC and 25-OHC) changes the effect of protofibrillar Aβ42 on Jurkat cells, I evaluated Aβ-42-induced changes in cytosolic calcium ions level with and without the oxysterols. Jurkat cells with 7-KC and 25-OHC at 5 and/or 10 µM were cultured in RPMI medium which contains ~0.425 mM calcium concentration and exposed to Aβ-42 protofibrils. For the purpose, a fluorescent calcium indicator (29), Fluo-3-AM, was employed to measure intracellular calcium level. In general, this indicator emits fluorescence after binding to calcium ions and that intensity indicates the amount of cytosolic calcium ions.

Firstly, I determined the changes induced by protofibrillar Aβ-42 on cytosolic calcium levels of Jurkat cells having basal cholesterol (control cells). There was an increase in the cytosolic calcium level when cell were exposed to the peptides as shown in fig 3.8. This increase was an indication towards the disruption in intracellular calcium homeostasis by protofibrillar Aβ-42. This changes is similar as mentioned in the previous reports where Aβ-42 was exposed to neuronal and fibroblast cells (58, 60).

When Jurkat cells were treated with 7-KC and 25-OH at 5 and/or 10 µM and then with the peptides, calcium level changed. Notably, addition of Aβ-42 peptides was done at the time of measurement. The results demonstrated that effect of oxysterols enhanced the changes induced by protofibrillar Aβ-42. Evangelisti et al. have suggested that oligomeric species of Aβ-42 results in calcium dyshomeostasis of fibroblasts from AD patients. Similarly, changes induced by 25-OHC was due to its potential to facilitate the peptide to insert through the cell membrane.

Recent studies proves that exposure to Aβ induces elevation in the cytosolic calcium level of cells by affecting the ion channels present in the membrane of calcium storing organelles and in plasma membranes. Furthermore, Aβ has potential to form pores in cell membranes, selectively for calcium ions (61). It has been reported that changes induced by cholesterol in the membrane fluidity allows it to advert Aβ-induced changes in intracellular calcium level in neurons (62) due to the fact that membrane fluidity is associated with pore formation in the membrane.

Previously, my group gave demonstrated that the 7-KC induces increase in the membrane fluidity, and thereby, could facilitates the peptide for pore-formation in membranes. In addition, 7-KC induced higher surface interaction between Aβ-42 and Jurkat cells. Moreover, it has been proposed that Aβ induces release of calcium ions from ER (63), where high accumulation of the

peptides causes ER stress, resulting in alteration in calcium channels in ER membrane, thereby, led to increase efflux of calcium ions (64) .

I have observed a remarkable increase in calcium level after addition of 25-OHC in Jurkat cells as shown in figure 3.8. In comparison with 7-KC, 25-OHC caused a greater release of the ions (see figure 3.9.). Thus effect of 25-OHC was more profound than 7-KC. In case of 24-OHC, mild increase in Ca^{2+} release was observed which was suggested to be from the cytoplasm after 24-OHC induced toxicity (65). 25-OHC elevates the calcium permeability in biological membranes (66, 67). Presumably, this permeability is the subsequent act on back and forth movement of 25- OHC between the leaflets of lipid bilayer (68). 25-OHC was found to produce toxicity in aortic smooth muscle cells which followed a calcium-dependent mechanism (69).

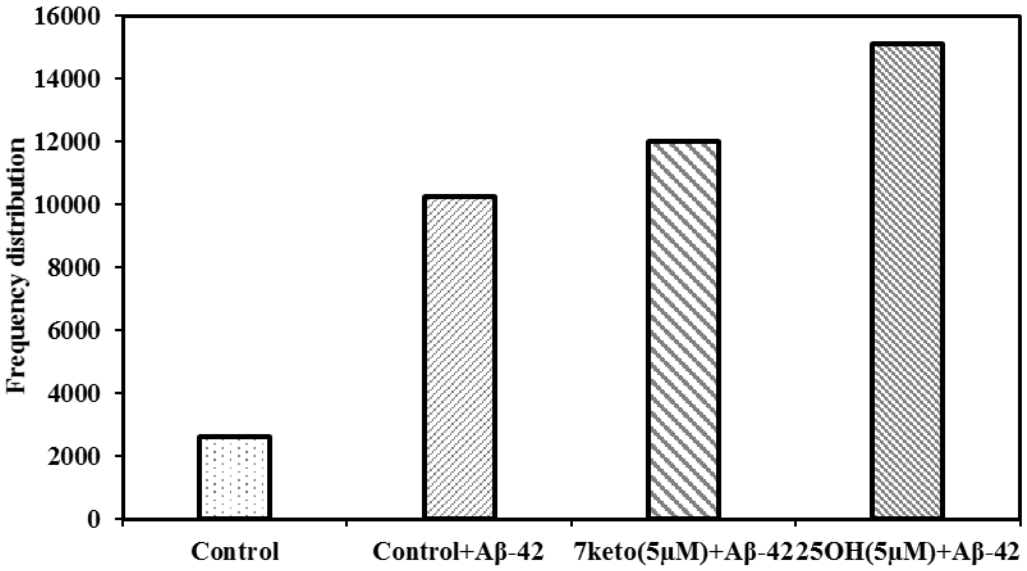


Figure 3.9. A) Microscopic images and B) Graphical representation of Intracellular calcium release after and before exposure of protofibrillar Aβ-42 for 1h in Untreated (used as control), 7-ketocholesterol and 25-hydroxycholesterol added cells using Flow cytometer (Beckmann Quanta SC). Scale bar=10 μm.

3.3.7. Risk factors for Alzheimer's disease

To summarize the findings of my study, I want to emphasize on the risk factors of Alzheimer's disease. It is an old age dementia, thus primarily, aging is the one of the most

important factors which gives rise to ROS as a primary factor. It will cause formation of lipid peroxidation products, specifically cholesterol derivatives (oxysterols). Oxysterols are known to be potential markers in neurological disorders. Previously, it was shown that 7-KC which caused greater surface interaction and now, I have demonstrated that 25-OHC helped in the endocytic transport of Aβ peptides.

Other than aging, changes in structure and function of proteins could cause their accumulation and misfolding. These changes in proteins can be achieved by a process known as glycation where reducing sugars are forming advanced glycation end-products (AGE). In vitro studies have shown that glycated Aβ peptides tend to aggregate faster as compare to its non-glycated form. Consequently, size of the aggregated species is larger which thus form stabilized Aβ peptides. These aggregated and misfolded peptides cause cell dysfunction and apoptosis. Receptor for advanced glycation end products (RAGE) are reported to involve highly in the endocytic transfer for Aβ peptides. It is highly expressed in brains of the patients of Alzheimer's disease. Production and accumulation of AGE species are also involved in metabolic conditions such as diabetes which is known to be a risk factor in the development of Alzheimer's disease. Diabetes is an ailment which is caused by glycation of insulin. Commonly, insulin is present in brain as an essential growth factor.

Thus, dysregulation in it may contribute to high blood sugar level in brain or damage to blood vessels which makes diabetes relevant to neurological disorders such as Alzheimer's disease and Parkinson disease. Monomeric forms of insulin advances amyloid aggregation. Similarly, glycosyl chains of GM1 which binds with Aβ peptides, reported to induce growth of Aβ. Internalization of Aβ in neural cells is relevant to the fact that GM1 is abundantly present in nervous system, comprising 6% of total phospholipids. In my study, GM1-CT-B interaction induced the negative curvature and thus endocytic transport of peptides.

AGE species are also correlated with aging and production of ROS, it was found that ribosylated amyloid aggregates caused a significant increase in ROS species and cell death, when exposed to cells (70). These products are found to increase in aged people and thus, prominent factor in age-related diseases such as diabetes and Alzheimer's disease. In particular, aging, oxidative stress, production of ROS and AGE species are integral parts in pathology of diabetes and Alzheimer's disease.

In my study, I have implicated oxysterols, aggregated species of Aβ peptides, glycosyl chains interaction which are found to be risk factors in Alzheimer's disease. I have demonstrated the importance of these factors in endocytic transport of Aβ peptides which will be the cause of ER stress. Next, I have used human neuroblastoma SH-SY5Y cell line to deepen the knowledge about role of oxysterols in the progression of Alzheimer's disease.

3.4. Conclusions

I have shown the important factors for the endocytic transport of the Aβ-42 using Jurkat cell line which appeared as an optimized model to understand the behavior of neural cells in AD. Notably, I had used protofibrillar species of peptide. My study was a comparative study involved use of different oxysterols to alter the composition of membrane. Use of CT-B was proposed when addition of oxysterols was not sufficient to observe endocytic transport of peptide. GM1 interaction with CT-B provided a negative curvature which helped in endocytosis in model membrane proven an important factor in living cells as well. Thus, 25-OHC and GM1-CT-B are considered to be vital for internalization of peptide inside the cell. This internalization of peptide was proved to proceed via microtubule (cytoskeleton) of the cell. Afterwards, Aβ-42 was co-localized with ER suggesting the final destination of the peptide to be ER. Role of these oxysterols in the progression in neurodegenerative studies such as Alzheimer's disease will further the understanding about the dementia affecting people in older age.

References

1. Song, S., Lee, H., Kam, T.-I., Tai, M. L., Lee, J.-Y., Noh, J.-Y., Shim, S. M., Seo, S. J., Kong, Y.-Y., Nakagawa, T., Chung, C.-W., Choi, D.-Y., Oubrahim, H., and Jung, Y.-K. (2008) E2-25K/Hip-2 regulates caspase-12 in ER stress–mediated Aβ neurotoxicity. *J. Cell Biol.* **182**, 675–684 [online] http://jcb.rupress.org/content/182/4/675 (Accessed 2008).

2. Resende, R., Ferreiro, E., Pereira, C., and Oliveira, C. R. (2008) ER stress is involved in Aβ-induced GSK-3β activation and tau phosphorylation. *J. Neurosci. Res.* **86**, 2091–2099
3. Costa, R. O., Ferreiro, E., Cardoso, S. M., Oliveira, C. R., and Pereira, C. M. (2010) ER stress-mediated apoptotic pathway induced by Aβ peptide requires the presence of functional mitochondria. *J. Alzheimer's Dis.* **20**, 625–636
4. Lana, E., Khanbolouki, M., Degavre, C., Samuelsson, E.-B., Åkesson, E., Winblad, B., Alici, E., Lithner, C. U., and Behbahani, H. (2016) Perforin Promotes Amyloid Beta Internalisation in Neurons. *Mol. Neurobiol.*, 1–14 [online] http://dx.doi.org/10.1007/s12035-016-9685-9 (Accessed 2016).
5. McMullen, T. P., Lewis, R. N., and McElhaney, R. N. (2004) Cholesterol–phospholipid interactions, the liquid-ordered phase and lipid rafts in model and biological membranes. *Curr. Opin. Colloid & Interface Sci.* **8**, 459–468 [online] http://dx.doi.org/10.1016/j.cocis.2004.01.007 (Accessed 2004).
6. Björkhem, I., and Meaney, S. Brain cholesterol: long secret life behind a barrier. [online] http://atvb.ahajournals.org/content/24/5/806.short.
7. Gamba, P., and G Testa, S. G. Oxidized cholesterol as the driving force behind the development of Alzheimer's disease. [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4473000/.
8. Yamanaka, K., and Y Saito, T. Y., Y. Urano… 24 (S)-hydroxycholesterol induces neuronal cell death through necroptosis, a form of programmed necrosis. [online] http://www.jbc.org/content/286/28/24666.short.
9. Morita, M., Hamada, T., Tendo, Y., Hata, T., Vestergaard, M. C., and Takagi, M. (2012) Selective localization of Alzheimer\textquotesingles amyloid beta in membrane lateral compartments. *Soft Matter* **8** [online] http://dx.doi.org/10.1039/c2sm07185a (Accessed 2012).
10. Phan, H. T. T., and T Hata, M. M., T. Yoda, T. Hamada… The effect of oxysterols on the interaction of Alzheimer's amyloid beta with model membranes. [online] http://www.sciencedirect.com/science/article/pii/S0005273613002101.
11. Chang, J. Y., and KD Phelan, J. C. Neurotoxicity of 25-OH-cholesterol on sympathetic neurons. [online] http://www.sciencedirect.com/science/article/pii/S0361923097004619.
12. Chang, J. Y., and KD Phelan, L. L. Neurotoxicity of 25-OH-cholesterol on NGF-

differentiated PC12 cells. [online] http://link.springer.com/article/10.1023/A:1022437000893.

13. Wohlfeil, E. R., and Campbell, W. B. 25-hydroxycholesterol increases eicosanoids and alters morphology in cultured pulmonary artery smooth muscle and endothelial cells. [online] http://atvb.ahajournals.org/content/19/12/2901.short.
14. Chang, J. Y., and JA Chavis, L. L., PD Drew Cholesterol oxides induce programmed cell death in microglial cells. [online] http://www.sciencedirect.com/science/article/pii/S0006291X98992373.
15. Choi, Y. K., Kim, Y. S., Choi, I. Y., Kim, S.-W., and Kim, W.-K. (2008) 25-Hydroxycholesterol induces mitochondria-dependent apoptosis via activation of glycogen synthase kinase-3β in PC12 cells. *Free. Radic. Res.* **42**, 544–553 [online] http://dx.doi.org/10.1080/10715760802146062 (Accessed January 1, 2008).
16. Ahonen, L., and FBR Maire, M. S., J. Kopra… Analysis of oxysterols and vitamin D metabolites in mouse brain and cell line samples by ultra-high-performance liquid chromatography-atmospheric pressure …. [online] http://www.sciencedirect.com/science/article/pii/S0021967314013703.
17. Morita, M., and M Vestergaard, T. H., M. Takagi Real-time observation of model membrane dynamics induced by Alzheimer's amyloid beta. [online] http://www.sciencedirect.com/science/article/pii/S0301462209002403.
18. Phan, H. T. T., and MC Vestergaard, K. B. Localization of amyloid beta (Aβ1-42) protofibrils in membrane lateral compartments: Effect of cholesterol and 7-Ketocholesterol. [online] http://onlinelibrary.wiley.com/doi/10.1016/j.febslet.2014.08.007/full.
19. Kayed, R., and E Head, J. T., TM McIntire… Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. [online] http://science.sciencemag.org/content/300/5618/486.short.
20. Town, T., and J Tan, R. F., M. Mullan T-cells in Alzheimer's disease. [online] http://link.springer.com/article/10.1385/NMM:7:3:255.
21. Hitomi, J., and DE Christofferson, A. N., J. Yao, A. Degterev… Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. [online] http://www.sciencedirect.com/science/article/pii/S0092867408013822.
22. Kiyokawa, E., and T Baba, N. O., A. Makino… Spatial and functional heterogeneity of

sphingolipid-rich membrane domains. [online] http://www.jbc.org/content/280/25/24072.short.

23. Dhingra, S., and M Morita, T. Y., MC Vestergaard… Dynamic morphological changes induced by GM1 and protein interactions on the surface of cell-sized liposomes. [online] http://www.mdpi.com/1996-1944/6/6/2522/htm.
24. Fukunaga, S., and H Ueno, T. Y., Y. Yano… GM1 cluster mediates formation of toxic Aβ fibrils by providing hydrophobic environments. [online] http://pubs.acs.org/doi/abs/10.1021/bi300839u.
25. Strober, W. Trypan blue exclusion test of cell viability. [online] http://onlinelibrary.wiley.com/doi/10.1002/0471142735.ima03bs111/full.
26. Dong, H., and SE Strome, D. S., H. Tamura… Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. [online] http://www.nature.com/nm/journal/v8/n8/abs/nm730.html.
27. Evangelisti, E., and D Wright, M. Z. Lipid rafts mediate amyloid-induced calcium dyshomeostasis and oxidative stress in Alzheimer's disease. [online] http://www.ingentaconnect.com/content/ben/car/2013/00000010/00000002/art00004.
28. Blank, N., and M Schiller, S. K., G. Wabnitz… Cholera toxin binds to lipid rafts but has a limited specificity for ganglioside GM1. [online] http://www.nature.com/icb/journal/v85/n5/abs/7100045a.html.
29. Takahashi, A., Camacho, P., Lechleiter, J. D., and Herman, B. (1999) Measurement of intracellular calcium. *Physiol. Rev.* **79**, 1089–1125
30. Evangelisti, E., and C Cecchi, R. C., C. Sgromo… Membrane lipid composition and its physicochemical properties define cell vulnerability to aberrant protein oligomers. [online] http://jcs.biologists.org/content/125/10/2416.short.
31. Yu, K., and Y He, L. Y., PKS Lam, RSS Wu… DE-71-induced apoptosis involving intracellular calcium and the Bax-mitochondria-caspase protease pathway in human neuroblastoma cells in vitro. [online] http://toxsci.oxfordjournals.org/content/104/2/341.short.
32. Olsen, B. N., and Schlesinger…, P. H. Perturbations of membrane structure by cholesterol and cholesterol derivatives are determined by sterol orientation. [online] http://pubs.acs.org/doi/abs/10.1021/ja8095224.

33. Kulig, W., and P Jurkiewicz, A. O., J. Tynkkynen… Experimental determination and computational interpretation of biophysical properties of lipid bilayers enriched by cholesteryl hemisuccinate. [online] http://www.sciencedirect.com/science/article/pii/S0005273614003678.
34. Tall, A. R., and P Costet, N. W. Regulation and mechanisms of macrophage cholesterol efflux. [online] http://www.jci.org/articles/view/16391.
35. Wolf, G. The role of oxysterols in cholesterol homeostasis. [online] http://search.proquest.com/openview/99853e121485b6e16e8f01038fee8410/1?pq-origsite=gscholar.
36. Björkhem, I. Do oxysterols control cholesterol homeostasis? [online] http://www.jci.org/articles/view/16388.
37. Joseph, S. B., and A Castrillo, B. L., DJ Mangelsdorf… Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. [online] http://www.nature.com/nm/journal/v9/n2/abs/nm820.html.
38. Kim, S. M., and CW Lee, B. K., YS Jung, SK Eo… 27-Oxygenated cholesterol induces expression of CXCL8 in macrophages via NF-κB and CD88. [online] http://www.sciencedirect.com/science/article/pii/S0006291X15301285.
39. Leonarduzzi, G., and S Gargiulo, P. G., G. Testa… S3-2-Modulation of cell signaling pathways by oxysterols in age-related human diseases. [online] http://www.sciencedirect.com/science/article/pii/S0891584914013380.
40. Wang, P., and J Weng, R. A. OSBP is a cholesterol-regulated scaffolding protein in control of ERK1/2 activation. [online] http://science.sciencemag.org/content/307/5714/1472.short.
41. Vurusaner, B., and P Gamba, G. T., S. Gargiulo… Survival signaling elicited by 27-hydroxycholesterol through the combined modulation of cellular redox state and ERK/Akt phosphorylation. [online] http://www.sciencedirect.com/science/article/pii/S0891584914003438.
42. Wang, H., Yuan, L., Ma, W., Han, J., Lu, Y., Feng, L., and Xiao, R. (2016) The cytotoxicity of 27-hydroxycholesterol in co-cultured SH-SY5Y cells and C6 cells. *Neurosci. Lett.* **632**, 209–217 [online] http://www.sciencedirect.com/science/article/pii/S0304394016306565 (Accessed October 6, 2016).
43. Dasari, B., and Prasanthi…, J. R. P. The oxysterol 27-hydroxycholesterol increases β-

amyloid and oxidative stress in retinal pigment epithelial cells. [online] https://bmcophthalmol.biomedcentral.com/articles/10.1186/1471-2415-10-22.

44. Brown, M. S., and Goldstein, J. L. Cholesterol feedback: from Schoenheimer's bottle to Scap's MELADL. [online] http://www.jlr.org/content/50/Supplement/S15.short.

45. Gorbenko, G. P., and Kinnunen, P. K. J. (2006) The role of lipid–protein interactions in amyloid-type protein fibril formation. *Chem. Phys. Lipids* **141**, 72–82 [online] http://dx.doi.org/10.1016/j.chemphyslip.2006.02.006 (Accessed June 2006).

46. Vetrivel, K. S., and Thinakaran, G. (2010) Membrane rafts in Alzheimer's disease beta-amyloid production. *Biochim. et Biophys. Acta* **1801**, 860–867 [online] //www.sciencedirect.com/science/article/pii/S1388198110000636 (Accessed August 2010).

47. Saavedra, L., Mohamed, A., Ma, V., Kar, S., and Chaves, E. P. de (2007) Internalization of -Amyloid Peptide by Primary Neurons in the Absence of Apolipoprotein E. *J. Biol. Chem.* **282**, 35722–35732 [online] https://doi.org/10.1074%2Fjbc.m701823200 (Accessed October 2007).

48. Singh, T. D., and SY Park, J. B., Y. Yun, YC Bae… MEGF10 functions as a receptor for the uptake of amyloid-β. [online] http://onlinelibrary.wiley.com/doi/10.1016/j.febslet.2010.08.050/full.

49. Choi, Y. K., and YS Kim, I. C., SW Kim, WK Kim 25-Hydroxycholesterol induces mitochondria-dependent apoptosis via activation of glycogen synthase kinase-3 β in PC12 cells. [online] http://www.tandfonline.com/doi/abs/10.1080/10715760802146062.

50. Hwang, P. L. H. Inhibitors of protein and RNA synthesis block the cytotoxic effects of oxygenated sterols. [online] http://www.sciencedirect.com/science/article/pii/016748899290077O.

51. Trousson, A., and S Bernard, P. P., P. Liere… 25-hydroxycholesterol provokes oligodendrocyte cell line apoptosis and stimulates the secreted phospholipase A2 type IIA via LXR beta and PXR. [online] http://onlinelibrary.wiley.com/doi/10.1111/j.1471-4159.2009.06009.x/full.

52. Christ, M., and B Luu, J. M., I. Moosbrugger, P. Bischoff Apoptosis induced by oxysterols in murine lymphoma cells and in normal thymocytes. [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1421833/.

53. Lange, Y., and J Ye, F. S. Movement of 25-hydroxycholesterol from the plasma membrane to the rough endoplasmic reticulum in cultured hepatoma cells. [online] http://www.jlr.org/content/36/5/1092.short.

54. Blanchard, B. J., Chen, A., Rozeboom, L. M., Stafford, K. A., Weigele, P., and Ingram, V. M. (2004) Efficient reversal of Alzheimer's disease fibril formation and elimination of neurotoxicity by a small molecule. *Proc. Natl. Acad. Sci. United States Am.* **101**, 14326–14332

55. Mattson, M. P. (2004) Pathways towards and away from Alzheimer's disease. *Nature* **430**, 631–639 [online] http://dx.doi.org/10.1038/nature02621 (Accessed August 5, 2004).

56. Kawahara, M., Kuroda, Y., Arispe, N., and Rojas, E. (2000) Alzheimer's β-amyloid, human islet amylin, and prion protein fragment evoke intracellular free calcium elevations by a common mechanism in a hypothalamic GnRH neuronal cell line. *J. Biol. Chem.* **275**, 14077–14083

57. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002) Molecular biology of the cell. new york: Garland science; 2002. *Class. Textb. now its 5th Ed.*

58. Mattson, M. P., and Chan, S. L. (2003) Neuronal and glial calcium signaling in Alzheimer's disease. *Cell Calcium* **34**, 385–397

59. Le, Y., Gong, W., Tiffany, H. L., Tumanov, A., Nedospasov, S., Shen, W., Dunlop, N. M., Gao, J.-L., Murphy, P. M., and Oppenheim, J. J. (2001) Amyloid ß42 activates a G-protein-coupled chemoattractant receptor, FPR-like-1. *J. Neurosci* **21**

60. Evangelisti, E., Zampagni, M., Cascella, R., Becatti, M., Fiorillo, C., Caselli, A., Bagnoli, S., Nacmias, B., and Cecchi, C. (2014) Plasma membrane injury depends on bilayer lipid composition in Alzheimer's disease. *J. Alzheimer's Dis.* **41**, 289–300

61. Anekonda, T. S., and Quinn, J. F. (2011) Calcium channel blocking as a therapeutic strategy for Alzheimer's disease: the case for isradipine. *Biochim. et Biophys. Acta* **1812**, 1584–1590

62. Kawahara, M., and Kuroda, Y. (2001) Intracellular calcium changes in neuronal cells induced by Alzheimer's ß-amyloid protein are blocked by estradiol and cholesterol. *Cell. Mol. Neurobiol.* **21**, 1–13

63. Ferreiro, E., Oliveira, C. R., and Pereira, C. M. (2008) The release of calcium from the endoplasmic reticulum induced by amyloid-beta and prion peptides activates the

mitochondrial apoptotic pathway. *Neurobiol. Dis.* **30**, 331–342

64. Popugaeva, E., and Bezprozvanny, I. (2013) Role of endoplasmic reticulum Ca2+ signaling in the pathogenesis of Alzheimer disease. *Front. Mol. Neurosci.* **6**
65. Kölsch, H., and D Lütjohann, A. T., I. Björkhem, ML Rao The neurotoxic effect of 24-hydroxycholesterol on SH-SY5Y human neuroblastoma cells. [online] http://www.sciencedirect.com/science/article/pii/S0006899398012748.
66. Boissonneault, G. A., and Heiniger, H. J. 25-Hydroxycholesterol-induced elevations in 45Ca uptake: Permeability changes in P815 cells. [online] http://onlinelibrary.wiley.com/doi/10.1002/jcp.1041250316/full.
67. Holmes, R. P., and Yoss, N. L. 25-Hydroxysterols increase the permeability of liposomes to Ca2+ and other cations. [online] http://www.sciencedirect.com/science/article/pii/0005273684900671.
68. Theunissen, J. J. H., and RL Jackson, H. K. Membrane properties of oxysterols. Interfacial orientation, influence on membrane permeability and redistribution between membranes. [online] http://www.sciencedirect.com/science/article/pii/0005273686904992.
69. Ares, M. P., and Pörn, M. I. Ca2+ channel blockers verapamil and nifedipine inhibit apoptosis induced by 25-hydroxycholesterol in human aortic smooth muscle cells. [online] http://www.jlr.org/content/38/10/2049.short.
70. Kong, F. L., and W Cheng, J. C., Y. Liang D-Ribose glycates β 2-microglobulin to form aggregates with high cytotoxicity through a ROS-mediated pathway. [online] http://www.sciencedirect.com/science/article/pii/S0009279711002559.

Chapter 4:

Role of 7-ketocholesterol and 25-hydroxycholesterol in the internalization of amyloid beta in SH-SY5Y cells

Abstract

Accumulation of amyloid beta (Aβ) in the intracellular spaces have been a major pathological feature in the progression of Alzheimer's disease (AD). Possibly, it may occur after uptake of the peptides form extracellular spaces of cell. Currently, it has become a new method in the pathogenesis of the disease. In this chapter, I have shown that externally added Aβ exhibit toxicity to the undifferentiated human neuroblastoma SH-SY5Y cell line in a time and concentration dependent manner. Exogenous Aβ have been associated with endoplasmic reticulum (ER) stress, possibly after internalization of the peptides with the aid of lipid rafts which are rich in cholesterol. I have also used cholesterol derivatives (oxysterols) to change the composition of the membrane and observed the changes induced by them on internalization of the peptides in undifferentiated SH-SY5Y cells. These findings will suggest the importance of oxysterols in the toxicity induced by amyloid peptides which will benefit in the pathology of AD.

Keywords: Amyloid beta-42, 25-hydroxycholesterol, 7-ketocholesterol, toxicity, internalization, undifferentiated human neuroblastoma SH-SY5Y cells, endoplasmic reticulum.

4.1. Introduction

Internalization of amyloid beta (Aβ) peptides is a crucial event in understanding the mechanisms of the pathology of Alzheimer's disease (AD), which is a neurodegenerative disorder that is one of the leading dementia affecting the world. Classically, intercellular Aβ-induced toxicity was given importance in the studies related to AD. But recently, uptake of extracellular Aβ peptides and then accumulation of Aβ within the cells have observed as an important step in the progression of AD. In the pathology of AD, it is still a question that whether Aβ peptides deposits straight within the cell or they come from outside the cell via internalization. Internalization of Aβ can proceed via multiple pathways: endocytic transport of the peptides, phagocytosis, pinocytosis, pore formation in the membrane, receptor-mediated endocytosis, via lipid rafts and their associated proteins. Lana et al. have suggested endocytosis to be the most dominating pathway for internalization of Aβ peptides (1). They have demonstrated suppression in the intake of Aβ peptides in both of its isomers after deterring the endocytic pathway in differentiated SH-SY5Y cells and human primary cortical neurons (hPCN). Another study have suggested micro-domains in the membrane, termed as lipid rafts, to be the major mechanism used by Aβ to internalize into the neurons (2).

Since lipid rafts are associated with internalization of proteins, components of these micro-domains (raft) in the membrane have substantial effect on the transport of protein through the membrane. Lipid rafts are composed of cholesterol, sphingolipids and phospholipids where levels of cholesterol have been linked with the high prevalence of AD in *in vitro* and *in vivo* studies (3–6) and GM1(monosialoganglioside) which is a sphingolipid bind to Aβ peptides and promotes their growth via seeding and acceleration by GM1-Aβ (7, 8). It was demonstrated that damage induced in lipid rafts could save cultured pheochromocytoma cells against toxicity induced by Aβ oligomers (9, 10). Among two isoforms of Aβ, Aβ-42 is more hydrophobic than Aβ-40, attributed to its hydrophobicity and thus possesses higher propensity to aggregate, which makes Aβ-42 more toxic. The hydrophobicity of peptides facilitates them to target neuronal cells; Aβ aggregates reported to interact with cell membranes, leading to pore formation in the bilayer (11). In addition, oligomeric and fibrillar species of Aβ bind to many membrane proteins, resulting in modification of protein function (12). Aβ-induced toxicity can generate via aggregation of the peptide at lipid rafts which in turn can cause membrane disruption or deformation by oxidative

damage (13, 14). In addition, GM1-Aβ interaction decreases membrane fluidity and activates APP processing, finally leading to excess production of the peptides (15).Moreover, oxidative damage has been associated with reactive oxygen species (ROS) which in turn is related with aging, risk factor in AD. Oxidative stress constitutes a crucial role in inflammation, apoptosis and neurodegeneration (16, 17). ROS, which is primarily produced in the mitochondria, leads to the free radical attack of membrane phospholipids and produces oxidative species of cholesterol. ROS also induces the loss of mitochondrial membrane potential, which causes the intermembrane protein, such as cytochrome c, expelled out of the mitochondria and finally stimulates caspase-3 (18, 19). Activation of caspase-3 has been linked to tailoring of DNA and thus, cell death.

In my study, I have focused on the role of oxidized derivatives of cholesterol in the endocytic transport of Aβ-42. I have incorporated oxysterols such as 7-ketocholesterol (7-KC) and 25-hydroxycholesterol (25-OHC) in the membrane of undifferentiated human neuroblastoma SH-SY5Y cells. In the previous chapter, biomimetic membranes and Jurkat cells were used a mimic to study the mechanism of Aβ-induced toxicity, whereas in this chapter, neuroblastoma SH-SY5Y cell line was employed. This cell line is a subclone from another cell line, SK-N-SH, and derived from bone marrow of a 4 year old female child (20). SH-SY5Y cells exhibit vigorous proliferation of cells attributed to their capability for regression (21). They have characteristics to present as adherent and floating cells in the medium.

There are a number of different risk factors which are intertwined in the development of AD, including upregulation in the production and polymerization of Aβ, aging, oxidative stress, advanced glycation end-products, hypertension, inflammation and hypercholesterolemia (ref). Among the risk factors in the pathology of AD, oxidative stress is linked with the accumulation of free cholesterol, previously found in AD brains. High concentration and accumulation of cholesterol may result in the production of oxidative products of cholesterol (oxysterols) enzymatically or by reactive oxygen species (ROS). Oxysterols have been suggested to exhibit toxic and pro-inflammatory effects. They have been found to produce disruption in smooth muscle cells (22, 23), endothelial cells (24, 25) and fibroblasts (26, 27). According to previous reports, 24(S)-hydroxycholesterol (24S-OHC) was found at high levels in AD patients (28). On the contrary, 27-hydroxycholesterol (27-OHC) and 24S-OHC exhibited neuroprotective and neurotoxic effects at different concentrations, in the presence of other toxic stimulant in

undifferentiated SH-SY5Y cells (29). In addition, oxysterols were suggested to decrease cell viability in leukemic T-cells, lymphoma cells and thymocytes (30–32) and also in neuronal cells (33, 34). Thus, toxicity induced by oxysterols depends on multiple conditions such as type and concentration of oxysterol used, cell line and incubation time. In addition, 7-KC, known as a neurotoxin (35) , decreased cell viability by 50% after 48 h incubation at 50 μM (36) . This sterol has a major contribution in atherosclerosis (37) and found to promote insertion of Aβ peptides into lipid bilayer .7-KC bears potential to accelerate exocytosis and enhances neurotransmitter release, thus causes neuroinflammation (38). It was also demonstrated as an inhibitor in the raft formation (39). On the other hand, 25-OHC is among the most important oxysterols produced via enzymatic oxidation. Oxidation in the alkyl chain present in the cholesterol produces compounds bearing additional -OH group possibly which makes them capable of crossing blood-brain-barrier (BBB). Although, 24S-OHC is the form which can cross BBB instead of cholesterol, 25-OHC shares similar structure with it. 25-OHC has been known as a potential regulator of biosynthesis of cholesterol (40) and suggested to be highly toxic at high concentrations (41). Specifically, 25-OHC lowers cell viability of thymocytes and RDM4 cells by 45% and 90%, respectively, at 10 μM in 24 h (30). In addition, this oxysterol reported to induce DNA fragmentation in these cells after overnight treatment. Moreover, it also exhibits an integral role in immune system and induces cytotoxicity in PC12 cells (34).

Another than oxysterols, when Aβ internalizes into the membrane it leads to toxicity, aggregation and increase in calcium ions. Previously, Demuro et al. reported about increase in intracellular calcium levels after Aβ introduction in SH-SY5Y cells (42). This increase in calcium ions can be associated to the release of ions from ER when the organelle is under stress.

With these understandings, I have used 7-KC and 25-OHC in my study with protofibrillar species of Aβ and undifferentiated human neuroblastoma SH-SY5Y cell line. In this chapter, I have demonstrated that protofibrillar Aβ-42 induces cytotoxicity in SH-SY5Y cells which efficiently enhanced in the presence of oxysterols in the membrane. In addition, Aβ-42 internalizes into the cell which promotes with longer incubation time and with incorporation of 7-KC and 25-OHC. Moreover, the peptides are co-localized with ER and possibly, chose microtubules as a pathway for peptide trafficking.

4.2. Experimental procedures

4.2.1. Materials-

25-hydroxycholesterol (25-OHC), 7-ketocholesterol (7-KC), bovine serum albumin (BSA), and trypan blue were obtained from Sigma-Aldrich (USA). Amyloid beta protein (Human, 1-42) (Aβ-42), Hilyte FluorTM 488-labeled (λex=503 nm, λex=528 nm) Aβ-42 and HiLyte Fluor-555-labeled (λex= 551 nm, λex= 567 nm) Aβ-42 were from Peptide Institute Inc. (Japan) and Anaspec, Inc. (USA), respectively. ER-Tracker Blue-White DPX, Oregon Green 488 taxol, Dulbecco's modified eagle medium (DMEM)/ F-12 (1:1), fetal bovine serum, peniciilin, streptomycin, Alexa Fluor 555 conjugated cholera toxin subunit B (CT-B) (λex= 555 nm, λem= 565 nm) and NucRed ® Live 647 (λex=638 nm, λem=686 nm) were from Invitrogen (USA). Phosphate buffer salts (PBS), and Tris (hydroxymethyl) aminomethane (Tris) were purchased from Takara Bio Inc. and Kanto-Chemical (Japan), respectively.

4.2.2. Cell Culture-

Human neuroblastoma SH-SY5Y cell line, a gift from Doshisha University (Kyoto, Japan) were cultured in medium containing DMEM/F-12 (1:1 mixture) supplemented with 10 % heat-inactivated fetal bovine serum and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin, in a humidified incubator at 37°C in the presence of 95% air and 5% CO_2. Cell were grown in 35X10 mm dishes at density of $2X10^5$ cells/ml and passaged twice a week using 0.25 % trypsin followed by EDTA treatment after 80% confluency. Cultures were grown in subcultures in 35 mm glass bottom dishes for microscopic observations, 96-well plates for intracellular calcium ions measurements and 24-well plates for cell viability experiments.

4.2.3. Preparation and addition of oxysterols-

Stock solution of oxysterols is prepared in ethanol and was diluted in non-serum medium as working solution. For the experiments, cells were treated with oxysterols for 24 h, 37°C to alter the membrane cholesterol level.

4.2.4. Preparation and addition of protofibrillar Aβ-42 –

200 μM Aβ-42 solutions were prepared by dissolving Aβ-42 powder in 0.02% (v/v) ammonia solution and stored at -80°C. Before experiments, Aβ-42 solution was diluted to 80 μM concentration with Tris buffer (20 mM, pH 7.4), and subsequently incubated at 37°C for 12 h. To prepare the protofibrillar species, fluorescence labeled Aβ-42 and normal Aβ-42 was mixed at 1:2 molar ratio before the incubation. SH-SY5Y cells were incubated with protofibrillar Aβ-42 at different concentrations for 2 and 24 h in humidified incubator at 37°C.

4.2.5. Measurement of cell viability-

Trypan blue exclusion assay was used to estimate cell viability upon the effect of Aβ-42 protofibrils. Untreated (Control), 7-KC and 25-OHC added cells cultured in 24-well plate were briefly washed with PBS, exposed to 100 nM and 1 μM protofibrillar Aβ-42 for 2 and 24 h. Thioflavin T assay showed that the confirmation of protofibrillar Aβ-42 did not significantly change in this condition (data was not shown). The cells were then trypsinized shortly and washed with PBS and treated with trypan blue at the final concentration of 0.01% (v/v) in PBS for 5 min at room temperature. The number of dead cells and viable cells were counted using a hemocytometer. The cell having a clear cytoplasm defined as a viable cell, while a dead cell is the one with a blue cytoplasm. Cell viability was calculated following the formation: cell viability=total viable cells/total cells. Data is expressed as mean ± standard deviation of at least three independent experiments.

4.2.6. Nucleus staining-

After treatment of Aβ, cells were washed with DMEM/F-12 (non-serum) and treated with NucRed Live 647, which binds with DNA, added 1 drop per mL for 15-20 min at 37°C.

4.2.7. Lipid raft staining and observation of Aβ-42-

Lipid rafts were labelled by treating cells with 1 μg/ml CT-B, lipid raft marker which binds with GM1 in lipid rafts, and 0.02% (v/v) BSA in PBS, followed by an incubation at 37°C for 10 min. Then cells were washed with PBS buffer.

4.2.8. Staining of microtubule-

To stain microtubule, Oregon Green® 488 Taxol was used for 5 min at 37°C in a humidified incubator in the presence of 5% CO_2. Then cells were washed twice with PBS.

4.2.9. Staining of Endoplasmic Reticulum-

ER organelle was labelled by treating cells with 1 μg/ml ER-Tracker (Blue-White DPX dye) in 0.02% BSA, followed by an incubation at 37 °C for 30 min. Then cells were washed with PBS buffer.

4.2.10. Microscopic observations-

After staining, glass bottom dishes were observed using Confocal Laser Scanning Microscope (Olympus FV-1000, Japan) using 60X oil-immersion objective lens at room temperature within 2-4 min at room temperature (RT).

4.2.11. Image processing and statistical analysis-

Images obtained by microscopic observations are processed using Olympus fluoview Version 4.2 and Image J software. Data is expressed as means ± standard deviations of three independent experiments. Statistical analysis of data was performed by ANOVA test and then with Bonferroni's test wherever applicable, P-values <0.05 were considered to be statistically significant.

4.3. Results and discussion

4.3.1. Changes in cell viability induced by amyloid beta (Aβ-42) and oxysterols-

To investigate the effect of toxicity induced by protofibrillar species of Aβ-42, cell viability was measured using trypan blue exclusion assay (43, 44). Trypan blue is one of those dyes which penetrates into the dead cell and turns the cytoplasm blue to distinguish the dead cells from live cells. It is a good and fast method to check the cell viability. It was assessed manually with haemocytometer and phase contrast microscope or with automated cell counter countess II FL.

Different concentrations of Aβ-42 were used to check its effect on the cell viability of undifferentiated neuroblastoma SH-SY5Y cells.

I had compared the effect of Aβ at 100 nM and 1 μM at different incubation times such as 2 and 24 h, incubation of protofibrils was carried out at 37°C (physiological temperature of human body). In the preceding chapters, I have used liposomes (biomimetic membranes) and Jurkat cells to understand the endocytic mechanism of Aβ-42. Although in Jurkat cells, toxicity induced by the peptides was not very prominent, but in undifferentiated neuroblastoma SH-SY5Y cells, Aβ-42 was harmful to cells at 1 μM after 2 h exposure which increased significantly when incubation period was 24 h as shown in fig. 4.1. When cells were incubated with Aβ-42 (1 μM) for a day, approximately 14% depression was observed in the cell survival. It was observed that after 2 h of incubation with Aβ at 100 nM could not cause severe damage in the undifferentiated neuroblastoma SH-SH5Y cells, which turned out to be slightly toxic and caused a small loss in the cell viability. According to previous reports, around 57% of the cells died after exposure to Aβ-42 peptides used as a mixture of fibril, protofibrils and oligomers (45).

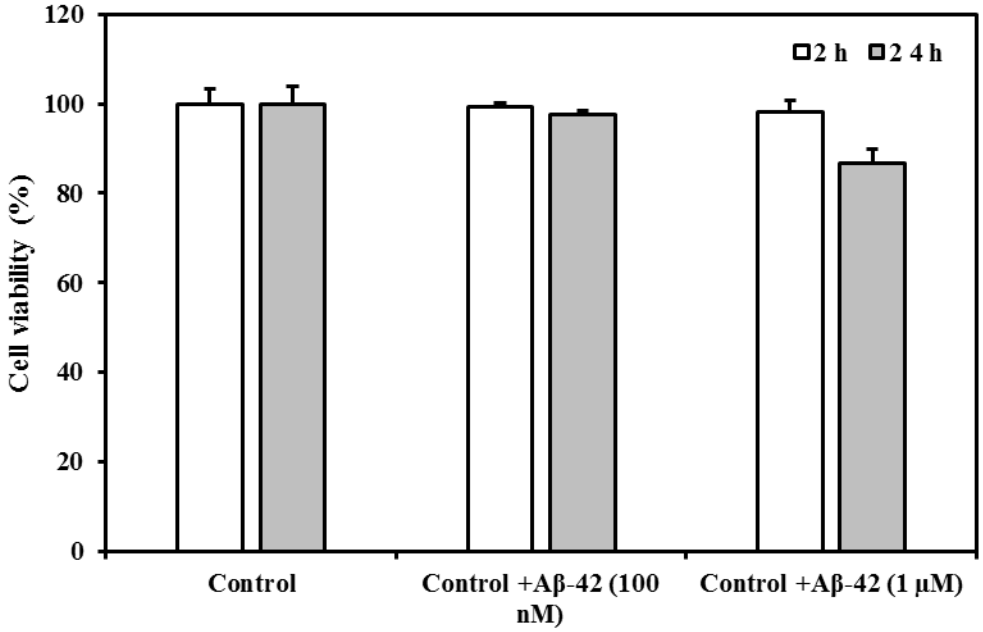


Figure 4.1. Cell viability of undifferentiated human neuroblastoma SH-SY5Y cells in control cells (basal cholesterol level), with and without the exposure of protofibrillar Aβ-42 at 100 nM and 1 μM after 2 and 24 h of incubation of cells with the peptides.

Then to determine the changes produced by alteration in the membrane cholesterol level, I had used oxysterols, 7-KC and 25-OHC at the concentration of 10 µM. Undifferentiated neuroblastoma SH-SY5Y cells were treated with the oxysterols for 24 h and since Aβ-induced toxicity was more pronounced at 24 h, thus cells were then treated in the presence and absence of amyloid peptides at 1 µM and 100 nM for 24 h. Incorporation of only oxysterols also lowers the cell viability of undifferentiated neuroblastoma SH-SY5Y cells (data was not shown). Previously, 7-KC and 25-OHC (at 5 µM) have proposed to lower cell viability within 48-72 h treatment in murine cancer cell lines (32). In addition, there was a further decrease in the cell viability when treated with oxysterols and Aβ-42 (1 µM) as shown in fig.4.2. On the contrary, in Jurkat cells, addition of oxysterols (7-KC and 25-OHC) and Aβ-42 could not reduce the cell viability effectively (as mentioned in Chapter 3), but alteration of membrane cholesterol in undifferentiated neuroblastoma SH-SY5Y cells increase Aβ-induced toxicity. Both of these oxysterols have exhibited toxicity which caused further 10-15% decrease in the cell viability where 7-KC together with Aβ-42 resulted total 25% decrease in the cell viability and 25-OHC exhibited almost 30% depression in the cell survival in the presence of Aβ-42 (1 µM). Chang and Liu have demonstrated the effect of oxysterols on the cell viability of cerebral granule cells, where they found 7-KC at 20 µg/mL causes 18% decrease in the number of viable cells when treated cells with the sterol for 48 h. In addition, 25-OHC killed 44% of the total cell population at 5 µg/mL in the exposure of 2 days (35). Moreover, in an experiment with sympathetic neurons, 25-OHC lowers cell viability to 50% at 4 µg/mL in 36 h (41).

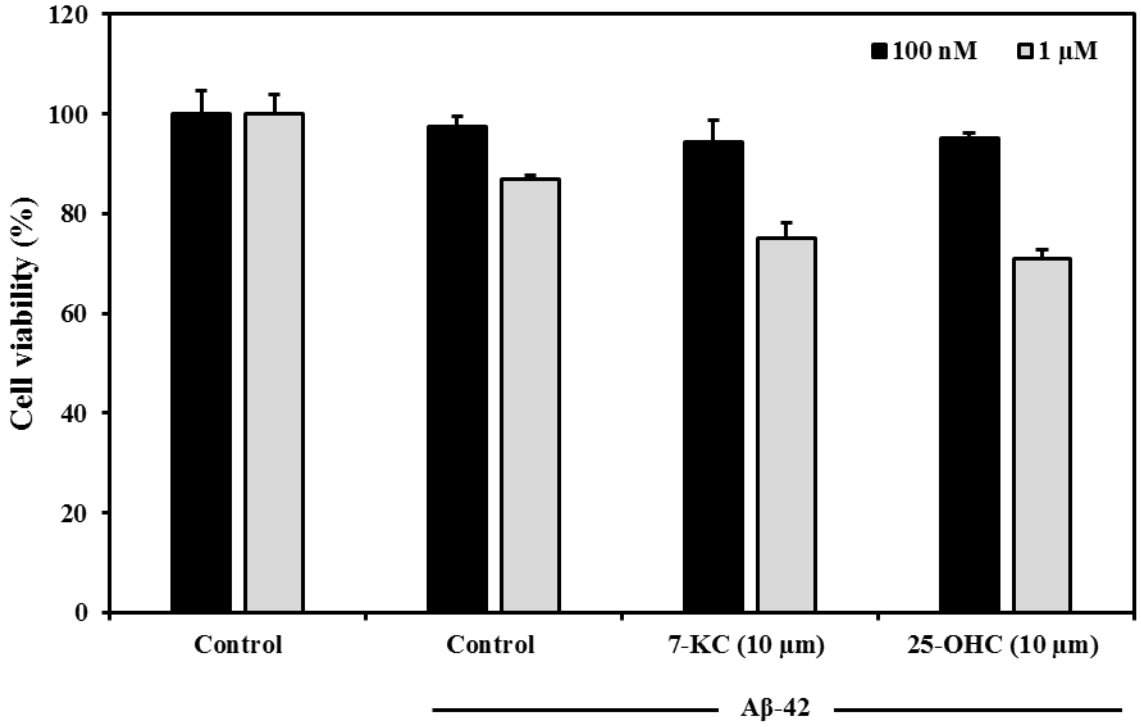


Figure 4.2. Cell viability of undifferentiated human neuroblastoma SH-SY5Y cells in control cells (basal cholesterol level), cells pretreated with 7-ketocholesterol and 25-hydroxycholesterol at 10 µM for 24 h, with and without the exposure of protofibrillar Aβ-42 at 100 nM and 1µM, after 24 h of incubation of cells with the peptides.

Notably, Aβ-42 at 100 nM was not very toxic to the undifferentiated neuroblastoma SH-SY5Y cells. Thus to avoid the malfunctions of the cell and its organelles while microscopic observations, 100 nM Aβ- 42 was used for the further experiments. Later, I had observed the changes in the cell viability in time-dependent experiments. Cells were pretreated with 7KC and 25-OHC for 24 h followed by incubation with protofibrillar Aβ-42 (100 nM) for 2 and 24 h. This particular concentration of the peptides was apparently non-toxic to the cells and so as in the case when cells were pretreated with oxysterols (Fig. 4.3.). After incubation periods, 2 and 24 h, cell viability remained ~99-96% and ~97-94% respectively.

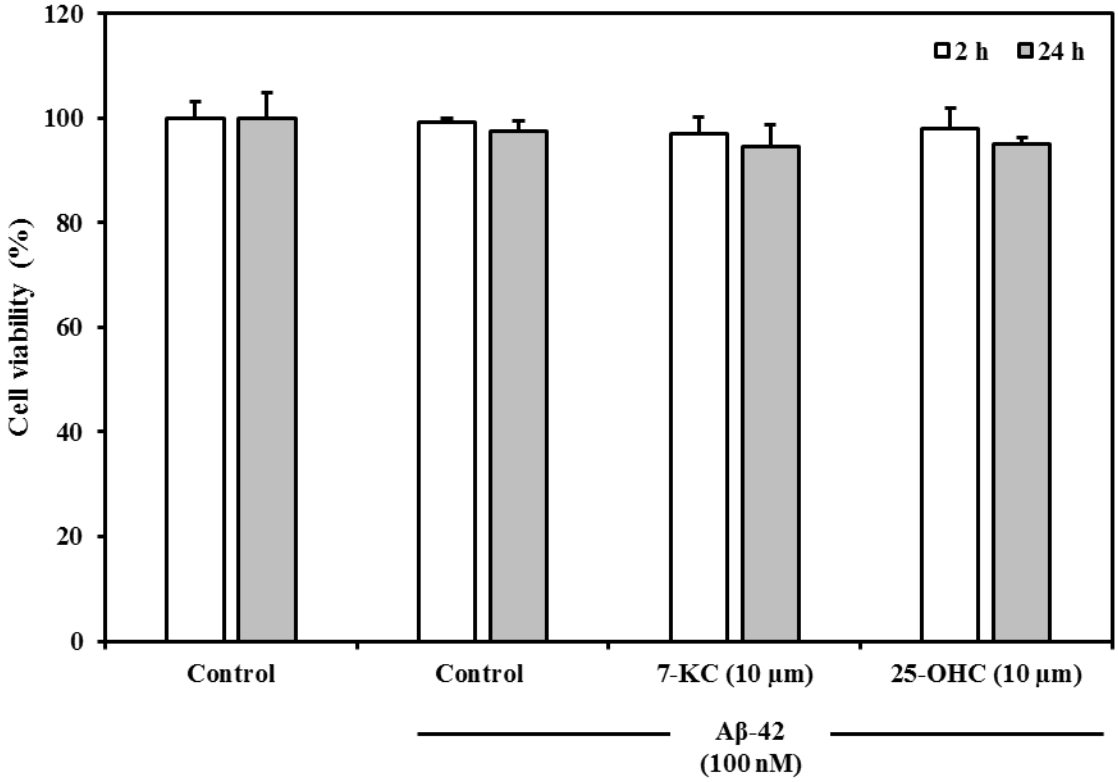


Figure 4.3. Cell viability of undifferentiated human neuroblastoma SH-SY5Y cells in control cells (basal cholesterol level), cells pretreated with 7-ketocholesterol and 25-hydroxycholesterol at 10 µM for 24 h, with and without the exposure of protofibrillar Aβ-42 at 100 nM, after 2 and 24 h of incubation of cells with the peptides.

Thus, exogenous Aβ-42 peptide is also harmful to the undifferentiated neuroblastoma SH-SY5Y cells in a concentration and time-dependent manner. This effect is enhanced in the presence of oxysterols in the cell membrane. Then to support this finding, next, I had observed the internalization of protofibrillar Aβ-42 in the presence and absence of oxysterols.

4.3.2. Internalization of amyloid beta (Aβ-42) in undifferentiated neuroblastoma SH-SY5Y cells-

Accumulation of Aβ peptides is a pathological hallmark in AD and the toxicity induced by these peptides is crucial in the progression of this neurological disorder. Recently, toxicity induced by Aβ peptides present in the extracellular spaces after their internalization considered to play an important role in the pathology of AD. Thus, I had observed influence of protofibrillar Aβ-42 on undifferentiated neuroblastoma SH-SY5Y cells. For the purpose of observation with confocal laser scanning microscope, cells were stained with NucRed dye which intercalates with the strands of DNA and emits red fluorescence. Samples were observed after 2 and 24 h of incubation with fluorescent species of Aβ-42 which exhibits green fluorescence. Unlike in the Jurkat cells, protofibrillar Aβ-42 was observed inside the undifferentiated SH-SY5Y cells after 2 h of incubation period as shown in fig 3.4A. In T cells, accumulation of peptides was observed on the surface of the cell membrane only. After 2 h, uptake of Aβ-42 was observed in ~25% of the total cell population. On the contrary, peptides were observed even after 15 min incubation with the cells sporadically (data was not shown). Previously it was reported that 7-KC induces higher accumulation of protofibrillar Aβ-42 than additional cholesterol and basal level cholesterol on the surface of Jurkat cell membrane (46). And as mentioned in Chapter 3, I found that 25-OHC together with CT-B caused endocytic transport of the peptides through Jurkat cell membrane. After the addition of oxysterols: 7-KC and 25-OHC in the undifferentiated SH-SY5Y cell membrane, there was an increase in the uptake of the peptides and then Aβ-42 was found in ~32-24% of the cells.

Later, when undifferentiated SH-SY5Y cells were incubated with the peptides for a longer period i.e. 24 h, protofibrillar Aβ-42 was internalized into 41% cells in cells with basal level of cholesterol. In addition, with pretreatment with oxysterols (7-KC and 25-OHC) for 24 h prior to incubation with Aβ-42 for 24 h, peptides were internalized in more cells as shown graphically in fig 4.4B. This increment may be attributed to the incorporation of oxysterols in the membrane. P. Gamba et al. have demonstrated that oxysterols (27-OHC, 24-OHC and 7β-OHC) elevates the adherence of Aβ-42 peptides at 1 μM in differentiated SK-N-BE cells after 48 h treatment with oxysterols succeeded by the incubation with Aβ-42 for 8 h (47).

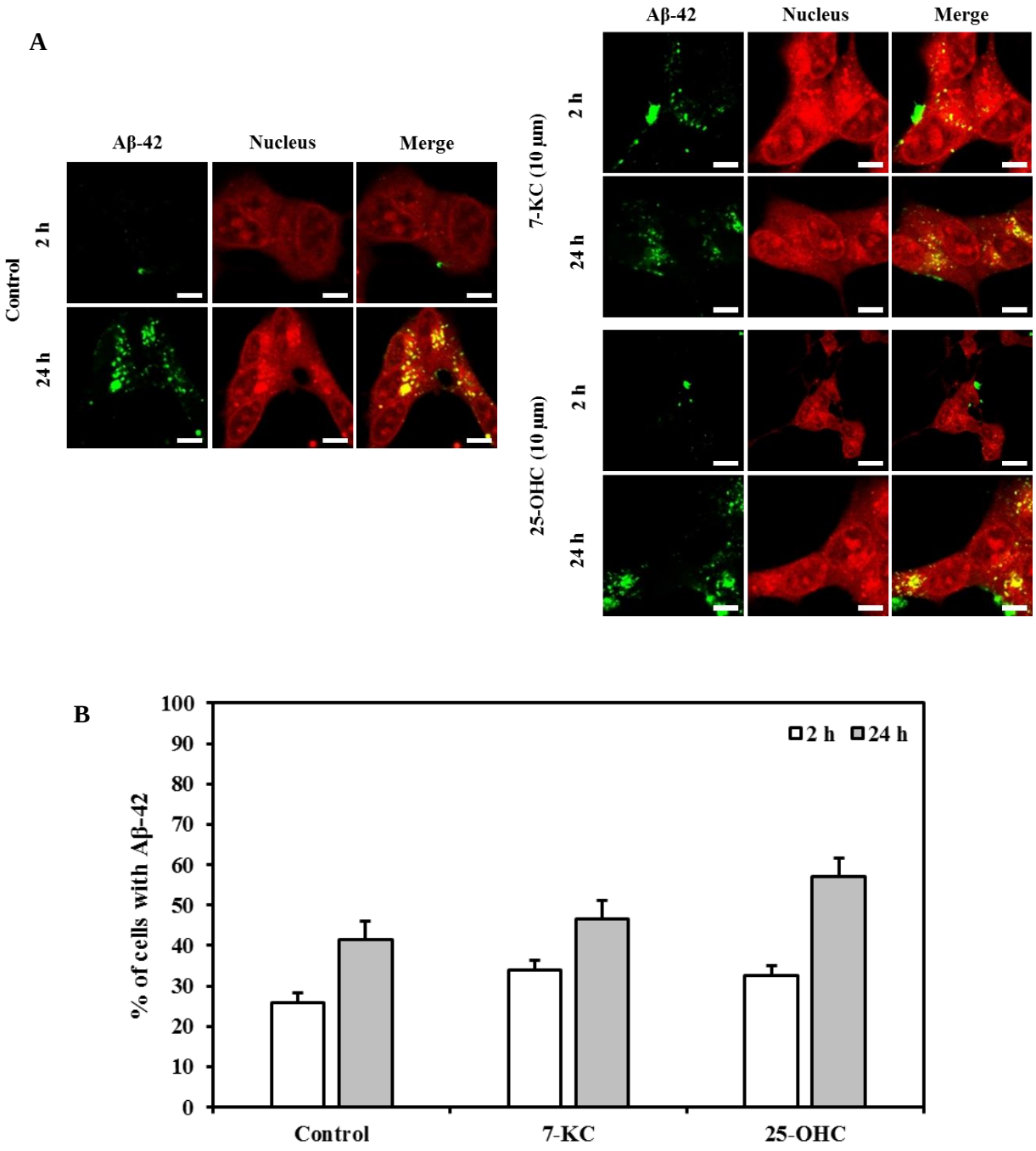


Figure 4.4. A) Microscopic images of the cells where Aβ-42 (HiLyte flor 488 nm), nuclei (Cy5) are represented by green and red fluorescence, respectively. **B)** Internalization of Aβ-42 (100 nM) in undifferentiated human neuroblastoma SH-SY5Y cells in control cells (basal cholesterol level), cells pretreated with 7-ketocholesterol and 25-hydroxycholesterol at 10 µM for 24 h, after 2 and 24 h of incubation of cells with the peptides. Scale bars = 10 µm.

4.3.3. Internalization of amyloid beta (Aβ-42) in the presence of cholera toxin B subunit (CT-B)-

Internalization of Aβ peptides have been associated with different pathways, one of them is through lipid rafts and lipid raft-associated proteins. Lipid rafts have been demonstrated as the binding site for exogenous peptides (12, 48) and Matsuzaki et al. have shown that GM1 accelerates the aggregation of Aβ peptides (49). I have also discussed the integral factors for endocytic transport of the peptide and discussed the crucial role of glycosyl chains involved in this phenomenon due to the fact that GM1-CT-B cause induction of negative curvature (50).

Here, using undifferentiated human neuroblastoma SH-SY5Y cell line, after the treatment with protofibrillar Aβ-42 for 2 and 24 h, cells were treated with lipid raft marker, CT-B. It was found that the peptides were internalized into ~17.6% after 2 h which increase with time and after 24 h of treatment, ~39% of the cells recruited Aβ-42 (fig 4.5.). A group of researchers have also suggested that only those oligomeric forms of amyloid which are adhered with the membrane have internalized into the cell. They have also focused on the aggregated species of the peptide where oligomers were internalized more prominently than fibrils (51). Another researchers have proposed that internalization of Aβ takes places at lipid rafts, probably after binding with the sphingolipid, GM1(52). Later, prior to treatment with the peptides, undifferentiated SH-SY5Y cells were treated with oxysterols (7-KC and 25-OHC at 10 µM) for 24 h. Then after brief washing, cells were incubated in medium containing protofibrillar Aβ-42 in time dependent experiment (for 2 and 24 h). In the presence of both oxysterols, in Jurkat cells, 7-KC enhanced the interaction of peptides at the surface of the cell membrane (46), whereas in 25-OH, with CT-B-GM1 interaction, Aβ-42 peptides were internalized into the cell. In the case of undifferentiated human neuroblastoma SH-SY5Y cells, both oxysterols showed similar behavior where Aβ-42 peptide were internalized into ~20% of total population in the presence of 7-KC and ~23% in 25-OHC added cells after 2 h of incubation with the peptides. Later, when incubation period lasted up to 24 h, Aβ-42 protofibrils were recruited into the cells in higher amount, ~40% of the total cells under the influence of 7-KC and ~47% in 25-OHC. These results were different from my expectation as Aβ-42 peptides were recruited in less cell as compare to when CT-B was not present. This can be attributed to the inhibitory effect of CT-B for the internalization of Aβ-42.

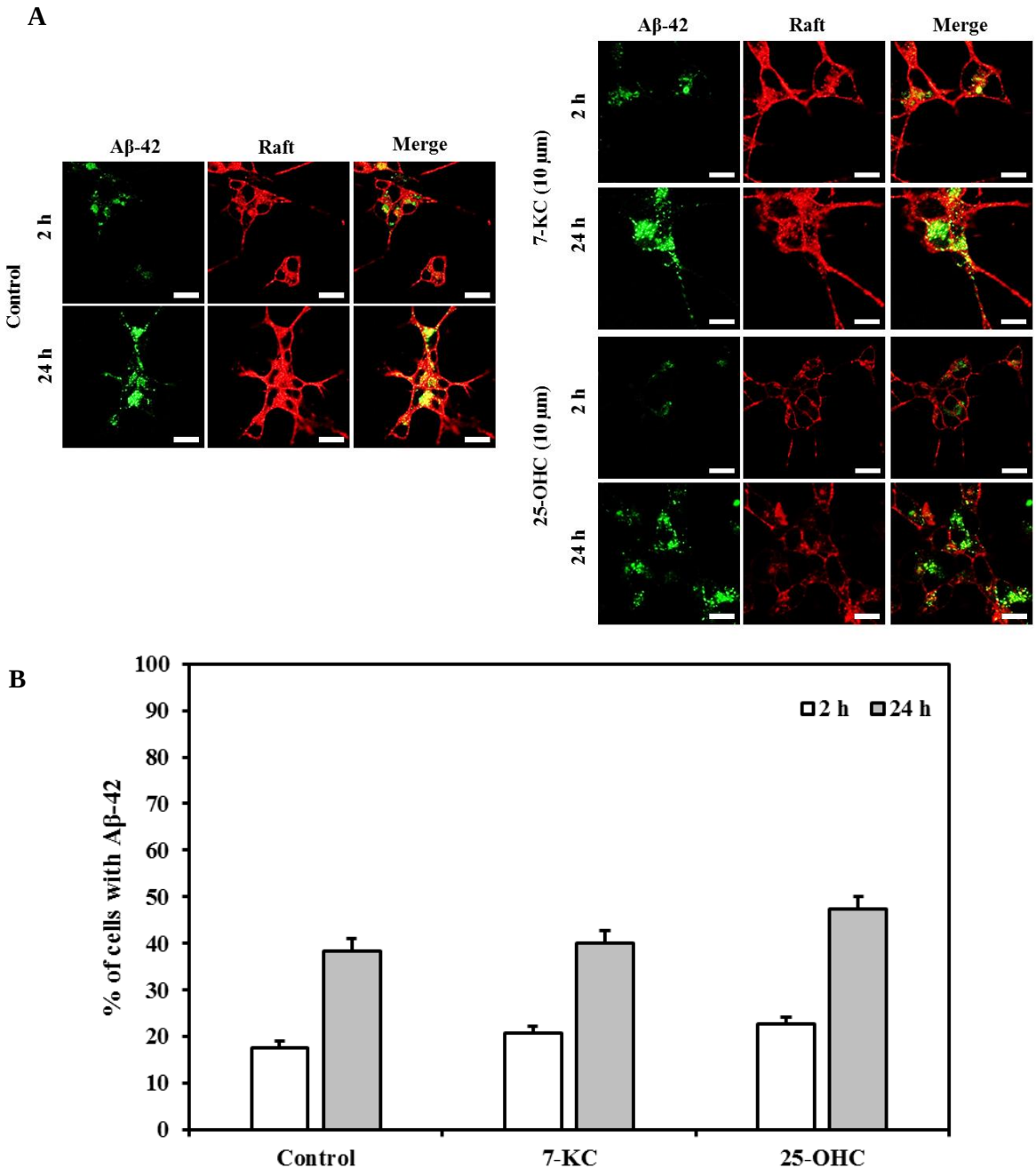
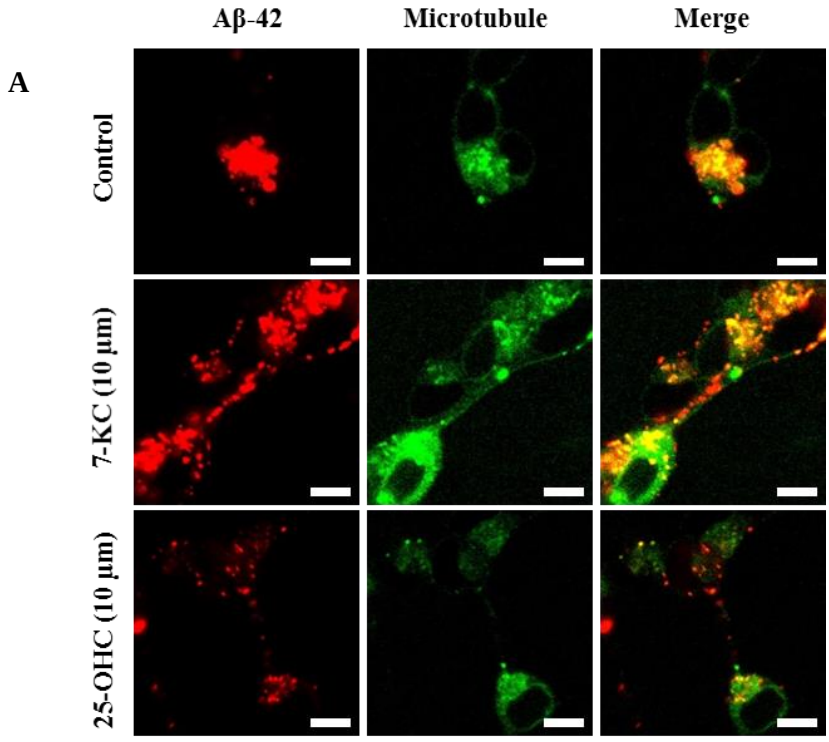


Figure 4.5. A) Microscopic images of the cells where Aβ-42 (HiLyte flor 488 nm), lipid raft (CT-B, 555 nm) are represented by green and red fluorescence, respectively. **B)** Internalization of Aβ-42 (100 nM) in the presence of cholera toxin B subunit (CT-B) in undifferentiated human neuroblastoma SH-SY5Y cells in control cells (basal cholesterol level), cells pretreated with 7-ketocholesterol (7-KC) and 25-hydroxycholesterol (25-OHC) at 10 µM for 24 h, after 2 and 24 h of incubation of cells with the peptides. Scale bars=10 µm.

4.3.4. Co-localization of amyloid beta (Aβ-42) with microtubules

Microtubules constitutes of heterodimers which are highly dynamic in nature, α- and β-tubulin are the components of microtubules which form hollow cylindrical structures. Microtubules are primarily involved in the motility of cells, regulates cell growth and plays key role in intracellular trafficking. Among various roles of microtubules, one of the important function is to deliver the protein to the cell organelles. In my study, Aβ peptides are added externally and supposedly they may reach to ER. In Jurkat cells, it was found that the peptides were using the microtubules as trail to reach ER. So, if in undifferentiated SH-SY5Y cells, Aβ-42 is localized with the microtubules, possibly in the neural cells, pathway chosen by peptide is via microtubules, even if proteins are added externally.

Thus, I have stained microtubules using Oregon green taxol (which is represented by green fluorescence) in pretreated cells with the peptides (represented by red fluorescence) and observed the presence of Aβ-42 along with microtubules (shown with the help of merge images) as shown in fig 4.6. The co-localization of protofibrillar Aβ-42 was observed in oxysterol added SH-SY5Y cells as well. It supports the fact that microtubules are responsible for protein trafficking when they are present in the interphase of the cell cycle.



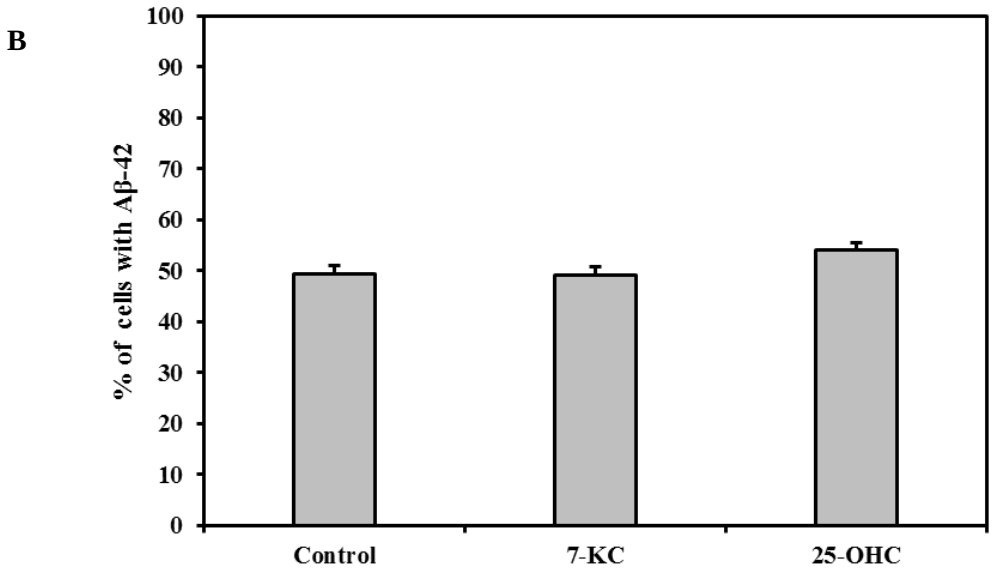


Figure 4.6. A) Microscopic images of the cells where Aβ-42 (HiLyte flor 555 nm), microtubule (Oregon green, 488 nm) are represented by red and green fluorescence, respectively. B) Co-localization of Aβ-42 (100 nM) with microtubules in undifferentiated human neuroblastoma SH-SY5Y cells in control cells (basal cholesterol level), cells pretreated with 7-ketocholesterol (7-KC) and 25-hydroxycholesterol (25-OHC) at 10 µM for 24 h, after 24 h of incubation of cells with the peptides. Values shown are means ±SE values. Scale bars=10 µm.

4.3.5. Localization of amyloid beta (Aβ-42) in endoplasmic reticulum (ER)-

Proteins like Aβ gets synthesized in our body, homeostasis of these proteins is very important, since dysfunction in the structure and functions of proteins will result into the progression of several diseases like AD. Endoplasmic reticulum (ER) is the principal organelle for appropriate folding of newly synthesized proteins where about 30% of total body proteins are formed. Whenever misfolded proteins accumulate in the lumen of ER, due to upregulation, in defense ER initiated Unfolded Protein Response (UPR), when these processes are uncontrollable organelle comes under stress which is known as ER stress. Thus presence of Aβ in ER has become a considerable marker in the pathology of AD. After treating the cells with 1 µM protofibrillar Aβ-42 for 2 and 24 h, I stained ER with Blue-white ER tracker for 30 min at 37°C.

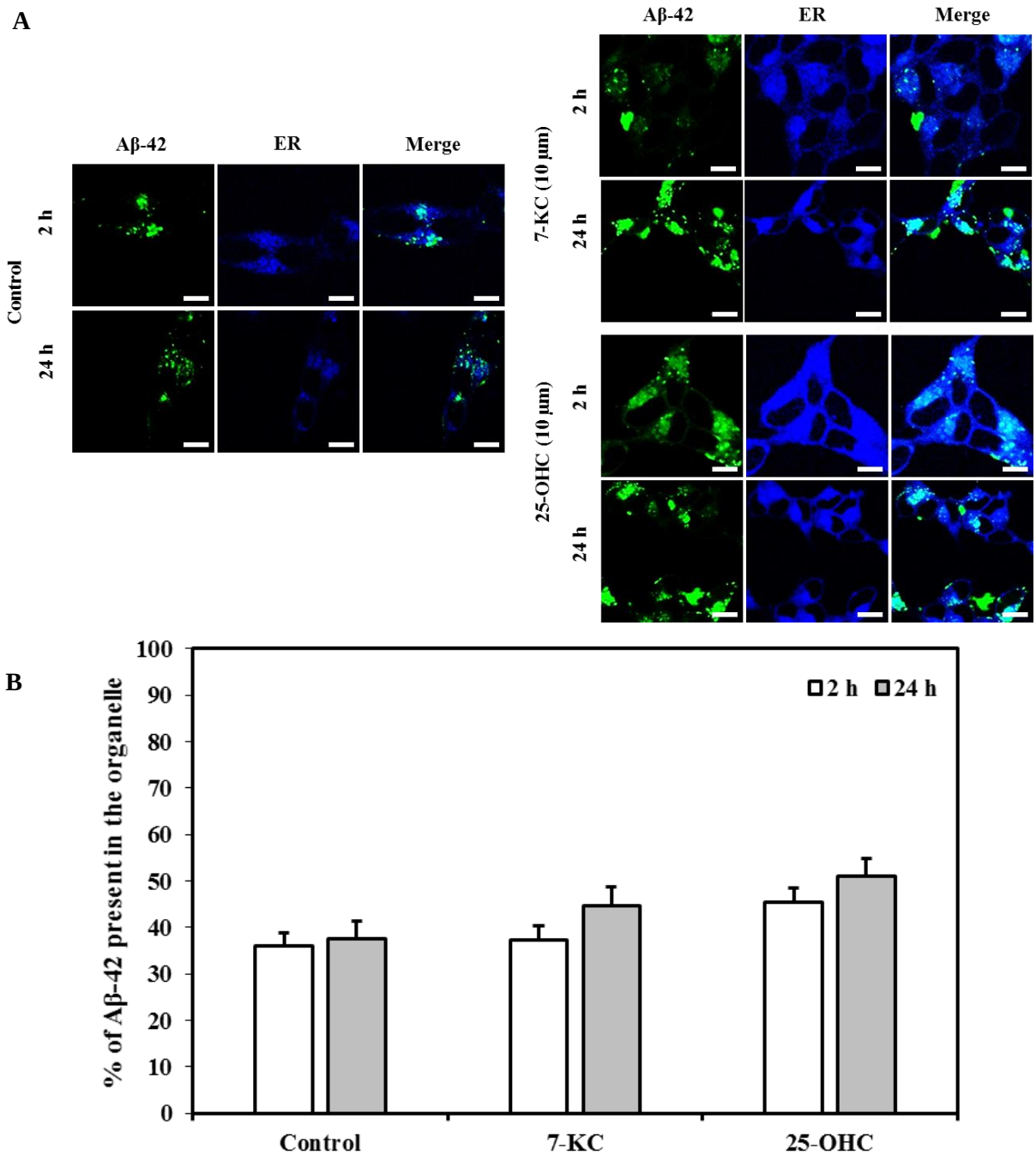


Figure 4.7. A) Microscopic images of the cells where Aβ-42 (HiLyte flor 488 nm), endoplasmic reticulum (ER) (DAPI filter) are represented by green and blue fluorescence, respectively. **B)** Internalization of Aβ-42 (100 nM) in endoplasmic reticulum (ER) in undifferentiated human neuroblastoma SH-SY5Y cells in control cells (basal cholesterol level), cells pretreated with 7-ketocholesterol (7-KC) and 25-hydroxycholesterol (25-OHC) at 10 µM for 24 h, after 2 and 24 h of incubation of cells with the peptides. Scale bars=10 µm.

It was observed that amyloid peptides (represented by green fluorescence) were internalized into the ER of undifferentiated SH-SY5Y cells. Thus after penetrating into the cells, Aβ-42 reaches to ER where it may cause ER stress. It was reported that Aβ can cause ER stress even when added externally in the cell (53–55). In addition, it may cause dysfunction in the mitochondria as it is closely related with ER. According to my speculation, this internalization may occurs through endocytic pathway. In Jurkat cells, it happened by the aid of 25-OHC and GM1-CT-B interaction.

After 2 h treatment of undifferentiated SH-SY5Y cells with Aβ-42, ~35% of the peptide was co-localized with ER which had increased to ~37% after 24 of incubation as shown in fig. 4.7. Since it was demonstrated that effect of oxysterols in the membrane is more prominent as compare to cholesterol as transport of oxysterols is much faster than that of cholesterol in the brain due to their rearrangement in the leaflets of the cell membrane attributed to the presence of additional hydroxyl group (-OH) (56), I had compared the influx of Aβ-42 in the presence and absence of oxysterols.

So I have pretreated the cells with two oxysterols (7-KC and 25-OHC) and then treated them with protofibrillar Aβ-42 for 2 and 24 h. It was observed that accumulation of exogenous Aβ-42 was increase in the presence of oxysterols after 2 h by 3-10% and 7-11% after incubation of 24 h.

4.4. Conclusions

As per general understanding, major pathway followed by extracellular Aβ-42 peptides to internalize into the membranes follows endocytosis. In my study, I have used undifferentiated human neuroblastoma SH-SY5Y cell line and altered level of cholesterol in the membrane using 7-KC and 25-OHC. Toxicity induced by Aβ-42 is associated with the concentration and aggregated species of the peptide, while in Jurkat cells, viability loss was not substantial. But, in SH-SY5Y cells, Aβ-42 induced prominent decrease in the viability of cells which was enhanced by the introduction of oxysterols in the membrane. Amount of internalized Aβ increased inside the cells which was more pronounced after addition of oxysterols (7-KC and 25-OHC) in the SH-SY5Y cells increased with higher incubation period. After internalizing into the cells, Aβ-42 was co-localized with ER which varies in a time-dependent manner. Thereby, aggregated species of the

peptide, type of oxysterols, kind and extent of glycosyl chains can be regarded as essential factors in the pathology of AD and facilitates the understanding about the mechanism behind the disease.

References

1. Lana, E., Khanbolouki, M., Degavre, C., Samuelsson, E.-B., Åkesson, E., Winblad, B., Alici, E., Lithner, C. U., and Behbahani, H. (2016) Perforin Promotes Amyloid Beta Internalisation in Neurons. *Mol. Neurobiol.*, 1–14 [online] http://dx.doi.org/10.1007/s12035-016-9685-9 (Accessed 2016).
2. Lai, A. Y., and McLaurin, J. (2011) Mechanisms of Amyloid-Beta Peptide Uptake by Neurons: The Role of Lipid Rafts and Lipid Raft-Associated Proteins. *Int J Alzheimers Dis* **2011** [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3010653/ (Accessed December 20, 2011).
3. Wolozin, B., Kellman, W., Ruosseau, P., Celesia, G. G., and Siegel, G. (2000) Decreased prevalence of Alzheimer disease associated with 3-hydroxy-3-methyglutaryl coenzyme A reductase inhibitors. *Arch. Neurol.* **57**, 1439–1443.
4. Jick, H. Z. G. L., Zornberg, G. L., Jick, S. S., Seshadri, S., and Drachman, D. A. (2000) Statins and the risk of dementia. *Lancet* **356**, 1627–1631.
5. Vetrivel, K. S., and Thinakaran, G. (2010) Membrane rafts in Alzheimer's disease beta-amyloid production. *Biochim. Biophys. Acta* **1801**, 860–867 [online] //www.sciencedirect.com/science/article/pii/S1388198110000636 (Accessed August 2010).
6. Kivipelto, M., Helkala, E.-L., Laakso, M. P., Hänninen, T., Hallikainen, M., Alhainen, K., Soininen, H., Tuomilehto, J., and Nissinen, A. (2001) Midlife vascular risk factors and Alzheimer's disease in later life: longitudinal, population based study. *Bmj* **322**, 1447–1451.
7. Kim, S.-I., Yi, J.-S., and Ko, Y.-G. (2006) Amyloid β oligomerization is induced by brain lipid rafts. *J. Cell. Biochem.* **99**, 878–889 [online] http://dx.doi.org/10.1002/jcb.20978 (Accessed 2006).
8. Okada, T., Ikeda, K., Wakabayashi, M., Ogawa, M., and Matsuzaki, K. (2008) Formation of toxic Aβ (1–40) fibrils on GM1 ganglioside-containing membranes mimicking lipid rafts: polymorphisms in Aβ (1–40) fibrils. *J. Mol. Biol.* **382**, 1066–1074.
9. Lin, M.-S., Chen, L.-Y., Wang, S. S. S., Chang, Y., and Chen, W.-Y. (2008) Examining the

levels of ganglioside and cholesterol in cell membrane on attenuation the cytotoxicity of beta-amyloid peptide. *Colloids Surfaces B: Biointerfaces* **65**, 172–177 [online] //www.sciencedirect.com/science/article/pii/S0927776508001367 (Accessed September 1, 2008).

10. Wang, S. S.-S., Rymer, D. L., and Good, T. A. (2001) Reduction in cholesterol and sialic acid content protects cells from the toxic effects of β-amyloid peptides. *J. Biol. Chem.* **276**, 42027–42034.
11. Sepulveda, F. J., Parodi, J., Peoples, R. W., Opazo, C., and Aguayo, L. G. (2010) Synaptotoxicity of Alzheimer Beta Amyloid Can Be Explained by Its Membrane Perforating Property. *PLOS ONE* **5** [online] http://dx.doi.org/10.1371%2Fjournal.pone.0011820 (Accessed July 27, 2010).
12. Verdier, Y., Zarándi, M., and Penke, B. (2004) Amyloid β-peptide interactions with neuronal and glial cell plasma membrane: binding sites and implications for Alzheimer's disease. *J. Pept. Sci.* **10**, 229–248 [online] http://dx.doi.org/10.1002/psc.573 (Accessed 2004).
13. Zampagni, M., Evangelisti, E., Cascella, R., Liguri, G., Becatti, M., Pensalfini, A., Uberti, D., Cenini, G., Memo, M., and Bagnoli, S. (2010) Lipid rafts are primary mediators of amyloid oxidative attack on plasma membrane. *J. Mol. Med.* **88**, 597–608.
14. Eckert, G. P., Wood, W. G., and Muller, W. E. (2010) Lipid membranes and β-amyloid: a harmful connection. *Curr. Protein Pept. Sci.* **11**, 319–325.
15. Knauer, M. F., Soreghan, B., Burdick, D., Kosmoski, J., and Glabe, C. G. (1992) Intracellular accumulation and resistance to degradation of the Alzheimer amyloid A4/beta protein. *Proc. Natl. Acad. Sci.* **89**, 7437–7441.
16. Moon, J. H., Kim, S. Y., Lee, H. G., Kim, S. U., and Lee, Y. B. (2008) Activation of nicotinic acetylcholine receptor prevents the production of reactive oxygen species in fibrillar β amyloid peptide (1-42)-stimulated microglia. *Exp. & Mol. Med.* **40**, 11–18.
17. Boldogh, I., and Kruzel, M. L. (2008) Colostrinin™: an oxidative stress modulator for prevention and treatment of age-related disorders. *J. Alzheimer's Dis.* **13**, 303–321.
18. Bras, M., Queenan, B., and Susin, S. A. (2005) Programmed cell death via mitochondria: different modes of dying. *Biochemistry* **70**, 231–239.
19. Pelicano, H., Feng, L., Zhou, Y., Carew, J. S., Hileman, E. O., Plunkett, W., Keating, M. J.,

and Huang, P. (2003) Inhibition of mitochondrial respiration a novel strategy to enhance drug-induced apoptosis in human leukemia cells by a reactive oxygen species-mediated mechanism. *J. Biol. Chem.* **278**, 37832–37839.

20. Biedler, J. L., Helson, L., and Spengler, B. A. (1973) Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. *Cancer Res.* **33**, 2643–2652.
21. Kuramoto, T., Werrbach-Perez, K., Perez-Polo, J. R., and Haber, B. (1981) Membrane properties of a human neuroblastoma II: Effects of differentiation. *J. Neurosci. Res.* **6**, 441–449.
22. Peng, S.-K., Tham, P., Taylor, C. B., and Mikkelson, B. (1979) Cytotoxicity of oxidation derivatives of cholesterol on cultured aortic smooth muscle cells and their effect on cholesterol biosynthesis. *Am. J. Clin. Nutr.* **32**, 1033–1042.
23. Imai, H., Werthessen, N. T., Subramanyam, V., LeQuesne, P. W., Soloway, A. H., and Kanisawa, M. (1980) Angiotoxicity of oxygenated sterols and possible precursors. *Science* **207** [online] http://science.sciencemag.org/content/207/4431/651.abstract (Accessed February 8, 1980).
24. Sevanian, A., Berliner, J., and Peterson, H. (1991) Uptake, metabolism, and cytotoxicity of isomeric cholesterol-5, 6-epoxides in rabbit aortic endothelial cells. *J. Lipid Res.* **32**, 147–155
25. Ramasamy, S., Boissonneault, G. A., and Hennig, B. (1992) Oxysterol-induced endothelial cell dysfunction in culture. *J. Am. Coll. Nutr.* **11**, 532–538.
26. Hessler, J. R., Morel, D. W., Lewis, L. J., and Chisolm, G. M. (1983) Lipoprotein oxidation and lipoprotein-induced cytotoxicity. *Arterioscler. Thromb. Vasc. Biol.* **3**, 215–222.
27. Astruc, M., Roussillon, S., Defay, R., Descomps, B., and de Paulet, A. C. (1983) DNA and cholesterol biosynthesis in synchronized embryonic rat fibroblasts: II. Effects of sterol biosynthesis inhibitors on cell division. *Biochim. Biophys. Acta* **763**, 11–18.
28. Lütjohann, D., Papassotiropoulos, A., Björkhem, I., Locatelli, S., Bagli, M., Oehring, R. D., Schlegel, U., Jessen, F., Rao, M. L., and von Bergmann, K. (2000) Plasma 24S-hydroxycholesterol (cerebrosterol) is increased in Alzheimer and vascular demented patients. *J. Lipid Res.* **41**, 195–198.
29. Emanuelsson, I., and Norlin, M. (2012) Protective effects of 27-and 24-hydroxycholesterol against staurosporine-induced cell death in undifferentiated neuroblastoma SH-SY5Y cells.

Neurosci. Lett. **525**, 44–48.

30. Christ, M., Luu, B., Mejia, J. E., Moosbrugger, I., and Bischoff, P. (1993) Apoptosis induced by oxysterols in murine lymphoma cells and in normal thymocytes. *Immunology* **78.**
31. Hietter, H., Bischoff, P., Beck, J. P., Ourisson, G., and Luu, B. (1986) Comparative effects of 7 beta-hydroxycholesterol towards murine lymphomas, lymphoblasts and lymphocytes: selective cytotoxicity and blastogenesis inhibition. *Cancer Biochem. Biophys.* **9**, 75–83.
32. Hwang, P. L. (1992) Inhibitors of protein and RNA synthesis block the cytotoxic effects of oxygenated sterols. *Biochim. Biophys. Acta* **1136**, 5–11.
33. Chang, J. Y., and Liu, L.-Z. (1997) 25-Hydroxycholesterol causes death but does not prevent nerve growth factor-induced neurite outgrowth in PC12 cells. *Neurochem. Int.* **31**, 517–523.
34. Chang, J. Y., Phelan, K. D., and Liu, L.-Z. (1998) Neurotoxicity of 25-OH-cholesterol on NGF-differentiated PC12 cells. *Neurochem. Res.* **23**, 7–16.
35. Chang, J. Y., and Liu, L.-Z. (1998) Neurotoxicity of cholesterol oxides on cultured cerebellar granule cells. *Neurochem. Int.* **32**, 317–323.
36. Kölsch, H., Lütjohann, D., Tulke, A., Björkhem, I., and Rao, M. L. (1999) The neurotoxic effect of 24-hydroxycholesterol on SH-SY5Y human neuroblastoma cells. *Brain Res.* **818**, 171–175.
37. Brown, A. J., and Jessup, W. (1999) Oxysterols and atherosclerosis. *Atherosclerosis* **142**, 1–28.
38. Ma, M.-T., Zhang, J., Farooqui, A. A., Chen, P., and Ong, W.-Y. (2010) Effects of cholesterol oxidation products on exocytosis. *Neurosci. Lett.* **476**, 36–41.
39. Brown, A. J., and Jessup, W. (2009) Oxysterols: Sources, cellular storage and metabolism, and new insights into their roles in cholesterol homeostasis. *Mol. Asp. Med.* **30**, 111–122 [online] //www.sciencedirect.com/science/article/pii/S0098299709000041 (Accessed June 2009).
40. Smith, L. L., and Johnson, B. H. (1989) Biological activities of oxysterols. *Free. Radic. Biol. Med.* **7**, 285–332.
41. Chang, J. Y., Phelan, K. D., and Chavis, J. A. (1998) Neurotoxicity of 25-OH-cholesterol on sympathetic neurons. *Brain Res. Bull.* **45**, 615–622.
42. Demuro, A., Mina, E., Kayed, R., Milton, S. C., Parker, I., and Glabe, C. G. (2005) Calcium Dysregulation and Membrane Disruption as a Ubiquitous Neurotoxic Mechanism of Soluble

Amyloid Oligomers. *J. Biol. Chem.* **280**, 17294–17300 [online] https://doi.org/10.1074%2Fjbc.m500997200 (Accessed February 2005).

43. Dong, H., Strome, S. E., Salomao, D. R., Tamura, H., Hirano, F., Flies, D. B., Roche, P. C., Lu, J., Zhu, G., Tamada, K., Lennon, V. A., Celis, E., and Chen, L. (2002) Tumor-associated B7-H1 promotes T-cell apoptosis: A potential mechanism of immune evasion. *Nat. Med.* [online] http://dx.doi.org/10.1038/nm730 (Accessed June 2002).
44. Strober, W. (2001) Trypan blue exclusion test of cell viability. *Curr. Protoc. Immunol.*
45. Famer, D., and Crisby, M. (2004) Rosuvastatin reduces caspase-3 activity and up-regulates α-secretase in human neuroblastoma SH-SY5Y cells exposed to Aβ. *Neurosci. Lett.* **371**, 209–214 [online] //www.sciencedirect.com/science/article/pii/S0304394004010924 (Accessed November 23, 2004).
46. Phan, H. T., Vestergaard, M. C., Baek, K., Shimokawa, N., and Takagi, M. (2014) Localization of amyloid beta (Aβ1-42) protofibrils in membrane lateral compartments: Effect of cholesterol and 7-Ketocholesterol. *FEBS Lett.* **588**, 3483–3490.
47. Gamba, P., Leonarduzzi, G., Tamagno, E., Guglielmotto, M., Testa, G., Sottero, B., Gargiulo, S., Biasi, F., Mauro, A., Viña, J., and Poli, G. (2011) Interaction between 24-hydroxycholesterol, oxidative stress, and amyloid-$\upbeta$ in amplifying neuronal damage in Alzheimer's disease: three partners in crime. *Aging Cell* **10**, 403–417 [online] https://doi.org/10.1111%2Fj.1474-9726.2011.00681.x (Accessed April 2011).
48. Williamson, R., Usardi, A., Hanger, D. P., and Anderton, B. H. (2008) Membrane-bound β-amyloid oligomers are recruited into lipid rafts by a fyn-dependent mechanism. *FASEB J.* **22**, 1552–1559.
49. Matsuzaki, K., Kato, K., and Yanagisawa, K. (2010) Aβ polymerization through interaction with membrane gangliosides. *Biochim. et Biophys. Acta* **1801**, 868–877 [online] //www.sciencedirect.com/science/article/pii/S1388198110000211 (Accessed August 2010).
50. Dhingra, S., Morita, M., Yoda, T., Vestergaard, M. C., Hamada, T., and Takagi, M. (2013) Dynamic morphological changes induced by GM1 and protein interactions on the surface of cell-sized liposomes. *Materials* **6**, 2522–2533.
51. Chafekar, S. M., Baas, F., and Scheper, W. (2008) Oligomer-specific Aβ toxicity in cell models is mediated by selective uptake. *Biochim. et Biophys. Acta* **1782**, 523–531.
52. Saavedra, L., Mohamed, A., Ma, V., Kar, S., and Chaves, E. P. de (2007) Internalization of -

Amyloid Peptide by Primary Neurons in the Absence of Apolipoprotein E. *J. Biol. Chem.* **282**, 35722–35732 [online] https://doi.org/10.1074%2Fjbc.m701823200 (Accessed October 2007).

53. Resende, R., Ferreiro, E., Pereira, C., and Oliveira, C. R. (2008) ER stress is involved in Aβ-induced GSK-3β activation and tau phosphorylation. *J. Neurosci. Res.* **86**, 2091–2099.
54. Costa, R. O., Ferreiro, E., Cardoso, S. M., Oliveira, C. R., and Pereira, C. M. (2010) ER stress-mediated apoptotic pathway induced by Aβ peptide requires the presence of functional mitochondria. *J. Alzheimer's Dis.* **20**, 625–636.
55. Song, S., Lee, H., Kam, T.-I., Tai, M. L., Lee, J.-Y., Noh, J.-Y., Shim, S. M., Seo, S. J., Kong, Y.-Y., and Nakagawa, T. (2008) E2-25K/Hip-2 regulates caspase-12 in ER stress–mediated Aβ neurotoxicity. *J. cell Biol.* **182**, 675–684.
56. Leoni, V., and Caccia, C. (2011) Oxysterols as biomarkers in neurodegenerative diseases. *Chem. Phys. lipids* **164**, 515–524.

Chapter 5:
General conclusions and future prospects

5.1. General conclusions

This study demonstrates several important risk factors for Alzheimer's disease (AD) such as aging, advanced glycation end products, different species of amyloid-beta (Aβ). These components are closely related with the necessary factors for the endocytic transport of Aβ. In this dissertation, role of membrane lipids in the interaction of Aβ with biomimetic and biological membranes have been discussed in detail. Oxysterols could facilitate the insertion of peptides with the cooperation of other factors. In general, toxicity, aggregation and internalization of Aβ peptide were enhanced in the presence of oxysterols.

In chapter 1, present facts about different risk factors for AD where part of membrane lipids, specifically oxysterols and aggregation of Aβ peptide and Aβ-induced toxicity were reviewed. Controversial studies about role of cholesterol and its oxygenated derivatives as cytoprotective and cytotoxic effect were discussed. Aβ-induced toxicity and its co-relation with the internalization of the peptide was explained to clarify the mechanism of internalization which pre-dominantly could occur via endocytosis. Based on this literature background, I proposed the research objectives of this study.

In chapter 2, use of model or biomimetic membranes showed that aggregation of Aβ is promoted by 25-hydroxycholesterol (25-OHC) to maintain the neurotoxic species of the peptide since rate of fibrillation was decreased in the presence of oxysterols. In addition, Aβ-42 localized into liquid disordered (Ld) region of the phase-separated membrane, thus enhanced the association of the peptide with the membranes.

In chapter 3, Jurkat T cells, type of leukemic cells were used as a mimic of neuronal cells to investigate the role of oxysterols, in the intracellular transport of the peptide. It was known that externally added Aβ could cause stress in the cell organelle, endoplasmic reticulum (ER) and I proposed that possibly it could occur when Aβ gets internalized in the cells through endocytic

mechanism. I have shown that presence of 25-OHC in the membrane promotes the insertion of Aβ-42. However, only 25-OHC was not sufficient to induce this transport, negative curvature induction was important which was achieved with the incorporation of cholera toxin B subunit (CT-B) in the system. Viability of the cells were affected by addition of oxysterols which increased the toxicity induced by Aβ-42. Perturbations in the calcium homeostasis was also observed which remarkably enhanced on the exposure of Aβ-42 and oxysterols where effect of 25-OHC was more than 7-ketocholesterol (7-KC) at similar conditions. Peptides were shown to be localized in ER and possibly transported via microtubules which are known to be responsible for the intracellular trafficking of the proteins (cytoskeleton).

In chapter 4, undifferentiated human neuroblastoma SH-SY5Y cells were used to assess the role of Aβ-induced toxicity by varying the reaction-time and concentration of the peptide. In addition, role of oxysterols such as 7-KC and 25-OHC in the Aβ-induced toxicity was demonstrated. I have shown enhanced effect of oxysterols in the peptide induced toxicity. Moreover, internalization of Aβ was shown to increase after the addition of both of the oxysterols which increased with longer incubation periods. In accordance with my hypothesis, I have shown that the peptide would reach to ER after internalizing into the cells. Use of these cells ensured the role of these oxysterols to be involved critically in the pathology of AD.

Use of biomimetic and biological membranes in the study have given me the opportunity to understand the mechanism behind Aβ interaction with the membranes in a better way. I have shown that oxysterols, glycosyl chains and nano-structures of the peptide are the risk factors in the pathology of AD. The findings of this study are beneficial to demonstrate the role of oxidative stress in cytotoxicity and neuroinflammation induced by Aβ peptides in AD pathology. It has been reported that the counteraction on oxidative stress by antioxidants and decrease in ROS generation may be a potential approach in the treatment of Alzheimer's disease.

5.2. Prospects of dissertation

From the findings obtained in this study, I propose further research prospects which would be benefit and advance the current understanding about neurodegenerative disorders like AD.

1. Investigating the effect of other oxygenated derivatives of cholesterol on Aβ-induced toxicity to neuronal cells. My studies have demonstrated the mediating role of oxysterols in Aβ interaction with model membranes and toxicity to Jurkat cells and undifferentiated human neuroblastoma SH-SY5Y cells. Further studies with differentiated cells could offer additional important information to advance our understanding about the role of oxysterols in Aβ-induced toxicity.

2. Unravelling the role and effect of multiple oxysterols by introducing them at one time could offer better understanding about the biological processes in the human body. It is known that oxysterols exhibit cytotoxic and cytoprotective properties, thus it will be interesting to study cooperative or competitive role of these compounds.

3. Elucidating the potential of antioxidants (natural polyphenols, capsaicin) to protect against the oxidation by metal such as copper which holds substantial impact in Aβ-induced toxicity. Antioxidants such as resveratrol, epigallocatechin gallate etc. have been reviewed as potential therapeutic candidates for AD (1–4). Capsaicin has been reported to promote the amyloidogenic pathway for APP production in in vivo studies (5). However, the mechanism behind the action of antioxidants against the metals in the progression of AD have not been studied in detail.

References

1. Baur, J. A., and Sinclair, D. A. (2006) Therapeutic potential of resveratrol: the in vivo evidence. *Nat Rev Drug Discov* **5**, 493–506 [online] http://dx.doi.org/10.1038/nrd2060 (Accessed June 2006).
2. Oomen, C. A., Farkas, E., Roman, V., van der Beek, E. M., Luiten, P. G. M., and Meerlo, P. (2009) Resveratrol Preserves Cerebrovascular Density and Cognitive Function in Aging Mice. *Front. Aging Neurosci* **1** [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2874408/ (Accessed December 9, 2009).
3. Saiko, P., Szakmary, A., Jaeger, W., and Szekeres, T. (2008) Resveratrol and its analogs:

Defense against cancer, coronary disease and neurodegenerative maladies or just a fad? *Mutat. Res. Mutat. Res.* **658**, 68–94 [online] //www.sciencedirect.com/science/article/pii/S1383574207000324 (Accessed January 2008).

4. Singh, N. A., Mandal, A. K. A., and Khan, Z. A. (2015) Potential neuroprotective properties of epigallocatechin-3-gallate (EGCG). *Nutr J* **15** [online] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4897892/ (Accessed June 7, 2015).
5. Pákáski, M., Hugyecz, M., Sántha, P., Jancsó, G., Bjelik, A., Domokos, Á., Janka, Z., and Kálmán, J. (2009) Capsaicin promotes the amyloidogenic route of brain amyloid precursor protein processing. *Neurochem. Int.* **54**, 426–430 [online] //www.sciencedirect.com/science/article/pii/S0197018609000308 (Accessed June 2009).

List of Achievements

Articles

1. **Neha Sharma**, KeangOk Baek, Huong T.T. Phan, Naofumi Shimokawa, Masahiro Takagi. Glycosyl chains and 25-hydroxycholesterol contribute to the intracellular transport of amyloid beta (Aβ-42) in Jurkat T cells (*FEBS Open Bio*, DOI-10.1002/2211-5463.12234)

2. **Neha Sharma**, Huong T.T. Phan, KeangOk Baek, Naofumi Shimokawa, Masahiro Takagi. Role of oxysterols in membrane mediated aggregation kinetics of amyloid beta (Aβ-42) (In preparation)

3. **Neha Sharma**, KeangOk Baek, Naofumi Shimokawa, Masahiro Takagi. Influence of oxysterols in the internalization of Alzheimer's amyloid beta (Aβ-42) in SH-SY5Y cells (In preparation)

4. **Neha Sharma**, Yuzuru Takamura, Mun'delanji C. Vestergaard. Electroanalysis of structure-dependent antioxidant activities of polyphenols (In preparation)

Presentations

International conferences

1. **Neha Sharma**, KeangOk Baek, Huong T.T. Phan, Naofumi Shimokawa, Masahiro Takagi. Effect of Oxysterols on the Interaction of protofibrillar amyloid beta (Aβ-42) with the Jurkat Cells, International Symposium on Fluctuation and Structure out of Equilibrium 2015, Aug 22nd 2015, Kyoto, Japan (Poster presentation).

2. **Neha Sharma**, KeangOk Baek, Huong T.T. Phan, Naofumi Shimokawa, Masahiro Takagi. Requirement of 25-hydroxycholesterol for endocytic transport of amyloid beta (Aβ-42) into the Jurkat Cells, Physics of Cells: From Molecule to Systems (PhysCell 2015), Sept 2nd and 3rd 2015, Bad Staffelstein, Germany. (Poster presentation)

3. **Neha Sharma**, KeangOk Baek, Huong T.T. Phan, Naofumi Shimokawa, Masahiro Takagi. Role of oxysterols in the endocytic transport of protein amyloid beta (Aβ-42) in Alzheimer's disease, 17th SPVM National Physics Conference, 2015 International Conference on

Applied Materials and Optical Systems and 2015 International Meeting for Optical Manipulation in Complex Systems Oct 23rd 2015, Cavite state university, Cavite, Philippines. (**Invited oral presentation**)

4. **Neha Sharma**, KeangOk Baek, Huong T.T. Phan, Naofumi Shimokawa, Masahiro Takagi. Enhancement of membrane interaction and endocytic transport of amyloid beta by oxidized derivatives of cholesterol, Alzheimer's Association International Conference (AAIC 2016), July 24th 2016, Canada. (Poster presentation)

Domestic conferences

5. **Neha Sharma**, KeangOk Baek, Huong T.T. Phan, Naofumi Shimokawa, Masahiro Takagi. Effect of oxidized derivatives of cholesterol on endocytic vesicular transport of amyloid beta (Aβ-42), 67th SBJ Annual meeting, Poster presentation, Oct 27th 2015, Kagoshima, Japan (Poster presentation).

6. **Neha Sharma**, KeangOk Baek, Huong T.T. Phan, Naofumi Shimokawa, Masahiro Takagi. The role of oxysterols in the interaction of amyloid beta (Aβ-42) with the Jurkat cells, 9th Symposium on Bio-relevant Chemistry 2015, Sept 10th 2015, Kumamoto, Japan. (Oral presentation).

7. **Neha Sharma**, KeangOk Baek, Huong T.T. Phan, Naofumi Shimokawa, Masahiro Takagi. Involvement of oxysterols in membrane interaction and vesicular transport of Amyloid beta, JAIST-SAST 2015, Nov 11th 2015, Entrance hall, Japan Advanced Institute of Science and Technology, Japan. (Poster presentation).

8. **Neha Sharma**, KeangOk Baek, Huong T.T. Phan, Naofumi Shimokawa, Masahiro Takagi. Interaction and Internalization of Amyloid beta (Aβ-42) with Jurkat cells in presence of oxysterols, 10th Symposium on Bio-relevant Chemistry 2016, Sep 7th 2016, Kanazawa, Japan. (Oral Presentation)

9. **Neha Sharma**, KeangOK Baek, Huong T.T. Phan, Naofumi Shimokawa, Masahiro Takagi., Endocytic transport of protofibrillar Amyloid beta (Aβ-42) in the presence of oxidized derivatives of cholesterol, 68th SBJ Annual meeting ,Sep 28th 2016, Toyama, Japan. (Poster presentation)

10. **Neha Sharma**, KeangOK Baek, Huong T.T. Phan, Naofumi Shimokawa, Masahiro Takagi.

Risk factors for Alzheimer's disease: crucial factors in the endocytic transport of Aβ-42, Jaist Japan India Symposium on Materials Science 2017, March 7th 2017, JAIST, Japan. (Poster presentation)

Awards

The Faculty Members Choice Award (Jaist Poster Challenge), October 11th 2014, Japan Advanced institute of Science and Technology, Japan.