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IN VIVO AND IN VITRO APPROACHES TO STUDY ALZHEIMER'S DISEASE

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder mainly causes cognitive impairments like deterioration in learning, reading, memory and cognition. The pathological hallmark associated with AD is the accumulation of amyloid beta (Aβ) peptides in the brain of the patients. The Aβ aggregation leads to plaque formation which is the main cause of neurodegeneration. The other characteristic which leads to AD involves cholinergic deficits and synaptic loss. In this review, the various *in vivo* and *in vitro* approaches are discussed to understand the pathophysiology of AD.

KEYWORDS: ***Alzheimer's disease, amyloid β, neurofibrillar tangles, gamma secretase, cholinesterase enzymes, acetylcholine receptors, GABA***

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder responsible for affecting around 33 million individuals worldwide. The incidence of this disease rises from 0.5% per year at age 65 to 8% per year at age 85 years and above, it mainly causes cognitive impairments like deterioration in learning, reading, memory (Perry E.K et al 1978). Various risk factors such as diabetes mellitus, obesity, homocysteinemia and hypertension leads to AD. This disease is characterized by abnormal fibrous tangles, β sheet plaques, cholinergic deficits, chronic neuroinflammation, nerve cell death, oxidative stress and inflammatory cascade (Everitt B.J and Robbins T.W, 1997, Masters C.L et al 1985, Rao S.G et al 2000). The defining pathologic hallmark of AD is accumulation of amyloid beta (Aβ) peptides in the brain of the patients along with the aggregation of neurofibrillary tangles leading to progressive neurodegeneration and slowly to death. The aggregation of Aβ is denoted by amyloid beta hypothesis which states that Aβ plaques or peptides are produced by enzymatic cleavage of amyloid precursor proteins (APP) and the enzymes responsible for such cleavage are beta (β) and gamma (γ) secretase enzymes (Selkoe D.J. 2001, Tanzi R.E and Bertram L, 2001, Ma X.H et al 2003). AD is also described by another hypothesis called as cholinergic hypothesis which states that neurodegeneration leads to cholinergic deficits which change the size and number of cholinergic neurons found in basal forebrain in humans and these cholinergic neurons are found to be reduced in AD patients compared to normal aging. Cholinergic neurons from the forebrain are responsible of carrying the role in cognitive functions that includes memory, sensory, attention and processing of thoughts. AD doesn't have any cure till date because the disease mechanism is not fully understood. Various scientists and researchers are unraveling the mystery of AD so that the suitable cure can be found for this disease. This review aims to cover the *in vivo* and *in vitro* approaches to study AD so that the pathophysiology of this disease could be understood and to find the cure for this disease.

PATHOLOGY OF AD

The degeneration of cholinergic neurons in the basal forebrain in AD leads to decreased levels of acetylcholine and the reduction in choline acetyl transferase, the rate limiting enzyme for the synthesis of acetylcholine thus in turn affects the cognitive and memory functions (Coyle J.T et al 1983). Cholinergic system has a major role in cognitive behavior, thus in AD which causes the muscarininc cholinergic receptors disrupt cognitive functions (Perry E.K et al 1978). Accumulation of

the Aβ aggregates extracellular deposits by the increased cleavage of APP by γ-secretases and leading to Aβ formation. Various hypothesis proposed that forebrain cholinergic functions are sensitive for cognition and memory and are prevented in AD to perform their daily function (Perry E.K et al 1978). Aβ peptide aggregation by cleavage of APP also leads to abnormal functions of cortical neurons hence causing memory impairment in AD (Chapman P.F et al 1999). Another study demonstrated that γ-aminobutyric acid (GABA) system plays a key role of controlling the muscuarinic actions in cognition and memory (Selkoe D.J. 2002). Even insulin is involved in priming the system in order to increase the activity of muscuraine signaling (Rao S.G et al 2000, Constantinidis C et al 2002). The disruption muscuraine signaling mechanism is caused by Aβ which directly leads to the defects in cognition and memory. Aβ also disrupts the mAChR activation, which prevents the production and formulation of amyloidogenic with negative regulation and also the accumulation of hyperphosphorylated tau into neurofibrillary tangles (Wozniak M et al 1993, Abbott M.A et al 1999).

CHOLINERGIC DEFICITS

As studied previously, basal forebrain of mammals contains a large collection of neurons and plays an important role in directing the neurons towards cortex and hippocampus regions and also containing choline acetyl transferase, the enzyme responsible for synthesis of acetylcholine (Zabroszky L and Duque A, 2000). The contribution of cholinergic from the basal forebrain plays a key role in mechanisms of cognitive functions such as memory and senses (Grothe M et al 2010, Zabroszky L et al 2012). In normal ageing and in AD levels of the neurons of basal forebrain are found to be reduced. In contrast to normal ageing in AD patients the initial progress of brain atrophy is much more accelerated due to neuronal and synaptic loss and also loss of cholinergic neurons from the basal forebrains in AD can be distinguished from normal ageing. Thus, it is considered the atrophy of the basalis forebrain and synaptic loss in the cortex and hippocampal regions these are the pathophysiological features in AD (Frank L et al 2006).

SIGNIFICANCE OF MOLECULAR GENETICS TO STUDY B AMYLOID AGGREGATION AS A CHARACTERISTIC IN *IN- VIVO* MODELS

The amyloid cascade hypothesis shows alternation in amyloid processing which is the major factor of pathogenic in AD. The genes which are involved in familial AD (FAD) strongly support the hypothesis of amyloid and these genes thus alleviate the production and aggregation of Amyloid β (Kang J et al 1987, Tanzi R.E et al 1991). Therefore identification of these genes which are related to FAD could provide a tremendous tool to determine the mechanism of the disease and thus production of transgenic models to study the pathogenicity. Although the mutation in APP gene is found to be the sole cause of pathogenicity in AD but other genes are also involved in amyloid production (Tanzi R.E et al 1987). These genes even have role in Aβ clearance such as presenilin gene which forms the active site of the gamma secretase complex which is involved in the cleavage of APP and leading to the aggregation of Aβ.

IN VIVO APPROACH

IN VIVO APPROACH RELIES ON STUDYING DIFFERENT VERTEBRATE or invertebrate animal models to demonstrate those features responsible for pathology in AD. Transgenic animal models provide various insights such as over expression of a target gene, knock outs, knock-ins, etc to study pathologic hallmarks in a particular disease (Yang F et al 2000). In order to investigate the entire mechanism causing pathogenesis, it is thus required that the genes in the animal model should be as expressed at the same level as it is seen *in vivo*. Therefore the phenotypes for an amyloid β aggregation should involve disease pathology with amyloid deposits and increasing age to be expressed in tissues or cells.

MOUSE AS A MODEL TO STUDY THE AMYLOIDOSIS THE MAJOR NEUROPATHY IN AD

MOUSE MODELS WITH CRUCIAL SIGNIFICANCE IN FAD

PDAPP

The first transgenic mice to be studied and which have shown AD related phenotype was PDAPP model. This model was engineered to show over expression of human APP model known as Indian mutation one controlled by platelet derived growth factor (PDGF-β). This model has been observed to show Aβ deposition, cerebral amyloid angiopathy, hippocampal atrophy, etc. Various observations were made at different ages and therefore results and expressions of AD characteristics were recorded. Even though this model was found to be perfect for expression of amyloid deposits but it failed to show all the neuropathology of human AD

or tau pathologies. Thus, others models were referred for the latter pathologies. (Moran P.M et al 1995, Masliah E et al 1996, Kobayashi D.T and Chen K.S, 2005).

Tg2576

Another APP model used to study the pathogenicity was Tg2576 mouse model and the expression of human Cdna APP in this model was five times the endogenous levels of APP in PDAPP model and was produced by double Swedish mutations in the mouse. This model was shown to produce higher amyloid production involving dystrophic neuritis, gliosis but lack the synaptic loss, neuronal loss and reduction in the size of hippocampus as in PDAPP. When studied at different age groups were demonstrated with memory deficits in behavioral tests in Morris water maze (Hsiao K et al 1996, Irizarry M.C. et al 1997).

APP23

This model was expressed with Swedish mutation under the influence of Thy-1.2 promoter. This model called APP23Tg is similar to Tg2676 and was shown to over express seven fold of APP and Aβ deposits during first few months. An increase in Aβ deposits were observed with age in size and number. Other than Aβ deposits, neuritic and synaptic degeneration or tau phosphorylations were also seen. After performing behavioral tests with different age groups it is shown to have aged dependent learning deficit with poor memory compared to Tg2676. (Sturchler- Pierrat C et al 1997, Bomemann K and Staufenbiel M, 2000, Sturchler-Pierrat C and Staufenbiel M, 2000).

APP-LONDON

This transgenic mice model expressing human amyloid β $PP_{V717I,}$ the London mutation have been produced, This was responsible to display increased level of amyloid β_{1-42} in the young mice and in aged mouse it was shown diffused plaque and neurofibrillary tangles unlike in PDAPP.

MOUSE MODELS EXPRESSING AMYLOID BETA FORMATION

As the majority of the patients suffer from sporadic forms of AD transgenic models expressing human (Aβ) precursor protein wild type are of the crucial importance than those model expressing mutated forms of APP.

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When this model over expressed with wild type human APP, it displayed no neurodegeneration. But, later, this mouse strain was treated with some another promoter resulting in an increased level of APP production. Vascular amyloid deposits were also detected in this mouse strain.

BRI-WT-AB42

A transgenic mice strain constructed expressed wild type Aβ1-42 together with BR1 a transmembrane protein. The transgene expressed showed high level of astrocytes were found in the mice but no neuropathology was detected in aged animals.

BEHAVIORAL TESTS OR ASSAYS FOR IN MOUSE MODELS

THE MORRIS WATER MAZE (MWM)

MWM is a necessary test in order to measure the hippocampal brain deficits. This test mainly functions on the ability of the animal model under test to be able to posses learning information. The common features of the tests are the ones where the mouse is provided with a particular task and capacity of the mouse to retain those features. And variations compared to this test could be observed in other tests. (Morris R.1984).

RADIAL ARM MAZE

This task provided with the different arms radiating from a platform and the rodents were trained to enter each arm not more than once and grab maximum amount of food or water. This task involves testing the working memory and reference memory. This test allows evaluation of both reference memory and working memory unlike the other tests. (de Toledo Morrell L et al 1984, Ikegami S. 1994, Morgan D et al 2000).

RADIAL ARM WATER MAZE (RAM)

This has been structured the way to escape the limitations as encountered previously in MWM and RAM. The exception to this method is that to search for the arm placed in water among several arms. The rodent is allowed to make use of spatial cues and working arm to know or recognize the platform located in water bath (Arendash G.W. et al 2001).

T-MAZE

This one is based on the cognitive behavioral testing. As the animal starts from the base of the T and chooses one of the arms towards the other end. There are two alternations involved. One is 'spontaneous alternation' two trials are allotted where usually the mouse prefers the other arm not utilized before showing reflection in memory. The other alternation 'rewarding alternation' can be

seen if the mouse is made hungry and then provided with food. But both the alternations are quite sensitive to the region of hippocampus and thus in symptoms of AD (Deacon R.M and Rawlins J.N, 2006).

DROSOPHILA AS A MODEL TO STUDY AMYLOID B AGGREGATION AND TAU NEUROFIBRILS.

Drosophila melanogaster has been used as a crucial and important model during the last decades to be studied for neurodegenerative disorders like AD. *Drosophila melanogaster* is one of the widely used vertebrate model system which exhibits various advantages such as a short life span, small size, large number of individuals and the last but not the least and most important characteristic of simplicity in genetic manipulations. Studies also revealed that on sequencing of the human and drosophila genome- 50% of the genes of *Drosophila* correspond to have homolog in humans (Greenspan R.J 2004, Iijima K et al 2004, Crowther D.C et al 2005).

THE UAS-GAL4 MODEL SYSTEM

Drosophila model system has UAS Gal 4 as their major advantage derived from *Sacchaeromyces cervisae* because this system involves the construction of various strains for expression of genes of interest ranging from different tissues to cell types. In this system the target gene is separated from its transcriptional activator so that the target gene remains silent in the absence of the activator. When the target gene is turned on various effects of misexpression including lethality could be studied. Thus these different activators expressing lines in the form of a library pool has been generated at Bloomington *Drosophila* stock Center at Indiana University (Brand A.H and Perrimon N,1993, Lohr D et al 1995, Phelps C.B and Brand A.H. 1998).

ELEGANS AS MODEL TO STUDY THE EXPRESSION OF AMYLOID B PEPTIDE

C. elegans vertebrate model system can be very advantageous tool for the drug discovery and also have been a perfect model to study basic pathways of complex neurological disorders such as AD. As many genes orthologs of human genes were expressed in this model. From the earlier studies this model has also been used to understand neuronal development at cellular level and previously among other transgenic models the nematode could not express a full length $A\beta_{1-40}$ aggregation but due to an N-terminal cleavage it expressed shortened $A\beta_{3-42}$ peptides, a more potent inhibitor of $A\beta_{1-40}$ aggregation (McGoll G et al 2009). *C. elegans* model system with its cost effective nature, well developed genetics, short life cycle and ability to show tau gene proteins makes it a suitable model to study AD. (Brennet S 1974, Sulston J.E and Horvitz H.R. 1977).

IN VITRO APPROACHES

In vitro approaches used to understand and find the treatment strategies of AD. The various methods integrated by the neurologists or researcher since last decade although any effective solution is yet to be attained. In order to study or inhibit the pathophysiolgical characteristics in AD causing neurotoxicity, hampering neuronal cells and other tissues in cortex and hippocampal region various *in vitro* assays have been established and identified. *In vitro* assays involved in screening of new lead molecules which can target different enzymes, Aβ and other drug target known in AD. Inhibition of cholinesterase enzymes such as acetylcholinesterase and butyrylcholinesterase have been considered by many scientists as suitable drug target by various synthetic drugs, natural herbs and phytocompounds for their anti-cholinesterase activity. Another most widely used *in vitro* approach is of using cell lines in order to investigate the amyloid formation or the protein fibril inhibition by performing cell viability assays. *In vitro* assays also involves measuring antioxidant activity of any products whether natural or synthetic compound.

TARGETING THE AB AGGREGATIONS

Formation of Aβ is mainly due to sequential cleavage of APP by two proteases γ and β-secretases at the N and C terminus of Aβ sequence. Another way of producing Aβ is by α- secretase which not only results in the processing of Aβ peptides but also forms a neurotrophic Aβ alpha peptides. According to numerous studies conducted, which states that aggregation and deposition of Aβ causes neuronal dysfunction. Aβ in the brain also leads to oxidative and neuronal destruction. Thus, by inhibiting the Aβ peptide formation is a major target if we have to find the cure for AD in future.

USING PRIMARY NEURONS AND CELL LINES TO STUDY AB CYTOTOXICITY

Cultured primary neurons or cell lines provide simple and economic means of studying the role of Aβ aggregation state and the mechanism of Aβ toxicity. These could also be used as a primary tool

for the screening of the inhibitors of Aβ toxicity *in vitro*. Simple, inexpensive, and reproducible methods which would allow easy access to the toxicity of Aβ preparations and correlating an Aβ aggregation or fibrillization with its cytotoxicity. Thus cultured cells are exposed to purified Aβ preparations (monomers, protofibrils, and fibrils) or crude preparations containing mixtures of heterogeneous Aβ species. Cell viability can determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay. In the case of primary neurons, subtle changes in neuronal viability can also be quantified using immunostaining (Hartley D.M et al 2010, Jan A et al 2010). A cell line with ability of differentiating into neurons are expressed or transfected with mutated genes in order to generate an AD model. Transfection of these mutated genes of APP into cells or isolation of fibroblastoma cells from the patients of Down syndrome and inducing pluripotent stem (iPS) cells all leads to aggregation of APP (Shi Y et al 2012).

DIFFERENT CELL LINES USED IN-VITRO

NEUROBLASTOMA/SH-SY5Y

Neuroblastoma (SH-SY5Y) cell line has been used to develop a model for Aβ cytotoxicity in AD. By expressing these cells to neuronal lineage, it has been an important key to understand the mechanism underlying the progression of AD (Zhang L et al 2006). Once these cells become mature they are provided with conditions like exposure to toxic Aβ causing neurodegeneration (Zhang L et al 2010).

HUMAN INDUCED PLURIPOTENT STEM CELLS (IPS)

This line have been primary derived from familial AD using primary human fibroblast and various factors required were transfected in order to produce iPS cells. From this two different clones were established using PS1 A246E and PS2 N141I mutations. IPS cells isolated from FAD patient had undergone neuronal differentiation and therefore it was possible to study the effects of presenellin mutations. Live human neurons showed increased $A\beta_{42}$ secretion, thus directly related to amyloid cascade hypothesis. Thus, these lines were found to be effective for drug validation (Yagi T et al 2011).

IMMORTAL RAT HIPPOCAMPAL CELL LINES

Cell lines are derived from embryonic rat hippocampus. The cells are made immortal by oncogene transduction using tsA58 and U19 tsa alleles belonging to simian virus40 to large tumors (Eves EM et al 1992). The use of this cell lines has two important characteristics on immortalization; conditional proliferation and capability to differentiate even after cessation of division to neurons. These cell lines are found to have paramount significance to study the pathogenesis of AD because hippocampal neurons are responsible for cognition and memory. Utilization of this cell line has an advantage over tumor cells as they do not have a malignant nature or lack of any cell lineage specificity (Romeo L et al 2009).

B AND Γ-SECRETASE INHIBITION

β secretase which is known as the first protease responsible for the processing of APP leading to Aβ production in the brain. Therefore inhibition of this protease is of paramount value in the treatment of AD. On the other hand another secretase known as γ-secretase which is also involved in generation of Aβ by APP cleavage and is considered as processing a number of type1 integral proteins also associated with Notch receptor (Kopan R and Ilagan X, 2004). Thus modulation is more preferred than inhibition of this enzyme. (Cole L and Vassar R, 2008).

B SECRETASE INHIBITION

Generating drugs for β secretase provided two different situations: Firstly, as this protease does not carry any functional problems unlike as observed in γ-secretase and abolishing the β secretase activity by genes has reduced the levels of Aβ production (Cai H et al 2001, Lou Y et al 2001). Therefore it was observed that the activity of β secretase can be attenuated without any serious side effects or consequences. Secondly, the problem associated with the production of inhibitors with the desirable drug properties because of no changing nature of active site of the protease (Ghosh A.K et al 2012). Although experiments conducted by using KMI-429 known as β amyloid cleaving enzyme (BACE) inhibitor into lateral ventricles and hippocampus of APP transgenic mice reduced Aβ production in animal and cell levels, but the drugs being employed are inappropriate as small peptides are not suitable for clinical applications. Therefore developmental of new drugs especially small molecules could be used to inhibit the APP dimerization.

BACE1 INHIBITION ASSAY

In order to measure the inhibitory activity of different compounds fluorescence resonance energy transfer (FRET) was used which involves use of multiwell spectrofluorometer. This assay involves

two fluorescent substrate groups. The rate of proteolysis was quantified by the intensity of fluorescence shown by the two different substrates. The inhibitory potency of the compounds used to inhibit BACE-1 was expressed as $IC_{50,}$ values which represent the concentration necessary for maximum inhibition or enzymatic activity (Song Y et al 2011).

Γ-SECRETASE INHIBITION

γ-secretase is a aspartyl protease membrane complex with presenelin. This enzyme is responsible for the production of Aβ by proteolytic cleavage of APP. Present research shows that γ-secretase have been one of the major target for the treatment of AD. The inhibitors used in the inhibition of γ-secretase are based on the fact that γ-secretase has a key role in the notch signaling pathway. Also, γ-secretase modulators have been used as a plausible therapeutics of AD. The modulators employed attenuate the Aβ production to less pathogenic peptides and decreases the proteolysis of APP compared to that of Notch (Jarrett J.T et al 1993, Saito T et al 2011).

CHOLINESTERASE (CHE) ENZYME INHIBITION IN-VITRO

For symptomatic treatment of AD by maintaining the levels of acetylcholine (ACh) in central nervous system (CNS) is one of the important parameter. Inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) is necessary steps to up regulate the levels of acetylcholine in CNS. Past decade has shown a wide range of compounds have been screened for having potential cholinesterase inhibition activity. In the past few years the major concern related to the treatment of AD has been the drug development, which could improve the central cholinergic functions. And this whole strategy has been based on the cholinergic hypothesis of AD which states that a hamper in the form of dysfunction is connected to the loss of cholinergic neurotransmission in the hippocampus and cortex region (Greig N.H 2001). In cholinergic transmission, levels of pyruvate dehydrogenase complex are alleviated and this decrease in pyruvate generation because of glucose depletion leads to acetyl CoA deficits in AD brains. The cholinergic hypothesis was the first to provide a strategy for treatment of cholinergic dysfunctions which led to the production of new drugs. Therefore inhibition of cholinesterase enzymes responsible for hydrolysis of ACh is the usual approach. Tacrine, donepzil, galantamine, rivastagamine, memantine are the drugs clinically used to inhibit ChE (Massoulie J 2002, Brazzolotto X et al 2012). Although these drugs were able to relieve AD patients by improved symptoms, however development of drugs which along with cholinesterase inhibition could also treat on other mechanisms such as neuroprotective effects are still on progress.

ELLMAN'S ASSAY

The inhibition of the cholinesterase enzymes demonstrated by screening the hydrolysis of acetylthiocholine on the basis of the colorimetric method of Ellman's assay (Ellman G. L 1961). The ability of the compounds to inhibit ChE enzymes can be measured or expressed as $IC_{50,}$ which can be used to quantify the concern necessary to decrease the maximum enzymatic inhibition by 50%. A color change in the reaction by development of an anionic molecules 2-nitro-4-thiobenzoate at wavelength of 412nm formed from the reaction of thiocholine with 5,5 dithiobiz-nitrobenzoic acid (Ellman G.L. et al 1961, Sinko . G et al 2007). The variation of absorbance at wavelength 412 nm depends on the substrate concentration and enzymatic activity of AChE or BChE.

BIOPHYSICAL APPROACH TO STUDY AB FORMATION IN-VITRO

Aβ aggregation mechanisms are important in order to devise any strategy for treatment of AD. Therefore, biophysical methods *in-vitro* allows us to study the mechanism underlying the aggregation of peptides or fibrils.

MASS SPECTROMETRY

Mass spectrometry (MS) is mainly utilized for measuring the mass of a molecule that can further be used for characterizing proteins and peptides. The principle involves the sample of interest is first introduced, ionized and transferred into the gas-phase in an ion source, the produced ions are separated in vacuum according to their mass-to-charge ratio (m/z) in a mass analyzer and are thereafter detected in a detector (Edmond de Hoffman V.S. 2002). The problems associated with transferring large biomolecules, such as proteins and peptides, into the gas-phase were resolved when two soft ionizations techniques were introduced in matrix-assisted laser desorption/ionization (MALDI) (Fenn J.B et al 1989) and electro spray ionization (ESI) (Karas M and Hillenkamp F, 1988). Using these techniques, fragmentation can be avoided and multiple charges can be imposed on molecules which will lower the m/z ratio making them easier to detect.

CIRCULAR DICHROISM SPECTROSCOPY

This technique is based on the fact that chiral molecules will react in a different manner with right and left polarized light. The sample such as proteins and peptides to be analyzed are illuminated with plane polarized light which will react with two beams polarized lights such as right and left of the same intensity. The chiral molecules present in the sample will absorb the two beams in two different ways and thus the light coming will be converted into elliptically polarized light. CD is not only used for measuring the difference in absorption of chiral molecules but also for determining conformational changes also utilized for estimation of secondary elements (Woody RW. 1995).

MALDI

The sample is first co-crystallized with a matrix. Then they form a combination of matrix and sample and together form a crystal and they are treated with laser. The matrix molecules will absorb the energy of the laser and will change the matrix into gas phase taking molecules of the samples along with it. In the gas phase the protons are transferred from the matrix to the sample developing ions that are later separated. This method is more compatible with non-solvent and is less sensitive to contaminating compounds (Vorm O et al 1994).

FLUORESCENCE SPECTROSCOPY

It is usually based on the fact that some flurophores or molecules are excited from their ground state to high energy state, then they are said to be in an excited state and a photon is emitted. When the molecules they turn to its original lower energy state then also a photon is emitted with a different wavelength (Lakowicz J.R. 1983)

INTRINSIC FLUOROSCENCE

In proteins or peptides, the aromatic side chains of tryptophan, tyrosine and phenylalanine are called as intrinsic fluorescence. And out of these amino acids tryptophan emits the highest fluorescence. To study the structural changes, the emission spectro sensitivity to binding of substrates can be employed as the binding is affected in protein-protein interaction and denaturation.

THIOFLAVIN T FLUORESCENCE

Thioflavin T (ThT), a cationic benzothiazole dye, is widely used for the detection of amyloid fibrils both in solution and in tissue. Upon binding to amyloid fibrils, this dye exhibits an enhanced fluorescence with an emission maximum at 482 nm and a new absorption peak at 450 nm (LeVine H. 2003). The mechanism behind this phenomenon and the exact structure that ThT binds to is not clear. It has been suggested that the dye, with a hydrophobic part linked to the polar benzothiazole group, could form micelles in aqueous solution and that binding of these micelles to the fibrils would cause the change in fluorescence (Khurana R et al 2005). Binding of monomeric ThT to channels within the β-sheet of the fibrils has also been suggested (Krebs M.R et al 2005).

TRANSMISSION ELECTRON MICROSCOPY (TEM)

This technique is a very important one which utilizes electrons instead of light. In this the resolution of light is not limited as in case of light microscopy with a focused beam of electrons very low wavelength is used. In order to gain an effective contrast, the samples needs to be negatively stained and stains most commonly use are heavy metals salts which will interact with the electron beam, thus when the electron passes through the sample of interest the electrons will display scatterings and those which remain unscattered draws an image on the detection plate which in turn will provide the densities of various parts of the sample.

ANTIOXIDANT ASSAYS IN-VITRO APPROACH

Oxidative stress occurs, due to disturbance in the balancing of certain factors that generate reactive oxygen species (ROS), and these factors exist as a part of regular physiology and also arise from other sources in the body. Oxidative stress is mainly responsible for development of many neurodegenerative diseases like AD, PD, etc. Another implication of the oxidative stress is observed in diseases like cardiovascular or cancer like diseases and therefore initiating the treatment with antioxidants is henceforth a preventive method for attenuating the oxidative stress. Reaction of antioxidants with free radicals employs various mechanisms such as hydrogen atom transfer (HAT), single electron transfer (SET) and in combination of both HAT and SET.

HAT

In this free radical removes one hydrogen atom from the substrate antioxidant and turns the antioxidant to a radical itself. This step or mechanisms was based on the bond dissociation enthalpy and this being an important step in determination of the antioxidant potential. HAT

based assays are oxygen radical absorbance capacity (ORAC) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS).

SET: This is based on the ionization potential which tests the antioxidant potential of the sample. In this antioxidant donates an electron to the free radical and turns into a radical cation. SET based assays involves 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) and ferric reducing antioxidant potential (FRAP).

DPPH ASSAY

This assay is usually more common for screening with natural products and is also performed easily in few steps compared to other methods. The methodology is that a molecule DPPH in order to prevent its dimerization undergo delocalization of the spare electron as a whole and this delocalization gives rise to a change in violet color, with a maximum absorption of 517 nm in the ethanol or methanol solution. When the following solution of DPPH is mixed with substrate, which then donates a hydrogen atom, it then produces a reduced form and loss of violet color is observed. To determine the antioxidant potential of a sample through this free radical scavenging, change in optical density of the DPPH radicals are observed (Brand- William W et al 1995).

FRAP ASSAY

This *in-vitro* antioxidant assay is responsible in determining the antioxidant potential by reducing the ferric irons. The method depends on reducing ferric irons and 2,4,6-Tripyridyl-s-Triazine (TPTZ) to ferrous irons. At absorption of 593nm the change is measured and the optical density readings are measured in a diode spectrophotometer. 3mM of prepared FRAP reagents is mixed with 100 mL of diluted sample, absorbance is recorded after 30 mins of incubation at 37 C. FRAP values can be monitored by comparing the absorption change of the mixture to the increasing concentrations of Ferric irons (Benzie IF and Strain JJ, 1999).

TEAC AND ABTS ASSAY

The Trolox Equivalent Antioxidant Capacity (TEAC) assay involves the formation of the ABTS radical cation from oxidation of 2, 2'-azinobis-3-ethylbenzothizoline-6-sulfonic acid (ABTS) by potassium persulfate. The addition of the pre-formed ABTS radical cation can then be reduced via the addition of compounds containing antioxidants. The reduction in ABTS radical concentration induced by a certain concentration of an antioxidant is related to that of Trolox and produces a TEAC value for that antioxidant, is a solution/mixture containing antioxidants. The change in absorption of ABTS is measured spectrophotometrically at 420 nm (Miller N.J et al. 1993).

ORAC ASSAY

ORAC assay is integrated in order to determine the antioxidant power of different chemical and organic substances. This assay utilizes the test tube analysis and involves agents such as b-PE or fluorescein. But later it was investigated that fluorescein has found to replace b-PE. Trolox (a water soluble analog of vitamin E) is used as a standard to estimate the Trolox equivalent. The ORAC assay it checks for the antioxidant power and the values are expressed in ORAC units and if the ORAC value is greater more effective the antioxidant power be. This assay is based on releasing free radicals AAPH and monitoring the decrease in fluorescence in the presence of free radical scavengers. This assay is being conducted at pH 7 and at an excitation wavelength of 485nm and emission wavelength of 520nm (Caldwell CR.2001).

CONCLUSION

There are different approaches to understand the pathophysiology of AD as mentioned above. Different researchers are using one or other approaches to understand the complex nature of AD. There are also different approaches to find the symptomatic relief, treatment and cure of AD. Looking at the scenario in past decades, combined *in vivo* and *in vitro* approach are required to find cure from this complex disease.

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