Studies on DNA Topology and DNA-Amyloid Beta (Aβ) peptides interaction in relevance to Alzheimer's Disease

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by

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DECLARATION

I hereby declare that the Ph.D. thesis entitled "Studies on DNA Topology and DNA-Amyloid Beta (Aβ) peptides interaction in relevance to Alzheimer's Disease" submitted to the University of Mysore, for the degree of Doctor of Philosophy is the result of work carried out by me under the guidance of Dr.K.S. Jagannatha Rao, Scientist, Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore during 2002-2005.

I further declare that the results contained in this thesis have not been previously submitted for any other degree or fellowship.

(S. Anitha)
Candidate

Dr. K.S. Jagannatha Rao
Scientist
Department of Biochemistry and Nutrition

CERTIFICATE

This is to certify that the Ph.D. thesis entitled "Studies on DNA Topology and DNA-Amyloid Beta (Aβ) peptides interaction in relevance to Alzheimer's Disease" submitted to the University of Mysore, for the degree of Doctor of Philosophy is the result of work carried out by Ms. S. Anitha in the Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore, under my guidance during the period 2002-2005.

(Dr.K.S.Jagannatha Rao)
Guide

Contents

Abbreviations

Aβ:	Amyloid beta
CA:	Control aged
CD:	Circular Dichroism
CY:	Control young
DSB:	Double stranded breaks
ELISA:	Enzyme linked immunosorption assay
EtBr:	Ethidium bromide
FASP:	Functional Assessment Staging Procedure
^{3}[H]:	Radioactive tritium
HBTR :	Human Brain Tissue Repository
MAD:	Moderately affected Alzheimer's disease
NBB :	The Netherlands Brain Bank
NFT:	Neurofibrillary tangles
SAD:	Severely affected Alzheimer's disease
sc DNA:	Supercoiled DNA
SP:	Senile Plaques
SSB:	Single stranded breaks
(TdT):	Terminal deoxynucleotidyl transferase
TEM:	Transmission Electron Microscope
thio T:	Thioflavine-T
TUNEL:	TdT-mediated dUTP Nick-End Labelling

Synopsis of the Thesis

Title of the Thesis: Studies on DNA topology and DNA-Amyloid Beta (Aβ) peptides interaction in relevance to Alzheimer's disease.

Name of the candidate: S.Anitha

Alzheimer's disease (AD) is a complex neurodegenerative disorder of multiple etiology. It is a devastating dementia, correlated with a region specific neuronal cell loss. The complex neuropathology includes cortical atrophy, formation of neurofibrillary tangles, deposition of amyloid β peptide (Aβ) in senile plaques, synaptic loss, oxidative stress, metal deposition, and apoptosis and all these coupled events lead to the neuronal loss. Despite progress in uncovering many of the factors that contribute to the etiology of the disease, the cause of the nerve cell death still remains unknown. It is very well accepted that conformational changes in the proteins namely Aβ play an important role in the neurodegeneration of AD, however little is known about the DNA conformational alteration. The changes in the genetic material of AD, so far documented are, i) mutations in genes, amyloid precursor protein (APP), presenilin-1 (PS-1) and presenilin-2 (PS-2), ii) shift in the DNA from a potentially transcribable euchromatic state to a condensed and probably heterochromatic state, iii) oxidative damage as revealed by damaged products namely 8-OH Guanosine and 8-OH Cytosine and iv) genetic imbalance caused by the tetraploidy status of the genome by the way of unusual replication before the cell death . However there is no data on DNA topology in AD brain. Hence the present study aimed to understand studies pertaining to DNA topology and Aβ-DNA interactions in relevance to neurodegeneration in AD brain.

DNA Topology in AD brain

Brain cells are very complex and have high level of gene expression, 3-4 times more than that found in liver and kidney. In brain cells where the level of gene expression is very high, accumulated DNA damage may have serious consequences in terms of altering genomic integrity consequently affecting the transcriptional fidelity and ultimately the survival of the organism. It was speculated that accumulated DNA damage in the brain might result in conformational changes in the DNA making it ineffective during transcription. Nevertheless the sequence and the DNA topology have been proposed to have a crucial role in DNA functional aspects like replication, transcription, and susceptibility of DNA for oxidative damage and mutations. Extensive studies on handedness and various high ordered structures were studied for the oligonucleotides, but little is known about genomic DNA topology with respect to non Watson-Crick right-handed B-DNA forms, their functional ability and possible implications in the pathogenic features in the brain.

Our study on genomic DNA isolated form the hippocampus region of the brain of AD affected patients revealed altered DNA conformation. Hippocampus was selected as it is one of the first brain regions affected in AD and is involved in the processing of short-term memory and spatial memory. The DNA was isolated form moderately affected (MAD), severely affected (SAD) AD brain and control aged (CA), and control young (CY) brain hippocampus. The Circular dichroism study revealed that SAD DNA showed a typical left-handed Z-DNA conformation whereas CY and CA brain DNA have the usual Watson-Crick B-DNA conformation. Interestingly, MAD DNA has modified B-DNA conformation (probable B-Z intermediate form). Immunochemical study with anti-Z-DNA IgG polyclonal antibody revealed that only the SAD DNA have the reactivity with the antibody, confirming left-handed Z-DNA conformation in SAD. The ethidium bromide binding pattern to DNA and melting temperature (Tm) profiles also revealed the conformational transition from B to Z- DNA in SAD brain. Agarose gel studies of CY and MAD DNA did not show any fragmentation. Both CA and

SAD DNA were fragmented as evidenced by their smear migration pattern. It is interesting to mention that even though there is no difference between CA and SAD DNA as both of them have smeared pattern, EtBr staining is faint for SAD DNA. And this is due to its Z-DNA conformation. The conformational alteration has also been seemed to follow the disease progression as MAD has B-Z intermediate form and SAD has Z-DNA form. The study was also extended to DNA isolated from other regions of the brain, superior frontal gyrus and parahippocampal gyrus, which are also shown to be implicated in the AD. However no conformational alteration in DNA was observed in these two regions. The results reveal that the conformational alteration seems to be region specific. Estimation of DNA single strand (by Nick Translation assay) and double strand breaks (by Terminal transferase assay) in the three regions of the control and AD brain revealed more breaks in hippocampus. The topological change in DNA, particularly the B to Z-transition in the hippocampus, will have tremendous implications in the functional biology of the brain cells e.g. transcription, replication and recombination. Based on the above results we hypothesized that AD etiological factors such as like Aβ, phosphorylated tau, metals and oxidative stress might play role in the conformational transition. AD specific proteins like Aβ and phosphorylated tau are lysine rich and studies have shown that lysine rich proteins favor the conversion of B→Z DNA transition. Tau was reported to bind to and stabilize the DNA. We investigated the Aβ binding to DNA.

Amyloid β (Aβ) peptide DNA interaction

Aβ, a hallmark feature of the senile plaques, is a proteolytic product of the transmembrane amyloid precursor protein. Evidence implicates a central role for Aβ in the pathophysiology of AD. According to the amyloid cascade hypothesis, the accumulation of Aβ deposits as amyloid plaques in the patient's brain is the primary event in the pathogenesis of AD. Under pathogenic conditions, the transition from a random coil to a β-sheet conformation in β-amyloid peptide causes deposits of the amyloid fibrils. The apparent role of Aβ, especially Aβ (1-

42) (the number in the bracket indicates the number of amino acids of the amyloid precursor protein sequence) is now considered as a unifying pathological feature of diverse forms of AD. *In vitro* studies have shown that Aβ (1-42) rapidly aggregates into fibrils and that extracellular fibrillar Aβ peptides induce apoptosis in cultured neurons. On the other hand, recent reports have demonstrated intraneuronal accumulation of Aβ (1-42) in AD vulnerable regions. Intraneuronal Aβ (1-42) accumulation has also been reported in transgenic mice expressing familial AD proteins. In the present study, we provided new evidence for Aβ immunoreactivity in the nucleus of apoptotic hippocampal cells of AD brain. Based on the evidence for nuclear localization of Aβ in AD brain, we hypothesized that Aβ might play a role in modulating DNA topology and possibly contribute to the B→Z-DNA helical transition associated with AD.

In the present study, we evidenced that the Aβ binds to supercoiled DNA (scDNA). Further Aβ (1-42) not only binds to scDNA but also is able to alter the superhelicity of DNA. An initial B→C transition was observed which gradually transformed into ψ-DNA, presumably reflecting a partial DNA collapse into a ψ-phase. In ψ-DNA, the DNA molecules are tightly packed in to toroidal superhelical bundle whose chiral sense is defined by the intrinsic DNA handedness. Specifically, the right-handed secondary conformations such as the B-DNA and C-DNA motifs stabilize a left-handed tertiary conformation. The ψ-DNA conformation induced by Aβ (1-42) is structurally closer to Z-DNA, which was observed in SAD brain. This evidently indicates that DNA topological change induced by Aβ is similar to the change seen in AD brain DNA. Other Aβ peptides like Aβ (1-16), Aβ(1-28) andAβ (1-40) caused modified DNA forms in sc DNA. Our study also provided intriguing observation of the sequence specificity of the Aβ peptide binding. The Aβ peptides showed preferential binding to AT rich sequences (poly d(AT) (AT)) and caused B→A DNA transition. Aβ (25-35), the hydrophobic region of Aβ (1-42) and another potent neurotoxic Aβ fragment has been shown to aggregate the sc DNA. Hence the results are novel interms of their DNA binding property.

Amyloid induced DNA damage: New toxic role

The proposed mechanisms for neurotoxicity for Aβ are diverse and there is no unifying mechanism for the toxicity and one of the mechanisms proposed was induction of apoptosis. Under *in vitro* conditions Aβ (1-42) is reported to be capable of inducing apoptosis in cultured cortical neurons. Neuronal cells treated with Aβ exhibit morphological and biochemical characteristics of apoptosis, including membrane blebbing, compaction of nuclear chromatin and internucleosomal DNA fragmentation. Interestingly studies also suggested that Aβ exert its toxic effect via activation of transcription factors. Studies also correlated between the DNA damage and amyloid deposits however the mechanism remains unclear.

Recent debate with in the AD community has focused on whether fibrillar (amyloid) or soluble oligomers of Aβ are active species of the peptide that ultimately cause the synaptic loss and dementia associated with AD. Studies suggest that Aβ-dependent toxicity can occur before significant extracellular accumulation; possibly involving soluble intracellular Aβ forms.

Our study provided fundamental contribution that Aβ peptide (1-42) could cause DNA damage directly. Agarose gel studies reveal that Aβ (1-42) caused open circular and linear form of DNA in sc DNA by inducing single strand and double strand breaks. Transmission Electron Microscopic (TEM) study also revealed formation of open circular and linear of sc DNA induced by Aβ peptide (1-42). Time dependent DNA nicking of Aβ (1-42) revealed that the soluble forms of Aβ (corresponding to low thio T binding) are more toxic than the fibrillar forms of Aβ (1-42) (corresponding to high thio T binding). Further metal ions Mg and Ca which solubilize the aggregated Aβ enhance DNA nicking ability leading to the hypothesis that soluble forms of Aβ are more toxic than the aggregated forms. Zn metal ion which is shown to promote the aggregation of the Aβ did not have any effect of DNA nicking of Aβ. The DNA

nicking was also shown by other Aβ peptides having different amino acid lengths like Aβ (1-16), Aβ (1-28), Aβ (1-40) and Aβ (1-43). However there exists subtle differences in the DNA nicking caused various peptides and are differentially modulated by metals. DNA nicking of Aβ was inhibited by aurintricarboxylic acid (ATA), a potent nuclease inhibitor. We propose based on the enhancement/inhibition of DNA nicking by metals and ATA respectively that histidine residues are implicated in the DNA nicking activity. Both metals and ATA bind to histidine residues. ATA prevents the formation of the protein-DNA interaction thereby inhibiting the DNA nicking. Metals by binding to histidine residues might result in solubilization of the Aβ and enhance toxicity.
The results evidenced a new toxic role of Aβ in terms of its direct DNA nicking activity and there by it alters the helicity of sc DNA. Our finding of DNA nicking activity of Aβ peptides has biological significance in terms of causing DNA damage.

In a nut shell the study provides the following contributions.

- Evidenced topological change in genomic DNA in the hippocampus region of AD brain.
- Shown Nuclear localization of Aβ in the nucleus in AD affected apoptotic hippocampal neurons.
- Aβ binds to supercoiled DNA and causes helicity change.
- Provided an evidence on DNA nicking in terms of single strand and double strand breaks by Aβ peptides.

The relevance of the above findings in understanding the neurodegeneration in AD have been discussed in the thesis.

CHAPTER - 1

1.1. Introduction

Alzheimer's disease (AD) is the most common form of dementing neurodegenerative disorder among elderly people. Dementia refers to brain disorder that affects person's ability to carry out daily activities. AD is malignant, reducing remaining life expectancy by almost half and raising the risk of death over five years threefold (cancer raises it fourfold). AD is a progressive mental deterioration manifested by memory loss, inability to calculate, visual-spatial disturbances, confusion and disorientation. AD involves parts of the brain that control thought, memory, and language and is characterized by selective primary neuronal and synaptic loss in the hippocampus and cerebral cortex that correlates clinically with dementia (Reviewed by Smith, 1998) (Fig. 1.1). The disease usually begins after age 60 yrs and risk goes up with age, while younger people may also get AD but it is much less common. About 5% of men and women aged 65-74 yrs have AD and nearly half of those aged 85 yrs and older may have the disease. However it is important to note that AD is not a normal part of aging. AD is named after Dr. Alois Alzheimer in the year 1906 (Alzheimer, 1907). Dr. Alzheimer noticed changes in the brain tissue of a woman who had died of an unusual mental illness. He found abnormal clumps (now called amyloid plaques or senile plaques) and tangled bundles of fibers (now called neurofibrillary tangles) in the brain. Today these tangles and plaques in the brain are considered signs or hallmarks of AD. A molecular genetic and histologic analysis of the neurophychiatric case described by Dr. Alzheimer has been reported (Graever et al., 1997).

It was not until the seminal work of Blessed and colleagues (Blessed et al., 1968) that the disease was recognized, not as a rare neurological disorder, but as the most common cause of dementia. In 1930, Divry succeeded in staining another pathologic structure, the SPs using the dye Congo Red, which bound to a component called amyloid on the basis of its physicochemical properties resembling those of polysaccharides. The intraneuronal aggregates called neurofibrillary tangles (NFTs) were shown by Kidd and co-workers at the electron microscopic level to be formed by paired helical filaments (PHFs)

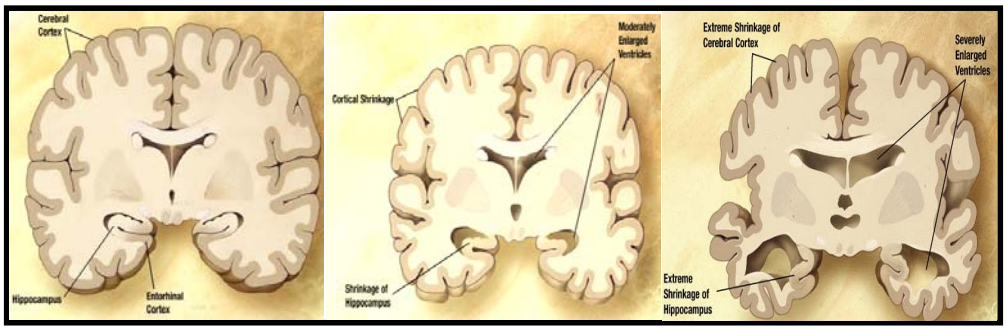

Figure. 1.1. AD begins in the entorhinal cortex, which is near the hippocampus and has direct connections to it. It then proceeds to the hippocampus, the structure that is essential to the formation of short-term and long-term memories. Affected regions begin to atrophy (shrink). These brain changes probably start 10 to 20 years before any visible signs and symptoms appear. (Courtesy: Alzheimer's Disease Education & Referral Center (ADERA), a service of the National Institute on Aging)

thin filaments of 10 nm in diameter (Kurt et al., 1997). It was not until the middle of the 1980s that the core protein component of PHFs was identified as microtubule-associated protein tau by the groups of Wisniewski in the U.S. and Brion in Belgium (Grunke-Iqbal et al., 1986; 1988; Brion et al., 1986). Close to the same time, Glenner and collaborators found that the amyloid deposits were composed of a 4-kDa peptide with a significant beta-sheet structure called beta amyloid (Aβ) (Glenner and Wong, 1984).

Survival of the disease is variable in patients with AD, and they usually die of infections. On an average, AD patients live from 8 to 10 yrs after they are diagnosed, though the disease can last for as many as 20 yrs. Histopathologic characteristic features seen in the brain of AD patients are the presence of SP with Aβ accumulation, NFTs and cerebrovascular amyloid deposits (Selkoe, 1989; Terry, 1994). Extensive neuronal damage and loss of synapses are also found in AD brain (Terry and Katzman, 1983). The kind of neuropathological changes which occur in AD also occur in other neurodegenerative disorders and to a lesser degree in normal aging (Hardy and Allsop, 1991). The cognitive deficits of AD have been attributed to abnormalities in the cerebral cortex and hippocampal formations in that NFTs

and SPs are prominent in these brain regions. Infact, the density of neuritic plaques in the cortex of AD patients at autopsy correlates with the severity of their cognitive defects (Blessed et al., 1968).

Nearly 5 million people in the United States (US) have been affected by AD. Estimates of nearly 22 million people worldwide with AD in near future exist. AD represents the fourth leading cause of death in the US and its profound morbidity has a major socioeconomic impact. In India and South Asian nations, cultural traditions took the onset of senility as a natural process of aging. Since the average life expectancy was rather short in these nations until a few decades ago, the AD was assumed to be an occurrence or the phenomenon of the Western nations. There is a greater recurrence of this disease now noticed in India, especially among the people of middle classes. Sadly, the belief that the AD is a Western phenomenon is not really true. A handful of prevalence studies emerging from the less developed countries have reported lower prevalence rates of dementia than in the industrialized countries. A low frequency of ApoE allele consistent with low prevalence of AD has been reported in India (Chandak et al., 2002). Given the latest United Nations population projections of the astounding figure of 1.1 billion elderly in the less developed countries by the year 2050, even the suggested low prevalence rate would predict a huge number of cases with dementia in the coming decades. Such an eventuality may pose enormous challenges for health and community care and create important socio-economic consequences. This suggests that the risk of dementia of AD in Indian population is fairly comparable to that in the US, although it is in variance with the published prevalence rates in India. Clearly, further prevalence studies aiming at population groups with different demographic characteristics (e.g., urban areas) are needed.

1.2. Symptoms

AD begins slowly and at first, the only symptom may be mild forgetfulness. In this stage, people may have trouble remembering recent events, activities, or the names of familiar people or things. They may not be able to solve simple

math problems. Such difficulties may be a bother, but usually they are not serious enough to cause alarm. However, as the disease goes on, symptoms are more easily noticed and become serious enough to cause people with AD or their family members to seek medical help. For example, people in the middle stages of AD may forget how to do simple tasks, like brushing their teeth or combing their hair. They can no longer think clearly. They begin to have problems speaking, under-standing, reading, or writing. Later on, people with AD may become anxious or aggressive, or wander away from home. Eventually, patients need total care.

1.3. Classification of AD

AD is usually divided into early-onset (presenile) dementia accounting for 25% of cases and late-onset (senile) dementia using the age 65 yrs at onset as the cut off age (Terry and Katzman, 1983), and it is also divided into familial and sporadic forms of disease according to family history (genetic background). Early-onset familial cases comprise 10% and late-onset familial cases 30% of all AD patients (Van Duijn et al., 1991). Most cases of AD are sporadic and familial AD patients only account for about 5% of all AD. Clinical and pathological findings are common to both sporadic and familial AD.

1.4. Diagnosis of AD

The diagnosis of AD is not based on defined qualitative features but rather is differentiated from normal aging based on the number of SPs in the cortical extracellular space and NFTs within the vulnerable neurons. Obviously, the study of a multisystem brain disorder with genetic and sporadic factors and involving essentially half of the aged population is complex. No definite early test or marker for AD exists. Various clinical, neuropsychological, biochemical, pharmacological, and genetic factors have been evaluated as tools for early diagnosis, but none of them has turned out to be unabridged. The clinical diagnosis of AD is based on criteria defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM III-R, American Psychiatric Association

1987) and criteria of the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association Work Group (NINCDS-ADRDA) (Mc Khann et al., 1984). The NINCDS-ADRDA criteria provide guidelines for clinical diagnosis of AD. Definite AD can be confirmed in neuropatholigical examination of the brain tissue either from biopsy or autopsy material. The criteria of the Neurophathology Task Force of the Conortium to Establish a Registry for Alzheimer's Disease (CERAD) (Mirra et al., 1991) have been commonly used to set the neuropathologic diagnosis of AD.

Today, the only definite way to diagnose AD is to find out whether there are plaques and tangles in brain tissue. To look at brain tissue, however, doctors must wait until they do an autopsy, which is an examination of the body done after a person dies. Therefore, doctors can only make a diagnosis of "possible" or "probable" AD while the person is still alive. At specialized centers, doctors can diagnose AD correctly up to 90% of the time. Doctors use several tools to diagnose "probable" AD, including: questions about the person's general health, past medical problems, and the history of any difficulties the person has carrying out daily activities, tests of memory, problem solving, attention, counting, and language, medical tests-such as tests of blood, urine, or spinal fluid, and brain scans. Some of these test results help the doctor find other possible causes of the person's symptoms. For example, thyroid problems, drug reactions, depression, brain tumors, and blood vessel disease in the brain can also cause AD-like symptoms. Some of these other conditions can be treated successfully.

1.4.1. The criteria for the clinical diagnosis of probable AD

-dementia established by clinical examination and documented by the Mini-Mental test (MMT), Blessed Dementia Scale, or some similar examination, and confirmed by neuropsychological tests;

-deficits in two or more areas of cognition;

-progressive worsening of memory and other cognitive functions;

-no disturbance of consciousness;

-onset between ages 40 and 90 yrs, most often after age 65; and

-absence of systemic disorders or other brain diseases that themselves could account for the progressive deficits in memory and cognition.

1.4.2. Criteria for diagnosis of AD

-the clinical criteria for probable AD and

-histopathologic evidence obtained by biopsy or autopsy.

Although familial AD gene testing has been done in a few families on a research basis (Lannfelt et al., 1995). However such testing needs to be done in the context of professional genetic counseling because of the involvement of the wide range of clinical, psychological and ethical as well as genetic issues. Modern imaging techniques have yielded tantalizing possibilities for determining whether an individual suffers from AD or some other form of dementia. These include positron emission tomography (PET) (Fig. 1.2), single photon emission tomography and functional magnetic resonance imaging (MRI) for measurement of brain functional activity, computer-assisted tomography hydrogen magnetic resonance spectroscopy (^{1}H MRS) and MRI for brain anatomy and magnetic resonance spectroscopy (MRS) for measurement of chemical composition (Helpern et al., 2004).

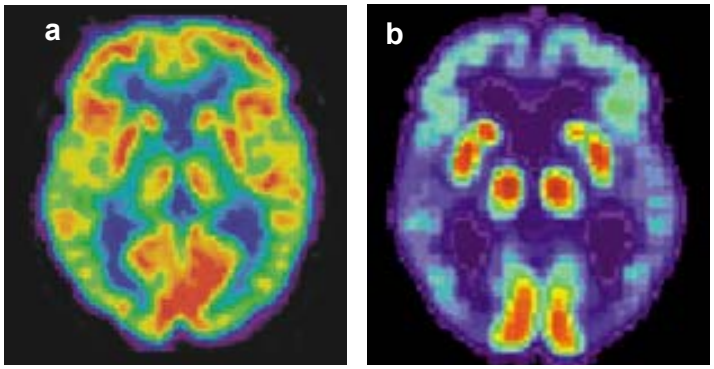

Figure. 1.2. PET Scan (glucose utilization) of (a) Normal Brain and (b) Alzheimer's Disease Brain. Red and Yellow indicate high level of glucose uptake in a living healthy person and a normal control subject. The Alzheimer's patient exhibits large decrease in energy metabolism in the frontal cortex (top of the brain) and temporal lobes (sides of the brain). (Courtesy: Alzheimer's Disease Education & Referral Center (ADERA), a service of the National Institute on Aging).

These techniques have been proved to be useful for positive diagnosis even in at risk individuals and in distinguishing AD from other causes of dementia, such as vascular and frontotemporal dementia (Leslie, 2002; Rapport, 2002; Schuff et al., 2002). Thus, in clinical practice, the diagnosis is based on typical features of the disease (gradual progression of intellectual and functional decline without other distinguishing features), and exclusion of other conditions causing dementia or cognitive dysfunction. Usually an AD diagnosis requires a follow-up of at least six months.

1.5. Genetic risk factors of AD

The genetics of AD is proving to be complex and the number of genetic risk factors associated with this disorder is increasing steadily (Bertram and Tanzi, 2004; Cacabelos, 2004). Recent progress in genetic analysis has made it possible to identify three genes that cause familial AD, which are the genes for amyloid precursor protein (APP) on chromosome 21, presenilin-1(PS1) on chromosome 14, and presenilin-2 (PS2) on chromosome 1(Seherrington et al., 1995; Levy-Lahad et al., 1995a and b; Rogaev et al., 1995; Goate et al., 1991). And the other factor is ApoE allele. ApoE 4 allele has been identified as susceptible genetic risk factor especially in late-onset AD patients.

1.5.1. Mutations in amyloid precursor protein (APP)

The APP gene is located on chromosome 21 (Goate et al., 1991) and a total of six mutations have been described so far, all of which lead to AD. The APP gene mutations are responsible for about 2% of all published cases of familial AD (Tanzi et al., 1996; Campion et al., 1996). The first clue pointing to the involvement of chromosome 21 in AD came from the observation that individuals with Down's syndrome, who have chromosome 21 trisomy, invariably develop clinical and pathological features of AD if they live over 30yrs (Mann et al., 1986). This supported a theory that chromosome 21 can underlie AD phenotype (Wisniewski et al., 1985; Mann, 1985). Sequencing of APP gene revealed mutations in the Aβ sequence. Native APP is a

transmembrane protein of 110 to 135 kDa, with a long extracellular N-terminal segment and a short intracellular C-terminal tail. The important cleavage product of the APP is a 39-43 amino acid peptide, the 4kDa Aβ (Glenner and Wong, 1984; Masters et al., 1985; Kang et al., 1987). Two thirds of sequence of the Aβ localizes to the N-terminal region of the APP and the remaining portion of the Aβ is contained within the transmembrane domain (Kang et al., 1987; Selkoe et al., 1988; Lemaire et al., 1989). APP is normally processed by protease pathways whose effectors are known as alpha (α), beta (β) and gamma (γ) secretase (Fig. 1.3). Depending upon the site of gamma secretase cleavage, the resultant peptides are typically either short (40 amino acids) or long 42/43 amino acid). The longer form of Aβ is thought to be the most amyloidogenic and to be critical in the onset and progression of AD.

Mutations in APP gene instigate overproduction of Aβ, an elevated synthesis of Aβ (1-42) compared to Aβ (1-40) or the synthesis of peptides containing single amino acid substitution. Studies have shown that this substitution triggers an accelerated protofibril formation, which represents an observation that links enhanced formation of neurotoxic assemblies with disease etiology (Lahiri and Greig, 2004).

1.5.2 Mutations in presenilins genes

Mutations of PS1 are the most common finding in patients with familial AD. At least 41 different mutations have been found in the PS-1 gene (Hardy, 1997). PS-1 mutations account for 30-50% of presenile AD families (Hutton et al., 1996). The PS-2 mutations (2% of all early-onset familial AD) are much rarer causes of early-onset familial AD than mutations in the PS-1. In familial AD families with the PS-1 gene mutation, the mean age of onset is earlier (45 yrs; range 29 to 62

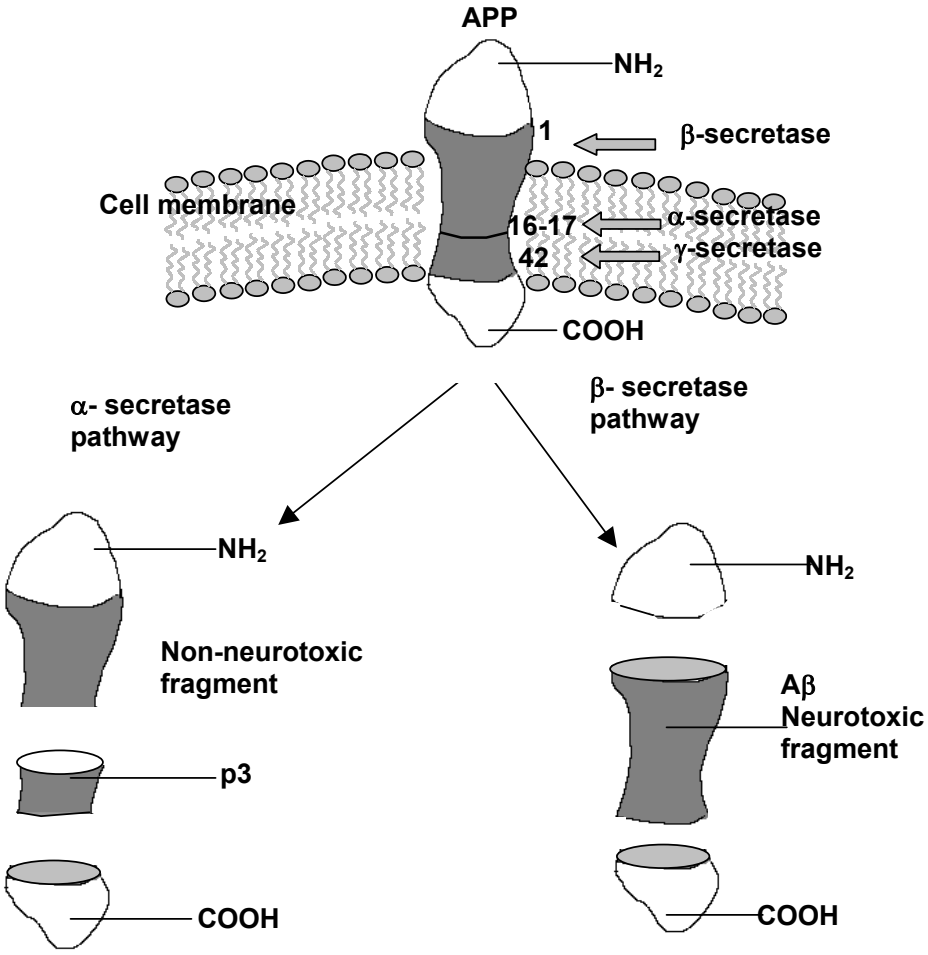

Figure. 1.3. APP cleavage pathway via α - and β-secretases. α-secretase cleaves between residues 16 and 17, producing soluble, non-neurotoxic segment. β-secretase cleavage pathway produces the Aβ (1-42) fragment, which is putatively neurotoxic. The cleavage of the transmembrane region has been proposed to occur inside lysosomes or in the trans Golgi network during the secretory process.

yrs) than in families with the PS-2 gene mutation (52 yrs, range 40 to 88 yrs) and in the APP gene mutation linked families (50 yrs; range 43 to 62 yrs) (Lendon et al., 1997). The mechanism of action of the PS1 and PS2 and their familial AD- associated mutations in AD are not yet known. However, a number of functions have been proposed for presenilins, including direct

involvement as a γ-secretase-like protease in the cleavage of membranous proteins such as APP and Notch, a role in intracellular trafficking and modulation of calcium homeostasis. Increasing evidence indicates that presenilins might also be involved in the modulation of apoptosis. PS1 is involved in normal APP processing therefore, mutations leading to PS1 with different altered sites appear to be responsible for the erroneous cleavage of APP and generation of Aβ (1-42). Further, PS1 interacts with Glycogen synthase kinase, one of the critical proteins involved in tau phoshphorylation. Thus PS1 mutations in some familial AD cases would increase tau hyperphosphorylation at sites that transform tau into protein lacking the ability to associate with the cytoskeleton and generate PHF.

1.5.3. Polymorphism in apoE

ApoE, a major lipoprotein in brain is a lipid and cholesterol transport protein that is synthesized within central nervous system (CNS). ApoE is involved in the growth and regeneration of both peripheral and central nervous tissues during development and following an injury in the CNS. Additionally, apoE is believed to play a pivotal role in the redistribution of lipid and cholesterol during membrane repair and has been postulated to be important for maintaining synaptic plasticity, especially after neuronal injury (Guillaume et al., 1996). In humans, apoE is a single gene with three major allelic variants (ε2, ε3 and ε4) encoding three protein isoforms (Weisgraber and Mahley, 1996).

The gene for apoE is located on the proximal arm of chromosome 19, in fact, in the very same region where a gene for late-onset familial AD is located (Pericak-Vance et al., 1991). These alleles determine apoE polymorphism, resulting in six possible phenotypes ε 2/2, 2/3, 2/4, 3/3, 3/4, and 4/4. ApoE ε 4 allele is recognized as a risk factor for late-onset familial (Strittmatter et al.,1993) and sporadic AD (Saunders et al., 1993) in a dose-dependent manner, that is, the risk increases with an increasing number of ε 4

alleles (Corder et al., 1993). In contrast, ε2 allele appears to have a protective effect for AD. The risk associated with ε 4 may lose its significance after a certain age and may no longer be a risk among the oldest old (Hyman et al. 1995). The exact role of apoE in the pathogenesis of AD is unknown, but isoform-specific differences have been identified in the binding of apoE to Aβ. It is supposed that apoE ε 2 and 3 stabilize the structures, whereas ε n 4 is a susceptibility factor leading to increased vulnerability or a cause of pathologic alterations (Strittmatter and Roses, 1995). Synaptosomes from mice with human apoE4 have shown to be significantly more vulnerable to the oxidative stress associated with Aβ (1-42) than synaptosomes from apoE3 and apoE2 mice. Besides AD, subjects carrying the ε 4 allele also have higher levels of total and low-density-lipoprotein cholesterol, a higher risk for myocardial infarct and coronary heart disease.

1.6. Environmental risk factors

The etiology of AD has not yet been fully understood. There probably is not one single cause, but several factors that affect each person differently. The finding that monozygotic twins may not both develop AD (Rapport et al., 1991) suggests that environmental factors also play major role in the development of AD. Age is the most important known risk factor as AD is an age dependent disorder and its prevalence increases with advancing age. The number of people with the disease doubles every 5 years beyond age 65 yrs. Recent research has identified 2 potential mechanisms related to aging that may contribute to the development of the disease. One is the concept that free radicals (reactive oxygen species (ROS)) produced during cellular respiration may play important role in the process of aging and in the development of AD. Another possible mechanism related to aging is messenger RNA. A novel type of mutations in 'vulnerable' dinucleotide repeats in messenger RNA has been discovered in AD patients (van Leeuwen et al., 1998). In this type of mutation a mutated transcript is produced from a correct DNA sequence by a process called "Molecular misreading". Molecular misreading can be regarded as a genetic biological source of transcript errors that may be involved in cellular

derangement in numerous age-related pathological conditions apart from AD. The resulting '+1 proteins' are prominent neuropathological hallmarks of AD and they are present in most elderly non-demented people also. This suggests that the dinucleotide deletions in the transcript could be one of the earliest events in the neuropathologenesis of AD and a factor in neuronal aging (van Leeuwen et al., 1998).

Besides age, the only risk factor consistently identified in epidemiological studies of AD is family history. Presence of an affected first–degree relative is associated with an approximately fourfold increased risk for AD (Larson et al., 1992), and a total lifetime risk of 23.4 to 48.8% (Farrer et al., 1995). In a number of studies, lifetime risk for first-degree relatives approached 50% (Farrar et al., 1995). Each child of an affected person is at 50% risk for inheriting the abnormal gene and expressing the disease if he/ she lives long enough. But in the case of late-onset AD no obvious inheritance pattern is seen. However, several risk factor genes may interact with each other to cause the disease. The only risk factor gene identified so far for late-onset AD is apoE. It is likely that other genes also may increase the risk of AD or protect against AD, but they remain to be discovered. Severe head trauma and previous depression may also increase the risk of AD (Mortimer et al., 1991; Van Duijn et al., 1991; Jorm et al., 1991). Head trauma or traumatic brain injury (TBI) is also associated with a predilection to the development of AD accounting for between 2% of AD cases (Mortimer et al., 1991), and this may be influenced by the presence of an apo ε4 allele. Carriage of ε4 variant of apoE gene (Mahley, 1988), and the presence of Down's syndrome, resulting in AD-like changes before the age of 40 yrs (Mann et al., 1986) are shown to be the risk factors. One hypothesis is that AD may represent a chronic active inflammatory disease. The brains of AD patients show evidence of mild active inflammation, including microglia and complement activation and the presence of inflammatory cytokines (McGeer and McGeer, 1995). Although the inflammation is likely secondary to more fundamental injuries it may participate in morbid cycle of tissue damage, as it does in systemic diseases like rheumatoid arthritis.

Several epidemiological studies have shown that women on estrogen replacement therapy may be less likely to be diagnosed with AD (Henderson, 1997; Henderson, 2004), and a few small trials claim improvement in female patients with AD who are taking estrogen. The wide spread effects of estrogen in the brain make these results plausible, although the mechanisms have not been fully elucidated. A seminal insight was provided by results of the Nun Study conducted by Snowden and Colleagues (Snowden et al., 1977) the presence of cerebral infarcts, even if small and scarce, raised the risk of dementia by as much as 20 times for those with AD-type lesions. This may be the most common mechanism by which vascular injuries contribute to dementia, since infarcts in the absence of AD-type lesions had few cognitive effects. Thus the vigorous treatment of hypertension and other vascular risk factors and the promotion of healthy diet and exercise could potentially reduce the incidence of dementia. Further low education, head injury and low folate levels are examples of potentially modifiable risk factors of AD (Katzman, 2004) .

1.7. Metal toxicity

There has been growing interest in the area of metals and their implication in AD. Evidences suggest that trace metal homeostasis plays a crucial role in the normal functioning of the brain and any disturbance in it, can exacerbate events associated with AD. Epidemiological evidence suggests that exposure to aluminium (Al) metal from drinking water is associated with increased incidence of AD (Martyn et al., 1989; McLachlan et al., 1996). Inoculation of Al into rabbit brain was demonstrated to produce NFT's resembling the NFT's of AD (Savory et al., 1999). Further Al exerts several neurotoxic effects which mimic that of AD (Veer et al., 2005). A significant piece of circumstantial evidence linking Al neurotoxicity to AD can be derived from a promising clinical trials involving the treatment of AD patients with Al ion chelator, desferrioxamine B (McLachlan et al., 1991). Al concentration was also shown to be elevated in NFTs and SPs. However for various reasons the role of Al in AD has become a matter of controversy. It was shown that Al has low DNA

binding affinity while it has higher affinity towards RNA (Schuurmans, 1990). They also found low Al levels in the nucleus. However, Lukiw et al. (1991) have shown increased amounts of Al in chromatin and they detected high concentration of Al (885.4 μg/g DNA) in DNA isolated from the neuronal nuclei of AD. Recent studies from our lab showed that Al not only strongly binds to DNA but also causes helicity change in DNA (Latha et al., 2002; Champion et al., 1998; Rajan et al., 1996).

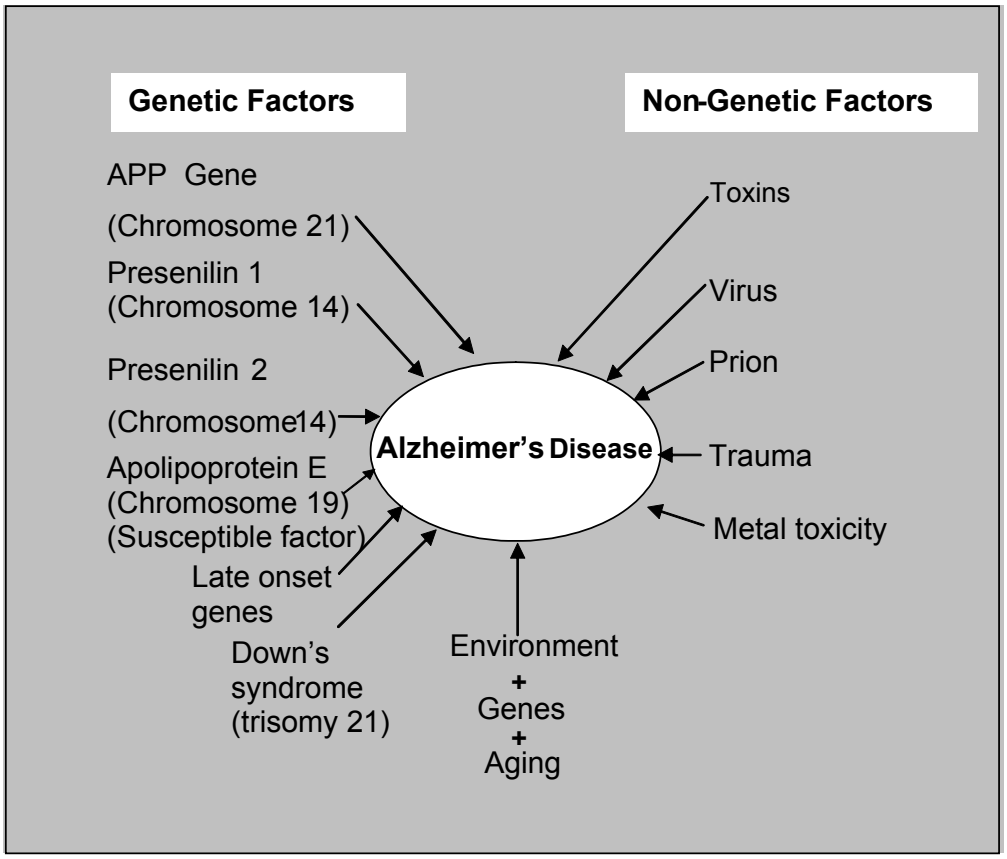

Figure. 1.4. Cross talk between genetic and non-genetic factors. It demonstrates the concept that the AD phenotype is etiologically heterogeneous. The four known genetic factors related to AD are shown on the left, as are additional probably involved genes. On the right are listed four potential non-genetic causes of AD that presently remain speculative and unproved. The bottom arrow in the figure suggests that the most common pathogenesis for AD in the general population may be a complex interaction between aging, genetic predisposition, and environmental factors.

Large body of evidence indicates that the homeostases of zinc (Zn), copper (Cu) and iron (Fe) and their respective binding proteins, are significantly altered in the AD brain (Atwood et al., 1998; Doraiswamy and Finefrock, 2004). There are two generic reactions of metals with reference to neurodegenerative diseases. Firstly, a metal-protein association leading to protein aggregation and secondly, metal-catalyzed protein oxidation leading to protein damage and denaturation. Aβ, a metalloprotein binds to metal ions Zn, Cu and Fe. Zn binding precipitates Aβ and also it may have a protective effect by displacing Cu and Fe enriched in Aβ plaques in AD. On the other hand, there is evidence that Zn can initiate plaque formation by its ability to bind to Aβ under non-acidic conditions and by creating the inflammation, which leads to acidity. Under acidic conditions, which exist in inflammed tissue, Cu displaces Zn (Cuajungco and Faget, 2003). Cu enhances β-sheet formation of amyloid-beta fibrils and this enhancement is potentiated by apoE (Huang, 1999), which in turn promotes the aggregation of Aβ that deposits as SPs. Also binding of redox active Cu and Fe to Aβ produces H_2O_2 that involves the reduction of these metal ions.

1.8. Neuropathology of AD

The background, pathophysiology, and pathology of AD don't constitute a single universally accepted concept either. The clinical heterogeneity of AD is a result of variation in the distribution, quality and severity of pathological changes in the brain. This diversity has even led to the assumption that AD might be considered as a convergent syndrome rather than a single disease. In brief, currently AD is considered to be a multifactorial disease, with a combination of aging, genetic aberration and/or environmental factors triggering the pathological cascade: accumulation of hallmarks of AD pathology, followed or accompanied by cytoskeletal and mitochondrial abnormalities, loss of neurons and synaptic connections, impaired cellular homeostasis, inflammatory reaction, and gliosis, which eventually lead to the clinical presentation of the disease to take place (Fig. 1.4).

Although cerebral atrophy is a typical manifestation of AD, it does not distinguish from AD accurately enough to be diagnostic; this applies to neuroimaging as well as gross inspection at post mortem. However, microscopic examination reveals the critical features of the disease- a cerebral cortex peppered with NFTs and SPs: the classic pathological changes considered as hallmarks of AD (Fig. 1.5. a & b) (Yankner, 1996).

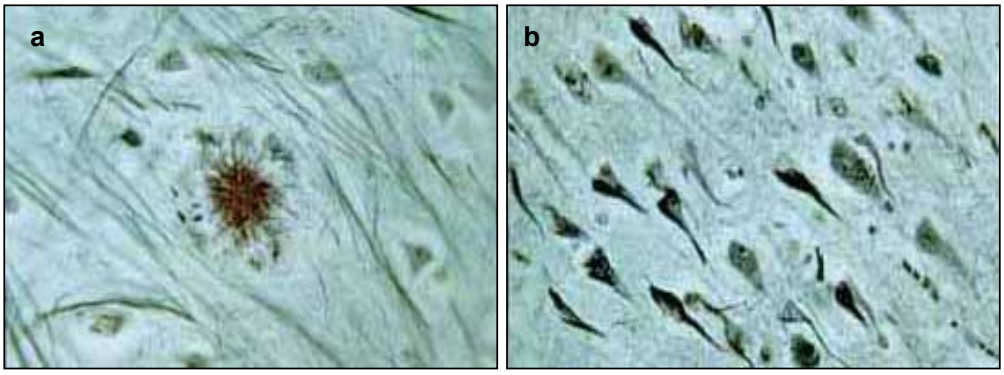

Figure. 1.5. Senile plaques (a) and Neurofibrillary tangles (b) of AD brain. (a)The brain of the Alzheimer's patient will show accumulations of a normal body-protein - beta-amyloid - which has been transformed into a form which is toxic to the brain. This transformed beta-amyloid is found between the nerve-cells of the brain and becomes surrounded by the remnants of the cells which it has killed forming so-called plaques. (b) The other marker of Alzheimer's Disease is known as a Neurofibrillary Tangle. This tangle is caused by the build-up of a protein called Tau inside the nerve-cells of the brain (Courtesy: www.alzforum.org)

1.8.1. Amyloid plaques or senile plaques

SPs are complex and consist of extracellular deposits of amyloid material and are associated with swollen, distorted neuronal process called dystrophic neurites. The term amyloid was coined by Virchow in 1854 being derived from amylose or amylon and means ‘starch like’. Amyloid is a generic term for the primarily extracellular accumulation of fibrillar protein deposits, which have unique tinctorial and structural properties. At least 20 unrelated normally nonfibrillar proteins are known precursors of amyloid (Westermark, 1997).

In AD, Aβ peptide aggregates and accumulates in the brain as diffuse and compact plaques. Diffuse plaques are not associated with degenerative changes; where as compact plaques composed of Aβ fibrils are associated with pathological changes in the surrounding brain parenchyma. The basis of this differential neuronal response is unclear but may relate to the different physical states of Aβ in diffuse and compact plaques (Yanker et al., 1991). The imbalance between Aβ production and Aβ clearance is the basis for the formation of amyloid plaques (Kowalska, 2004)

Aβ, a self aggregating peptide, is derived by proteolytic cleavage of APP. While it was originally thought that Aβ represents an abnormal cleavage product, Aβ has since been established as a normal product of neuronal APP metabolism, found in the cerebral spinal fluid (CSF) and serum of healthy individuals (Haass, 1992; Shoji, 1992). APP and its isoforms are distributed evenly in neuronal cell bodies and their axons and dendrites. The APP – positive neuronal processes showed mesh-like networks and in AD mesh-like networks are generally decreased (Shoji et al., 2000). Differential activity between three different secretase, α, β and γ, at their specific cleavage sites yields a number of different products, including Aβ(1–40) and Aβ(1–42) (Sisodia, 1992). While Aβ (1–40) is the predominant product of this proteolytic pathway, Aβ(1–42) is far more fibrillogenic in vitro and is the major Aβ species present in the core of SP (both AD and non-AD related) (Burdick, 1992; Jarrett and Lansbury, 1993). The deposition of Aβ(1–40) and Aβ(1–42) into SP likely begins with the nucleation of soluble Aβ(1–42) into fibrils followed by accumulation of normally soluble Aβ(1–40)(Jarrett and Lansbury, 1993). Micro-environmental changes in the brain, such as pH, metal ion (Cu, Fe and Al) availability and oxidants, likely impact upon Aβ structural conformation and its deposition in amyloid plaques (Atwood, 1998; Smith, 1997). As of late a great deal of attention has also been focused on the fact that soluble forms of amyloid that are pre-fibrillar may also be involved in AD pathogenesis (Lambert, 1998). Nevertheless, soluble forms of Aβ, including oligomers, correlate quantitatively with the number of SP (Teller, 1996).

The destructive nature of Aβ is evident from a close examination of histological preparations in the immediate vicinity of SPs revealing degenerative dendritic processes surrounding and infiltrating the plaques (Geddes, 1986). Additionally, regions severely affected by disease, including the hippocampus and frontotemporal cortices, show colocalization between Aβ plaques and neuronal cell death (Rogers and Morrison, 1985). This lead investigators to explore whether Aβ is toxic to neurons in both in vitro culture assays and in the intact brain of animals. The results of these experiments at first seemed extremely contradictory, stemming from lot-to-lot variability in the peptide and the lack of proper control over whether Aβ was aggregated into fibrils of β-sheet conformation (Cotman et al., 1992). Nonetheless, it is now established that fibrillation of Aβ is required to obtain neurotoxic effects (Lorenzo and Yankner, 1996; Pike, 1993) and that it is inherently toxic to neurons and clonal cell lines in culture (Pike, 1991; Yankner, 1990). Toxicity of the peptide has been shown to reside in between amino acids 25 and 35 (Pike, 1995). The neurotoxicity of the peptide in vivo was likewise assessed by infusion of the peptide in a variety of animal models. Notably, intracortical injection of Aβ(1–42) or Aβ(25–35) fragments into aged rats or primates produced lesions reminiscent to those seen in AD patients (Kowall, 1992). In *vivo* injection of Aβ into the brain also led to neurodegeneration and dystrophic neurites in hippocampal neurons (Pike, 1991 and 1992).

The source of Aβ toxicity has yet to be established, however, a number of theories have been advanced. The possibility that Aβ may act through a cell surface receptor and thereby trigger an intracellular signaling cascade, while not being eliminated as a possibility, is supported by little experimental evidence. Numerous studies have since supported the idea that an oxidative event is critical for Aβ toxicity (Mattson, 1995). It is thought that the peptide is capable of generating reactive oxygen species (ROS), which is supported by the fact that Aβ peptides can induce the generation of H_2O_2 (Hensley, 1994; Huang, 1999), can stimulate inflammatory cells to produce ROS (Akama, 1998; Butterfield, 1996; Meda, 1995) and that neurotoxicity can be attenuated by administration of anti-oxidants and free radical scavengers, such as Vitamin

E (Behl, 1992). The prooxidant potential of Aβ is also supported by *in vivo* evidence where deposits are associated with oxidative damage (Atwood, 2001; Smith, 1994) and such damage is, like Aβ deposition (Selkoe, 1996), viewed as an extremely proximal event in disease pathogenesis (Nunomura, 1999 and 2000). However, while it is clear that Aβ, either directly or indirectly, promotes oxidative stress and that toxicity can be attenuated by anti-oxidants, the precise mechanism by which amyloid deposition leads to increased oxidative stress remains elusive. Indeed, while studies have suggested that the neurotoxicity of aggregated Aβ is mediated by its ability to induce oxidative stress via the spontaneous generation of free radicals and ROS (Hensley, 1994), this proposition has been questioned on theoretical and methodological grounds (Dikalov, 1999; Turnbull, 2001). Instead, it now appears that the oxidant effects of Aβ are mediated by its interaction with redox-active metals such as Fe and Cu since metal chelation treatment of Aβ significantly attenuates toxicity (Rottkamp, 2000). Significantly, Aβ has an unusually high affinity for both Fe and Cu (Atwood, 1998; Cuajungco, 2000) and is capable of reducing these metals with subsequent production of H_2O_2 and oxidized amyloid (Huang, 1999). The relevance of this mechanism to disease pathogenesis is highlighted by the association of redox active metals with SPs in AD (Sayre, 2000; Smith, 1997). In addition, the deposition of this normally soluble cellular protein promotes a chronic inflammatory response of the AD brain, whereby activated microglia release ROS as part of the respiratory burst (Atwood, 2001). Therefore, Aβ likely has much to answer for with regards to the oxidative damage observed in the AD brain. Aβ (1-42) and Aβ (25-35) were also shown to cause marked oxidative damage to mitochondrial DNA (Pappolla et al., 1999). Further, studies also revealed increased mitochondrial DNA oxidation in AD (Mecocci et al., 1994).

1.8.2. Neurofibrillary tangles

NFT is the most consistent post-mortem characteristic of AD, consisting of phosphorylated fibrillary proteins aggregated within the neuronal cytoplasm.

Tau protein (consisting of 441 amino acids) incorporation into PHFs is a pathognomonic sign of AD. In 1986, Brion and coworkers showed that antibodies against the cytoskeletal protein tau labeled NFTs; these studies were complemented by findings in several laboratories that tau is the main component of PHF (Kosik et al., 1986). NFTs composed of arrays of PHFs are present mainly in the hippocampus, entorrhinal cortex, and amygdala. PHFs are anomalous structures generated by self-aggregation of hyperphsophorylated forms of tau protein that form a compact filamentous network (Maccioni and Cambiazo, 1995). Tau, a member of the microtubules associated protein family, is a phosphoprotein with multiple phosphorylation sites and widely expressed in brain. The primary function of tau is to maintain microtubule stability (Geula, 1998), which plays an important role in axonal transport and morphogenesis.

The neurofibrillary changes in AD may present as NFTs in nerve cell bodies and apical dendrites, and neuropil threads in remote dendrites and in the abnormal nerve endings associated with SPs. Nerve cells with NFTs and synapses within the region covered by SPs are believed to undergo degeneration, become dysfunctional and result in AD. The NFTs developed within the soma of the neuron, get converted into extraneuronal structures, and are finally engulfed and degraded by astrocytes, after degeneration of the parent cell (Braak and Braak, 1991). The development of tangles is a major and possibly the main mechanism of neuronal death in AD (Gomez- Isla et al., 1998). Some groups of neurons are preferentially affected by tangles in AD. For example NFTs frequently occur in areas of the hippocampus that are involved in processing experience prior to storage as permanent memories. This correlates with the clinical deficits observed in the early stages of AD in learning and in the creation of new memories, as well as with the relative preservation of established memories. The neurons at the basal forebrain that provide most of the cholinergic innervations to the cortex are also permanently affected resulting in the cholinergic neurotransmitter deficits.

The discovery of tau as the major subunit of PHFs in AD has markedly increased interest in understanding the structure and function of this protein. In a normal neuron, tau is localized in the axons and neuronal soma (Binder et

al., 1985). The tau and tangle hypothesis argue that in AD, the normal role of tau in stabilizing microtubules is impaired and indeed, in diseased neurons microtubules are gradually replaced by tangles. At present much of what is known regarding tau hyperphosphorylation and cytoskeletal degeneration has come from studies aimed at understanding the neurotoxic effects of Aβ (Yanker, 1990; Lovestone, 1997). Understanding how tau is produced and processed is important to researchers as they attempt to understand how AD unfolds in the human brain. Tau processing also may serve as a target for the development of new therapies to treat the disease. In AD, aggregated tau is hyperphosphorylated and phosphorylation of tau reduces its ability to bind microtubules (Frank et al., 2003). It is the incorporation of excess phosphate groups i.e hyperphosphorylation, which lead to the formation of PHF/tau. Currently promising efforts are underway to establish phosphorylated tau in CSF as a putative disease specific biological marker of AD (Lu and Kosik, 2001; Rachakonda et al., 2004; Olsson et al., 2004). In normal brain, equilibrium between phosphorylation and dephosphorylation of tau modulates the stability of the cytoskeleton and consequently axonal morphology. Amyloid fibril formation has been shown to be one factor associated with tau protein phosphorylation in AD (Busciglio et al., 1995). Further, the pattern of tau phosphorylation correlates with a loss of neuronal integrity and with the severity of dementia (Augustinack et al., 2002). NFTs account for between 2.2 and 17.2% of neuronal loss in AD (Kril et al., 2004).

Immunohistochemical experiments show that tau exists in nuclei of various cell lines, such as human neuroblastoma and human retinoblastoma. In the nucleus, as detected by Tau-1 monoclonal antibody, tau localizes to the nucleolar organizer regions (NORs) (Loomis et al., 1990; Haque et al., 1999; Greenwood et al., 1995). However, whether or not tau is associated with DNA in mature brains *in vivo* needs further study. Further, studies have shown that tau can bind both eukaryotic (calf thymus, plasmid DNA) and phage DNA and synthetic polynucleotides of arbitrary nucleotide sequence when they were double stranded and single stranded DNA did not bind to tau. Hence tau appears to bind to ds DNA and induced DNA conformational bending (Corces

et al., 1980; Hua and He, 2000; Hua and He, 2002 a and b), however the function of tau in the neuronal nuclei is unknown. Microtubule-associated proteins have been shown to have higher affinity for DNA than for microtubules and their removal from the microtubules by DNA causes microtubule breakdown (Corces et al., 1980). Separation of tau from LA-N-5 nuclei in the chromatin fraction has indicated the direct or indirect association of nuclear tau with DNA (Greenwood et al., 1995).

1.9. Oxidative stress

Several different studies have provided evidence implicating oxidative stress as a major pathogenic mechanism in AD. The concept of oxidative stress refers to a state in which oxidant production surpasses the endogenous antioxidant capabilities leading to oxidative molecular damage of the tissue. Such a state can be achieved either by increased production of cellular oxidants and/or decreased concentrations of cell antioxidants including glutathione, Vitamin E, ascorbate, the glutathione peroxidase system, superoxide dismutase (SOD), and catalase. Overproduction of reactive oxygen and nitrogen species such as superoxide, hydrogen peroxide, nitric oxide, and peroxynitrite occurs in AD and is considered to mediate cellular damage and signal apoptosis.

The rationale to examine oxidative stress in aging brain is based on the following aspects a). Brain has high content of easily peroxidizable unsaturated fatty acids (especially high in 20:4 and 20:6 fatty acids); b). Brain requires very high amounts of oxygen per unit weight (about 20% of the total amount used in humans; c). Brain has a high content of both Fe and ascorbate (i.e they are key ingredients in carrying out membrane lipid peroxidation) and d). Brain is not highly enriched in antioxidant protective defenses and this then adds to its otherwise readily poised potential for oxidative damage. While most of the radicals are sequestered in the mitochondria, oxidative insult is exacerbated by age, metabolic demand, and disease conditions such as AD.

Free radicals produced during oxidative stress are speculated to be important in AD and other neurodegenerative diseases (Smith et al., 1995). Free radicals produce tissue damage through multiple mechanisms, including excito-toxicity, metabolic dysfunction, and disturbance of intracellular homeostasis of calcium. Over the past decade, modification to virtually all classes of biomacromolecules indicative of oxidative stress has been described in association with the susceptible neurons of AD: a). DNA and RNA oxidation is marked by increased levels of 8-hydroxyl-2-deoxygaunosine (8OHG). DNA repair deficiency is also noted in AD since higher levels of DNA breaks, DNA nicking and fragmentation are observed in AD patients (Mecocci et al., 1994; Mecocci et al., 1997; Nunomura, 1999; Kadioglu et al., 2004). b).Oxidative modification of proteins is marked by significantly elevated levels of protein carbonyl and widespread nitration of tyrosine residues (Good et al., 1996; Smith et al., 1997). Some specifically oxidized proteins have recently been identified by proteomics (Castegna et al., 2003) and it is notable that many are either enzymes that are related to ATP generation or enzymes involved in glycolysis. Therefore, oxidative modification may lead to metabolic impairment in AD. Moreover, crosslinking of proteins, by oxidative process, may lead to the resistance of the lesions to intracellular and extracellular removal even though they are extensively ubiquitinated (and this resistance of NFTs to proteolysis might play an important role in the progression of AD (Crass et al., 1995). c). Lipid peroxidation is marked by higher levels of thiobarbituric acid reactive substances (TBARS), malondialdehyde (MDA), 4-hydroxy-2-transnonenal (HNE) and isoprostane and altered phospholipid composition (Butterfield, 2001) and d). Modification to sugars is marked by increased glycation and glycoxidation (Castellani et al., 2001; Vitek et al., 1994).

Increasing evidence suggests that the very earliest neuronal and pathological changes characteristic of AD show indication of oxidative damage (Perry et al., 1998; Pratico et al., 2001) indicating that oxidative stress represents a very early contributor to the disease. A systematic examination of the spatiotemporal relationship between the presence of oxidative modification and the hallmark AD lesions at early AD stages suggest that

many markers of oxidative damage are present in susceptible neurons even without neurofibrillary pathology (Nunomura et al., 1999). Further, there is marked accumulation of 8OHG and nitrotyrosine in the cytoplasm of cerebral neurons from Down Syndrome cases in their teens and twenties, which temporally precedes Aβ deposition by decades (Nunomura et al., 2000). Some specific mechanisms proposed for generation of ROS in AD include direct generation by Aβ, activation of microglia by Aβ and possibly electron transport chain defects (Mattson and Goodman, 1995). In AD, ROS have been proposed to interact with amyloid protein to create SPs and amyloid may generate oxygen radicals within the membrane and damage membrane lipids and other proteins (Mattson and Goodmann, 1995).

1.10. Apoptosis

Apoptosis, a programmed cell death by intrinsic mechanism to regulate cell population, characterized by cell shrinkage and nuclear condensation and fragmentation, has been shown to occur extensively in brains from patients with AD. In addition to NFTs and SPs, abundant apoptotic neuronal and glial cells are another pathological hallmark of AD (Su et al., 1994; Cotman and Anderson, 1995; Smale et al., 1995; Lucassen et al., 1997).

Increasing evidence points towards a role for apoptosis in AD. This evidence comes from both *invitro* and *invivo* studies (Smith et al., 1996; Sugaya et al., 1997; Su et al., 1996; Anderson et al., 1995). Two families of proteins have been shown to play a major role in initiating and regulating apoptosis. The first is a family of cysteine proteases, the caspases, which cleave substrates following the aspartic residue and bring about cell death through proteolysis of proteins vital to cellular homeostasis (Cohen, 1997, Nicholson and Thornberry, 1997). The second, is the Bcl-2 family of proteins (Barinaga, 1994; Clearly et al., 1986; Martin et al., 1994) which comprises both antiapoptotic and proapoptotic members and plays an important role in regulating caspases activation (Jacobsen, 1996). The Bcl-2 onco-protein has a protective effect against apoptotic and necrotic cell death (Clearly et al., 1986; Garcia et al., 1992; Hockenbery, 1995; Reed, 1995; Steller, 1995).

Bcl-x is a recently discovered member of the Bcl-2 family of protein (Barinaga, 1994; Frankowski et al., 1995). It has two spliced forms, Bcl-x_s and Bcl-x_l (Minn et al., 1995), while Bcl-x_s promotes apoptosis, Bcl-x_l has a similar apoptosis sparing effect to Bcl-2 (Barinaga, 1994; Minn et al., 1996). Another member of the family is the Bax protein, which is believed to promote apoptosis. The four members of the family are known to hetero and homodimerize with known inhibiting each other's action. Therefore in a cell that expresses various amounts of these proteins, the fate of the cell may be dependent upon the ratio of these proteins (Barinaga, 1994; Oltvai et al., 1993). The absence of the apoptosis-sparing Bcl-2 protein from neurons in the hippocampus of AD patients may indicate that these neurons are vulnerable to apoptotic stimuli. Although the Bcl-x protein expression did not seem to be clearly linked to any individual pathological feature in AD. Strong positive signal for Bcl-x was never detected in controls and overall labeling intensity showed an increase with accumulation of AD-related pathology. It was shown that Bcl-x related phenomenon might possibly be involved in the death or survival of the neurons in AD-affected regions of the brain. The presence of this apoptosis promoting protein in these structures and the absence of Bcl-2 or Bax which are capable of hereto-dimerizing with Bax and counteracting its effect, indicate that the cells with Bax-positive processes in neuritic plaques are likely to be prone to cell death. These results strengthen the evidence that apoptosis-related phenomena are related to cell death in AD and related disorders. However, the pathway involved in triggering apoptotic cell death may be different in different cells and may also differ from one patient to another suggesting that these phenomenon are heterogenous. NFTs formation, Aβ deposits, high concentration of APP, caspase-3, the PS 1 and 2 gene and nitric oxide are considered to be important triggers of neuronal and glial apoptosis (Ugolini et al., 1997).

1.11. DNA fragmentation

The etiopathogenesis of AD is poorly understood. One of the mechanisms responsible for cellular death in AD might be an accumulation of DNA damage

(strand breaks). Mullaart et al. (1989) found that at least a twofold higher level of DNA damage in cortex from individuals with AD as compared to control. The appearance of apoptotic cells in AD brains was analyzed mainly by the method of deoxynucleotidyl transferase dUTP end labeling (TUNEL) staining which indicates cells with fragmented DNA (a phenomenon resulting from DNA fragmentation in apoptotic cells). Several reports show that AD brains exhibit more abundant TUNEL staining than age-matched control brains and that in neurons, the TUNEL signal colocalizes with intracellular Aβ (Su et al., 1994; Anderson et al., 1995). DNA fragmentation was found frequently in cell bodies containing NFT's, as observed using either the anti-tau antibody or the anti-NFT antibody. There was tendency for more strongly anti-tau stained cells to exhibit DNA fragmentation more frequently (Sugaya et al., 1997). Damage to nucleic acids caused by ROS includes base modification, single-strand breaks, and double-strand breaks if singe-strand breaks, and double strand breaks are in close proximity. The predominant marker of oxidative damage is the hydroxylated nucleoside 8-hydroxy-2'-deoxygaunosine (8OHdG). A relatively high basal level of 8OHdG is detected in the brain of control and AD subjects (Gabbita et al., 2001; Lyras et al., 1997, Mecocci et al., 1994). There are conflicting reports as to whether 8OHdG is elevated in nuclear DNA in any AD brain region (Gabbita et al., 2001) but an increase is detected in mitochondrial DNA (Mecocci et al., 1994). Elevated levels of oxidative DNA damage were also found in lymphocytes from patients with AD (Morocz et al., 2002; Kodioglu et al., 2004). In addition increased oxidative damage to RNA in neurons throughout the brain of AD subjects is observed (Nunomura et al., 1999). Evidence for widespread single-and double-strand breaks in AD brains has been provided by in situ labeling methods (Lassmann et al., 1995; Stadelman et al., 1998; Su et al., 1994). The relative contribution of free radical mediated DNA cleavage, ongoing or incomplete DNA repair process or endonuclease cleavage as part of an apoptotic cascade to the generation of DNA strand breaks is not known. Whether DNA fragmentation accumulation over the course of disease or occurs within the perimortem period is yet to be established. A correlation between DNA fragmentation and

Aβ deposition was made in cortex and midfrontal gyrus from patients with AD implicating Aβ as one of the factors of cell injury (Colurso et al., 2003).

1.12. Therapeutic strategies

AD is a very complex disorder and there probably is not one single cause, but several factors are important to describe the etiology of disease. Therefore, combination of compounds, which act as more than one target site, could be useful for the treatment. Owing to the beneficial effects of of α-tocopherol and ascorbic acid in reducing the free radical load in hippocampus and cerebral cortex (Bano and Parihar, 1997), a considerable interest in the use of these vitamins for treatment of AD has been made. Both α-tocopherol and ascorbic acid have been shown to slow the progression of the disease and reduce the risk for AD (Reiter, 1997). Relatively long term antioxidant treatment may delay the onset of disease. Up-regulation of multiple ROS-scavenging enzyme capacities, which may be accomplished with various neurotrophins (Spina et al., 1992) may provide a mechanism for the prevention of neurotoxicity. Cholinergic drugs are routinely used in the treatment of AD to improve cognitive functions. These drugs together with multiple antioxidants have been proposed to be more effective in the treatment of AD than the individual agents alone (Prasad, 2000).

Preventions of mitochondrial oxidative damage and mitochondrial mutations have received considerable interest for the treatment of neurodegenerative disorders (Byrne, 2002). Infusion of nerve growth factor (NGF) in aged animals up regulates cholinergic activity in the basal forebrain and striatum and improves learning and memory as well as motor performance (Mendoza-Ramirez et al., 1995). Estrogens have been shown to modulate neurotransmission, acting as a free radical scavenger and activating nuclear estrogen receptor in intracellular signaling (Behl and Holsboer, 1999). It prevents the formation of Aβ by promoting the non-amyloidogenic α-secretase processing of APP (Xu et al., 1998). Another hormone insulin which has been shown to regulate phosphorylation of tau protein that underlies neurofibrillary lesions in the brains of AD patients and also affects the metabolism of Aβ

(Gasparini et al., 2002) highlighted new potential target for therapeutic intervention. Nonsteroidal anti-inflammation drugs, which inhibit the synthesis of prostaglandins, reduce the rate deterioration of cognitive functions in patients with advanced AD. Natural agents of food supplements that could have multiple properties (e.g., anti-inflammatory, antioxidant, improving mitochondrial energetics, etc.) and cross blood-brain barrier may apparently prevent or delay or maintain the individuals at their higher level of functioning. There is a growing interest in the use of polyphenolic antioxidants to reverse age-related decline in neuronal signal transduction and in cognitive and motor behavior deficits. Extracts of Ginkgo biloba has been shown to have beneficial effects on cognitive function. Neuroprotective properties of extracts of *Asparagus racemosus*, *Convolvulus pleuricauas* and *Withania somnifera* against free radicals induced damage in different brain regions in experimental animals have been investigated (Parihar and Hemnani, 2003 and 2004). However, the mechanisms underlying their neuroprotective effect are not fully understood. Clinical efficacy and potential toxicity of active compounds in trials require further assessment.

Blocking the proteolytic machinery that produces Aβ(1-42) is the preferred strategy. This could be accomplished either by reducing formation of APP or by inhibiting proteolysis of APP to Aβ(1-42). Inhibition of β- and γ-secretase activities and stimulation of α-secretase activity have been suggested to be the most promising strategy for neuroprotection (Citron, 2000). Complete inhibition of either β or γ- secretase alone should be sufficient to block the Aβ production completely. The generation of secretase specific protease inhibitors that penetrates the blood-brain barrier is a challenge for drug discoveries. However, the drug that blocks the function of γ- secretase might not be effective in patients developing AD by factors other than Aβ(1-42) accumulation. The toxic forms of Aβ might be soluble oligomers. Therefore, agents that prevent Aβ nucleation could be more effective than those that merely block Aβ deposition (Wolfe, 2002). The anti-Aβ immunization either active or passive holds great promise in future for treating or even preventing AD (Schenk 2002; Hock et al., 2002).

However, for some people in the early and middle stages of the disease, the drugs tacrine (Cognex), donepezil (Aricept), rivastigmine (Exelon), or galantamine (Reminyl) may help prevent some symptoms from becoming worse for a limited time. Another drug, memantine (Namenda), has been approved for treatment of moderate to severe AD. Treating these symptoms often makes patients more comfortable and makes their care easier for caregivers. Developing new treatments for AD is an active area of research. Now, researchers are studying the NSAIDs celecoxib (Celebrex) and naproxen to find out if they can slow the onset of the disease.Research has shown that vitamin E slows the progress of some consequences of AD by about 7 months. Scientists now are studying vitamin E to learn whether it can prevent or delay AD in patients with MCI. Recent research suggests that ginkgo biloba may be of some help in treating AD symptoms. There is no evidence that ginkgo will cure or prevent AD. Scientists now are trying to find out whether ginkgo biloba can delay or prevent dementia in older people. Curcumin, the active constituent of turmeric was also shown to have therapeutic approach to ameliorate the inflammation and progression of AD (Giri et al., 2004)

AIMS AND SCOPE OF THE STUDY

It is very well accepted that conformational changes in proteins play an important role in some diseases. Moreover, AD and other neurodegenerative disorders like Parkinson's disease and prion diseases are widely acknowledged as Protein Conformational Disorders. However, information on the conformational manifestation of eukaryotic DNA or their biological significance with reference to gene regulation and expression is limited. As genomic integrity is essential for the living organism as any damage or structural change of the genome will affect the transcriptional fidelity and ultimately the survival of the organism. It was speculated that accumulated DNA damage in the brain might result in conformational changes in the DNA making it ineffective during transcription.

In the present study we looked in to the lacunae and aimed to see whether there is any conformational change in the DNA in the AD brain. This is of considerable interest as biological function is often correlated with structure at molecular levels. The role of AD etiological molecules like Aβ in modulating the DNA was also evaluated. The following objectives have been undertaken for the study.

1. To study DNA topology, stability and nucleotide specific DNA damage in brain regions like hippocampus, superior frontal gyrus and parahippocampal gyrus in normal and AD brain tissue.
2. To understand Aβ (1-42) induced changes in supercoiling and superhelicity in DNA. To study Aβ peptides (1-16, 1-28 and 1- 40) interaction with sequence specific Oligomers, poly d(GC).(GC) and poly d(AT). (AT).
3. To study the mechanism of Aβ induced DNA damage.

The results of these studies are presented in the following chapters.

CHAPTER - 2

2.1. Chemicals

The following chemicals have been used

Supercolied Plasmid DNA (pUC 18 scDNA) (Cesium chloride purified, 90% supercoiled structure) and DNA molecular weight markers, proteinase K and RNAse were purchased from Bangalore Genei, India. Amyloid beta peptides, Aβ(1-42), Aβ(1-16), Aβ(1-40), Aβ (1-28), TRIS (hydroxymethyl) aminomethane (Tris buffer) and HEPES buffer (N-[2- hydroxyethyl] piperazine-N' - [2-ethanesulphonicacid]), aurintricarboxylic acid (ATA), trifluro ethanol (TFE), deoxyribonucleotides, 3,3' Diamino benzidine (DAB), poly d(GC).d(GC) and poly d(AT).d(AT) were purchased from Sigma Chemical Co., USA. Aβ (1-42) was also purchased from r-Peptides (USA). Anti-Aβ (1-42) antibody was obtained from Chemicon International, USA. Anti Z-DNA antibody was a gift from Prof. Hasan (Hasan et al.,1995). Oligonucleotides, d(GCCCATGGC) and d(CCGGGCCCGG) were synthesized in Prof Vishwamithra's lab at Indian Institute of Sciences, India (Champion et al., 1995). Apoptosis detection kit for the TUNEL technique was procured form Promega, Maidson, WI (Rao et. al, 1998). *Escherichia coli* DNA polymerase I, terminal deoxynucleotidyl transferase, and [^{3}H] TMP (40 Ci/mmol) were purchased from Amersham (U.K). Copper grids (300 mesh size) were purchased from Sigma chemicals. Uranyl acetate was purchased from B.D.H laboratory chemicals division. All other chemicals, sodium chloride (NaCl), sodium acetate, sodium dodecyl sulphate (SDS), EDTA, phenol, chloroform, isoamylalcohol were of analytical grade and were purchased from SISCO chemical laboratories, Mumbai, India.

2.2. Brain samples

In the present investigation DNA was isolated from postmortem human brain tissues from patients with AD and age –matched controls (with no history of long-term illness, dementia, or neurological disease). Hippocampus region of the human brain was obtained form Human Brain Tissue Repository (HBTR), India and The Netherlands Brain Bank (NBB), Netherlands. Further

parahippocampal gyrus and superior frontal gyrus were also provided by the NBB. At both HBTR and NBB the postmortem interval on average was 6 h. The Functional Assessment Staging Procedure (FASP) (Reisberg, 1998) assessed the clinical severity of the moderate AD and severe AD. This information was collected from Medical history of the patients from HBTR. The brain autopsy samples were subjected to regular AD analysis by HBTR and NBB by regular neuropathological guidelines (Mirra et al., 1993).

2.2.1. Clinical and neuropathological diagnostic criteria applied in the rapid autopsy system

Human brain material is obtained via the rapid autopsy system of the NBB, which supplies post-mortem specimens from clinically well documented and neuropathologically confirmed cases. Autopsies are performed on donors from whom written informed consent has been obtained either from the donor or direct next of kin. The demented patients are clinically assessed and the diagnosis of "probable Alzheimer's disease " is based on exclusion of other possible causes of dementia by history, physical examination and laboratory tests. The clinical diagnosis is performed according to the NINCDS-ADRDA criteria (McKahn et al., 1984) and the severity of the dementia is estimated according to the Global Deterioration Scale (Reisberg et al., 1982). The control subjects have no known history or symptoms of neurological or psychiatric disorders.

Once the brain is removed it is macroscopically examined and immediately dissected following a standard protocol. The specimens are rapidly frozen in liquid nitrogen and stored at – 80^0C. All cases are neuropathologically confirmed using conventional histopathological stains on formalin-fixed specimens. The diagnosis is based on the presence and distribution of the classical hallmarks for each of the disease groups investigated. The NBB uses a scoring system in which the density of SPs, NFTs, disrupted interneuronal-network (dINN), neuropil threads, congophylic plaques and vessels are estimated in Bodian and Congo stains in 4 neocortical areas; frontal; temporal; parietal and occipital. For the staging of the various pathological hallmarks a

combination of a grading system and the Braak staging is applied to all specimens (Mirra, 1997; Braak and Braak, 1991; Braak et al., 1996; Ravid et al., 1998). In addition, apoE allele frequency is determined for each case as a possible risk factor for AD (Nielsen et al., 2003). All cases in the disease groups and controls are well matched for various factors, both ante-mortem and post-mortem. Ante-mortem factors include age, sex, agonal state, seasonal alterations, circadian variation, clock time of death and medication. Post-mortem factors include post-mortem delay, fixation and storage time and laterality (Ravid et al., 1992, 1993; Ravid et al., 1995). Samples are also controlled for quality by monitoring the agonal state of the deceased prior to death (Ravid et al., 1992; Ravid et al., 1995).

2.3. Isolation of DNA from brain tissue

Genomic DNA from brain tissues was isolated by phenol-chloroform extraction protocol using the following steps.

1. Tissue pieces were transferred into an autoclaved porcelain mortar and pestle (all glass wares, mortar, pestle etc have been autoclaved before using them in order to avoid bacterial contamination).
2. Liquid nitrogen was poured into the mortar and the tissue was allowed to freeze.
3. Tissue was grind thoroughly with pestle with frequent additions of liquid nitrogen.
4. Sufficient quantity of liquid nitrogen was poured into the mortar and was swirled. Tissue homogenate was transferred into a sterile tube and the liquid N_2 was allowed to evaporate (used a sterile spatula to transfer the powdered tissue into a graduated tube).
5. Lysis buffer (50mM Tris-HCl (pH 8.0), 10mM EDTA (pH 8.0), 100mM NaCl) was added into the tube along with 15μg per mL of proteinase K and 2% SDS final volume. (1 mL of lysis buffer was used for every 500 mg of tissue. (Note1: Lysis buffer should be pre warmed, 2: Add Proteinase K after first 2 h, optimum: 3 h)).

6. The homogenate was incubated at 37^0 C in a water bath for 12-16 h or over night.
7. After the completion of incubation, the incubated lysate was transferred to an autoclaved 50 mL conical flask and equal volume of tris-saturated phenol (pH 8.0) and mixed thoroughly, either manually or mechanically for 10min.
8. The lysate was centrifuged for 10min at 10,000 rpm at 13^0C.
9. The supernatant was collected into a fresh autoclaved 50 mL conical flasks and ½ volume of Tris-saturated phenol and chloroform: isoamyl alcohol was added and mixed thoroughly. (1 part phenol: 1 part chloroform (C) and isoamylalcohol (IA) mixture (C:IA=23:1).(Note: Tris-saturated phenol was stored in amber colored bottles at low temperature to avoid oxidation of phenol).
10. The supernatant and Tris-saturated phenol-chloroform mixture was centrifuged at 5000 rpm at 4^0C.
11. The upper aqueous layer was collected into a fresh tube and $1/30^{th}$ volume of sodium acetate (pH 5.5) and equal volume of chilled absolute ethanol were added.
12. DNA was precipitated by slowly swirling the tube manually.
    Note: Pre-cooled tubes were used and DNA was transferred into another tube containing 70% alcohol for washing.
13. DNA was washed twice with 70% alcohol and once with absolute alcohol to remove excess salt and vacuum dried.
14. The vacuum dried DNA was dissolved in 1mL of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). The DNA isolated from cells contains RNA also and has to be removed by digesting the preperation with RNAse enzyme. RNAse solution should be kept in boiling water for 10min so as to inactivate any DNAse, because the RNAse may contain DNAse also. (RNAse can also be added before Proteinase K treatment, and incubate at 37^0Cfor 1h and then start Proteinase K (Add 1μg of RNAse /mL of lysis buffer for the 30min)).

2.4. Quantification of DNA

DNA was quantified by recording its optical density (OD) at 260 nm by UV spectrophotometer by following relation.

OD 260nm x 50 x Dilution factor = μg/mLof DNA

Low salt and alkaline buffer are used in order to achieve reproducible A 260nm.

2.4.1. Spectrophotometric equivalents of DNA

The following table (table1) depicts the spectrophotometric equivalents ofdouble and single stranded DNA.

Nucleic acid (One A 260nm)	Amount	Molarity (in nucleotides)
Double stranded (ds) DNA	50μg/ml	0.15 mM
Single stranded (ss) DNA	33μg/ml	0.10mM

Table 1. Correlation between A 260nm and the concentration of DNA.

2.4.1. Estimation of purity of DNA

Absorbance at 280 (A 280nm) nm indicates presence of protein and A 260/A 280 nm ratio denotes the purity of the sample, in terms of protein contamination. A260/A 280 ratio should be 1.5 to 1.8 in order the DNA to be pure. The following table provides the absorbance ratios for DNA and protein sample mixture

Sl. No	% DNA	% Protein	260/280 ratio
1	100	0	1.8
2	75	25	1.4
3	50	50	1.1
4	25	75	0.8
5	0	100	0.7

Table 2: Correlation between purity of DNA and the 260/280 nm ratio.

2.5. Circular Dichroism (CD) spectroscopy

Dichroism is the phenomenon in which light absorption differs for different directions of polarization. In circularly polarized light the magnitude is constant and the direction is modulated. Circular Dichroism (CD) is a special kind of absorption spectroscopy so it occurs at energies where normal absorption occurs. CD investigates are sensitive to structure. The instruments needed are similar to normal absorption instrument, although optical elements are added to produce the polarized light. As a practical matter, it is important to investigate the normal absorption first to ensure that the absorption of solution is not more than 1.0 so that at least 10% of it is transmitted for the instrument to use in determining the Dichroism.

2.5.1. CD of biological molecules

A solution of randomly oriented molecules will be optically active if the molecules are asymmetric. Biopolymers are asymmetric and of course they show optical activity. The optical activity can be seen as the rotation of linearly polarized light due to the differences in refractive index for the two types of circularly polarized light (called optical rotatory dispersion) or by the differences in absorption for two types of circularly polarized light (called circular dichroism). The two phenomena are related and in modern work, CD is measured because it monitors the effect of absorption bands one at a time. In contrast optical rotation is more complicated. It measures the combined effect of all the bands that gives rise to a refractive index. Both right and left-handed circularly polarized light obey Beer's Law and CD is defined as the difference in extinction coefficients

$$\Delta A\ (\lambda) = A_L\ (\lambda) - A_R\ (\lambda)$$

$$= [\varepsilon_L(\lambda) - \varepsilon\ (\lambda)]\ lc = \Delta lc$$

Where subscript denotes the type (handedness) of the light. For historical reasons commercial spectrographs present CD as ellipticity, θ. Ellipticity in degrees is related to the difference in absorbance by $\Delta A = \theta / 32.98$.

The corresponding expression for $\Delta\varepsilon$ is molar ellipticity in deg.dl/mol.dm, $[\theta]$= 3298 $\Delta\varepsilon$ which includes an arbitrary factor of 100.

2.5.2. Electronic CD of nucleic acids

The CD of biopolymers is usually measured in the region of electronic absorption. The nucleotides have an intrinsic asymmetry due to chiral sugar. The interactions of the strong $\pi \rightarrow \pi$ transition of the chromophoric bases with the higher energy transition in the sugar yield a CD of low intensity. Formation of a helical structure is a super asymmetry that gives rise to degenerate interactions between chromophoric bases and results in intense CD spectra. The CD will be characteristic of the conformation of the Nucleic acids, and will depend on base composition, because each base has different transition dipoles. 10.4 base pair B-form is the normal conformation of DNA in aqueous solution at moderate salt. The 10.2 base pair B-form is formed in aqueous solution at high salt in solvent system with a high concentration of methanol and for DNA wound around histone cores. These conformations are easily identified by their very different CD spectra. The positive and negative couplet at about 280nm and 240nm and the intense positive CD at about 190nm are characteristic of 10.4 bp B-DNA form. Collapse of the long wavelength 280nm CD band is the hallmark of the 10.2 bp B-DNA form.

The more intense positive band at 270nm coupled with the negative CD at 210nm and the extremely intense positive CD at 185nm are characteristic of the A-form. Double stranded RNA has a very similar CD spectrum. Poly.d(GC).d(GC) will form the left handed Z-DNA form under certain conditions of salt or alcohol. The Z-form sometimes has negative band at 290nm, but its hallmark is an intense negative band at about 195nm. The CD spectra were recorded on a JASCO J 700 Spectropolarimeter at 25^0 C, with 2mm cell length. DNA structures were characterized using the references of Gray et al. (1978), Hanlon et al. (1975) and Shin et al. (1984).

2.6. Enzyme linked immunosorption assay (ELISA)

Anti-Z-DNA polyclonal antibody was used in ELISA test by following the method of Hasan et al. (1995). In brief, 20 μg of DNA (120 μM (Phosphate)) was used for ELISA studies. DNA dissolved in 0.01M HEPES buffer (pH 7.4) was added along with Anti-Z-DNA polyclonal antibody to micro-titer plates and kept over night at 4^0C . The titer plates were pre-treated with protamine sulfate to facilitate the binding of DNA. Anti-Z-DNA polyclonal antibody was a gift from Prof. Hasan, India. The absorbance of DNA -antibody complex was recorded at 410 nm. The oligomers d(GCCCATGGC), a classical B-DNA and d(CCGGGCCCGG), a classical Z-DNA have been used as negative and positive controls respectively in ELISA test and the structural conformations of these two oligomers were reported using CD and X-ray diffraction studies (Champion, 1995, Champion et al., 1998).

2.7. EtBr binding to DNA and scatchard plots

The binding of EtBr to DNA and DNA protein complexes was measured in 0.01M HEPES buffer (pH 7.0) using F-2000 HITACHI spectrofluorometer by taking 1:1 (w/w) DNA/ EtBr before measuring fluorescence emission. DNA/EtBr solutions were excited at 535 nm, and emission intensity was monitored at 600 nm using HITACHI F-2000 Fluorescence Spectrophotometer.

The amount of EtBr bound and the average number of base pairs per bound EtBr molecule were calculated using Scatchard's equation. The fluorescence was measured using a constant amount of DNA with increasing EtBr against the blank containing no DNA. The measurements were performed keeping the excitation at 535 nm and emission at 600 nm. The path length was 10nm. The amount of EtBr bound to DNA was calculated using the independent binding equation of Scatchard (Scatchard, 1949).

The concentration of bound EtBr concentration (C_b) was calculated from the relation:

$$C_b = Ct\ (I-I_0)/(V-1)I_0,$$

Where Ct is the known total EtBr concentration (picomoles) in the dye-DNA mixture, I is the observed fluorescence intensity at any point of dye-DNA mixture, I_0 is the observed intensity of the identical concentration of EtBr in the absence of DNA, and V is the experimentally determined ratio of the fluorescence intensity of totally bound EtBr to that of free EtBr. Free EtBr concentrations (C_f) were obtained from the relationship $Ct = C_b + C_f$. Ct, C_b and C_f are expressed in picomoles. The amount of bound EtBr/nucleotide (r) was calculated by

$$r = C_b\ (\text{pmoles}) / [\ \text{DNA bp}]_{\text{total}}\ (\text{pmoles}).$$

A plot was made for r vs r/ C_f and the point where the straight line intersects the axis r was defined as the maximum amount of dye bound per nucleotide (n).

2.8. Agarose gel studies

DNA mobility and damage analyses studies were done using the Agarose gel. The electrophoresis was carried out using TAE (Tris –acetic acid-EDTA) conductive media. The percentage of the agarose gels was varied depending on molecular weight of the DNA (lower the molecular weight of DNA higher the percentage of the gel and vice versa). DNA was electrophoresed at 4V/cm at room temperature in TAE buffer. The gels were stained with EtBr (0.1μg/ml) for UV photography.

The topoisomers separation of scDNA and complexes were carried out in presence of chloroquine a drug, which mimics the action of Topoisomerase 1enzyme. Chloroquine was added to samples and also to the running buffer.

2.9. Preparation of linear form of supercoiled DNA (pUC 18)

Linear form of supercoiled DNA was prepared using the restriction enzyme *Eco*RI. The reaction buffer for *Eco*RI contains 50mM Tris HCl (pH 8.0), 100mM NaCl, 10mm $MgCl_2$ and 5mM β-mercaptoethanol. The restriction enzyme: DNA: reaction volume was 1unit: 1μg: 50μl. (One unit is defined as the amount of enzyme required to produce a complete digest of 1μg of lambda DNA in a reaction volume of 50μl in 60 min under optimal conditions of salt, pH and temperature). The mixture was incubated at 37^0C for 60 min. *Eco*RI recognizes GAATTC sequence.

2.10. Quantification of apoptosis in hippocampal neurons: TUNEL method

15-micron brain sections were cut from the hippocampal region of 3 normal and 6 AD affected human brains. Brain sections were first stained for apoptosis using fluorescence TUNEL (TdT-mediated dUTP Nick-End Labelling) technique (Rao et. al, 1998) and co-stained for Aβ(1-42) using monoclonal antibody and developed by 3,3'-diaminobenzidine (DAB) reagent. Co-localization of Aβ in non-apoptotic cells was also carried out. The apoptotic detection system utilizes fluorescein, which measures the fragmented DNA of the apoptotic cells by the catalytic incorporation of fluorescein-12-dUTP at the 3'-OH DNA ends with the enzyme Terminal deoxynucleotidyl transferase (TdT). The latter forms a polymeric tail by applying the TUNEL assay. The fluorescein-12-dUTP-labelled DNA is then visualized directly by fluorescence microscopy. Sections were photographed at 520 nm under 400x magnification using Fluorescence microscope. The slides were scanned using Adobe Photoshop 3.0 and a Nikon scanner (Nikon LS-3510 AF, 365-MM film scanner, # 6070192). The captured images were used for quantification. The cells, which were stained with flourescein (green color), were counted as positive for apoptosis based on the following criteria as reported earlier by Rao et al. (1998): (1) DNA fragmentation with no indication of chromatin margination or other morphologic

changes in the nucleus, (2) Markedly condensed nuclei with a nuclear diameter of 2.5 µm or more, (3) Swollen nuclei containing cytoplasmic fragments of DNA, and (4) Intracellular or extracellular chromatin fragments. A minimum of 18 fields were photographed at an initial magnification of 400x from Hippocampal (CA1) regions of each brain section. Six sections from each brain were evaluated. A total area of 1 mm^2 for each section was evaluated. The number of apoptotic cells was quantified by two independent observers, and the inter-observer variability approximated 2 percent. The number of apoptotic cells was represented as cells/mm^2.

2.10.1. Co-localization of apoptosis and Aβ immunoreactivity

The hippocampus sections (obtained from NBB) were first stained for apoptosis using TUNEL procedure followed by co-staining for Aβ immunoreactivity using anti-Aβ monoclonal antibody and reactivity was developed using ABC kit. In brief, the sections, pre-stained for apoptosis were incubated overnight at 4^0C with anti-Aβ (1-42) antibody. The bound immunoglobulins were detected using the avidin-biotin-peroxidase complex method (Vecta-stain ABC kit, Vector Laboratories, Burlingame, Calif.) and visualized by incubation with 0.05% DAB. The co-localization of apoptosis and Aβ in single hippocampal neuron in CA1 region was imaged by Laser fitted Confocal microscope.

2.11. Estimation of single strand breaks (SSB)

Single strand breaks (SSB) in DNA induced by Aβ peptides were determined through a Nick Translation type of reaction using *E. Coli* DNA Polymerase I (Klenow Fragment). DNA polymerase I is known to add nucleotides at the 3'-OH end of a single strand break, generated by various means, using the other strand as template (Mosbaugh and Linn, 1982). It is essentially a nick translation type of a reaction and if one of the deoxynucleotide triphosphates (TMP) is labelled, then the incorporation of radioproperty into substrate DNA would be proportional to the number of SSB present in the DNA sample. 1 µg

of supercoiled DNA was incubated with 100μM of each peptide with and without 1mM $MgCl_2$ in 10mM Tris-NaCl buffer (pH 7.4) for overnight. During the standardization of the assay, using the cos T fragment of λ phage DNA with a known number of SSB, it was noticed that ~375 TMP residues are added to each 3'-OH groups (SSB). As the incubation mixture contains all the four deoxynucleotides and only one of them is radiolabelled ([^{3}H] TMP), the total number of nucleotides added to the 3'-OH is taken as four times the number of TMP residues, i.e. 1,500 (375 x 4).The number of TMP residues added at each 3'-OH are calculated from the mole percentage of [^{3}H] TMP into DNA and Avogadro's number. Thus each picomole of TMP incorporated would be equivalent to 6x 10 ^{11} molecules. This number is divided by 375 to arrive at the number of 3'-OH groups (SSBs) present in the DNA sample taken for assay, which would be equivalent to 1.6x 10 ^{9}. The assay mixture consisted of the following in a total reaction volume of 50μl: 40mM Tris HCl (pH 8.0), 1 mM β-mercaptoethanol, 7.5 mM $MgCl_2$, 4 mM ATP, 100μM each of dATP, dCTP, and dGTP and 25μM dTTP, 1μCi of ^{3}[H]TTP and 1μg of DNA and 1 unit of *E. Coli* DNA polymerase I (1 unit is the amount of enzyme activity that leads to an incorporation of 10 nmol of total nucleotide into the acid-perceptible fraction in 30 min under the given assay conditions). Incubation was at 37^{0}C for 20 minutes.

SSB were estimated by following the incorporation of ^{3}[H]-thymidine into DNA. After incubation of DNA (treated with Aβ peptides) with *E.coli* polymerase I for 30mins, the DNA was precipitated by adding 10% TCA containing 10 mM Na pyrophosphate in the presence o 100μg of Calf Thymus DNA and bovine serum albumin (BSA) act as carrier molecules. Pellet was separated after centrifugation at 12000 rpm for 15 sec and dissolved in 400 μl NaOH (0.2 N) by vortexing. DNA was reprecipitated in 10%Trichloro acetic acid (TCA) containing 10 mM Na pyrophosphate, which was separated by centrifugation at 6000 rpm for 5 min. The DNA precipitated was washed twice with 5% TCA, and 95% ethanol on Glass Fibre (GF 6). The filters were dried at room temperature for ~12 h and counted for radioproperty in BRAY'S mixture (containing 4gms/litre of PPO, 200 mg/ liter POPOP in Dioxan) in

Beckman LS1800 Liquid scintillation counter. The values are expressed as DPM incorporated into the 1μg of DNA.

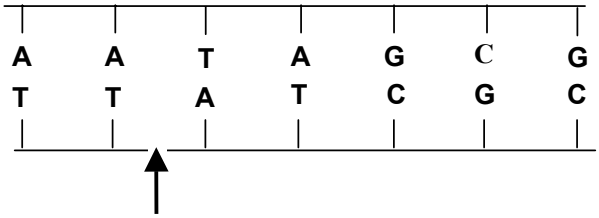

Figure. 2.1. Single-stranded break, one of the most frequent consequential end points of various damages.

2.12. Estimation of double stranded break (DSB)

Terminal transferase has the ability to add deoxynucleotides at 3' ends of duplex DNA, and the conditions for optimal chain length at 3' blunt ends of a double-stranded DNA have been worked out by Deng and Wu (1983). Under these conditions it was shown that ~ 50 nucleotides are added at each 3' –OH groups and double stranded breaks (DSB) are calculated in a similar way as in the case of SSB. The assay mixture for terminal transferase assay consisted of the following in a total reaction volume of 50μl: 100mM sodium cacodylate buffer (pH 7.0), 1mM $CoCl_2$ 0.2 mM DTT, 1μCi of [^{3}H] TPP, 1μg of DNA and 1 unit of the enzyme (1unit is the amount of enzyme activity that leads to an incorporation of 1nmol of a given nucleotide into acid-insoluble products in 60min at 37^{0}C). Incubation was at 37^{0}C for 30min. Each femtomole of TMP incorporated is equivalent to 1.2 x 10^7 3' ends or half that number minus one DSB. The rationale behind this calculation is depicted in fig 2.2 and 2.3.

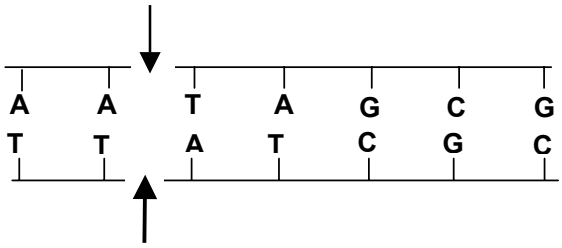

Figure. 2.2. Double-stranded break, an occasional end point of oxidative damages.

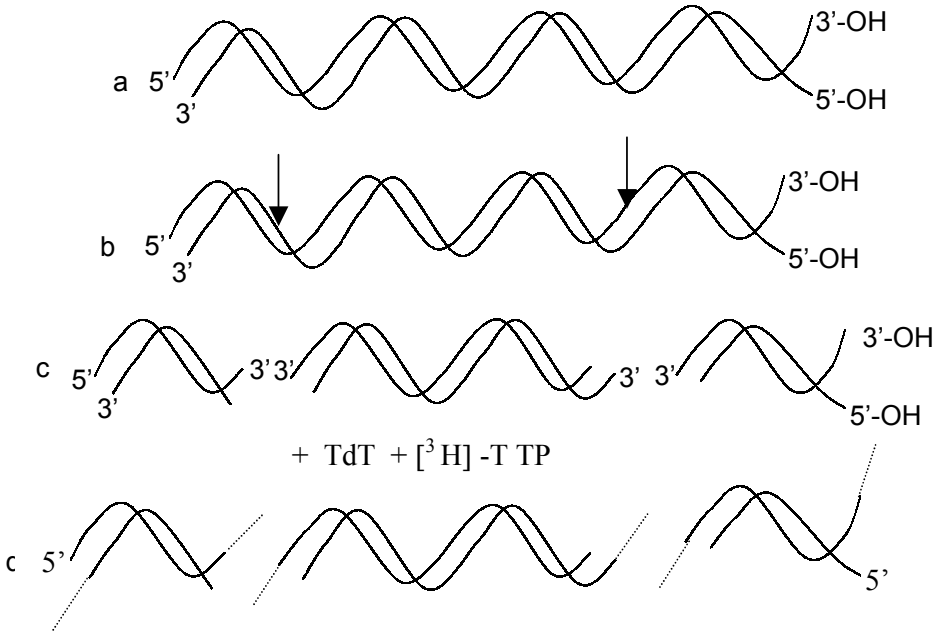

Figure. 2.3. Strategy for calculating the number of DSBs present in genomic DNA using terminal transferase activity. a. Intact linear double-stranded DNA molecule and has two terminal 3'-OH groups. Supposing that two DSBs occurred in that molecule, as shown by arrows, the result would be three pieces of double-stranded DNA with the generation of four more terminal 3'-OH groups. The number of such terminal 3'-OH ends can be assessed by labeling those ends with a radioactive nucleotide using terminal transferase activity. As can be seen, the DSBs that were present in the DNA sample would be equivalent to half the number of terminal 3'-OH groups minus one.

2.13. DNA denaturation: melting temperature profiles

The melting curves for DNA and DNA protein complexes were carried out in 0.01 M HEPES buffer (pH.7.4) by recording UV absorbance at 260 nm at different temperatures (25-95^{0}C, 1^{0}C / min) using a Gilford Response II UV spectrophotometer and Ultrospec 3110 *pro* UV/Visible Spectrophotometer (amersham pharmacia biotech) fitted with thermostat control. Tm values were determined graphically from the absorbance versus temperature plots. The temperature point at which there is a 50% absorbance shift is taken as melting

temperature (Tm) of the DNA sample. The precision of Tm values estimated from variance in three repeated experiments was ± 0.05^0C.

2.14. Electron microscopy of DNA complexes

DNA and DNA-protein complexes were observed under JEOL 1010 Transmission Electron Microscope (TEM). Carbon- coated copper grids (300 mesh size) were glow discharged for 1-2 min (~ 150 millitorr: discharge curent, 2-3 mA), dipped in absolute alcohol for 1 s and were air dried. A drop (5μl) of DNA sample solution was placed on the grid and was allowed to dry in air for 30 min. A second drop was applied after blotting the first drop with filter paper. This process was repeated 4-6 times after which the grids were negative stained by adding a drop of 1% uranyl acetate (pH 5.1) on the grid and blotted with a filter paper after 10 s. The grids were completely dried so as to avoid moisture for EM examination (Thomas and Bloomfield, 1985).

2.15. Thioflavine-T assay for protein aggregation

Aggregation of the Aβ peptides was analyzed using thioflavine-T (thio-T). The induced fluorescence of thio-T has been widely applied to study amyloid formation because of its sensitivity and practicality (Naiki et al., 1989). Fluorescence enhancement is assumed to be common to amyloid fibrils. There is also evidence that thio-T binds to soluble oligomers of Aβ (LeVine, 1993) An aliquot of the peptide (2.0μM) from the stock was collected at different time intervals and treated with 100μM thio-T in 10mM Tris-HCl (pH 7.0). Aggregation was measured by recording fluorescence (λex = 446 nm, λem = 482 nm).

CHAPTER - 3

3.1. Introduction

Brain cells are very complex and have high level of gene expression, 3-4 times more than that found in liver and kidney. In brain cells where the level of gene expression is very high, accumulated DNA damage may have serious consequences in terms of altering genomic integrity consequently affecting the transcriptional fidelity and ultimately the survival of the organism (Bhaskar and Subba rao, 1994). It was speculated that accumulated DNA damage in the brain might result in conformational changes in the DNA making it ineffective during transcription. Information on the conformational manifestation of eukaryotic DNA or their biological significance with reference to gene regulation and expression is limited. This is of considerable interest as biological function is often correlated with structure at molecular level. The changes in the genetic material of AD, a devastating neurodegenerative disorder, documented are: mutations in APP, PS-1, and PS-2 genes (Tanzi et al., 1992; Levy-Lahad et al., 1995; Hutton et al., 1996), shift in the DNA from a potentially transcribable euchromatic state to a condensed and probably heterochromatic state (Crapper et al., 1979), oxidative damage as revealed by damaged products namely 8-OH guanosine and 8-OH cytosine (Smith et al., 1996; Lyras et al., 1997; Sugaya et al., 1997), and genetic imbalance caused by the tetraploidy status of the genome by way of unusual replication before cell death (Yang et al., 2001 and 2003). Microarray analysis study also revealed differential gene expression in AD compared to normal aging in cortex (Ricciarell et al., 2004) DNA molecule is dynamic and polymorphic in nature. Though the right-handed B-form is the most predominant conformation, non B-type conformations also occur in biological systems like Z-DNA, triple helix, tetraplexes, hairpin, cruciform etc., under various conditions (Pohl and Jovin, 1972; Wang et al., 1979; Klysik et al., 1981; Saenger, 1983; Rich, 1993). Both the sequence and the DNA topology have been proposed to have a crucial role in DNA functional aspects like replication, transcription, and susceptibility of DNA for oxidative damage and mutations. Extensive studies on handedness and various high ordered structures were studied for the oligonucleotides (Latha et al., 2002; Cooke et al., 2004) but little

is known about genomic DNA topology with respect to non Watson-Crick right-handed B-DNA forms, their functional ability and possible implications in the pathogenic features in the brain. Therefore the present study pertains to assess the conformation of genomic DNA isolated from hippocampus* (moderately (MAD) and severely (SAD) affected AD patients), parahippocampal gyrus** and superior frontal gyrus*** of control and AD brain. Note*: Hippocampus is part of the limbic system, the emotional system of the brain (in charge of transferring information into memory. It is located inside the temporal lobe and plays a part in memory and navigation. The name derives from its curved shape, which supposedly resembles that of a seahorse (Greek:hippocampus). Hippocampus is first and severely affected region, before other parts of the cortex and later, the frontal lobes too. And this part of brain appears to be absolutely necessary for making new memories. Memory is usually the first thing to start to falter in AD.

The parahippocampal gyrus**, located at the medial temporal lobe, is a key structure in declarative memory processing. The cortical areas that form the parahippocampal gyrus are vulnerable to pathological changes in AD, and its entorhinal and perirhinal subdivisions are both the most heavily damaged cortical areas and the focus for disease onset (Van Hoesen et al., 2000).

Frontal lobes play a major role in the planning and execution of movements. The most anterior region of the frontal lobe is called the prefrontal cortex and includes superior frontal gyrus***, the middle frontal gyrus and the inferior frontal gyrus. This area takes part in the higher aspects of motor control and planning and execution of behavior, tasks that require the integration of information over time. The frontal lobes are reduced in size in AD patients as the result of degeneration of synapses and death of neurons (Mattson, 2004).

3.2. Results

3.2.1. Circular Dichroism (CD) studies

Conformation of DNA was analyzed using CD spectroscopy. The spectra of all the control young (CY) (n=8) and control aged (CA) (n=8) hippocampal brain DNA have right-handed B-DNA conformation with characteristic positive peak at 274 nm, and a negative peak at 245 nm (Fig. 3.1a and b). In MAD hippocampal brain DNA (n=8), CD spectra showed a modified B-DNA with a predominant positive peak at 274 nm, and a positive shoulder peak at 290 nm with a negative peak at 242 nm (Fig. 3.1c). The CD spectra of SAD brain DNA (n=8) have two negative peaks at 245 nm and 290nm, and a positive peak at 260 nm: a characteristic feature of Z-DNA (Fig. 3.1d). CD data was analyzed for all individual DNA samples.

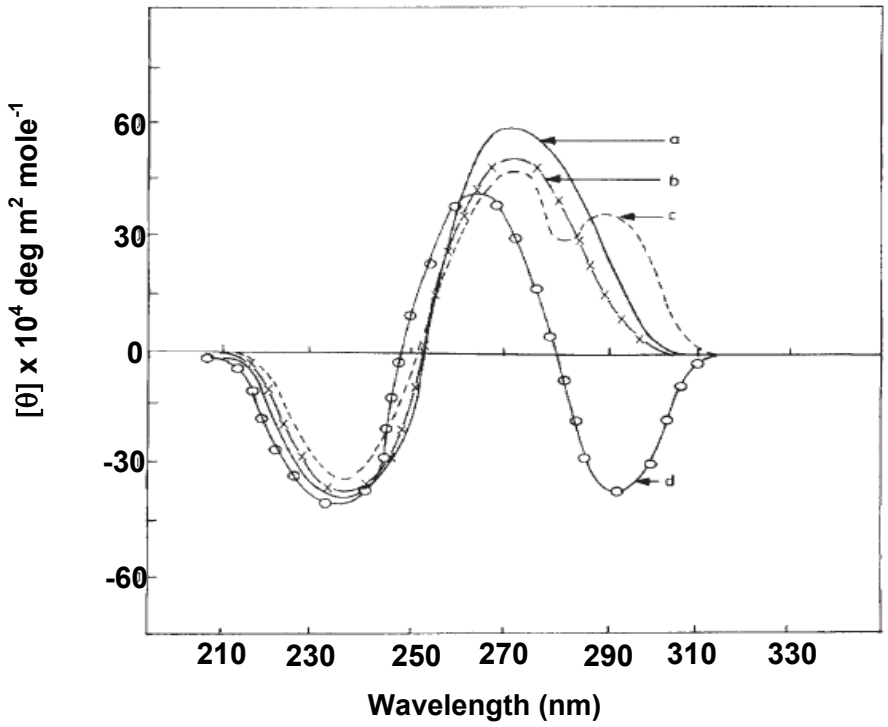

Figure. 3.1. CD spectra of hippocampal DNA (60 μM (Phosphate)) in 0.01 M HEPES buffer (pH.7.4). CD was done on eight samples each of CY, CA, MAD, and SAD DNA. One spectrum from each group has been represented. (a) CY brain DNA; (b) CA brain DNA; (c) MAD brain DNA and (d) SAD brain DNA. Each spectrum represents average of four recordings.

Since the individual CD spectra of each group were the same, which differed only in the magnitude of the positive and negative peaks with a magnitude change of $\pm$ 5(θ) X 10^4 deg m^2 $mole^{-1}$, we present the CD spectra of one sample from each group. DNA structures were characterized following the guidelines of Gray et al. (Gray et al., 1992). In control young and aged brains, four each were male and female. Control brains were obtained from persons who died of either cardiac arrest or by accident. In MAD, six were male and two were female brains. In SAD, four brains were male and four were female.

CD spectra of control (n=4), and AD affected (n=8) parahippocampal gyrus and control (n=4), and AD affected (n=7) superior frontal gyrus DNA revealed B-DNA conformation, and hence only representative CD spectra are provided (Fig. 3.2 and 3.3).

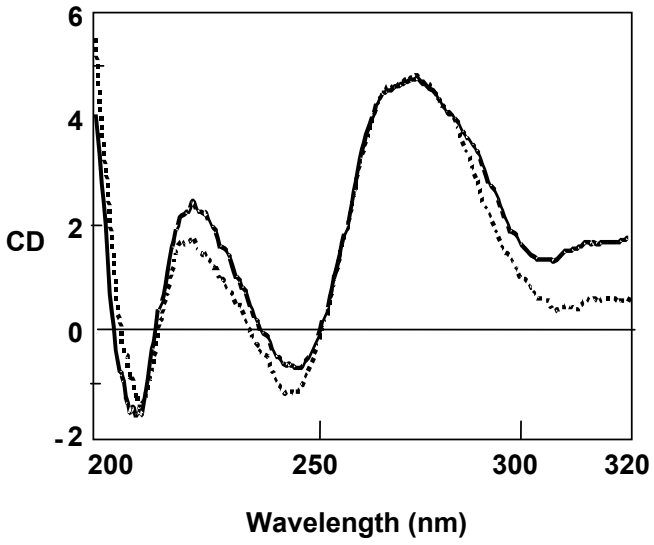

Figure. 3.2. CD spectra of parahippocampal DNA. The recordings were performed 0.01 M HEPES buffer (pH.7.4). Solid line represents AD and dashed line represents control DNA. Both revealed B-DNA conformation. Each spectrum represents average of 4 recordings.

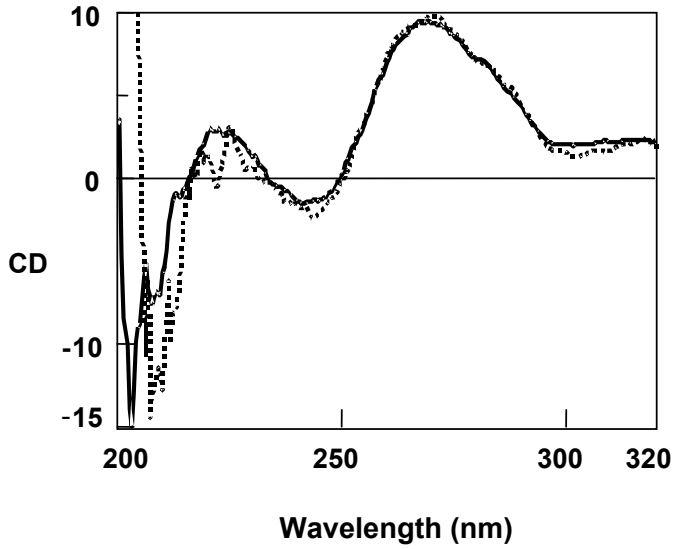

Figure. 3.3. CD spectra of superior frontal gyrus DNA. The recordings were performed 0.01 M HEPES buffer (pH.7.4). Solid line represents AD and dashed line represents control DNA. Both revealed B-DNA conformation. Each spectrum represents average of 4 recordings.

3.2.2. ELISA studies

Immunochemical study with anti Z-DNA IgG polyclonal antibody (Fig. 3.4) revealed that only SAD DNA (Fig. 3.4d) have the reactivity with the antibody, indicating left-handed Z-DNA conformation in SAD. Oligonucleotides d(GCCCATGGC), a classical B-DNA and d(CCGGGCCCGG), a classical Z-DNA have been used as negative and positive controls respectively. MAD, parahippocampal gyral and superior frontal gyral DNA of AD did not show any reactivity towards anti Z-DNA antibody.

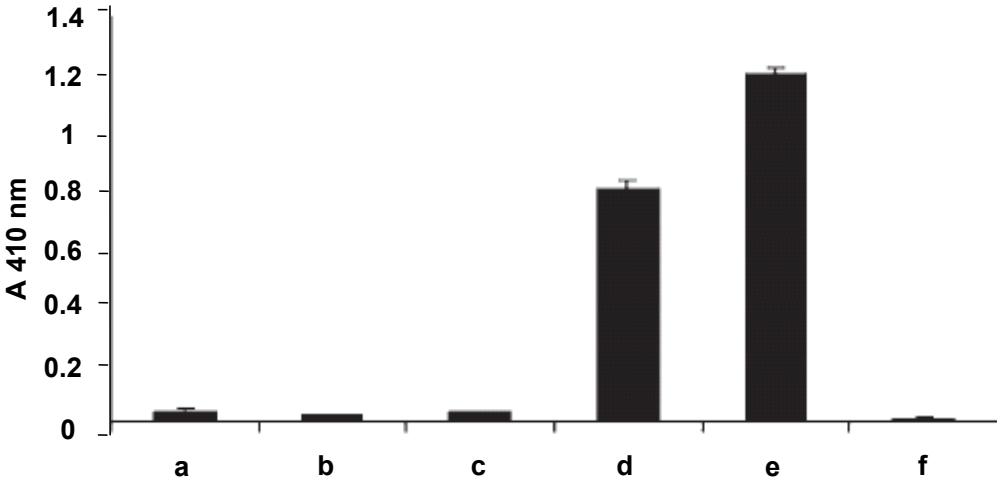

Figure. 3.4. Immunochemical assay for Z-DNA using anti Z-DNA polyclonal antibody. ELISA was done on eight samples each of CY, CA, MAD, and SAD DNA (120 μM [Phosphate]). (a) CY brain DNA; (b) CA brain DNA; (c) MAD brain DNA; (d) SAD brain DNA; (e) d(CCGGGCCCGG); (f) d(GCCCATGGC). The values are mean ± SD.

3.2.3. Melting temperature studies

Melting temperature (Tm) data indicated that CY and CA brain hippocampal DNA have Tm of 65^0C ±1.5^0C and 59^0C±1.2^0C respectively (Fig. 3.5a and 3.5b). SAD DNA as expected for Z-DNA, have high Tm 79^0C±1^0C, since Z DNA is stable as well as a high- energy conformer (Fig. 3.5d). MAD DNA have an unusual bi-phasic melting profile which is biologically a rare phenomenon with two Tm values: 54^0C ±1.8^0C and 84^0C ±1.3^0C, and thus MAD DNA shows a clear- cut violation of all or none response of long duplex DNA (Fig. 3.5c). The major difference between B-DNA and Z-DNA is the distance between phosphate groups on the opposite strands of the molecule, which is comparatively greater for B-DNA than that of Z-DNA. This is the possible reason for high Tm for SAD DNA.

Z-DNA molecule has varying conformation of phosphate groups in the backbone. The two forms of Z-DNA, Z-I and ZII have been identified for the cstal structure of d (C_p G_p C_p G_p C_p G_p). The distance between the two closest phosphate groups in ZI and ZII were 7.7 and 8.6 A^0 respectively. The P…P (Phosphate-Phosphate) separation across the double helix is 17.4 A^0 for

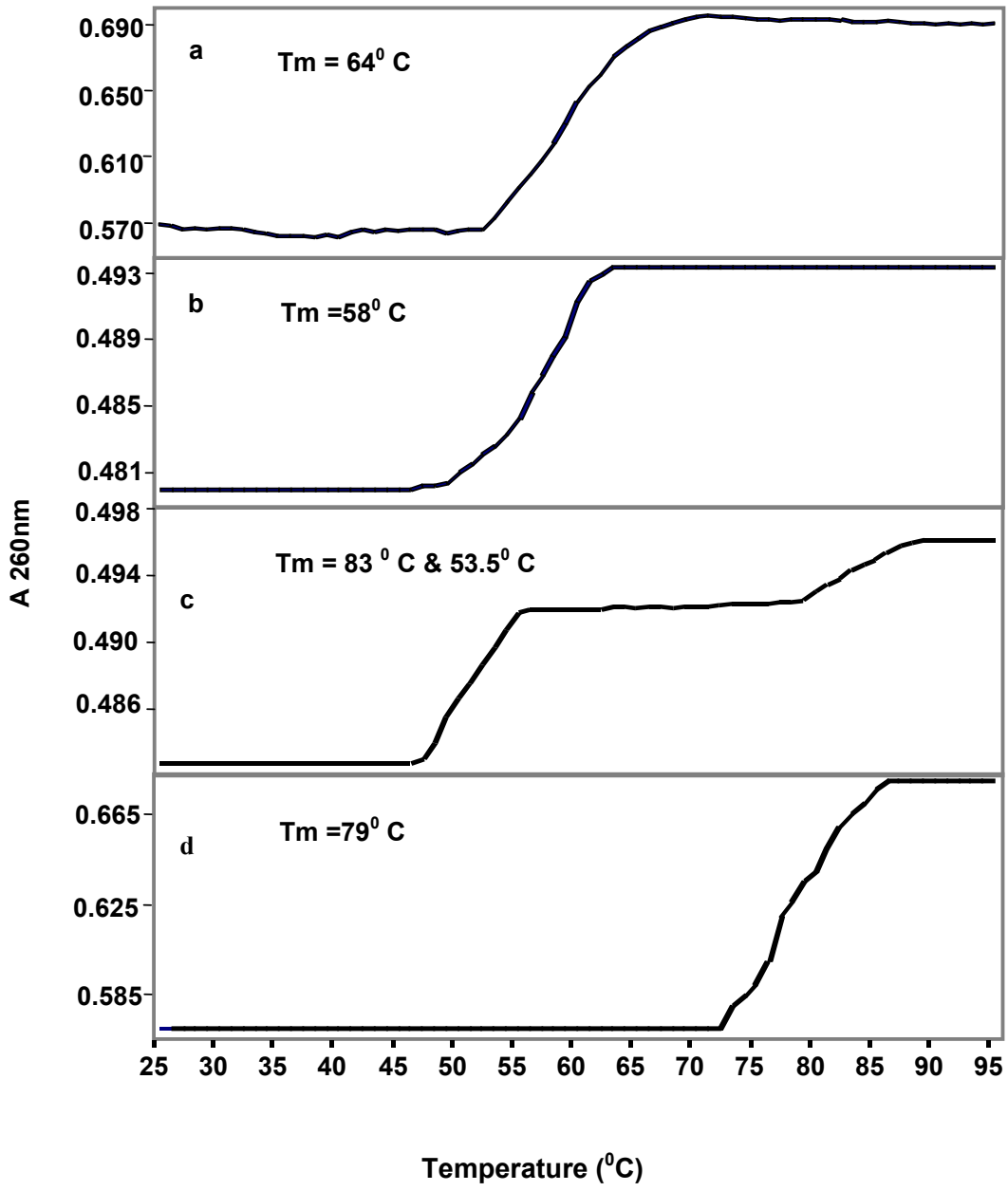

Figure. 3.5. DNA melting temperature profiles of control (young and aged) and AD Hippocampus in 0.01M HEPES (pH 7.4). (a) CY brain DNA; (b) CA brain DNA (c) MAD and (d) SAD brain DNA.

B-DNA, and 16.2A^0 for ZII. Thus ZII conformation is likely to be found at B-DNA and Z-DNA interface (Wang et al., 1981). Based on the biphasic Tm, the presence of a strong shoulder positive peak at 290nm and its high EtBr binding affinity (discussed in the following section), it is concluded that a major portion of DNA present in moderately affected AD brain might be in ZII conformation (B-Z intermediate conformation). The Tm of control and AD affected parahippocampal gyrus were 60 $\pm1^0$C and 61$\pm1^0$C respectively indicating no significant alteration in Tm of control and AD DNA. In case of superior frontal gyrus, the control and AD affected showed Tm of 61.5$\pm$1.5 and 59.5$\pm$1.5^{0}C respectively. Both parahippocampal gyrus and superior frontal gyrus (control and AD affected) obeyed all-or-none response similar to that shown in control hippocampal DNA.

Increased Tm values of DNA have been observed in aging brain and are taken to indicate a more condensed conformation of DNA (Kurtz et al., 1974; Chaturvedi and Kanungo, 1985; Bhaskar and Subba Rao, 1994). It was also proposed that with age the brain cell DNA undergoes changes in the structure resulting in more condensed conformation (Bhaskar and Subba Rao, 1994). In our study we observed significant change in the Tm values of DNA only in the hippocampus, where altered, rigid DNA conformation also observed supporting the above concept. However we did not observe such trend in the parahippocampal gyrus and superior frontal gyrus.

3.2.4. EtBr binding studies

EtBr binding study has provided supporting evidence for Z-DNA conformation in hippocampus. Figure 3.6 indicates the fluorescence emission intensity of EtBr bound to DNA. The EtBr emission intensity was high for MAD DNA and low for SAD DNA compared to both CY and CA DNA, the DNA concentration being same in all the cases. We presume that the high EtBr fluorescence in MAD is attributed to the possible existence of B-Z intermediate form of DNA. It has been reported that EtBr binding is significantly high in DNA having a B-Z junction compared to normal B-DNA (Suh et al., 1991). The B-Z junctions are local regions of structurally distorted DNA, which result in favorable binding of EtBr. We further confirm the Z-DNA conformation in SAD with the supporting

evidence that the EtBr has less affinity for left handed Z-DNA (Walker et al., 1985).

3.2.4.1. Scatchard plots

The amount of EtBr bound to DNA (EtBr bound / bp of DNA) was calculated using the independent binding equation of Scatchard (Scatchard, 1949). Results showed that there exists differential EtBr binding to DNA from three different regions (Fig. 3.7). Among the three regions tested hippocampus showed lesser EtBr bound per base pair and we conclude that this is due to the formation of Z-DNA conformation. Few representative Scatchard plots from control and SAD hippocampus have been presented (Fig. 3.8 and 3.9).

AD affected superior frontal gyral and parahippocampal gyral DNA showed less EtBr bound per base pair than that of controls. This can be attribited to the DNA fragmentation in the AD affected DNA samples compared to the control.

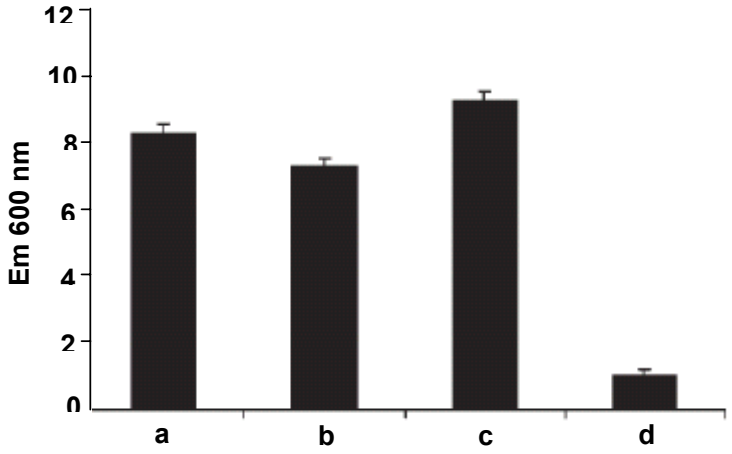

Figure. 3.6. DNA-EtBr complex (2:1w/w) fluorescence intensity in 0.01M HEPES buffer (pH.7.4). Fluorescence was done on eight samples each of CY, CA, MAD, and SAD DNA of Hippocampus. 1 μg of DNA (3 μM [Phosphate]) from each sample was used for fluorescence studies. (a) CY brain DNA; (b) CA brain DNA; (c) MAD brain DNA; and (d) SAD brain DNA. The values are mean ± SD.

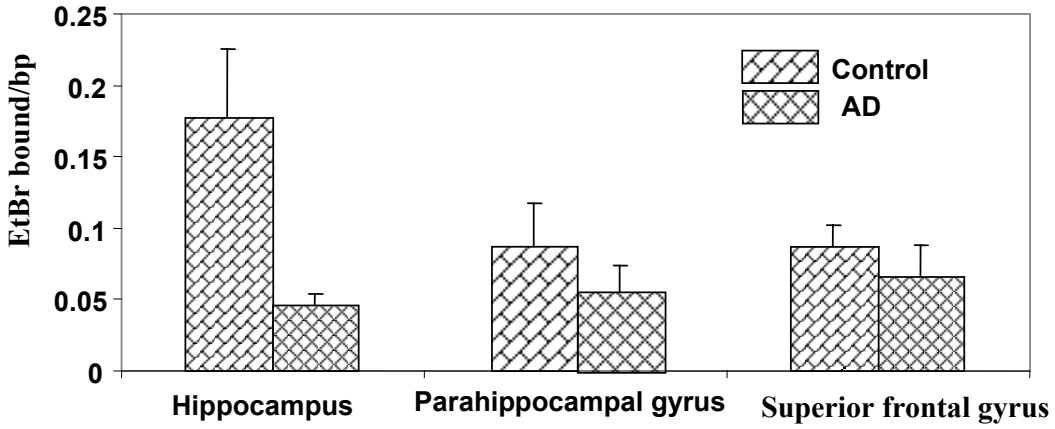

Figure. 3.7. Amount of EtBr to DNA (EtBr bound per base pair of DNA) derived from the Scatchard plots in Hippocampus (CA and SAD), Parahippocampal gyrus and Superior frontal gyrus DNA. (Details are provided in the materials and methods). The values are mean ± SD.

3.2.5. CD spectroscopy of EtBr and brain DNA complex

The CD spectra of the hippocampal DNA and EtBr complex i.e. CY-EtBr, CA-EtBr, MAD-EtBr and SAD-EtBr complex (EtBr: DNA w/w ratio: 1:2) obtained are all similar to that of the "EtBr-B-DNA" reference spectra (Walker et al., 1985) (Fig. 3.10). As both CY, and CA DNA were in B-DNA conformation their EtBr complex spectral peaks are exactly the same. Hence only the CA-EtBr complex spectrum has been presented in figure. 3.4. The DNA-EtBr complex spectrum showed that the ellipticity of bound ethidium goes through a minimum at ~ 250nm and ~ 290nm. The EtBr could reverse the Z-DNA, and B-Z intermediate form to B-DNA. This is in accordance with Walker et al. (1985) and Lamos et al. (1986) results, who suggested that CD spectra of left-handed polynucleotides change towards that characteristic of a right-handed DNA-EtBr complex. Although the three DNA- EtBr complex spectra are similar in shape, there is a slight change in the magnitude.

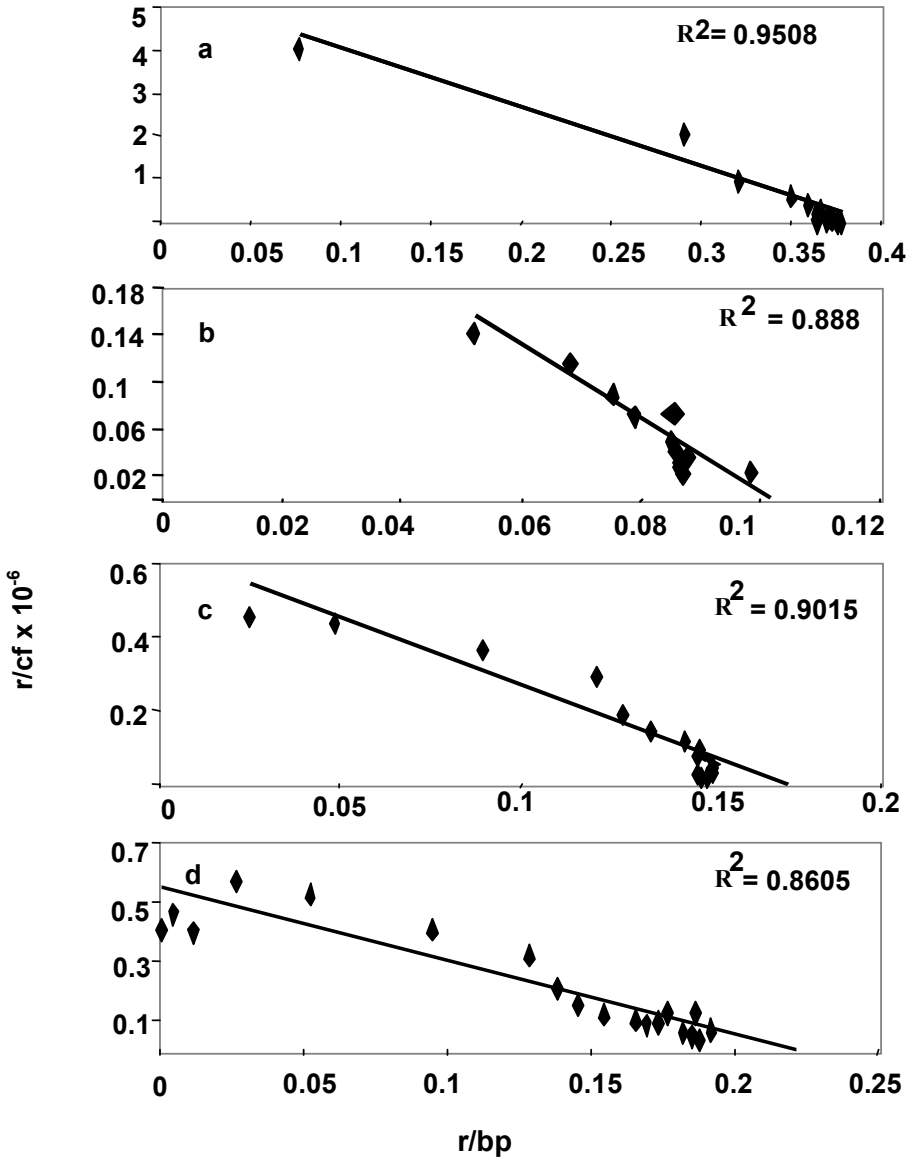

Figure. 3.8. Scatchard plot of EtBr binding to control hippocampal DNA (a-d represent samples from different brains).

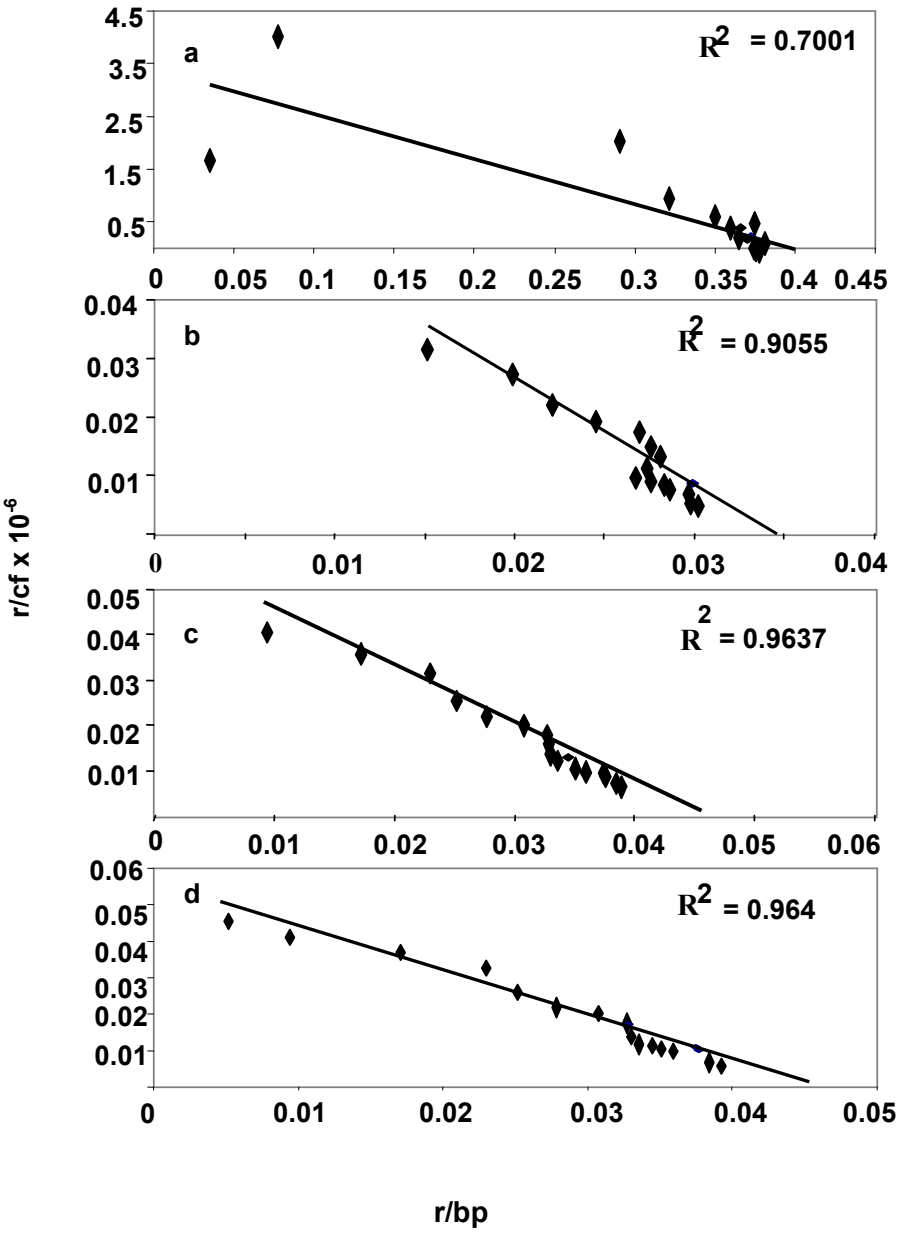

Figure. 3.9. Scatchard plot of EtBr binding to AD (SAD) hippocampal DNA (a-d represent samples from different brains).

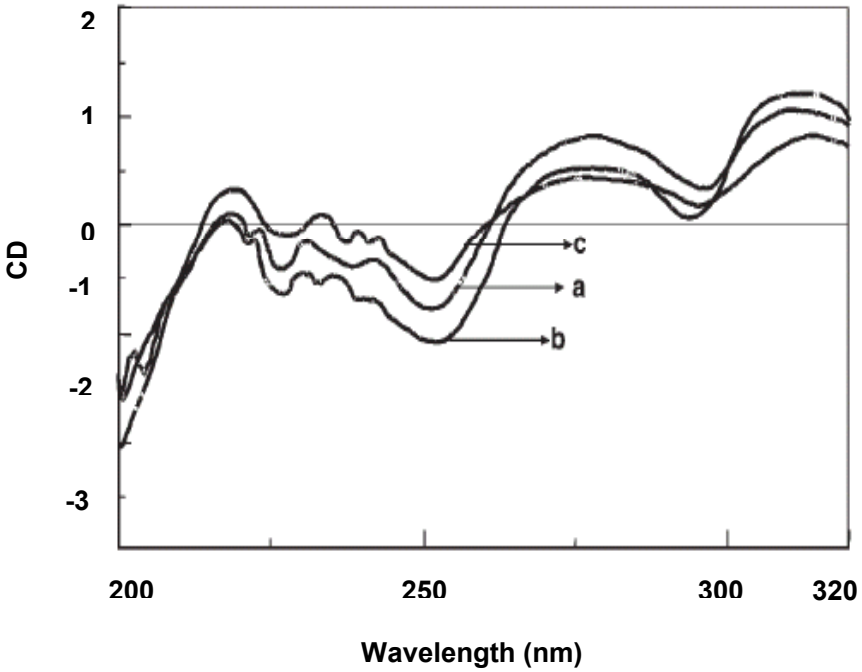

Figure. 3.10. CD spectra of DNA-EtBr complex (2:1w/w) in 0.01M HEPES buffer. (a) CY brain DNA + EtBr; (b) MAD brain DNA + EtBr; and (c) SAD brain DNA + EtBr. CD was done on eight samples each of CY, CA, MAD, and SAD DNA of hippocampus. One from each sample was has been represented.

It has been suggested (Gupta et al., 1983 and Shafer et al., 1984) that EtBr could intercalate into left-handed poly (dG-dC). poly (dG-dC). But Walker et al. (1985) and Lamos et al. (1986) using fluorescence-detected CD study suggested that CD spectra of left-handed polynucleotides change directly towards that characteristic of a right-handed DNA-EtBr complex without evidence for intercalation into left-handed polymer. Furthermore, our study showing the apparent low binding of EtBr to SAD and supports the above concepts of Lamos et al. (1986). The differential pattern of DNA-EtBr complex CD spectra is due to changes in the rotation angle between adjacent base pairs and unwinding of DNA. In the case of parahippocampal and superior frontal gyral (both control and AD affected) DNA and EtBr complex CD spectra were similar to each other (hence figures were not represented).

3.2.6. DNA fragmentation analysis by agarose gel electrophoresis

Agarose gel studies of CY (lane b, Fig. 3.11) and MAD DNA (lane c, Fig. 3.11) did not show any fragmentation. Both CA and SAD (lanes d and e Fig. 3.11) DNA were fragmented as evidenced by smear pattern. It is interesting to mention that even though there is no difference between CA and SAD DNA as both of them have smeared pattern, EtBr staining is faint for SAD DNA.

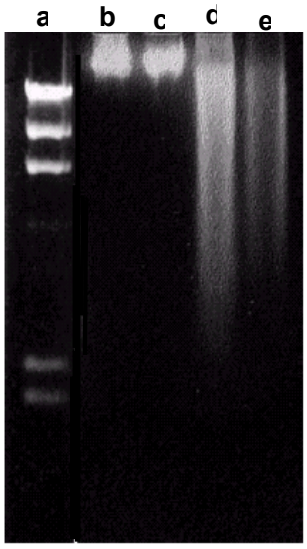

Figure. 3.11. Agarose Gel electrophoretic analysis of DNA damage in the Hippocampus of controls and AD brain. Gel studies were done on eight samples each of CY, CA, MAD and SAD and one DNA sample form each group has been presented. 2 μg of DNA from each sample was used for gel studies. (a) 1kb DNA marker; (b) CY brain DNA; (c) MAD brain AD DNA; (d) CA brain DNA and (e) SAD brain DNA.

And this is due to its Z-DNA conformation. Since all the DNA samples from each group have the similar migratory pattern, we provided one sample from each group, which revealed the basic migratory pattern of their respective group. One control parahippocampal gyral DNA sample (lane j, Fig. 3.12) was damaged among four samples, and in the case of AD affected samples three

samples (lanes a, d and i, Fig. 3.12) showed smear pattern of migration and the six samples were intact. All the control samples (n=4) of superior frontal gyral DNA samples were intact and four (lanes a, b, d and e, Fig. 3.12) of seven AD affected samples were damaged. It is interesting that all control superior frontal gyrus samples were intact and damage was seen only in the case of AD superior frontal gyrus (Fig. 3.13). The damage was analyzed by the disappearance of the genomic DNA band corresponding to the 800-900 bp DNA maker.

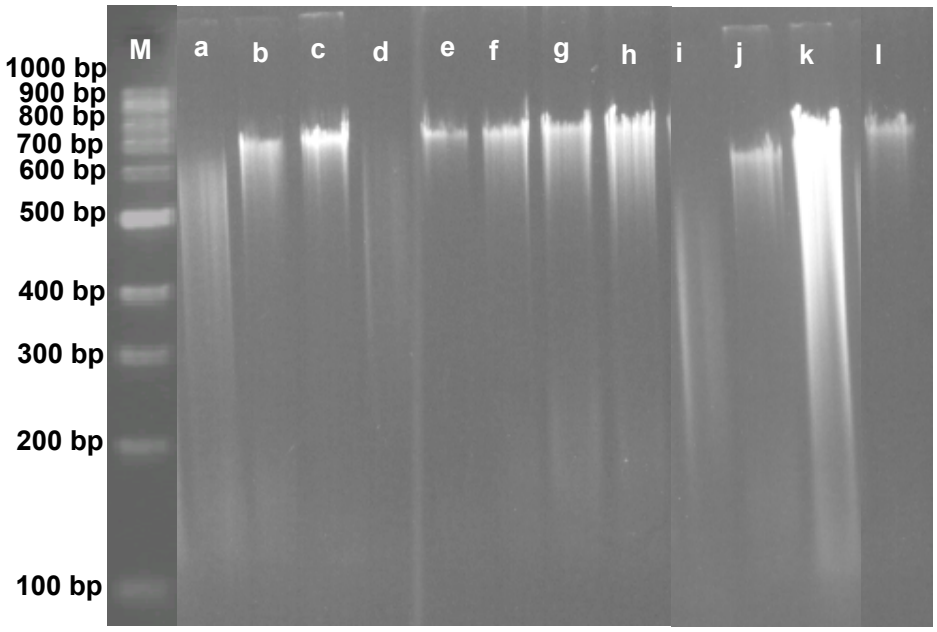

Figure. 3.12. Agarose gel (1.5%) electrophoretic analysis of DNA damage in the Parahippocampal gyrus of AD (a-i) and control (j-l) brain. 2 μg of DNA from each sample was loaded. M: 100 bp DNA marker.

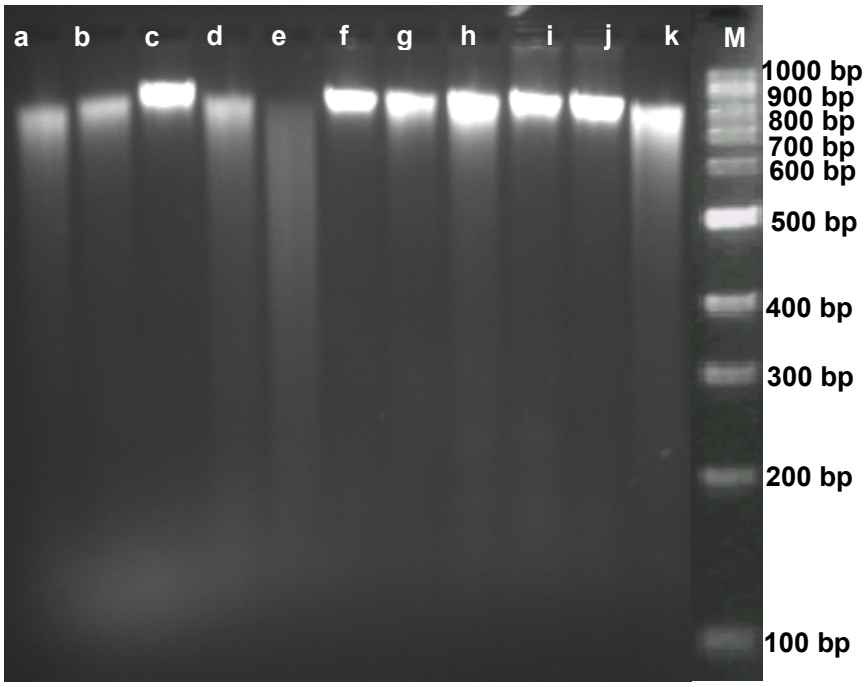

Figure. 3.13. Agarose gel (1.5%) electrophoretic analysis of DNA damage in the Superior frontal gyrus of controls (h-k) and AD brain (a-g). 2 μg of DNA from each sample was loaded. M: 100 bp DNA marker.

3.2.7. Estimation of single strand breaks (SSB) and double strand breaks (DSB)

Single stranded breaks (SSB) were assessed by the incorporation of ^{3}[H] - TMP into DNA samples using *E.coli* DNA polymerase 1 in a nick transnational assay. During standardization of the assay conditions with a plasmid DNA (cos T fragment of λ phage) having known umber of single strand breaks, it is found an average of 1500 nucleotides are added at each of the 3'-OH group. From this it is inferred that each picomole of TMP incorporated is equivalent to $1.6x10^{9}$ 3'-OH groups or SSB. It has been shown that incorporation increases with age of the brain in neuronal as well as astroglial DNA. Thus the DNA damage in the form of SSB is significantly increased in AD superior frontal gyrus compared to control (Fig. 3.14). In AD hippocampus as previously shown in agarose gel, both CY and SAD DNA showed strand breaks. Of the three regions parahippocampal region showed less strand breaks.

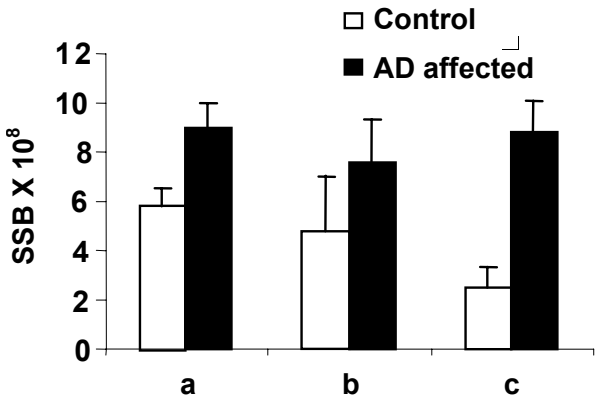

Figure. 3.14. Assessment of Single Stranded Breaks (SSB). (a) Hippocampus (CA and SAD); (b) Parahippocampal gyrus and (c) Superior frontal gyrus DNA from control and AD affected brains. Assay was carried out through Nick Translation type incubation with *E.Coli* -polymerase. The values are mean ± SD.

Double stranded breaks (DSB) in DNA are appraised by using terminal deoxy nucleotidyl transferase enzyme. From the conditions of incubation (Deng and Wu, 1983) it is assumed that about 50 TMP residues are added at each of the 3'-ends of the duplex DNA. From this it is calculated that each femtomole of TMP, incorporation would be equivalent to 1.2x 10^7 3' ends or half that number minus one DSB. From the data it is clear that AD hippocampal, and superior frontal gyral DNA suffer from more DSB (Fig. 3.15).

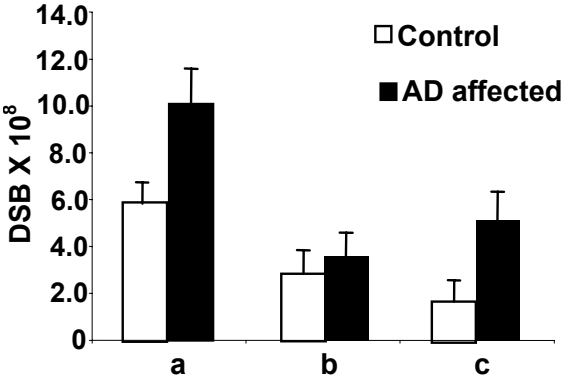

Figure. 3.15. Assessment of Double Stranded Breaks (DSB). (a) Hippocampus (CA and SAD); (b) Parahippocampal gyrus and (c) Superior frontal gyrus DNA from control and AD affected brains. Assay was carried out using Terminal transferase. The values are mean ± SD.

Previous studies also reported high incidence of nuclei with either SSB or DSB DNA breaks in AD hippocampus (Adame et al., 1999). It was reported by Mecocci et al, (1994) that oxidative damage to mitochondrial DNA is increased in AD. In our study however, mitochondrial DNA makes up an insignificant percentage (1-2%) of total brain DNA, not enough to be the cause of our increased value. Therefore we conclude that the increased damage involves nuclear DNA, and increased damage appears focused in the hippocampus.

3.3. Discussion

Hippocampus is a brain region playing a key role in the learning and memory process. The hippocampus was selected as it is one of the first brain regions affected in AD and is involved in the processing of short-term memory and spatial memory. Our study evidenced the presence of left-handed Z-DNA conformation in the AD brain hippocampal DNA. Superior frontal gyral and parahippocampal DNA did not show conformational alteration. The topological change in DNA, particularly the B to Z-transition in the hippocampus, will have tremendous implications in the functional biology of the brain cells e.g. transcription, replication and recombination (Sinden, 1994). The B-Z intermediate form observed in MAD and Z-DNA in SAD reveal that the topological transition is AD progression dependent. The potentially Z-DNA conforming sequences are highly dispersed through out the human genome (Hamada and Kakunaga, 1982; Schroth et al., 1992). It has been speculated, based on immunohistochemical data, that Z-DNA is about one-seventh as abundant as B-DNA (Soyer- Gobillard, 1990; Gagna et al., 1997). In particular interest with AD, it is observed from the Human Genome Sequence that the Z-DNA conforming GC rich sequences are observed in 5´ regions of AD specific genes like PS1, PS2, and apoE (Rogaev et al., 1997; Levy-Lahad et al., 1995a; Breslow et al., 1982). It is interesting to mention that some of these genes have been over expressed in AD and have significant role in AD pathogenesis. It was reported that Z-DNA formation excludes nucleosome formation and could affect the placement of nucleosome as well as organization

of chromosomes (Garner et al., 1987). It was also suggested that a distinct class of human genes that may potentially be transcriptionally regulated by a mechanism that couples Z-DNA with nuclear factor (Champ et al., 2004). We strongly feel that the observed changed conformation of DNA is also relevant under cell system and open up new avenues for further understanding of the neurodegenerative mechanisms.

DNA fragmentation has been one of the crucial steps in B→Z DNA conversion since the fragmentation reduces the high activation energy barrier required to induce this conformation. Studies have shown that radiation induced DNA damage i.e. strand breaks can lead to B → Z conformational transition assisted by metals. The smaller metal ion concentration could induce this conversion in DNA with strand breaks than in the absence of strand breaks. It is attributed to the reduction in the high activation energy barrier in the metal ion driven B → Z conformation (Trumbore et al., 1994). It also provides a clue as to why SAD-DNA could go to Z-form from B-form, probably due to DNA fragmentation and assisted by the metals like Zn, Cu, Fe, and Al, which are elevated in AD brain (Rao et al., 1999). However, absence of conformational alteration in superior frontal gyrus and parahippocampal gyrus ruled out the sole involvement of DNA damage to conformational change. Further we propose that DNA damage along with other etiological factors might play role in DNA conformational alteration.

Recently it was shown that lysine rich proteins favor the conversion of B→Z DNA transition and further it was found that methylated DNA could be easily converted from B to Z DNA by the lysine rich proteins (Takeuchi et al., 1994). The altered DNA structure in the hippocampus, whether reflects the pathogenic event or represents an adaptation to cell death cannot be ascertained now. It is interesting to hypothesize that AD specific proteins like Aβ and phosphorylated tau are lysine rich and probably have a role in inducing B→Z transition in AD brain (Fig. 3.16). And it has been shown that some DNA-binding proteins have amino acid sequences that favor Z-DNA. We propose that Aβ, tau and Al interaction with DNA cause formation of intermediate structure, found in MAD and oxidative stress, altered metal homeostasis and cell shrinkage might be responsible for further change of

DNA conformation from intermediate forms to the Z-DNA, observed in the SAD. DNA damage in the presence of metals has also been shown to favor the conformational transition in DNA. We propose that all these factors which occur in AD might be responsible for the observed changes in DNA. However the conformational transition from B-DNA to Z-DNA in SAD whether a pathological feature or an adaptation can not be ascertained now. In the later study to test our hypothesis, we carried out interaction of Aβ with DNA.

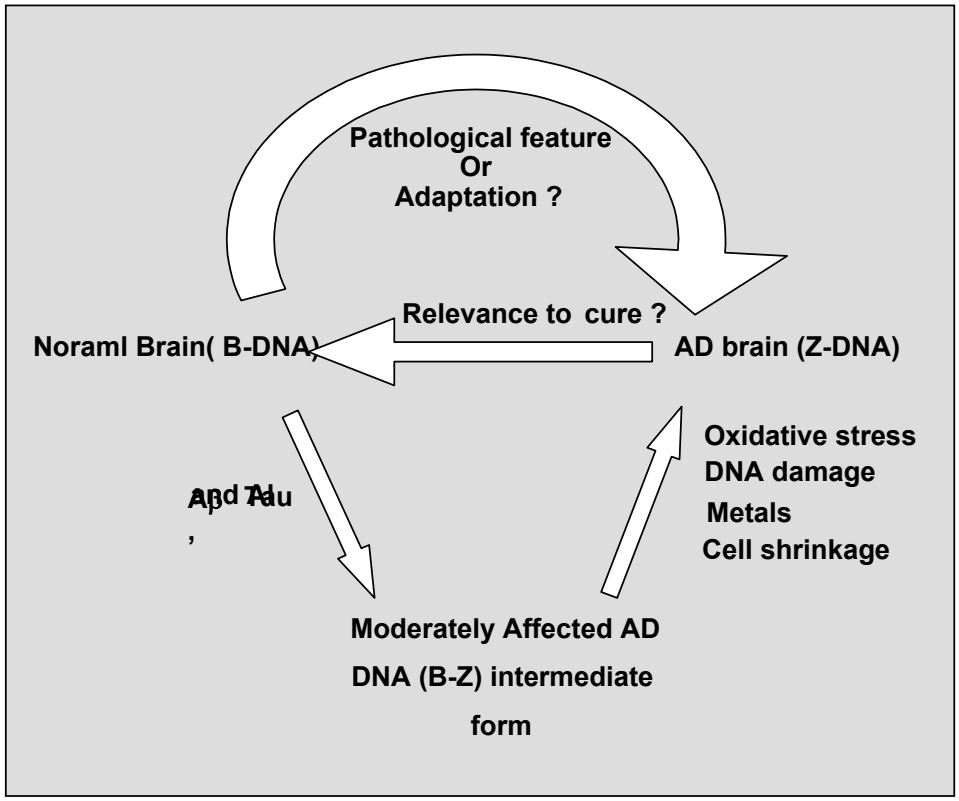

Figure. 3.16. A hypothesis on the complexity of DNA topology in Alzheimer's brain.

The implications of the Z-DNA in relevance to AD have been highlighted below.

3.3.1. Biological implications of Z-DNA and its neurochemical implications in relevance to AD

The significant pathophysiological events hypothesized in AD pathology are: distorted nucleosome formation, terminal differentiation and DNA damage. However there is no explanation at the cellular level why AD brain hippocampal cells adopt this unusual phenomenon. The presence of Z-DNA in AD brain could explain the above phenomenon. Brief structural information of Z-DNA is as follows: Z-DNA is a condensed DNA with left-handed conformation (Fig. 3.17). It has only minor groove and thus alters ligand-binding ability. Z-DNA is basically classified as ZI and ZII types; ZI and ZII are classified based on phosphate group orientation. In ZI conformation, two hydrogen atoms of the guanine N_2 amino group are involved in hydrogen bond formation. One bond to cytosine O_2 to which it is paired and another to a water molecule. This water molecule, in turn, forms a hydrogen bond with phosphate oxygen linked to deoxygaunosine 3'hydroxyl group.

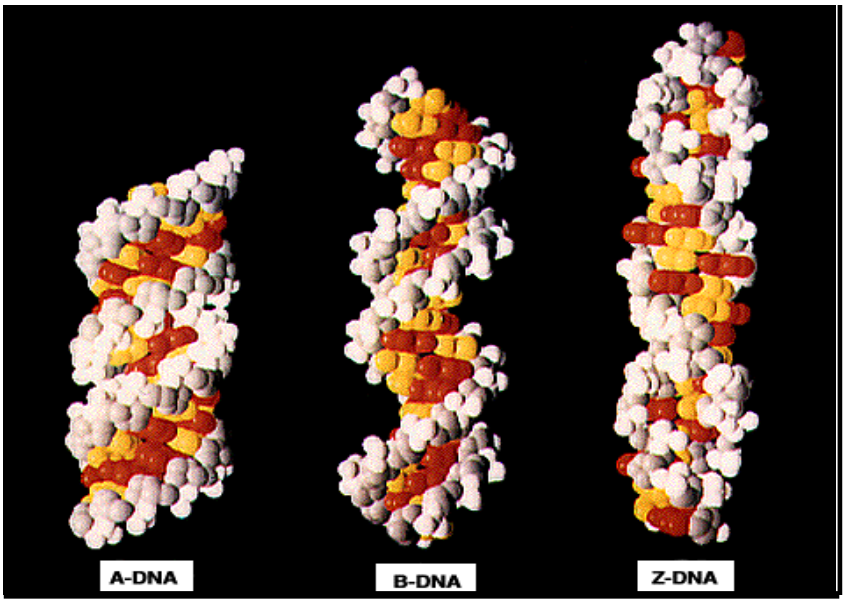

Figure 3.17. Space filling models of three different forms of DNA.

Thus ZI conformations will have this bridging water molecule. In ZII conformation there is also a hydrogen- bonding-bridge but it involves a chain of two water molecules linking N_2 amino group and phosphate oxygen. Furthermore, the distances between the two closest phosphate groups in ZI and ZII were 7.7 and 8.6 A^0 respectively. The P….P separation across the double helix is 17.4 A^0 for B-DNA, while the values are 14.5 and 16.2 A^0 for ZI and ZII respectively (Wang et al., 1981; Rich et al., 1993). This suggested that the ZII conformation might be an interface of B-and Z-DNA. Furthermore, DNA present in moderately affected AD brain may be in ZII conformation and this may be attributed to biphasic Tm and a strong shoulder positive CD peak at 290nm.

3.3.2. Z-DNA and nucleosome formation

Extensive information is available on the role of point mutations, abnormal proteins (Aβ, presenilins, phosphorylated and aggregated tau) and various environmental factors (Al, head injury etc) involved in AD neurodegeneration (Rao et al., 1998; Terry, 1994). Limited headway has been made on the therapeutic aspect of AD because of the complexity of the disorder. Studies have been done concerning modulation in the genetic material. Studies have shown DNA fragmentation through oxidative damage (Cotman and Su, 1996; Sugaya et al., 1997), point mutations and changes in the integrity of chromatin structure (Crapper et al., 1979; Lukiw et al., 1989) but no information on the helical nature of DNA. Crapper et al., (1979) made interesting findings with reference to chromatin structure and reported a shift in the folding of DNA from a potentially transcribable euchromatic state to a condensed and probably heterochromatic state. These changes may play a role in altering chromatin conformation, which might result in reduced availability of genetic information to endogenous RNA polymerases.

Results also indicated reduced accessibility of micrococcal nuclease to the nucleosome linker region in Alzheimer brain chromatin. Lukiw et al. (1989)

showed that chromosomal linker histones are also considered to be partly responsible for the packing of chromatin into inactive, condensed states and thereby allowing them to act as general gene repressors. They also observed an inverse relation between the abundance of linker histones and gene activity in AD. Lewis et al. (1981) reported that nucleosome repeat length appears to be the same in control and AD chromatin. Furthermore, they showed a reduction in mononucleosome in comparison with dinucleosomes and larger oligomers in AD brain. However no specific explanation was provided for the differences in kinetics and distribution of the release of nucleosomes. It was reported that Z-DNA formation excludes nucleosome formation (Garner and Felsenfled, 1987) thereby affects the transcriptional ability. Further selective messenger RNA has been shown to be reduced in AD, which has been speculated to a functional deficit of gene expression in AD (McLachlan et al., 1988). Further, Z-DNA conformation has implications in nucleosome organization and assembly thus affecting the function of chromatin (Garner and Felsenfled, 1987). DNA bending is a crucial factor in nucleosome assembly. In B-DNA, negatively charged groups are evenly distributed thus favoring positive histone interaction and good nucleosome assembly. In Z-DNA, negative charges are not uniformly distributed due to the zig-zag array of backbone phosphates. So the poly (GC.GC) or poly (G.Cmet) in Z-DNA conformation excludes nucleosome formation. This charge distribution is not favorable for stable nucleosome structures. There is only one report stating that nucleosomes can also be prepared by a core containing the Z-form of poly (dG).(5meC) (Miller et al., 1985). It has also been shown that Z-DNA can be an insitu marker for transcription (Cerna et al., 2004). It was shown by Garner and Felsenfled (1987) that histone wrapping around left handed Z-DNA is energetically costly, hence it is not possible to get stable nucleosomes in a cell system. Restriction endonucleases and methylases are incapable of cleaving their respective recognition sites in Z-DNA conformation (Wholrab and Wells, 1987). The extent of DNA adopting Z- conformation stretches of DNA will exist in the Z-form in the cell. These short Z-DNA conformation stretches are sufficient enough to bring about altered patterns of nucleosome placement

(Garner and Felsenfled, 1987). Most non B-DNA conformations are likely to have both an altered twist and reduced ability to enter the nucleosomes. This biophysical explanation will provide molecular hypothetical evidence that Z-DNA conformation in AD has great relevance in nucleosomes placement and gene function. This argument is likely to open up new exploration to determine the role of DNA topology in neurodegeneration in AD.

3.3.3. Z-DNA and terminal differentiation

The study suggests that the AD brain cells possibly undergoing terminal differentiation pattern may prefer to adopt the Z-DNA conformation in their genomic DNA. Z-DNA is involved in both transcriptional activation and inactivation depending upon cell physiological condition (Herbert and Rich, 1996). Gagna et al. (1999) reported that left-handed Z-DNA may be a transcriptional enhancer and be influenced by the destructive effects of terminal differentiation, thus making DNA transcriptionally inactive. The nicking-denaturation of double stranded Z-DNA initiating and enhancing transcriptional ability of Z-DNA conformation in AD cells undergoing neurodegeneration needs to be explored. This information will provide greater insight in understanding molecular mechanisms involved in AD pathogenesis.

3.3.4. Z-DNA and DNA damage

DNA damage plays an important role in the genomic instability and neuronal cell death. Studies on DNA damage in AD brain showed that the levels of oxidatively damaged cytosine and guanine (G^*) bases, namely 5-hydroxycytosine and 8-hydroxyguanine were significantly higher in AD brain than in normal brain (Lyras et al., 1997). The main questions are: why G^* -specific oxidation is greater in AD brain and does the DNA's helical nature have any role in this specificity? It was theoretically postulated that the G^* base present in DNA would be more susceptible to hydroxyl radical induced DNA damage if the conformation of DNA is Z –form rather than B or A forms because of greater exposure of bases (Michak et al., 1995). The other cause is that purines are the most susceptible bases for oxidative damage, and the

repair of the oxidized purines is slower than that of pyrimidines and that among oxidized guanine derivatives 8OHG has the slowest repair kinetics (Jaruga and Dizdaroglu, 1996; Gabbita et al., 1998) also found that guanine is the most vulnerable base in oxidative stress to the brain and suggest that damage to this base could possibly cause functional changes in AD. Guanine is attacked preferentially upon oxidation because it has the lowest oxidation potential of the four bases (dG 1.29V, dA 1.42V, dC 1.6V, dT 1.7V) (Steenken and Jovanovic, 1997). This results from the fact that guanine has an electron rich purine structure, which enables it to react with radicals easily (Ames et al., 1981). In addition guanine also acts as a hot 'spot' for electron migration (Melvin et al., 1998). The oxidation of guanine generally results in the formation of 8-oxo-G, which is produced abundantly *in vivo* and used as biomarker of oxidative damage. 8OHG is more reactive towards radicals than unmodified base because of its low potential and represents a hot spot and 'electron sink' for oxidative DNA damage. Our present finding on the presence of Z-DNA in AD brain supports the experimental findings on the presence of GC^{*} rich oxidized products in AD brain (Lyras et al., 1997).

Therefore the study provides an insight into the conformational alterations of genomic DNA and the associated implications in relevance to AD

CHAPTER - 4

4.1. Introduction

Aβ, a hallmark feature of the SPs, is a proteolytic product of the transmembrane APP (Kang et al., 1987), a protein of unestablished cellular function that has the general motif of a surface receptor. Evidence implicates a central role for Aβ in the pathophysiology of AD. Mutations in APP and presenilin PS-1 genes lead to elevated secretion of Aβ, especially the more amyloidogenic Aβ(1-42). Native APP is a transmembrane protein of 110 to 135 kDa, with a long extracellular N-terminal segment and a short intracellular C-terminal tail. The important cleavage product of the APP is a 39-43 amino acid peptide, the 4kDa Aβ. Two-thirds of sequence of the Aβ peptide localizes to the N-terminal region of APP and the remaining portion of the Aβ peptide is contained within the transmembrane domain. Amyloid burden in the brain is correlated with dementia, that Aβ deposits are found in regions of the brain susceptible to neurodegenerative processes. The extent of accumulation of Aβ in SPs correlates with the severity of dementia in AD (Flood et al., 1991). Additionally, Aβ is a known killer of neurons *in vitro*. Aβ (1-42) peptide and its fragments, Aβ(1-16) and Aβ(1-28) (Fig.4.1) are reported to play a critical role in inducing the pathology seen in AD (Kang et al., 1987; Chen et al., 1996). The apparent role of Aβ, especially Aβ(1-42) is now considered as a unifying pathological feature of diverse forms of AD (Selkoe, 1996). The neurotoxicity of insoluble Aβ aggregates has been widely reported (Clements et al., 1996; Huang et al., 2000; Demeester et al., 2001). A direct association has been shown to exist between the aggregation state of Aβ and neurotoxicity (Pike et al., 1993). *Invitro* studies have shown that Aβ(1-42) rapidly aggregates into fibrils and that extracellular fibrillar Aβ peptides induce apoptosis in cultured neurons (Pike et al., 1993). On the other hand, recent reports have demonstrated intraneuronal accumulation of Aβ(1-42) in AD vulnerable regions (Gouras et al., 2000; Mochizuki et al., 2000). Intraneuronal Aβ(1-42) accumulation has also been reported in transgenic mice expressing FAD proteins (Wirths et al., 2001) as well as in transgenic mice showing accelerated neurodegeneration without extracellular amyloid deposition (Chui

et al., 1999). It has also recently been established that soluble extracellular β-peptide is normally produced in cultured cells and human biological fluids (Shoji et al., 1992; Haass et al., 1992; Busciglio et al., 1993).

Our results as highlighted in the previous chapter have evidenced a left-handed Z-DNA conformation in severely affected AD brain hippocampal cells whereas normal hippocampal brain DNA exhibited usual B-DNA conformation and moderately affected AD brain DNA exhibited a B-Z intermediate conformation (Anitha et al., 2002). Based on these findings, we hypothesized that AD associated molecules like Aβ, tau and Al may play a pivotal role in modulating DNA topology in AD brain (Anitha et al., 2002). Al and tau have been shown to bind to DNA (Rajan et al., 1996; Champion et al., 1998; Hua and He, 2000; Hua and He, 2002). The puzzling question was, however, about the nuclear localization of Aβ in order to bring about changes in DNA topology. Two previous studies reported Aβ immunoreactivity in the nuclear envelops of P19 cells (Grant et al., 2000) and intranuclear accumulation of Aβ in AD brain (Gouras et al., 2000). In the present study, we provided new evidence for Aβ immunoreactivity in the vicinity of DNA in hippocampal cells from AD brain. Based on the evidence for nuclear localization of Aβ in AD brain, we hypothesized that Aβ might play a role in modulating DNA topology and possibly contribute to the B→Z helical transition associated with AD (Anitha et al., 2002). To investigate the above hypothesis experimentally, we have studied the interaction of Aβ peptides (Aβ (1-42), Aβ (1-40), Aβ (1-16) and Aβ (1-28)) with supercoiled DNA (scDNA). It is quite evocative to study plasmid scDNA as a model system, in view of the observation that a vast array of small scDNA packets have been found to be present in animal and human cells and are known to be involved in gene expression (Bauer et al., 1980). These superhelical packets are proposed to be analogous to the plasmid DNA supercoiling. Hence the results can be correlated or extended to human brain genomic DNA in order to provide an insight in explaining the possible role of Aβ in the progression of AD pathology with reference to DNA topology. The present study attempts to investigate the potential role of Aβ in terms of DNA helical alteration. The understanding

of complex biology of Aβ peptides with respect to DNA topological transition might provide an avenue to explore new insight in to AD pathology.

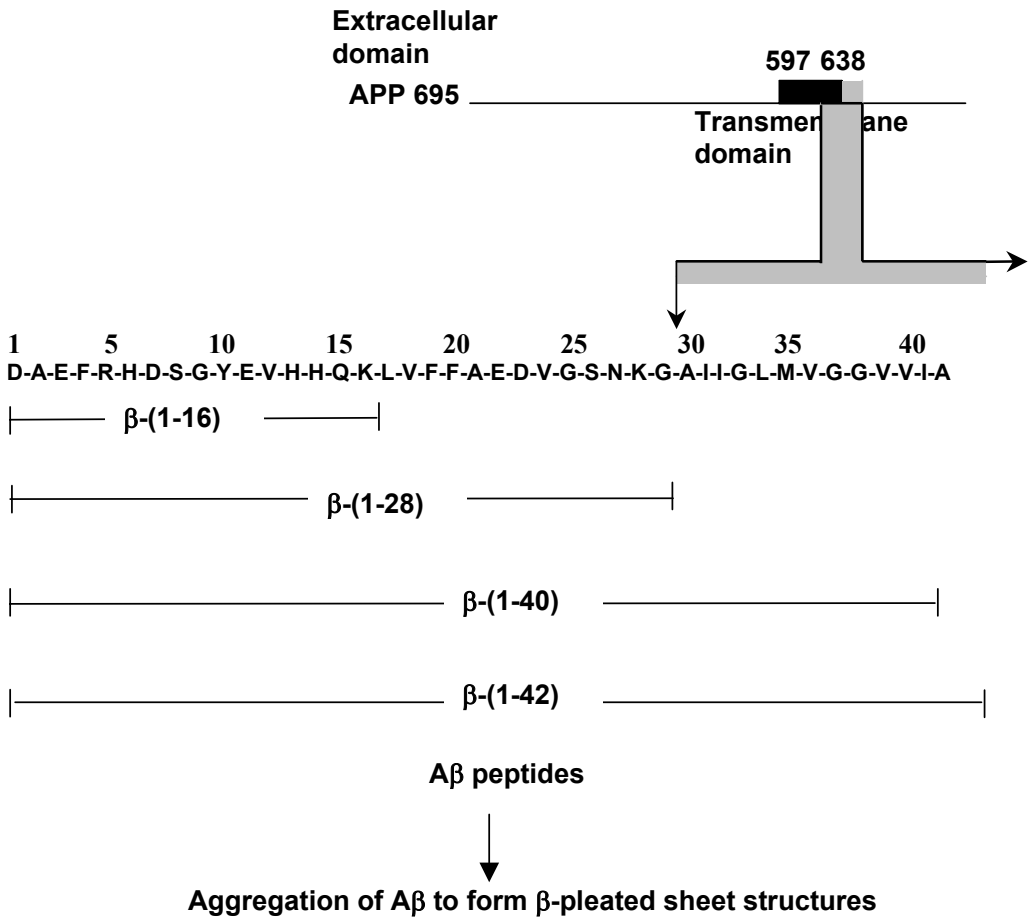

Figure. 4.1. Amino acid sequences of the four peptides Aβ (1-16), Aβ (1-28), Aβ (1-40), and Aβ (1-42) investigated in this study. The major Aβ peptide component of amyloid plaques contains 42 amino acid residues and is referred to as Aβ (1-42). The Aβ (1-28) and Aβ (29-42) peptides occupy the extracellular and transmembrane regions of APP and Aβ (1-42).

4.2. Results

4.2.1. CD studies

4.2.1.1. Interaction of Aβ peptides with Supercoiled DNA (sc DNA)

The potential role of Aβ in modulating DNA topology has been examined by CD spectroscopy. CD spectra of sc DNA alone showed B-DNA conformation with a characteristic positive peak at 275nm and negative peak at 245nm (Fig. 4.2a). Substantial changes in the CD spectra of sc DNA were observed upon interaction with Aβ(1-42) ($0.8x10^{-7}$ to $0.8x10^{-4}$M). At Aβ(1-42) / DNA ratios lower than 0.1, a DNA secondary structural transition from the native B-DNA to the C motif was observed. The spectral changes involved decrease in positive peak at 275 nm with no concomitant change in the magnitude of the negative peak at 245nm (Fig. 4.2b). The spectral modifications of the positive peak were continuous with the increasing Aβ(1-42) concentration. The 'limit C-DNA motif' is characterized by a small positive CD peak at 275 nm and a long negative signal at 245 nm. However, on the addition of higher concentration of Aβ(1-42) (Aβ(1-42) / DNA ratio > 0.1) the negative CD band extended in the non-absorbing region in the form of CD tails, with a large CD magnitude compared to the intrinsic CD of scDNA which is intriguing. This spectral change presumably reflects the asymmetric compaction of scDNA by Aβ(1-42) to form ψ (+) DNA. This pattern of CD signal (Fig. 4.2c) is a typical characteristic of ψ (+) form of DNA (Shin et al., 1984). All the spectra showed isodichroic point at the cutting point of 215 nm. Examination of DNA ellipticities as shown in figure. 4.2d and 4.2e indicated the transformation of native B-DNA to C-DNA to ψ -DNA in scDNA induced by Aβ(1-42). Thus Aβ(1-42) caused B→C→ ψ conformational transition in scDNA. The Aβ(1-40) peptide, which differs from Aβ(1-42) by short of two N-terminal amino acids, however showed different pattern of conformational alteration. In this case the magnitude of both the positive peak (275 nm) and negative peak (245 nm) have been reduced by the peptide interaction (Fig. 4.3). Increasing concentrations of the peptide resulted in the disappearance of large positive peak at 190nm, a strong B-DNA peak. In a similar fashion Aβ(1-28) peptide also interacted with scDNA resulting in

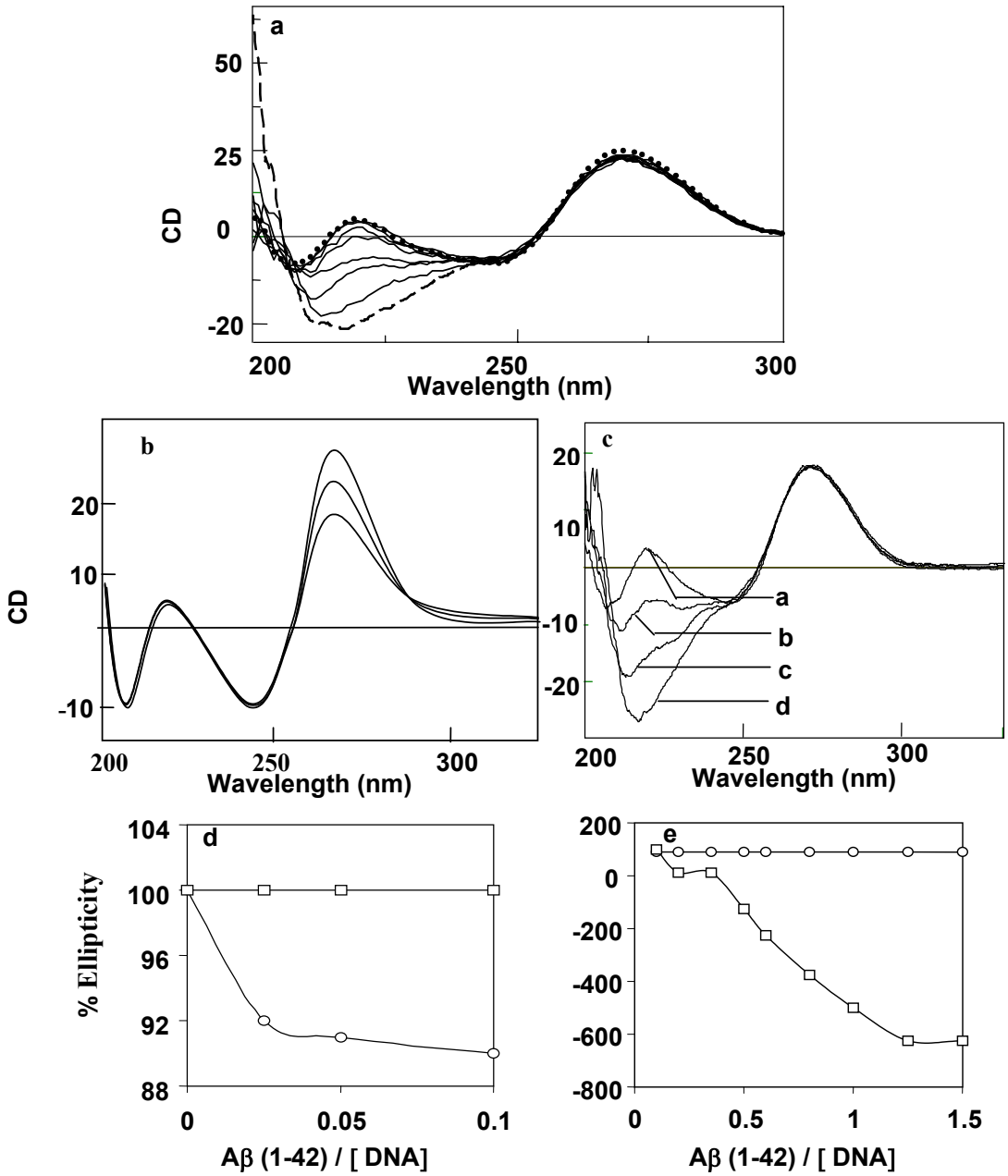

Figure. 4.2. The interaction of Aβ (1-42) with sc DNA: CD spectra of sc DNA titrated against increasing concentration of Aβ (1-42) ($0.8x10^{-7}$ to $0.8x10^{-4}$M) (a) Dotted line represents sc DNA alone and dashed line represents highest concentration of the peptide ($0.8x10^{-4}$M) used in the study. The changes in the ellipticities at 275nm (-0-) and at 220nm (-□-) were expressed as percentage of its value for control (native B-DNA) and plotted against molar ratio of [Aβ (1-42)] / [sc DNA] (d and e respectively). (b) The native B-DNA was transformed to a 'Limit C-motif' at a [Aβ (1-42)] / [sc DNA] molar ratio lower than 0.1. (c) At higher [Aβ (1-42)] / [sc DNA] ratio (>0.1), The C-DNA was further converted into a asymmetrically condensed ψ-DNA.

decrease of both positive and negative bands magnitude at 275 nm and 245 nm respectively. The CD spectra showed an isobestic point at 210 nm (Fig. 4.4). The CD spectral perturbations by Aβ imply involvement of both hydrophobic and hydrophilic interactions of the peptide with DNA. Next we examined the effect of the different concentrations ($0.8x10^{-7}$M to $0.8x10^{-4}$M) of the shorter length Aβ, Aβ(1-16) on scDNA conformation. Higher concentration ($0.8x10^{-4}$M) of Aβ(1-16) modified the scDNA to an unusual altered B-form (Fig. 4.5). Addition of Aβ(1-16) caused a decrease in the intensity of the positive peak centered around 275 nm, with a concomitant reduction of the negative peak around 245 nm. However, there was a significant increase in the negative band intensity at 205 nm, indicating a modification in the usual B-secondary structure. The cutting point has been shifted from 220 nm to 224 nm as the concentration of Aβ (1-16) is increased. All the spectra have two isodichroic points at 228 nm and 252 nm.

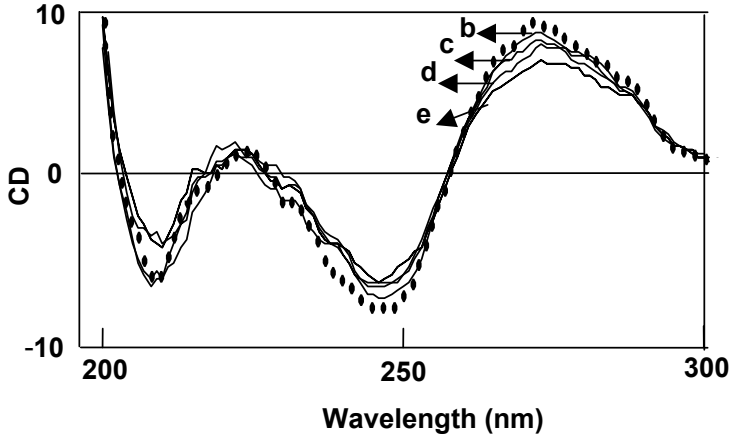

Figure. 4.4. CD spectra of interaction of Aβ (1-28) with sc DNA. $25x10^{-6}$g scDNA alone spectra (Dotted line); (b) sc DNA+Aβ (1-28) ($6x10^{-6}$M); (c) sc DNA + Aβ(1-28) ($17x10^{-6}$M); (d) sc DNA + Aβ (1-28) ($30x10^{-6}$M) and (e) sc DNA + Aβ (1-28) ($45 x10^{-6}$M).

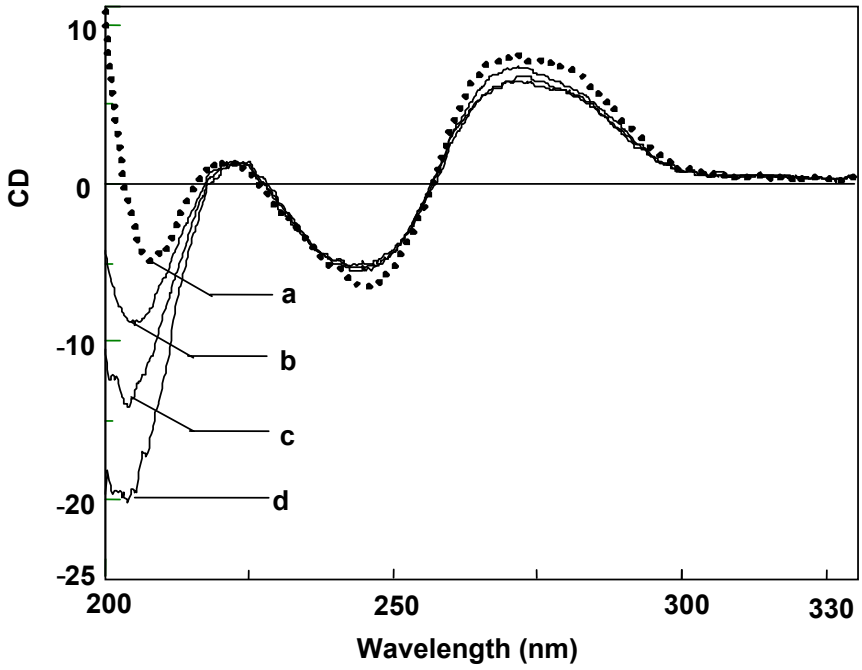

Figure. 4.5. CD spectra of interaction of Aβ (1-16) with sc DNA: Aβ(1-16) peptide binding to sc DNA results in the modified B-DNA conformation. (a) $25x10^{-6}$g sc DNA alone; (b) sc DNA+ $5x10^{-7}$M Aβ(1-16); (c) sc DNA+ $1x10^{-5}$M Aβ(1-16); and (d) sc DNA+ $0.8x10^{-4}$M Aβ(1-16).

4.2.1.2. Interaction of Aβ peptides with poly d(GC).(GC) and poly d(AT).(AT)

In order to understand GC and AT sequence specific requirement of the peptide binding, we interacted the Aβ peptides with poly d(AT).(AT) (GC content 0%) and poly d(GC).d(GC) (GC content 100%). Normal B-DNA conservative spectrum is observed for both poly d(GC).(GC) and poly d(AT).(AT) alone. The study provided interesting insight into the binding affinity of peptide with DNA. In case of poly d(AT).(AT) interaction of Aβ peptides showed significant alteration in the CD spectra where as no significant interaction have been noticed in poly d(GC).(GC). The smaller peptide Aβ (1-16) caused B→A DNA conformation in poly d(AT).(AT). This has been characterized by the shift in the cross over point of poly d(AT).(AT) alone at 259 nm to 253 nm by the

addition of the peptide (Fig. 4.6a). Aβ (1-28) (Fig. 4.6b) caused a cross over shift from 259 nm to 256 nm and Aβ (1-40) (Fig. 4.6c) effected significant cross over shift from 259 nm to 252 nm. Aβ (1-42) (Fig. 4.6d) has caused shift only at the high concentration tested from 259 nm to 257 nm. All the peptides showed decrease in both positive and negative CD bands. The group of the spectra obtained on interaction with Aβ peptides (all the peptides) showed an isobestic point at 269nm.

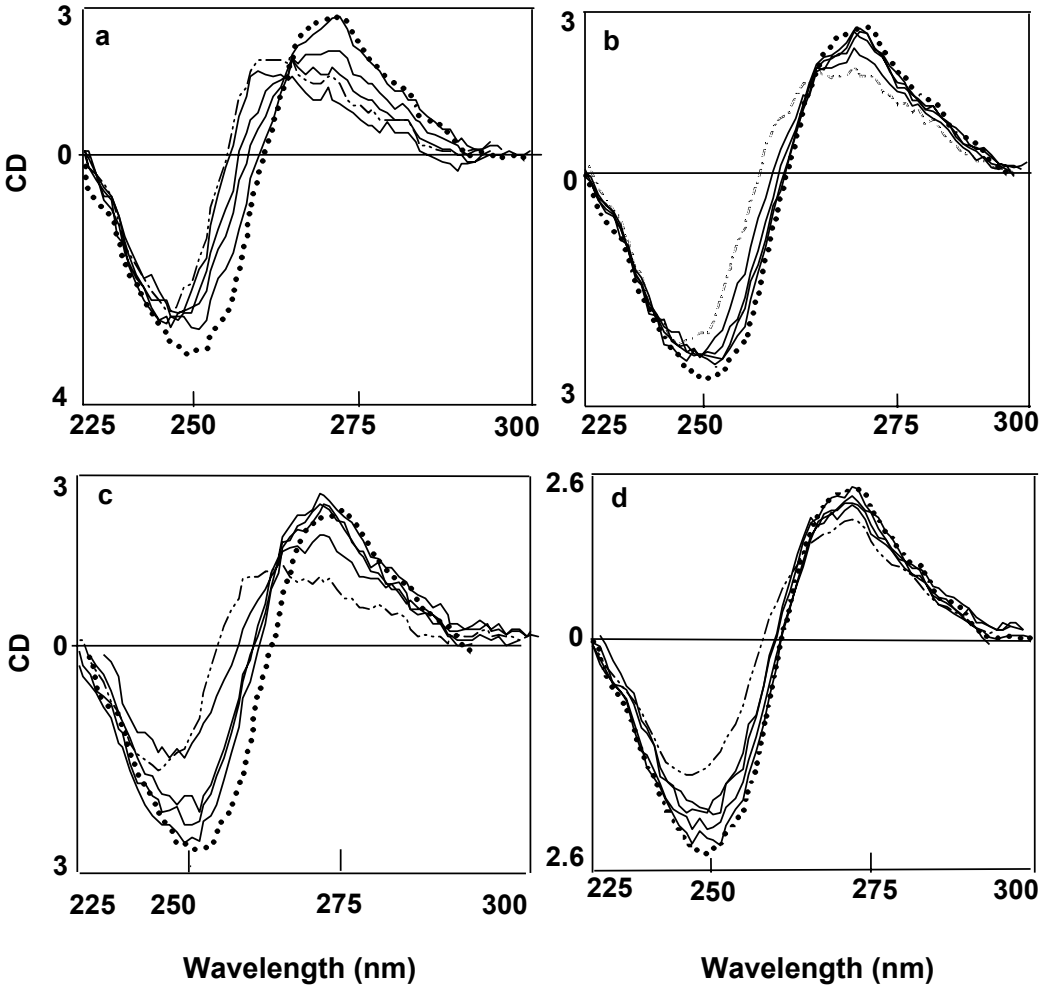

Figure. 4.6. The interaction of Aβpeptides (0.6x10^{-6} to 14.0x10^{-6} M) with poly d(AT).(AT): (a) poly d(AT).(AT) + Aβ(1-16); (b) poly d(AT).(AT)+Aβ(1-28); (c) poly d(AT).(AT)+Aβ(1-40); and (d) poly d(AT).(AT)+Aβ(1-42). Dotted line represents poly d(AT).(AT) DNA only spectra. Dashed line represents the highest concentration of peptide used in the study.

In case of poly d(GC).(GC), Aβ peptides did not show significant alteration in the CD spectra (Fig. 4.7). Results reveal that Aβ peptides bind strongly to AT specific sequences than GC sequence.

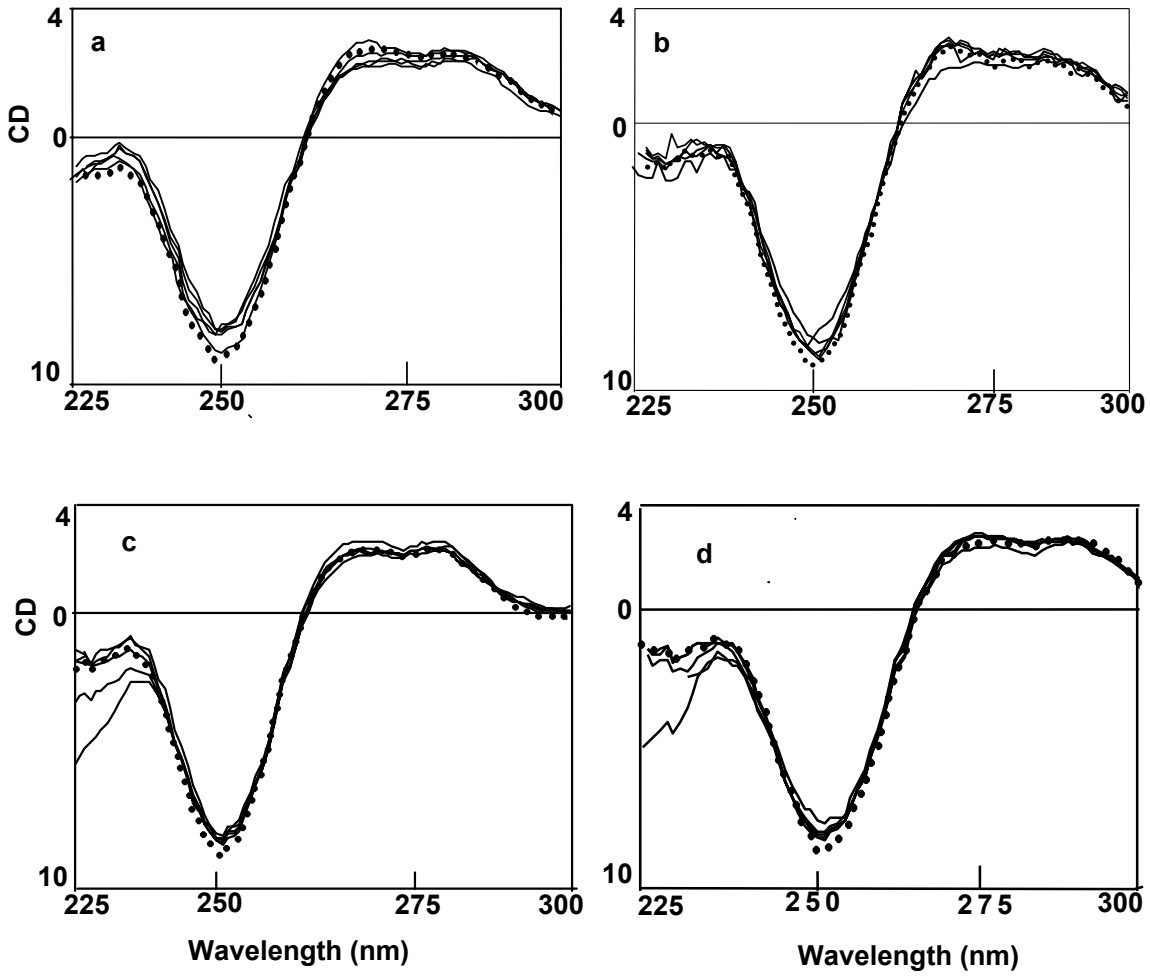

Figure. 4.7. The interaction of Aβ peptides (0.6x10^{-6} to 14.0x10^{-6} M) with poly d(GC).(GC): (a) poly d(GC).(GC) + Aβ(1-16); (b) poly d(GC).(GC)+Aβ(1-28); (c) poly d(GC).(GC)+Aβ(1-40); and (d) poly d(GC).(GC)+Aβ(1-42). Dotted line represents poly d(GC).(GC) DNA only spectra.

4.2.2. Agarose gel electrophoresis studies

These studies were carried out to monitor uncoiling process of scDNA. scDNA (1µg) was incubated with Aβ(1-42), Aβ(1-40), Aβ(1-28), and Aβ(1-16) ($0.8x10^{-4}$M) for overnight at 37^0 C in 10mM Tris-NaCl-EDTA buffer (pH 7.0) and subjected to agarose gel electrophoresis (Fig.4.8). All the Aβ peptides have been shown to alter the superhelicity by partially uncoiling the DNA (lanes b, c, d and e respectively). The reduction in the intensity of supercoiled band and enhanced intensity of the open circular form band revealed the uncoiling of the DNA. The result implicates that Aβ peptides acted on the supercoiled band of scDNA and relaxed it to the open circular form.

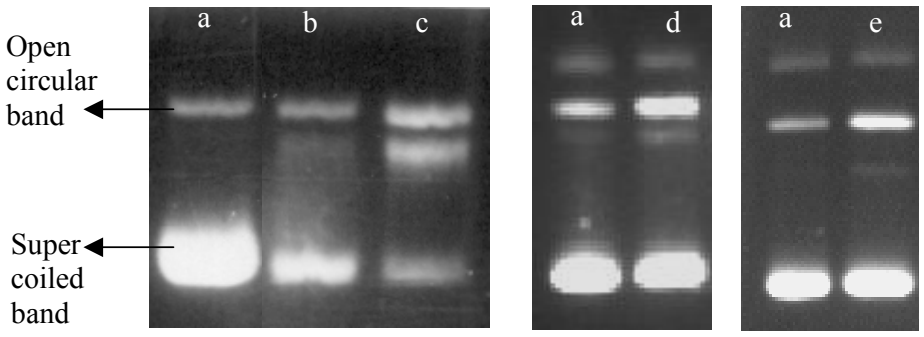

Figure. 4.8. Agarose (1%) gel study of interaction of Aβ peptides with sc DNA. scDNA was incubated with Aβ peptides for 12 h and was separated on agarose gel (1%) electrophoresis**.** scDNA alone (Cesium chloride purified) showed >90% superhelicity (lane a) as represented by intense supercoiled band. Aβ (1-42) caused clear cut appearance of an extra band between the supercoiled band and open circular form (lane, c). Aβ (1-16), Aβ (1-28) and Aβ (1-40) ($0.8x10^{-4}$M) partially uncoiled the sc DNA (lanes b, d and e respectively). DNA concentration loaded in all lanes was 1µg and electrophoresis was carried out at 4V/cm at room temperature. The gel was stained with EtBr.

4.2.2.1. Effect of chloroquine on Aβ - sc DNA interaction

Clioquinol is a drug which mimics Topoisomerase 1 in its action. We studied the sensitivity of the DNA-Aβ complexes to chloroquine induced topoisomers separation and to obtain information on the stability of the complexes, gel

electrophoresis was carried out in the presence of chloroquine (Fig.4.9). A fair degree of sensitivity was observed towards chloroquine for DNA+Aβ complexes (lanes b to e) compared to DNA alone (lane a). The topoisomers separation was observed for scDNA at 1μg/mL of chloroquine, while Aβ-DNA complexes were relaxed to topoisomers at much lower amount of chloroquine (0.8 μg /mL). The chloroquine acts only on the supercoiled form of DNA. Therefore Aβ peptides which act on supercoiled band resulting in open circular form conferred sensitivity to chloroquine.

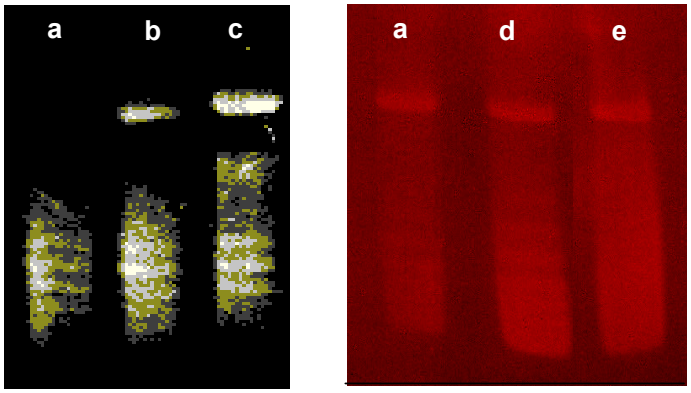

Figure. 4.9. Sensitivity for chloroquine induced topoisomers separation. Sc DNA was incubated with chloroquine in the absence and presence of Aβ peptides. The effective topoisomers separation was observed for scDNA at 1μg /mL of chloroquine (lane a), while Aβ (1-42), Aβ (1-40), Aβ (1-28), and Aβ (1-16) ($0.8x10^{-4}$M) -DNA complexes (lanes b, c, d and e respectively) were relaxed to topoisomers at lower chloroquine level of 0.8μg /mL of sample volume.

4.2.3 EtBr binding studies

4.2.3.1 Effect of EtBr on Aβ - scDNA interaction

The quantification of uncoiling pattern of scDNA induced by, Aβ peptides was also studied by measuring EtBr fluorescence intensity (at 1:1 w/w). Fluorescence intensity of the scDNA has shown to be altered by the interaction of the Aβ peptides. Aβ (1-42), Aβ (1-40) and Aβ(1-28) affected decreased fluorescence intensity. The fluorescence intensity for scDNA+Aβ (1-42) complex (Fig. 4.10b) was 35.8 % less compared to sc DNA alone (Fig. 4.10a) and this may be attributed to ψ DNA conformation. Aβ (1-40) has

caused a slight decrease (5%) (Fig. 4.10c) and Aβ (1-28) caused a significant reduction (29.5%) (Fig. 4.10d) in sc DNA fluorescence. However, Aβ (1-16) enhanced EtBr fluorescence by 29.5% (Fig. 4.10e) and this may be due to modified B-form of the sc DNA.

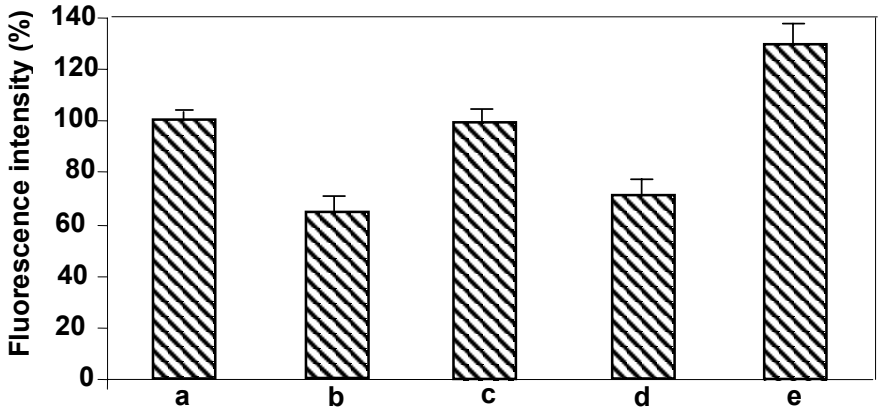

Figure. 4.10. Effect of Aβ peptides on sc DNA-EtBr fluorescence: Equal amounts (w/w) of sc DNA and EtBr was used as model to study the effect of Aβ peptides on EtBr intensity. EtBr intensity also provides information on DNA conformation. The uncoiling of scDNA was quantified by measuring the EtBr fluorescence intensity of 1:1 (w/w) DNA/EtBr solutions. The solutions were excited at 535nm and emission monitored at 600nm. (a) scDNA; (b) scDNA+ Aβ (1-42); (c) scDNA +Aβ (1-40); (d) scDNA +Aβ (1-28); (e) scDNA +Aβ (1-16). Values are expressed as mean ±SD.

4.2.3.1 Effect of EtBr on Aβ - poly d (AT).d(AT) and Aβ-poly d(GC).d(GC) interaction

All the Aβ peptide caused decreased EtBr fluorescence of poly d(AT).(AT) (Fig. 4.11). This may be due to the conformation alteration from B→A DNA as A-DNA has less EtBr binding than the B-DNA (Champion et al., 1998). However in case of poly d (GC).(GC), Aβ peptides facilitated EtBr binding and thereby increased the fluorescence intensity (Fig. 4.12).

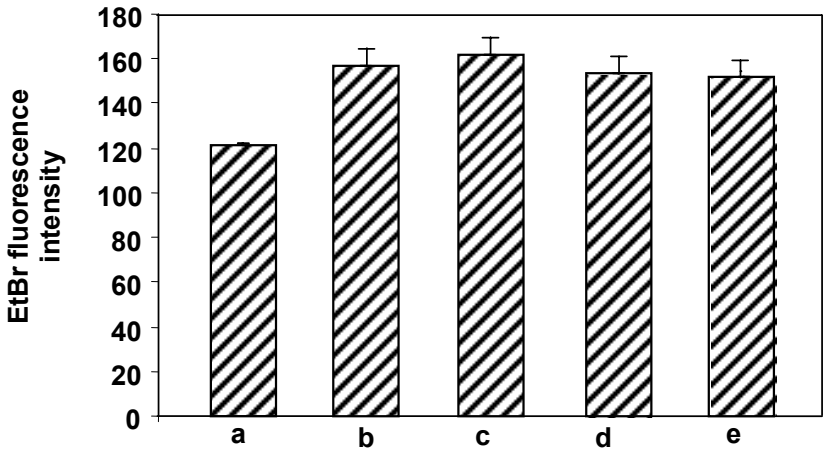

Figure. 4.11. Effect of Aβ peptides on poly d(AT).(AT)-EtBr fluorescence: Equal amounts (1:1w/w) of DNA (poly d(AT). d(AT)) and EtBr were used to study the effect of Aβ peptides ($14.0x10^{-6}$) on EtBr fluorescence. (a) poly d(AT).(AT) alone; (b) poly d(AT).(AT) + Aβ (1-16); (c) poly d(AT).(AT)+Aβ (1-28); (d) poly d(AT).(AT)+Aβ (1-40); and (e) poly d(AT).(AT)+Aβ (1-42). The solutions were excited at 535 nm and emission was monitored at 600 nm. Values are expressed as Mean ±SD.

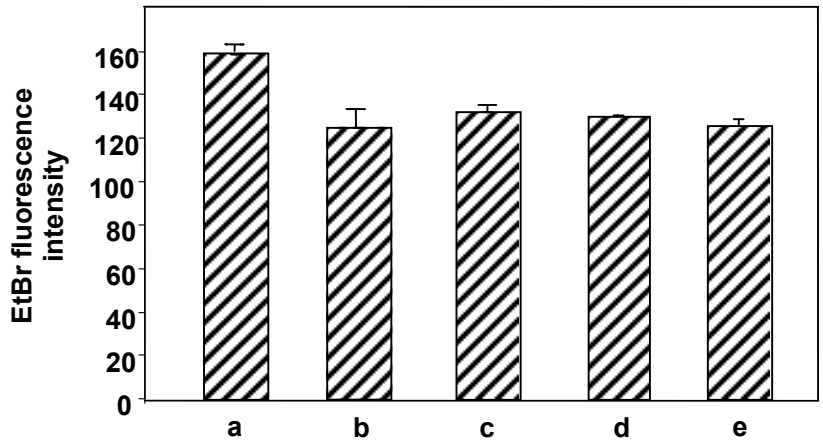

Figure. 4.12. Effect of Aβ peptides on poly d(GC).(GC)-EtBr fluorescence: Equal amounts (1:1 w/w) of DNA (poly d(GC). d(GC)) and EtBr were used to study the effect of Aβ peptides ($14.0x10^{-6}$) on EtBr fluorescence. (a) poly d(GC).(GC) alone; **(**b) poly d(GC).(GC) + Aβ (1-16); (c) poly d(GC).(GC)+Aβ (1-28); (d) poly d(GC).(GC)+Aβ (1-40); and (e) poly d(GC).(GC)+Aβ (1-42). The solutions were excited at 535 nm and emission was monitored at 600 nm. Values are expressed as Mean ±SD.

4.2.4. Melting temperature profiles

Melting temperature study provides insight on the stability of DNA. Thus in the next set of experiments, we examined the melting profile of scDNA in the absence or presence of Aβ peptides. The T_m value for scDNA was 59.5^0C (±0.5) and its melting profile revealed an unusual monophasic pattern (Fig. 4.13a). The T_m for sc DNA+Aβ (1-42) complex showed a typical biphasic melting pattern with two T_m values (59^0C ± 1 and 88^0C± 1.5) (Fig. 4.13e). This is the first report studying the melting pattern of ψ (+) DNA induced by Aβ (1-42). Aβ (1-16) did not alter the T_m at lower concentrations (10^{-6}M) while, it enhanced the T_m to 71.4^0C (±0.5) at higher (10^{-4}M) concentration. scDNA+ Aβ(1-16) complex showed a usual monophasic melting profile (Fig. 4.13d). Aβ (1-28) (Fig. 4.13c) and Aβ1-40) (Fig. 4.13d) at a concentration of 10^{-4}M caused non-cooperative melting temperature profiles in scDNA. The different melting temperature profiles of scDNA caused by various Aβ peptides reveal that Aβ peptides alter melting profiles in a sequence dependent manner.

To further explore the nature of the unusual monophasic melting profile of scDNA and to see the effect of uncoiling the scDNA on its Tm, we studied the melting pattern of the scDNA in the presence of chloroquine. There was no change in the Tm (59.5^0C (± 0.5) in scDNA treated with chloroquine at 1μg/mL (topoisomers separation concentration as confirmed by Agarose gel) and 10μg/mL (complete relaxation of superhelicity to linear DNA) concentration. These results indicate that the unusual monophasic melting profile observed in case of scDNA may not be due to the supercoiling, because the same melting pattern was obtained also with linearised scDNA.

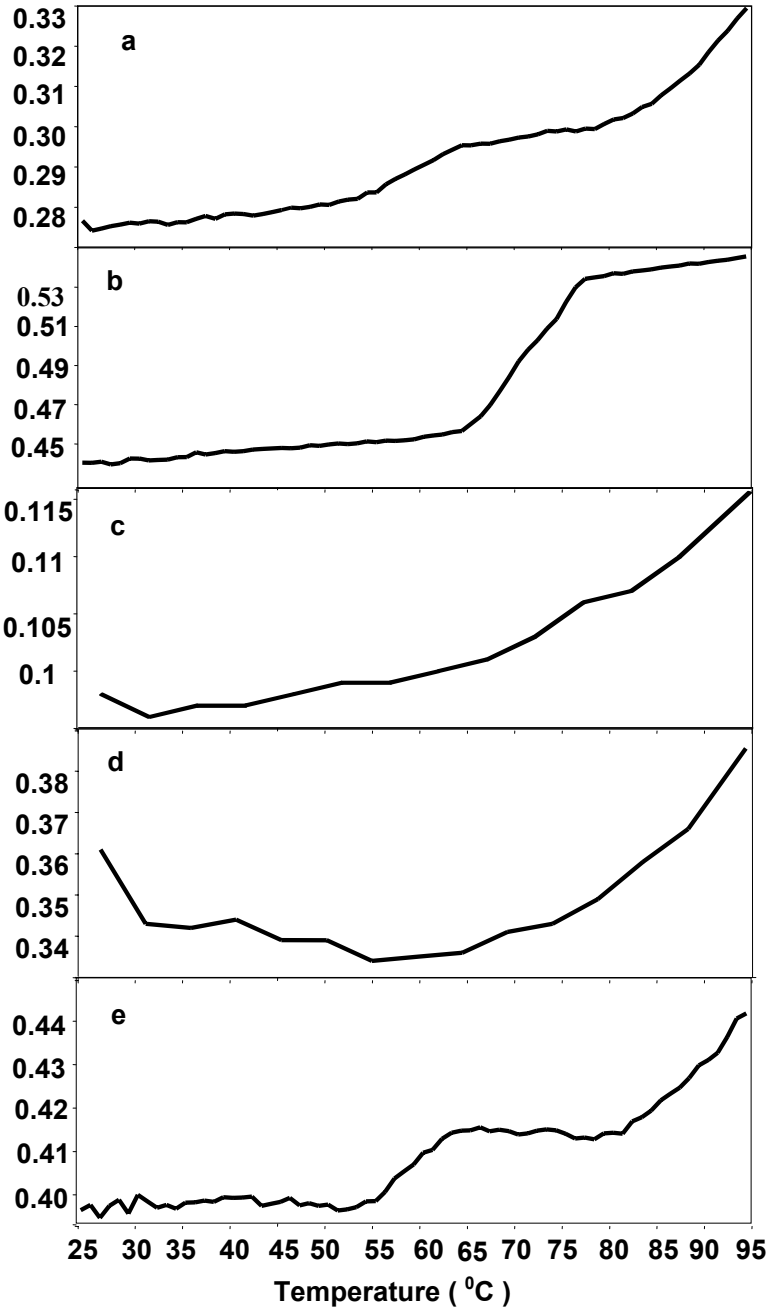

Figure. 4.13. Effect of Aβ peptides on sc DNA meting temperature profiles. The UV absorbance at 260 nm was recorded for scDNA and scDNA + Aβ peptides at different temperatures (25-95^0 C). The melting curves were recorded in 10^{-2} M HEPES, pH 7.0 in UV- spectrophotometer with thermostat. (a) sc DNA alone; (b) sc DNA + Aβ (1-16); (c) sc DNA + Aβ (1-28) ; (d) sc DNA + Aβ (1-40); and (e) sc DNA + Aβ (1-42).

4.2.5. Localization of Aβ in the nuclei of CA1 neurons in hippocampal region of brain

In previous chapter we showed that hippocampal DNA obtained from postmortem AD brain samples has a preferentially left-handed Z-DNA conformation (Anitha et al., 2002) and our present results show that Aβ induces similar (?) conformational changes *in vitro*. Moreover, it was shown that Aβ induces apoptosis in cell lines and rabbit brains (Selkoe, 1996; Clements et al., 1996; Huang et al., 2000). Thus we hypothesize that Aβ is presented in the neuronal nuclei of affected AD brain areas where it may induce DNA conformational changes. These changes may in turn lead to apoptotic neuronal death. In an attempt to examine whether Aβ (1-42) is presented in neuronal nuclei in AD brains and explore its relationship to apoptosis, the presence of Aβ (1-42) immunoreactivity and apoptotic nuclei (TUNEL positive) were examined in neurons of the hippocampal CA1 region of normal and AD brains. The number of Aβ and or TUNEL positive cells counted per mm^2 of brain section are given in table (Table. 4.1). Quantification of data revealed that in normal hippocampal brain sections 25% of the cells were apoptotic while in the case of AD brain, about 75% of the cells were apoptotic. When normal brain hippocampal sections were tested for co-localization of apoptosis (TUNEL positive) and Aβ immunoreactivity, we could not find any TUNEL positive cell, which was also Aβ positive. But in the AD brain sections out of 100 TUNEL positive cells counted, 50% of cells were found to be positive also for Aβ. These results suggest that Aβ is deposited in the vicinity of DNA in the nuclear region of AD cells. In case of normal brain sections Aβ(1-42) immunoreactivity was not observed in either apoptotic and non-apoptotic hippocampal neurons. Figure. 4.14a presents a confocal image of a single representative neuron showing absence of Aβ deposition in an apoptotic nucleus from hippocampal section of normal brain. Figure. 4.14b shows the localization of Aβ immunoreactivity in the apoptotic nucleus of hippocampal neuron in CA1 region. We could not find any TUNEL positive cell, which was also Aβ positive. But in the AD brain sections out of 100 TUNEL positive cells

counted, 50% of cells were found to be positive also for Aβ. These results suggest that Aβ is deposited in the vicinity of DNA in the nuclear region of AD cells. In case of normal brain sections Aβ (1-42) immunoreactivity was not observed in either apoptotic and non-apoptotic hippocampal neurons. Figure. 4.14a presents a confocal image of a single representative neuron showing absence of Aβ deposition in an apoptotic nucleus from hippocampal section of normal brain. Figure. 4.14b shows the localization of Aβ immunoreactivity in the apoptotic nucleus of hippocampal neuron in CA1 region.

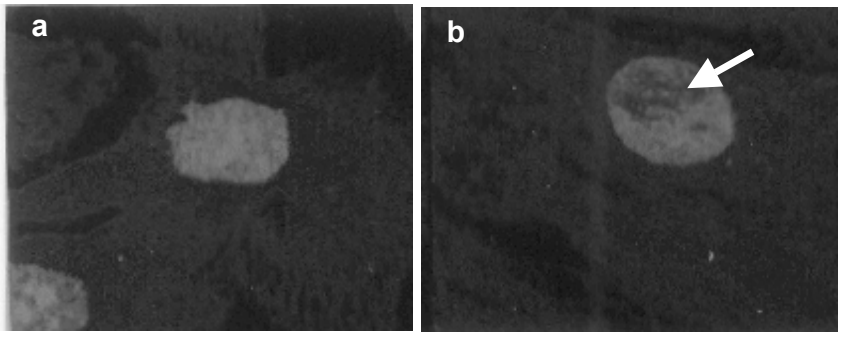

Figure. 4.14. Localization of Aβ in human normal and AD brain hippocampal samples: 15 micron frozen sections were cut from hippocampal brain regions and stained for apoptosis using fluorescence TUNEL technique and co-stained for Aβ (1-42) using monoclonal antibody and developed by DAB reagent. Co-localization of apoptosis and Aβ immunoreactivity in the viscinity of DNA in a nucleus of hippocampal neuron in the CA1 region was imaged by Confocal microscopy. (a) Normal brain apoptotic nuclei showing absence of Aβ immunoreactivity. (b) AD brain apoptotic nuclei from hippocampal region showing Aβ immunoreactivity. Arrow indicates Aβ immunoreactivity.

Brain region	**Normal**			**AD**		
Hippocampus	**TUNEL**	**Aβ**	**TUNEL+Aβ**	**TUNEL**	**Aβ**	**TUNEL+Aβ**
	15±1.0	**0**	**0**	**100±5.0**	**75±2.0**	**50±4.0**

Table. 4.1. Number of Apoptotic and Aβ positive cells in normal and AD affected human brain samples. TUNEL and Aβ positive cells in hippocampal sections were counted microscopically in mm^2. The results presented were obtained from sections of each of 3 brains for normal and 6 sections of each of 6 brains for AD. Values are expressed as Mean ±SD.

4.3. Discussion

AD is associated with several complex neuropathological events like deposition of Aβ in SPs, abnormal phospharylation of tau in NFTs, oxidative stress and DNA damage (Iqbal et al., 1994; Lyras et al., 1997). It is of interest to mention that Aβ (Grant et al., 2000; Gouras et al., 2000) was found to be localized in the nuclei. Our team first time evidenced the presence of left-handed, rigid Z-DNA in severely affected AD brain and a B-Z intermediate DNA conformation in moderate AD hippocampus (Anitha et al., 2002). In contrast, normal young and aged brains have usual right-handed Watson-Crick DNA conformation (Anitha et al., 2002). It has also been hypothesized that the prime etiological factors of AD like Aβ, tau and Al (a highly debatable etiological factor) might be playing a role in right handed to left handed DNA helical change associated with AD. Based on this DNA conformational change, we proposed an explanation to the unusual phenomenon like nucleosome mis-assembly, G* specific DNA oxidation, terminal differentiation and altered gene expression associated with AD brain (Anitha et al., 2002; Anitha et al., 2001). There were few reports on the ability of Al and tau binding to DNA (Karlik et al., 1980; Rao et al., 1993; Rao and Diwakar, 1993; Hua and He, 2000; Hua and He, 2002). It was also evidenced that Al could strongly bind to AT* specific oligomers (Rajan et al., 1996). In the present study, we first time evidenced the interaction of Aβ with scDNA. We found that Aβ (1-42) not only binds to scDNA but also is able to alter the conformation of DNA. An initial B→C transition was observed which gradually transformed into ψ-DNA, presumably reflecting a partial DNA collapse into a ψ-phase (Zuidam et al., 1999). In ψ-DNA, the DNA molecules are tightly packed in to toroidal superhelical bundle whose chiral sense is defined by the intrinsic DNA handedness. Specifically, the right-handed secondary conformations such as the B and C motifs stabilize a left-handed tertiary conformation (Reich et al., 1994).

Such a tightly packed left-handed DNA organization exhibits negative CD signals whose magnitude is larger than that characterizing dispersed DNA molecules which lack a tertiary structure. The ψ-DNA conformation induced by Aβ (1-42) is structurally closer to Z-DNA, which was observed in severely

affected AD brain (Anitha et al., 2002). Studies by Thomas and Thomas (1989) clearly showed that ψ-DNA, an ordered, twisted, tight packing arrangement of the double helix, is structurally and immunologically closely related to Z-DNA family. It is left handed in conformation like Z-DNA. This evidently indicates that DNA topological changes induced by Aβ is similar to the changes seen in AD brain DNA. Our study also provided intriguing observation of the sequence specificity of the Aβ peptide binding. The peptides showed preferential binding to AT rich sequences and caused B→A DNA transition, similar to binding of Al. However though the peptides did not cause significant CD spectral perturbation in case of poly d(GC).d(GC), they facilitated EtBr binding to DNA indicating its interaction with of poly d(GC).d(GC) also. These protein-DNA complex conformations (altered B-DNA, A-DNA, ψ- DNA, B-C-A complex) are energetically weak and are likely to go into Z-DNA conformation as reported in AD brain (Anitha et al., 2002). Vast arrays of small scDNA packets have been found to be present in animal and human cells and are known to be involved in gene expression (Bauer et al., 1980). Our study also clearly shows that Aβ peptides relax the sc DNA partially. Thus the present study hearsay for the first time that Aβ uncoils sc DNA besides bringing about helicity changes. Another interesting feature observed was sensitivity of sc DNA+Aβ complexes to chloroquine. This observed sensitivity indicates a possible alteration in DNA replication and gene expression in the cells.

The possible complex role of Aβ in modulating DNA helicity in relevance to AD is proposed in the figure. 4.15. We propose that Aβ peptides along with other etiological factors might modulate DNA topology in AD brain. The complex conformational changes (ψ-DNA, B→A; B→C or B-C-A, altered B) observed experimentally in scDNA induced by Aβ peptides, are presumably the early events in AD pathology. Because, we reported in moderately affected AD brain the DNA has a B-Z intermediate conformation and other intermediary complex conformations might possibly exist (Anitha et al., 2002; Anitha et al., 2001). In the later stage the secondary factors such as oxidative stress, cell shrinkage, ionic imbalance, polyamines and metals are likely to

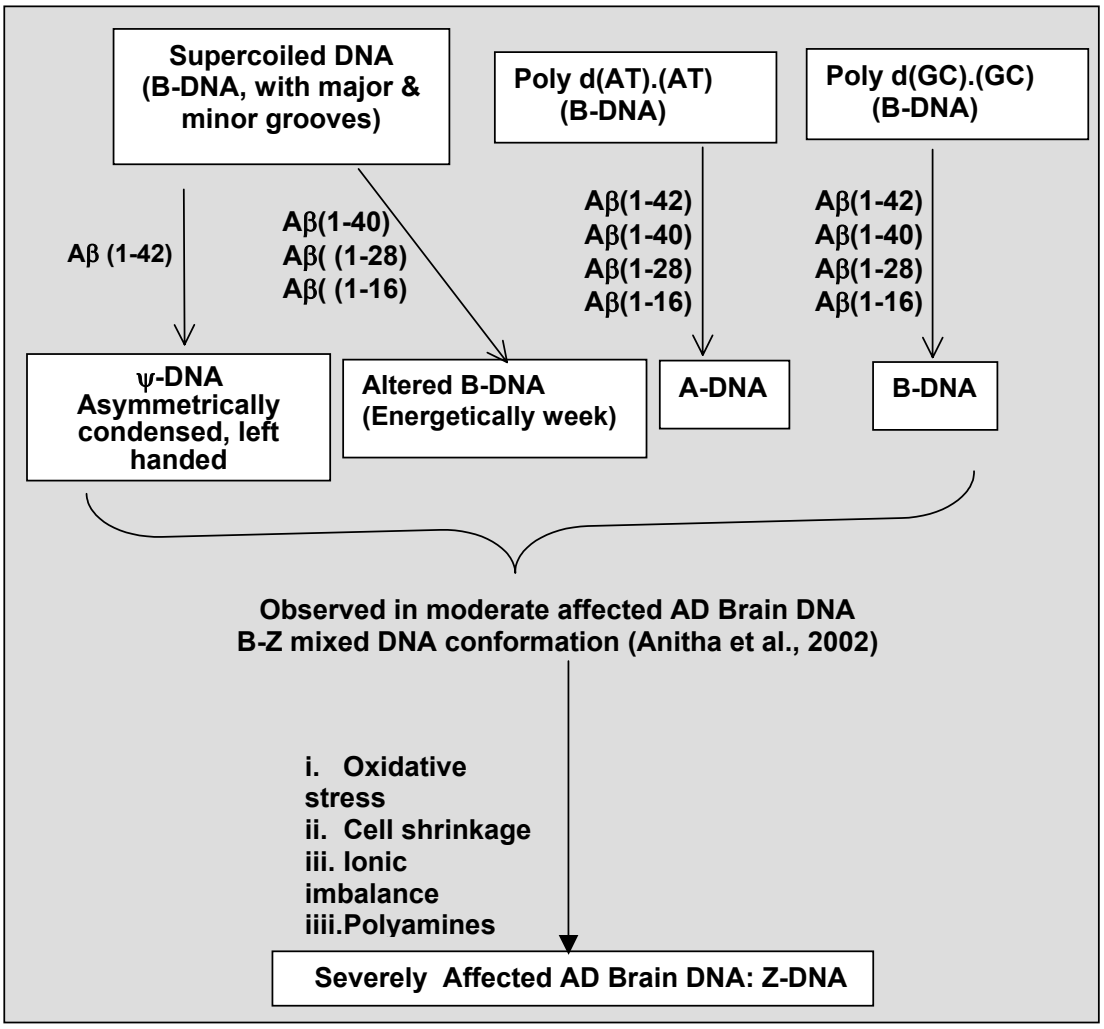

Figure. 4.15. Possible role of Aβ peptides in modulating DNA topology in relevance to AD. Aβ peptides could cause conformational alteration in sc DNA and poly d(AT) (AT). These modified conformations might further undergo alterations under various conditions like oxidative stress, cell shrinkage, ionic imbalance and polyamines.

play role to convert these intermediate complex conformations to rigid, and left- handed Z-DNA.

Accordingly we reported the presence of left-handed Z-DNA in severely affected AD brain (Anitha et al., 2002). We propose that DNA topological changes also play a role in AD progression.

These novel observations may have important implications for aiding in our understanding of toxicity of Aβ in terms of its direct role in altering DNA conformation and its relevance to neurodegeneration occurring in AD.

CHAPTER - 5

5.1. Introduction

Amyloid assembly into fibrillary structure is a defining characteristic of AD. Accumulating evidence suggests that Aβ peptides contribute to the neurodegeneration in AD (Selkoe, 1991; Hardy and Higgins, 1992; Hardy and Selkoe, 2002; Ohyagi et al., 2004). The SPs of AD is a complex extracellular lesion composed of a central deposit of amyloid (the core) surrounded by activated microglia, fibrillary astrocytes and dystrophic neuritis (Masters et al., 1985). Aβ is constitutively produced by cells of the CNS, and is present in normal and AD CSF and plasma. Under pathogenic conditions, the transition from a random coil to a β-sheet conformation in β-amyloid peptide causes deposits of the amyloid fibrils (Salomon et al., 1996; Pappolla et al., 1998). Deposits of Aβ (1-40)/(1-42) fibrillar aggregates in the brain as SP and as amyloid angiopathy are among the key pathological hallmarks of AD (Selkoe, 1997 & 2001). Several lines of evidence suggest that Aβ may play a key role in the pathogenesis of AD including *in vitro* evidence that Aβ may be toxic to neurons and cultured cells and *in vivo* evidence for neuronal degeneration from exposure to Aβ (reviewed in Selkoe, 1996; Chen et al., 1996). Although genetic data indicate a central role for Aβ in the etiology of AD, the molecular form associated with the neurodegeneration process has not been definitely identified. The number of amyloid plaques does not correlate in number or distribution with neurodegeneration or clinical dementia. Thus recent debate with in the AD community has focused on whether fibrillar (amyloid) or soluble oligomers of Aβ are active species of the peptide that ultimately cause the synaptic loss and dementia associated with AD (Lansbury, 1999; Lacor et al., 2004; Walsh and Selkoe, 2004). The intracellular presence of potentially amyloidogenic fragments of Aβ remains a subject of intensive investigation (Wilson et al., 1999). Studies suggest that Aβ-dependent toxicity can occur before significant extracellular accumulation, possibly involving intracellular Aβ accumulation. Studies with neuronal cell culture have demonstrated that Aβ can accumulate intracellularly (Wertin et al., 1993) after either endogenous Aβ production or uptake of extracellular Aβ and contribute to plaque formation

(D'Andrea et al., 2001). Intracellular Aβ dimers have also been detected in primary neurons and in neuronal cell lines (Walsh et al., 1999). All these data unequivocally support the concept of intracellular existence, and toxicity of Aβ. The proposed mechanisms for neurotoxicity for Aβ are diverse and there is no unifying mechanism for the toxicity and one of the mechanisms proposed was induction of apoptosis. Under *in vitro* conditions Aβ (1-42) is reported to be capable of inducing apoptosis in cultured cortical neurons (Loo et al., 1993; Cotman et al., 1994). Synthetic Aβ peptides are shown to trigger the degeneration of cultured neurons through activation of an apoptotic pathway (Forloni et al., 1993; Anderson et al., 1995; Nakagawa et al., 2000). Neuronal cells treated with Aβ exhibit morphological and biochemical characteristics of apoptosis, including membrane blebbing, compaction of nuclear chromatin and internucleosomal DNA fragmentation. Esposito et al. (2004) reported that Aβ, especially intracellular Aβ counteracts the antiapoptotic function of its precursor protein and primes proapoptotic pathways. Interestingly studies by Baron et al. (1999) suggested that Aβ exert its toxic effect via activation of transcription factors (Baron et al., 1999). The authors reported that Aβ (25-35) induced the expression of the growth arrest and DNA damage-inducible gene (gadd 45) implicated in the DNA excision-repair process (Baron et al., 1999). In particular, they observed strong up regulation of gadd45, indicating DNA damage in Aβ cytotoxicity suggesting that DNA strand breaks occurred rapidly in cells exposed to Aβ which may be a critical event in Aβ neurotoxicity (Baron et al., 1999). Furthermore, aurintricarboxylic acid (ATA) a nuclease inhibitor was shown to prevent apoptotic DNA fragmentation and delays cell death caused by Aβ (Loo et al., 1993). Moreover DNA damage suggestive of apoptosis was increased 30 fold in neurons, oligodendrocytes, and microglia of AD brains (Lassmann et al., 1995). Taken together, the above findings strongly link the Aβ and DNA damage. Our previous study (Hegde et al., 2004) along with other studies (Culvenor et al., 1998; Grant et al., 2000; Gouras et al., 2000; Ohyagi et al., 2004) reported the nuclear localization of Aβ and it's DNA binding property. We also showed that the Aβ alters the superhelicity of the sc DNA. The objective of the present study is to elucidate the molecular mechanism by which Aβ alters the superhelicity of sc DNA.

5.2. Results

5.2.1. Evidence for linearization of supercoiled DNA (sc DNA) by Aβ (1-42)

The strategy used for the identification of Aβ (1-42) action on DNA is through agarose gel, Electron Microscopic (EM) study and estmiation of single strand breaks. Figure 5.1 presents the sc DNA damage induced by Aβ(1-42). Aβ (1-42) ($0.8x10^{-4}$M) interaction with sc DNA resulted in linearization of sc DNA. Lane a, Figure 5.1 represents sc DNA alone with 80% supercoiled DNA as shown by the intense supercoiled band (form I). The gel picture depicts that the peptide first induces relaxed circular form as shown by the increased intensity of the open circular band (form II) and then linearization of the DNA (form III) (lane b, Fig. 5.1). Identification of the linear DNA was made using the comparative migration of the *EcoR*I treated linearized sc DNA (Fig. 5.1c). Sc DNA has one restriction site for the *EcoR*I restriction enzyme. *EcoR*I treated linearised sc DNA also served as a control for double stranded breaks also. We propose that the appearance of the open circular form of DNA by Aβ is through the single strand nicks and then leading to linearization and perhaps by double stranded breaks as a result of cumulative single stranded nicks in sc DNA. The peptide-DNA interactions were carried out in 10mM Tris-NaCl-EDTA buffer (pH.7.0) for 12 h. The EM study enabled to visualize the open circular form and linear forms of DNA induced by Aβ(1-42) in sc DNA (Fig. 5.2). Figure 5.3a represents the EM picture of sc DNA alone with supercoiling. Figure 5.3b shows the open circular and linear forms of the scDNA induced by Aβ(1-42). The EM images of open circular and linear forms have been enlarged in Fig. 5.2c and 2d respectively. The physical association of the fibrillar form of peptide with DNA was also observed in EM picture (Fig. 5.2e).

The nicking of the Aβ (1-42) occurred in a concentration dependent manner (lanes b-e, Fig. 5.3). As shown in the Fig. 5.3 the increasing concentration of Aβ (1-42) (10-200 μM) resulted in a gradual decrease in the supercoiled band

intensity with simultaneous increase in the relaxed and linear form of DNA. Since the gel was run in 0.7% gel both open circualar form and linear form ran together in a single band (formII). The quantitative analysis of the electrophoretic assay has been presented in the Figure 5.3B has been represented in Figure 5.3C.

The role of metals on DNA nicking of Aβ was studied using calcium (Ca^{2+}), magnesium (Mg^{2+}) and zinc (Zn^{2+}) ions. The DNA nicking of the Aβ (1-42) has been enhanced in the presence of Mg^{2+}, a divalent ion, and a requirement for various DNA degrading nucleases (lane d, Fig. 5.4). Mg^{2+} enhanced the Aβ induced nicking property thereby resulting in increased formation of the relaxed circular and linear form DNA. Ca^{2+} also enhanced the Aβ induced nicking activity as shown by the increased open circular form (lane e, Fig. 5. 4). However, Zn^{2+} did not have any effect on Aβ induced nicking activity (lane c, Fig. 5.4).

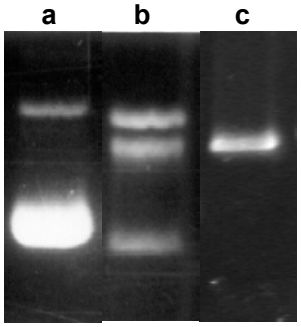

Figure. 5.1. Effect of Aβ (1-42) binding to sc DNA on 1.4 % Agarose gel. (a) sc DNA alone (Cesium chloride purified) showed >90% superhelicity; (b) Aβ(1-42) ($0.8x10^{-4}$M) partially uncoiled and linearized the scDNA; (c) Linearized supercoiled DNA (EcoR1 digested). DNA concentration loaded on all lanes was 1μg and electrophoresis was carried out at 4V/cm at room temperature. The samples were stained with EtBr (1μg/ml).

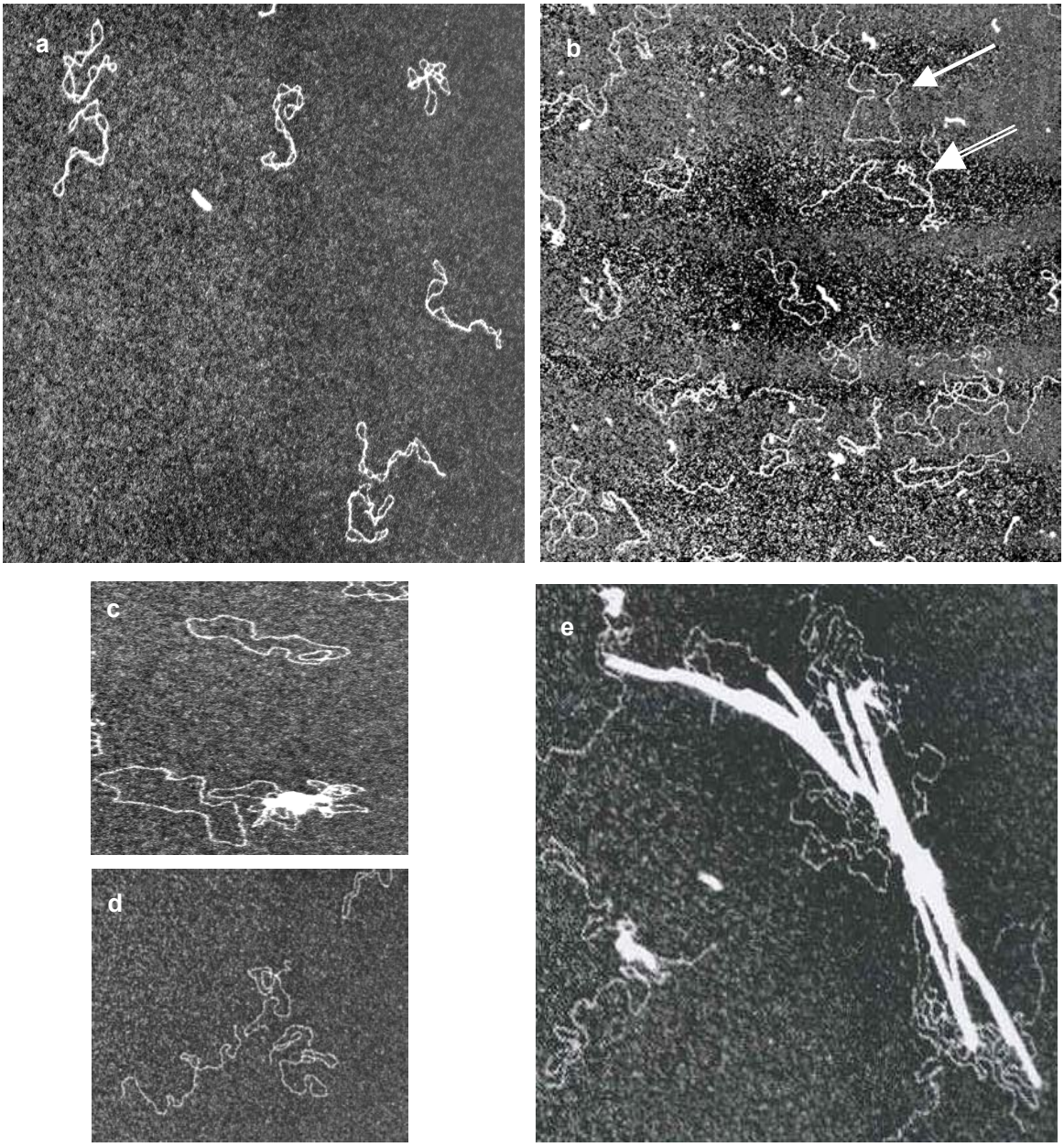

Figure. 5.2. Electron microscopic analysis of Aβ (1-42) interaction with sc DNA. (a) sc DNA alone; (b) sc DNA + Aβ(1-42). The single and double lined arrows represent the open circular and linear form respectively caused by the Aβ; (c) Enlarged portion of the open circular form of sc DNA; (d) Enlarged portion of the linear form of sc DNA; and (e) Physical association of Aβ(1-42). For EM study 1μg of sc DNA was incubated with 0.1 x 10^{-6} M Aβ(1-42) in 10mM Tris-NaCl-EDTA buffer (pH.7.0) at 37^{0}C for 12 h.

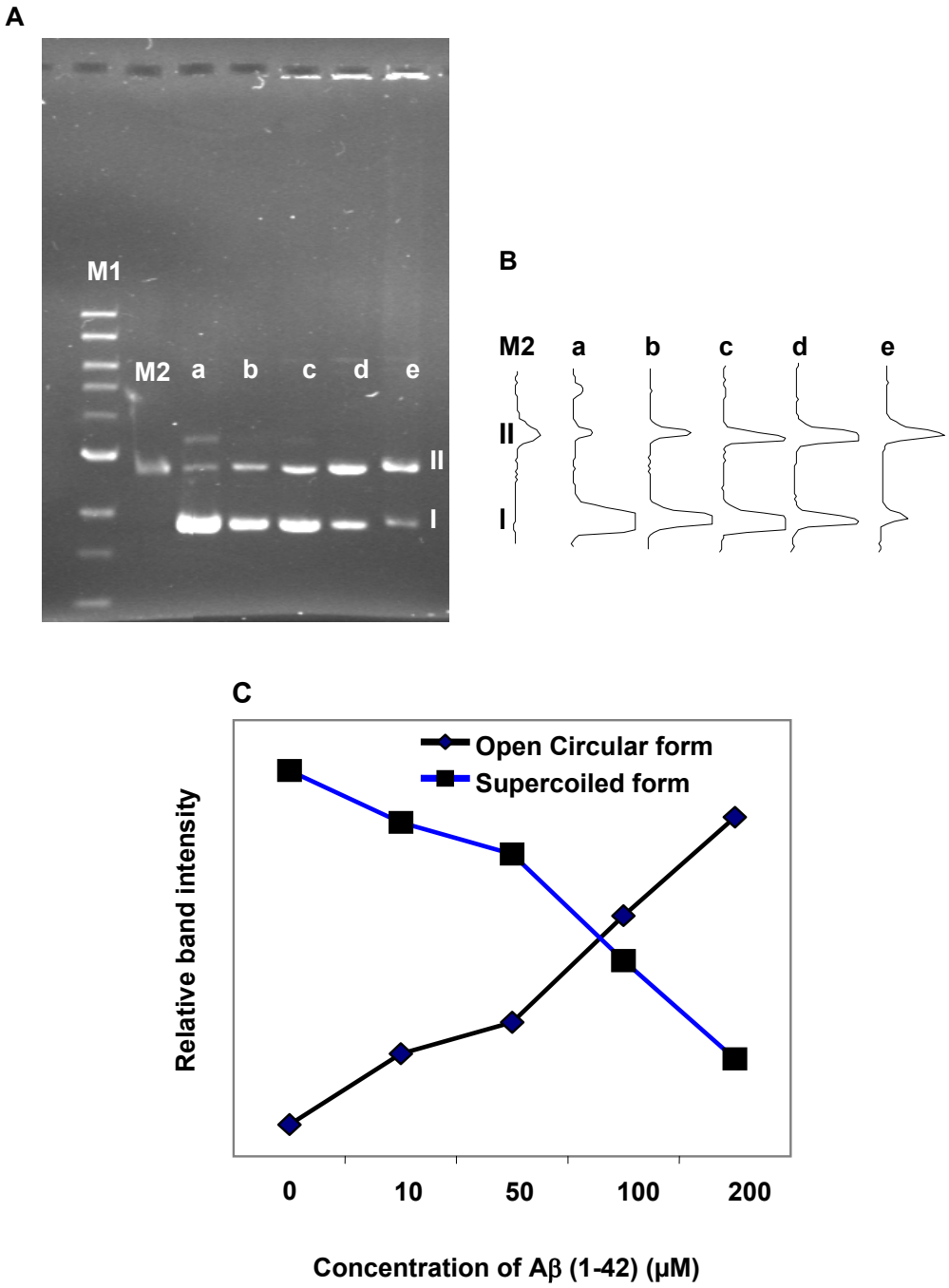

Figure. 5.3. Effect of concentration of Aβ (1-42) on sc DNA. (A) Agarose gel electrophoresis (0.5%): M1, 1Kb marker; M2, Linearized supercoiled DNA (*EcoR*1 digested); (a) scDNA alone; (b) scDNA + Aβ (1-42) ($10x10^{-6}$ M); (c) scDNA + Aβ (1-42) ($50x10^{-6}$ M); (d) scDNA + Aβ (1-42) ($10x10^{-5}$ M); (e) scDNA + Aβ (1-42) ($20x10^{-5}$ M). **(B)** Representation of DNA bands in the form of peaks and **(C)** Densitometry of the above gel: Correlation of relative amounts of supercoiled (form I) and open circular and linear form (form II).

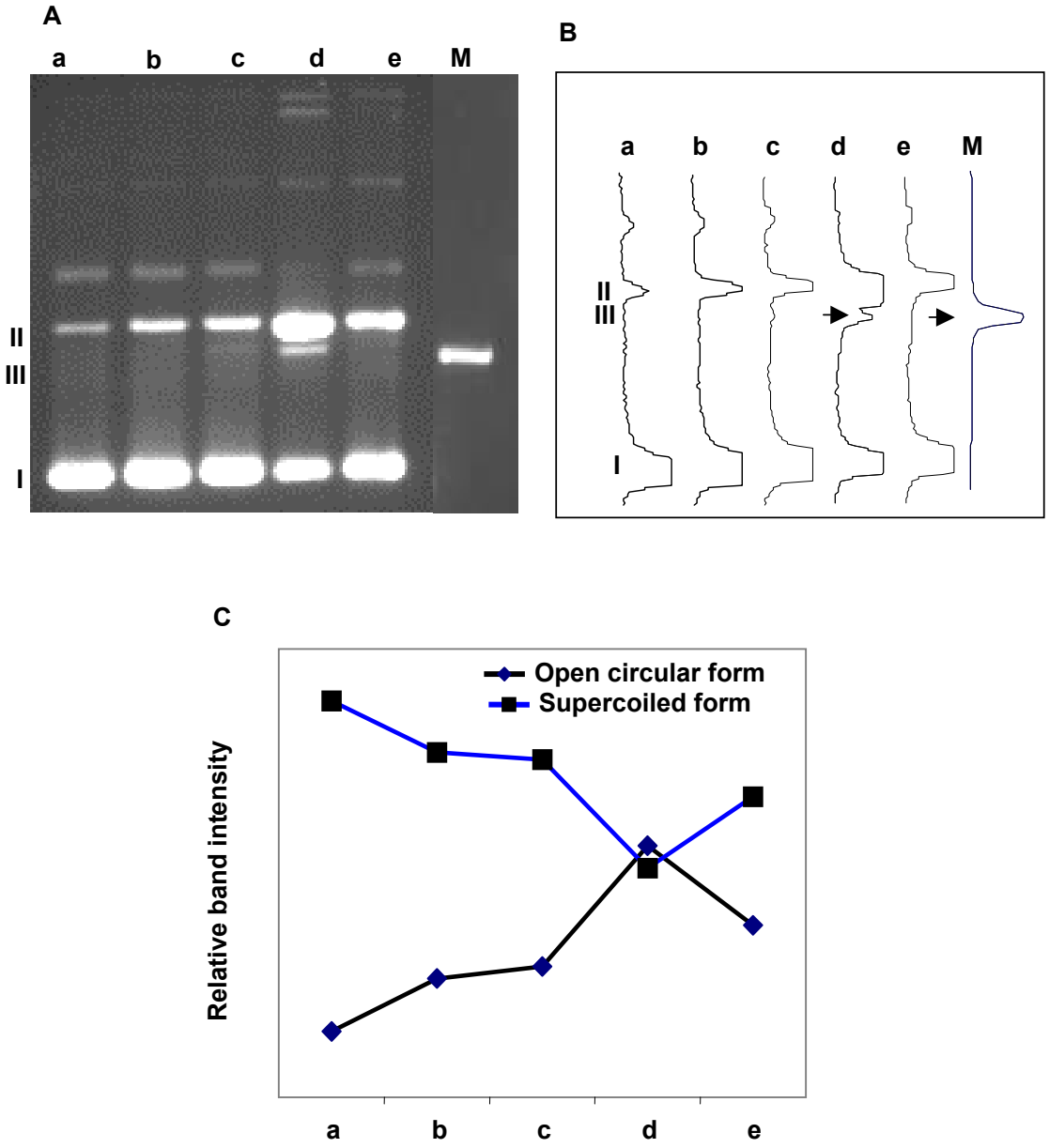

Figure. 5.4. Effect of Ca, Mg and Zn on Aβ (1-42) – sc DNA interaction. (A) Agarose gel electrophoresis (1%): (a) sc DNA alone; (b) sc DNA + Aβ (1-42) ($50x10^{-6}$ M) ; (c) sc DNA + Aβ (1-42) ($10x10^{-6}$ M)+ 1mM $ZnCl_2$; (d) sc DNA + Aβ (1-42) ($50x10^{-6}$ M)+ 1mM $MgCl_2$; (e) sc DNA + Aβ (1-42) ($50x10^{-6}$ M)+ 1mM $CaCl_2$; (M) *EcoR*I treated linearised sc DNA. (**B**) Representation of the DNA bands in the form of peaks: Arrows represent the peaks corresponding to the linearised sc DNA (form III). (**C**) Densitometry of the above gel: Correlation of relative amounts of supercoiled (form I) and open circular (form II). Aβ (1-42) was freshly dissolved in water.

5.2.2. Effect of aging of peptide (Aβ 1- 42) on DNA nicking

The conformational changes of Aβ from random coil to β-sheet (McLaurin et al., 1998) and subsequnt fibril foramtion (Harper et al., 1997) can take from hours to days depending on the particular peptide batch. Therefore we studied the DNA nicking activity of Aβ at different time intervals of incubation in relation to its aggregation state. Aβ (1-42) was dissolved in Milli Q water at a stock concentration of 1mg/ml and incubated (without stirring) for 6 days at 37^0C. At different time intervals an aliquot of the peptide stock solution was collected to look for the DNA nicking activity and also fibrillization/ aggregation state. The aggregation of the peptide was monitored using thioflavine- T flourescence (see materials and methods). Aβ (1-42) of varius incubation time intervals was incubated with sc DNA in 10mM Tris-NaCl-EDTA (pH.7.0) buffer for overnight and subjected to agarose elcetrophoresis. The agarose gel study revealed that Aβ (1-42) caused only open circular form till 8 h of incubation while at 24h and afterwards incubated Aβ (1-42) caused linearization of the DNA (Fig. 5.5). The corresponding thioflavine- T fluorescence of the Aβ was shown to be gradually decreased from 0h to 32h and increased there upon (Fig. 5.6). The 0h thioflavin-T fluorescence was higher because of the non-specific aggregates. The maximum DNA nicking activity corresponds to the lower thioflavine T-flourescence. However Aβ (1-42) continued to linearize the DNA till the 6^{th} day of incubation which has higher thioflavine –T fluorescence. EM study of Aβ (1-42) revealed fibrillar structure only at 124 h incubation and not at 32 h of incubation (Fig. 5.7).

5.2.3. Protection against Aβ induced DNA nicking

Protection against Aβ (1-42) induced DNA nicking was studied using aurintricarboxylic acid (ATA) and trifluoroethanol (TFE). ATA is a powerful inhibitor of proteins whose biological function depends on the formation of complex with nucleic acid. ATA prevents the formation of a protein-nucleic acid complex (Gonzalez et al., 1980). ATA prevented Aβ (1-42) induced nicking Fig. 5.8). Furthermore it could also partially prevent Ca^{2+} and Mg^{2+} induced DNA nicking (Fig. 5.9).

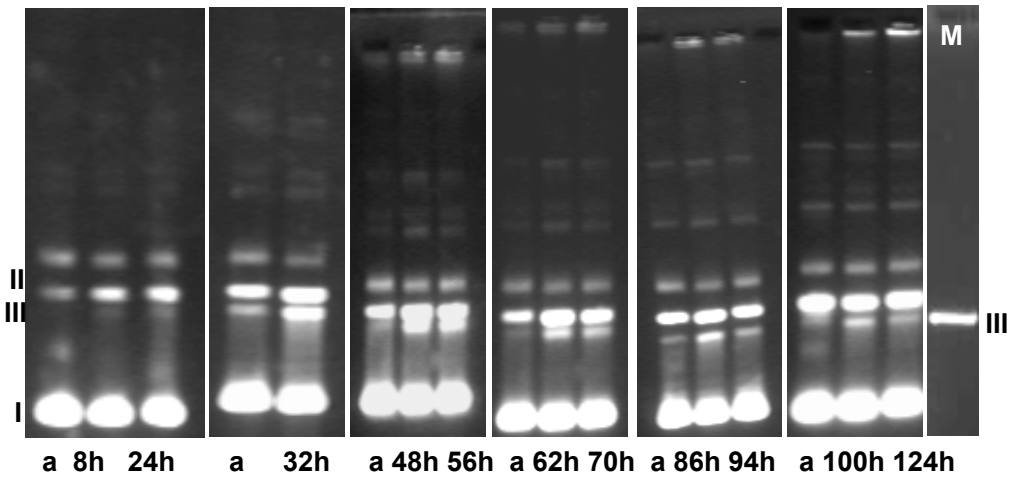

Figure. 5.5. sc DNA nicking activity of Aβ (1-42) at different time intervals of incubation: Agarose gel electrophoresis. a: sc DNA alone. 8, 24, 32, 48, 56, 62, 70, 86, 94, 100 and 124 resprestnt the age (in hours) of the Aβ peptide. Aβ of different age was incubated with the sc DNA for overnight. M: EcoRI treated linearized sc DNA. Aβ (1-42) is dissolved in Milli Q water at 1mg/mL concentration. I, II and III represent supercoiled, open circular and linear forms of sc DNA respectively.

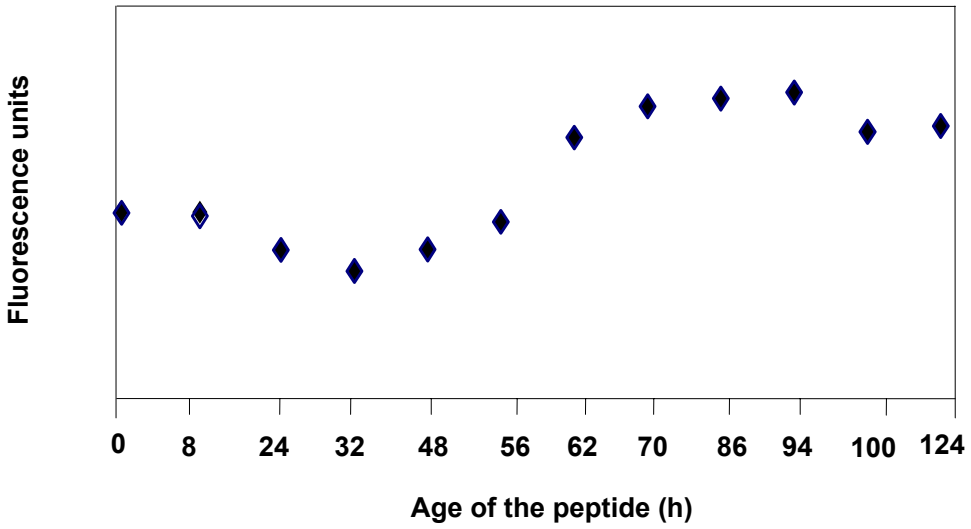

Figure. 5.6. Thioflavine-T fluorometric assay of Aβ (1-42). Aβ (1-42) dissolved in Milli Q water (200μM) was incubated at 37 ^{0} C for 124 h. At an appropriate time, an aliquot of the solution (0.5 μM) was mixed with thioflavine-T (100μM). Fluorescence intensity was monitered at 482 nm with exictation at 450 nm in 10mM Tris –HCl buffer (pH.7.0).

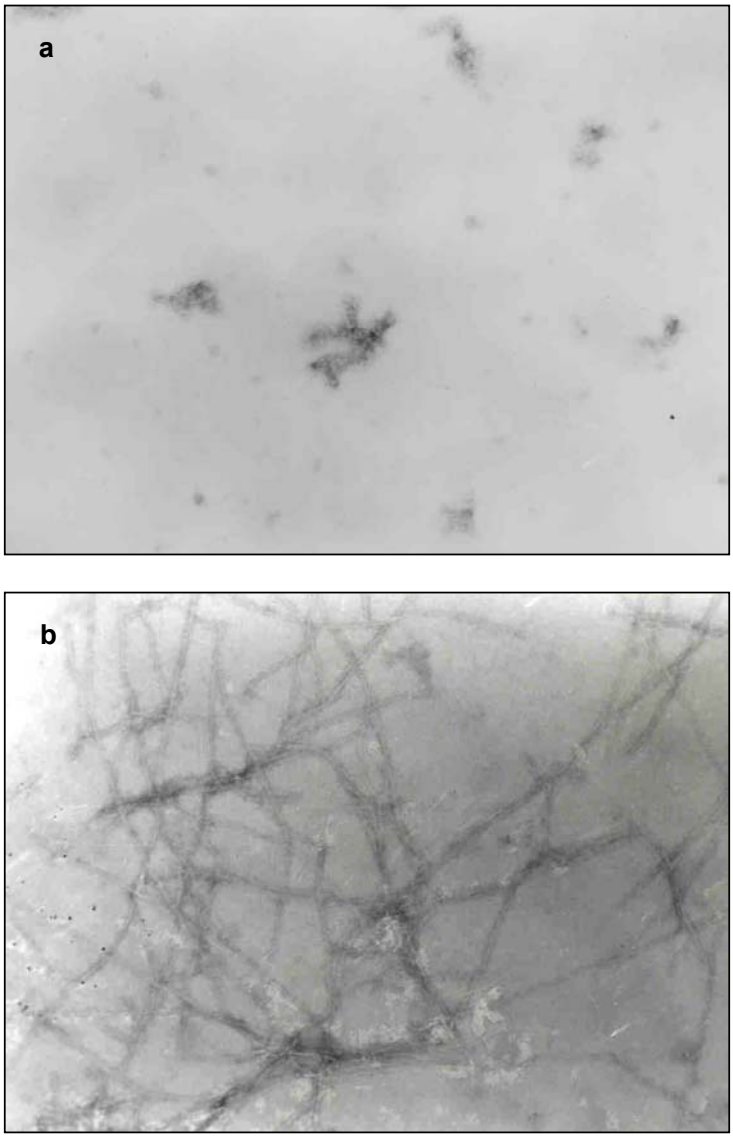

Figure 5.7. Electron Microscopic pictures of Aβ (1-42). Aβ (1-42) dissolved in Milli Q water (200μM) was incubated at 37 ^{0} C. (a) Aβ (1-42) at 32 h of incubation and (b) Aβ (1-42) at 124 h of incubation. Aβ (1-42) at 124 h of incubation clearly showed fibrillar network.

TFE also protected the Aβ (1-42) induced nicking (Fig. 5.10). Aqueous solution containing TFE favors α-helical structure in Aβ peptides (Barrow et al., 1992). Therefore both ATA and TFE protected the Aβ (1-42) induced DNA nicking. Pike et al (1993) observed the disappearance of neurotoxicity by reversing the aggregation process by dissolving aged Aβ (1-42) in TFE, which promotes α-helical structure. Similarly in our study, DNA nicking activity of Aβ was inhibited by its dissolution in TFE.

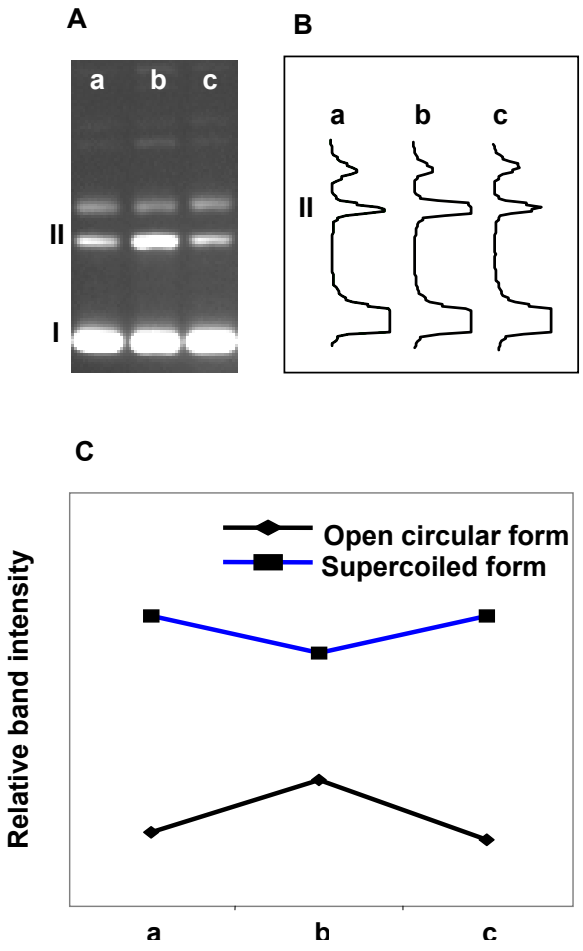

Figure 5.8. Effect of ATA on Aβ (1-42)-sc DNA interaction. (A) Agarose gel (0.75%) electrophoresis: (a) sc DNA alone (1μg); (b) sc DNA + Aβ (1-42) ($50x10^{-6}$ M); (c) sc DNA + Aβ (1-42) ($50x10^{-6}$ M) + ATA (2×10^{-4} M). **(B)** Peak representation of the DNA bands. **(C)** Densitometry of the above gel: Correlation of relative amounts of supercoiled (form I) and open circular (form II).

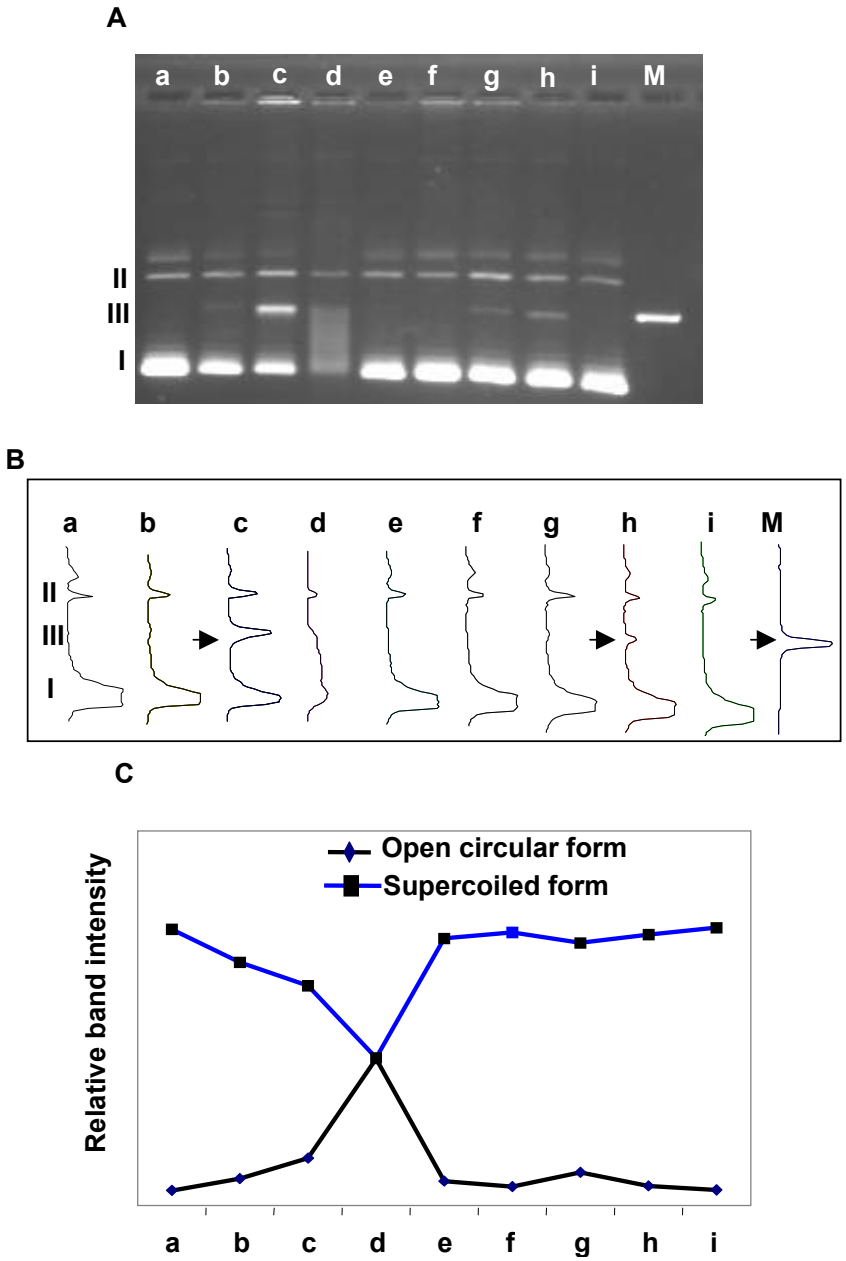

Figure 5.9. Effect of ATA on Aβ (1-42) –sc DNA interaction in presence of metals. (A) Agarose gel electrophoresis (1.2%): (a) sc DNA alone (1μg); (b) sc DNA + Aβ (1-42) (75 x10^{-6} M); (c) sc DNA + Aβ (1-42) (75x10^{-6} M) + 1mM $CaCl_2$; (d). sc DNA + Aβ (1-42) (75x10^{-6} M) 1mM $MgCl_2$; (e) sc DNA + Aβ (1-42) (75x10^{-6} M) 1mM $ZnCl_2$;(f) sc DNA + Aβ (1-42) (75x10^{-6} M) + 200μM ATA ; (g) sc DNA + Aβ (1-42) (75x10^{-6} M) + 1mM $CaCl_2$ + 200μM ATA; (h) sc DNA + Aβ (1-42) (75x10^{-6} M) + 1mM $MgCl_2$ + 200μM ATA;(i) sc DNA + Aβ (1-42) (75x10^{-6} M) + 1mM $ZnCl_2$ + 200μM ATA and (M) linearized supercoiled DNA (*EcoR*1 digested). **(B)** Representation of the DNA bands in the form of peaks. Arrows represent the peaks corresponding to the linearised sc DNA (form III). **(C)** Densitometry of the above gel: Correlation of relative amounts of supercoiled (form I) and open circular (form II). Aβ (1-42) was 36 h old (incubated at room temp in MQ water).

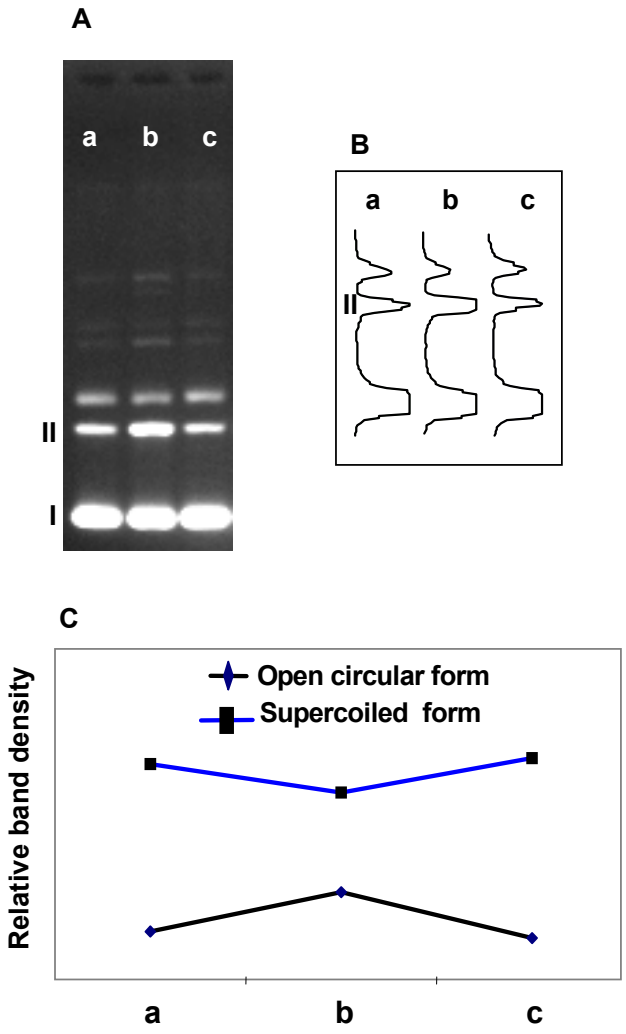

Figure. 5.10. Effect of TFE on Aβ (1-42) -sc DNA interaction. (A) Agarose gel electrophoresis (0.75%): a) sc DNA alone (1μg); (b) sc DNA + Aβ (1-42) ($50x10^{-6}$ M); (c) sc DNA + Aβ (1-42) ($50x10^{-6}$ M) dissolved in TFE (50%). **(B)** Representation of the DNA bands in the form of peaks. **(C)** Densitometry of the above gel: Correlation of relative amounts of supercoiled (form I) and open circular (form II).

5.2.4. CD spectral analysis of Aβ (1-42) peptide

Aβ (1-42) CD spectral analysis showed that fresh Aβ (1-42) (dissolved in water) has β-sheet conformation (Fig. 5.11a) where as aged peptide (32 h old) has aggregated β-sheet conformation (Fig. 5.10b). Aβ (1-42) in 50% TFE showed α-helical conformation (Fig. 5.11c). The results reveal that β-sheet conformation of Aβ (1-42) is toxic rather than the α-helix.

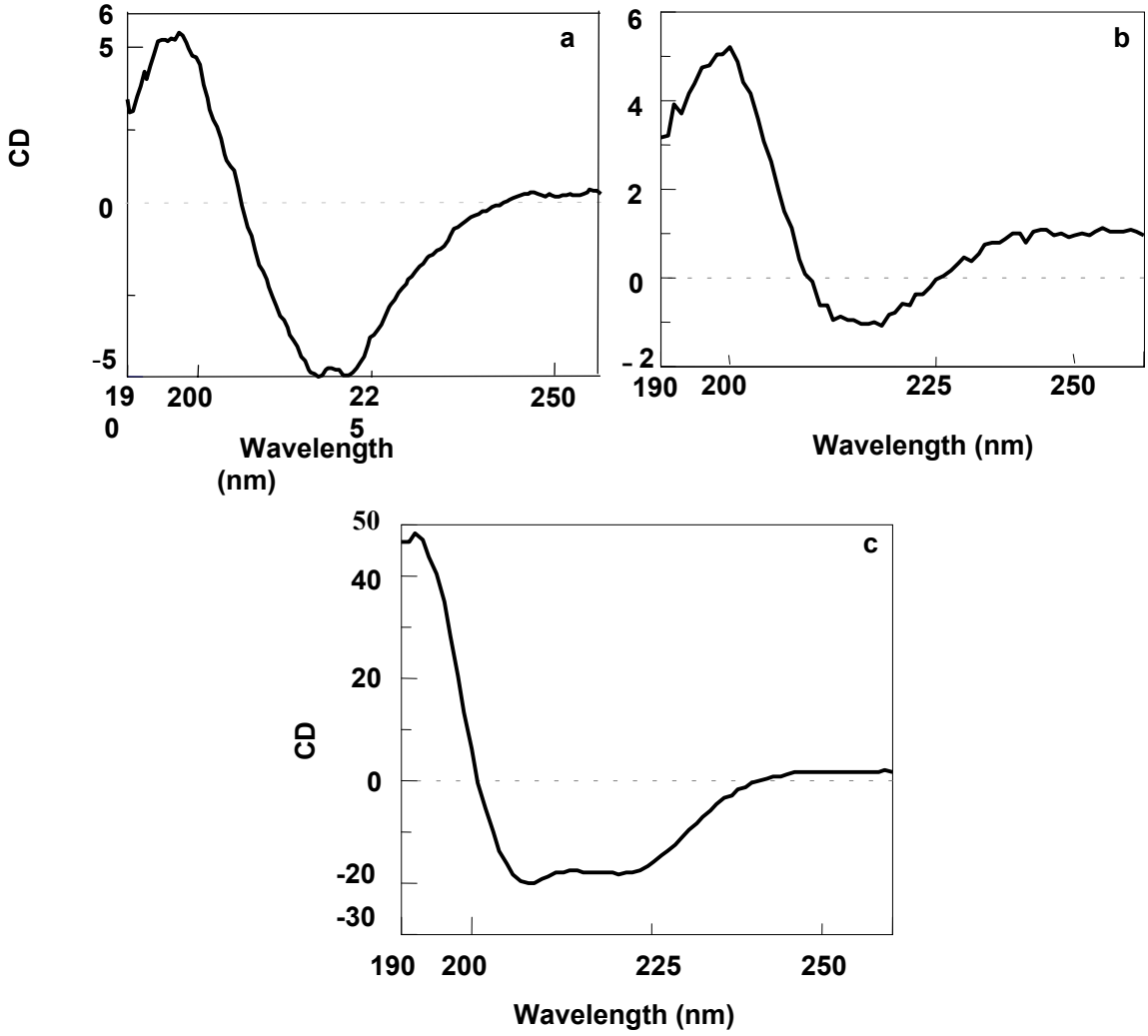

Figure. 5.11. CD spectra of Aβ (1-42). (a) Aβ peptide (fresh) dissolved in water showing β-sheet structure. (b) Aged Aβ peptide dissolved in water showing aggregated β structure. (c) Aβ dissolved in 50% TFE showing α-helical structure.

5.2.5. Whether the nicking property is specific for Aβ (1-42) peptide?

The effect of other Aβ peptides like Aβ (1-28), Aβ (1-16), Aβ (1-25-35), Aβ (1-40), and Aβ(1-43), on DNA has also been checked. Smaller length peptides Aβ (1-16) and Aβ (1-28) both caused DNA nicking.

Aβ (1-28) at the concentration tested ($50x10^{-6}$M) caused open circular form (Lane b, Fig. 5.12). All the three metal ions caused linearisation of DNA in the order Ca >Mg >Zn as revealed by the densitometric analysis (Fig. 5.12C). In this case Ca^{2+} showed more nicking among the three metals (Lane d, Fig. 5.12).

In the present study Aβ fragment (1-16) used ([Gln^{11}] Aβ(1-16)) has Glutamine instead of Glutamic acid at 11th position (Sigma Cat No: A 4309). The modified Aβ(1-16) also could induce nicking in sc DNA (Fig. 5.13). Both Ca^{2+} (Lane e, Fig. 5.13) and Mg^{2+} (Lane f, Fig. 5.13) enhanced the activity. Zinc did not show any effect (Lane g, Fig. 5.13). Its quite interesting that the aged modified Aβ(1-16) (90 days at 4^0C) achieved remarkable DNA nicking activity and resulted in DNA smear (lane b, Fig. 5.14). Both Ca^{2+} (Lane d, Fig. 5.14) and Mg^{2+} (Lane e, Fig. 5.14) absolutely degraded DNA and we could not observe any DNA staining in the agarose gel. However Zn^{2+} did not alter the nicking activity (Lane f, Fig. 5.14). ATA could protect the DNA nicking caused by modified Aβ (1-16) (Lane c, Fig 5.14).

CD spectral analysis of Aβ (1-16) and Aβ (1-28) revealed random coil secondary conformation (Fig. 5.15 A and B respectively). Random coil conformation of the peptides also seems to exert DNA nicking as revealed by the above two peptides.

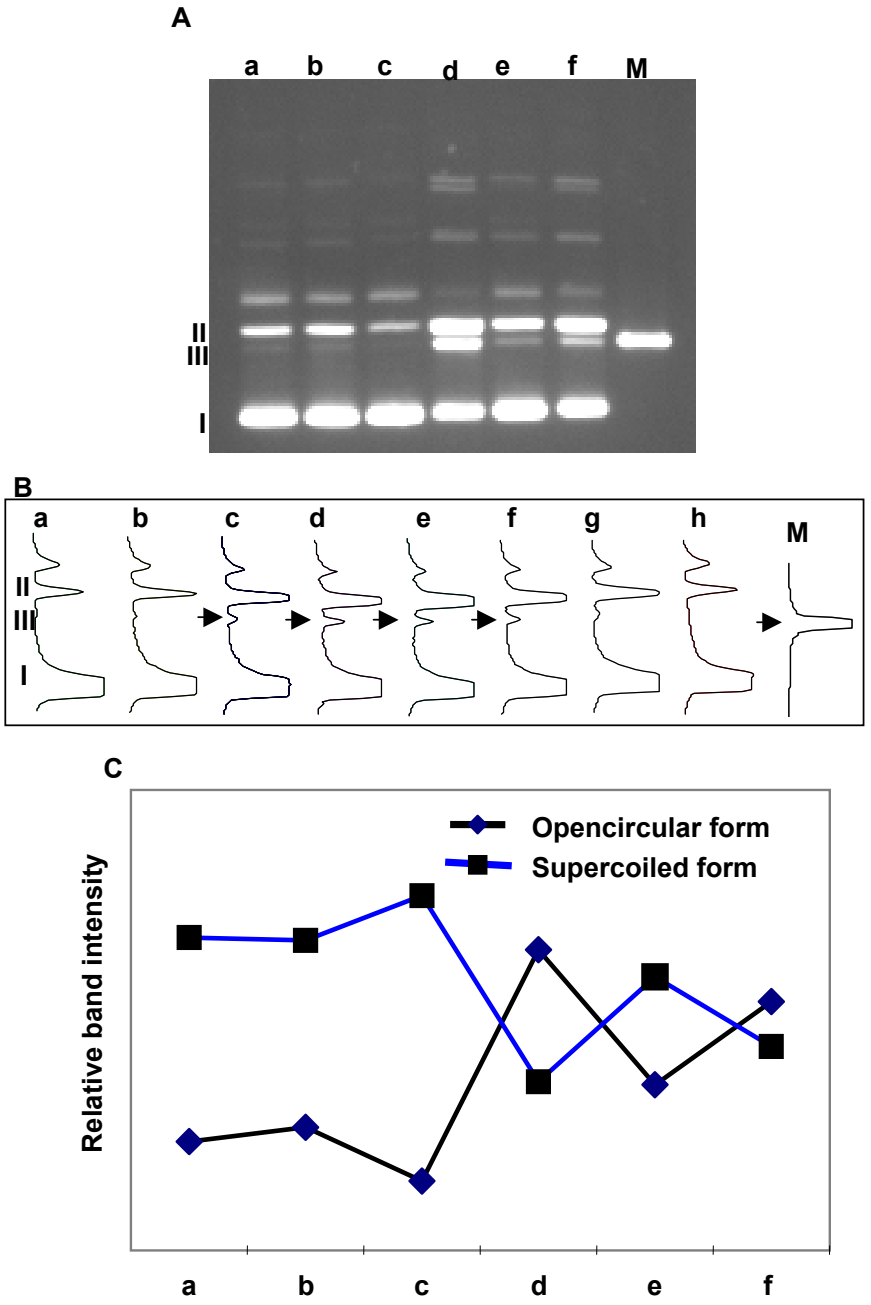

Figure. 5.12. Effect of metals on Aβ (1-28) binding to sc DNA. (A) Agarose electrophoresis (1.2%): (a) sc DNA alone (0.8μg); (b). sc DNA +Aβ(1-28) ($50x10^{-6}$M); (c) sc DNA +Aβ(1-28) ($50x10^{-6}$M)+ 2% SDS; (d) sc DNA +Aβ(1-28) ($50x10^{-6}$M)+ 1mM $CaCl_2$; (e) sc DNA +Aβ(1-28) ($50x10^{-6}$M)+ 1mM $ZnCl_2$; (f) sc DNA +Aβ (1-28) ($50x10^{-6}$M)+ 1mM $MgCl_2$ and (M) linearised supercoiled DNA (*EcoR*1 digested). **(B)** Graphical representation of supercoiled (form I), open circular (form II) and linearised (form III) bands. Arrows indicate the linearised DNA form (form III) induced by Aβ (1-28). **(C)** Densitometry of the above gel: correlation of relative amounts of supercoiled (form I) and open circular forms (form II).

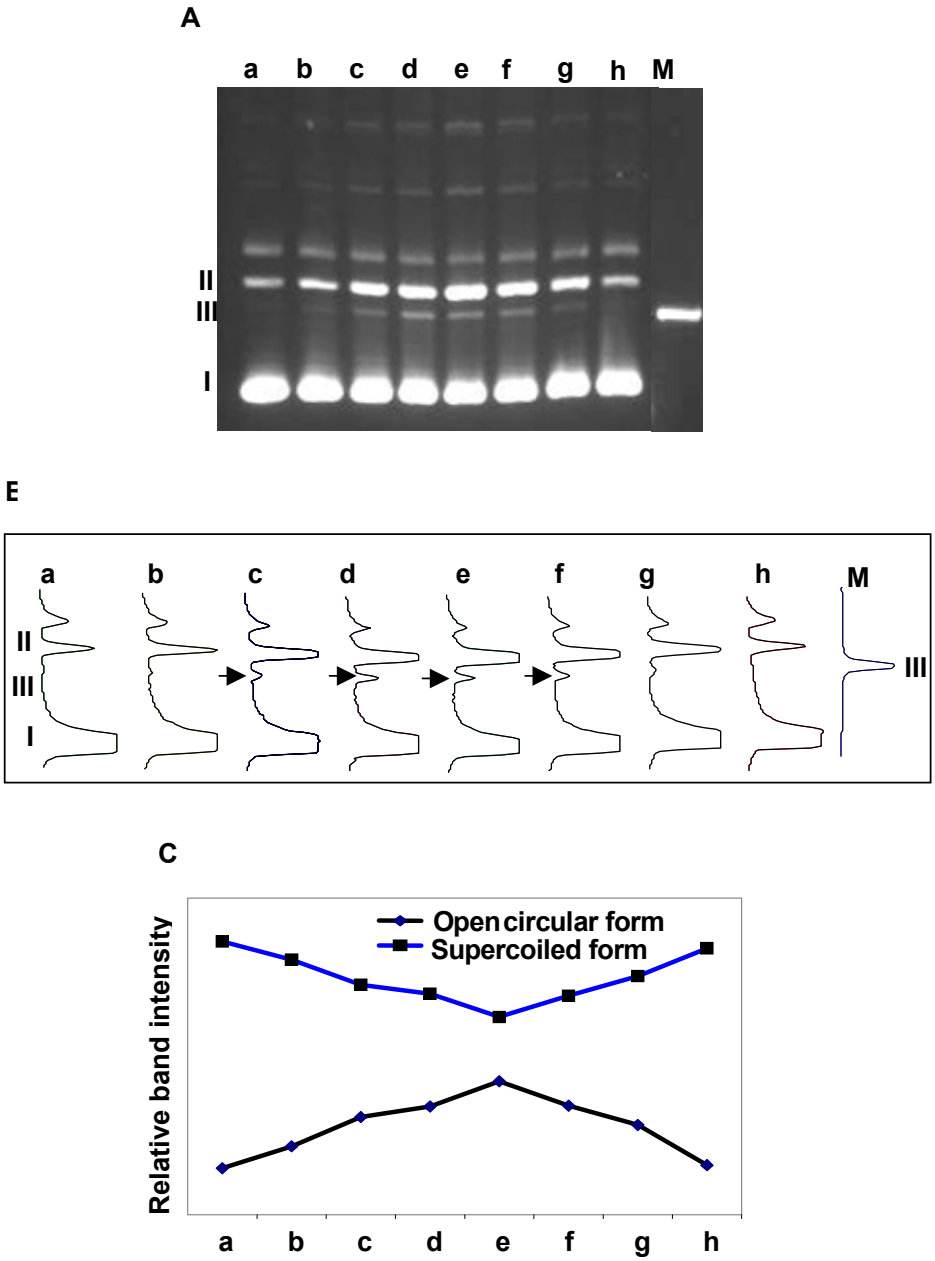

Figure. 5.13. Aβ (1-16) binding to sc DNA. (A) Agarose gel electrophoresis (1.2%): (a) sc DNA alone (1.0μg); (b) sc DNA +Aβ(1-16) ($10x10^{-6}$M); (c) sc DNA +Aβ(1-16) ($50x10^{-6}$M); (d) sc DNA +Aβ(1-16) ($75x10^{-6}$M); (e) sc DNA +Aβ(1-16) ($50x10^{-6}$M) + 1mM $CaCl_2$; (f) sc DNA +Aβ(1-16) ($50x10^{-6}$M) + 1mM$MgCl_2$; (g) sc DNA +Aβ(1-16) ($50x10^{-6}$M) + 1mM $ZnCl_2$ and (h) sc DNA +Aβ(1-16) ($75x10^{-6}$M) + ATA (2 x 10^{-4} M). Aβ (1-16) was freshly dissolved in water. **(B)** Representation of the DNA bands in the form of peaks. Arrows indicate the linearised DNA form (form III) induced by Aβ (1-16). **(C)** Densitometry of the above gel: correlation of relative amounts of supercoiled and open circular forms.

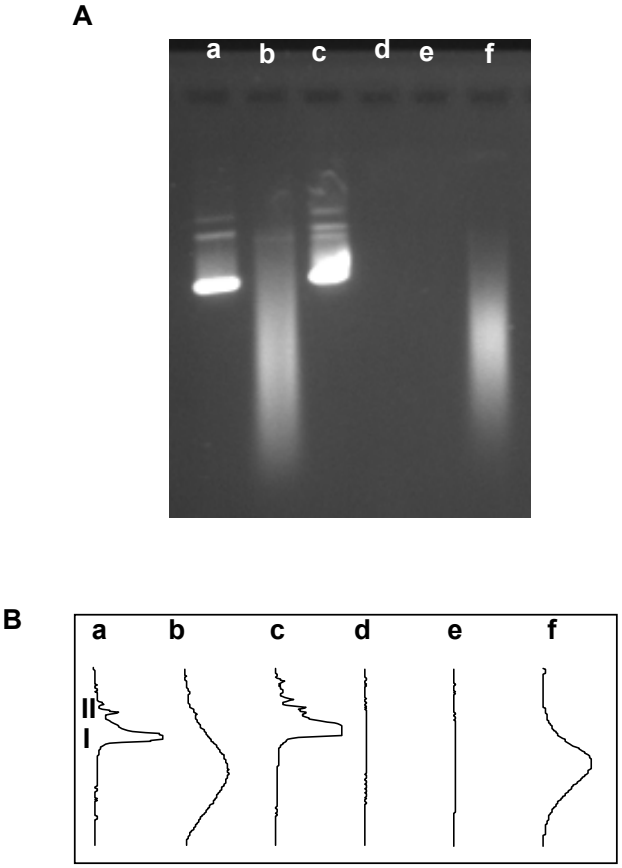

Figure. 5.14. Effect of aged peptide (60 days at 4^0C) Aβ (1-16) on sc DNA nicking activity. (A) Agarose gel electrophoresis (1.2%): (a) sc DNA alone; (b) sc DNA + Aβ (1-16)) ($75x10^{-6}$ M); (c) sc DNA + Aβ (1-16) ($75x10^{-6}$ M)+ ATA (2×10^{-4}M); (d) sc DNA + Aβ (1-16) ($50x10^{-5}$ M) +1mM $CaCl_2$; (e) sc DNA + Aβ (1-16) ($50x10^{-5}$ M)+1mM $MgCl_2$; and (f) sc DNA + Aβ (1-16) ($75x10^{-5}$ M)+1mM $ZnCl_2$. Aβ (1-16) in the presence of Ca and Mg completely degraded DNA so we could not find the DNA in the lanes d and e. (B) Representation of the DNA bands in the form of peaks: b, d, e and f lack corresponding form I and form II bands of sc DNA alone because of the smear damage caused by Aβ (1-16).

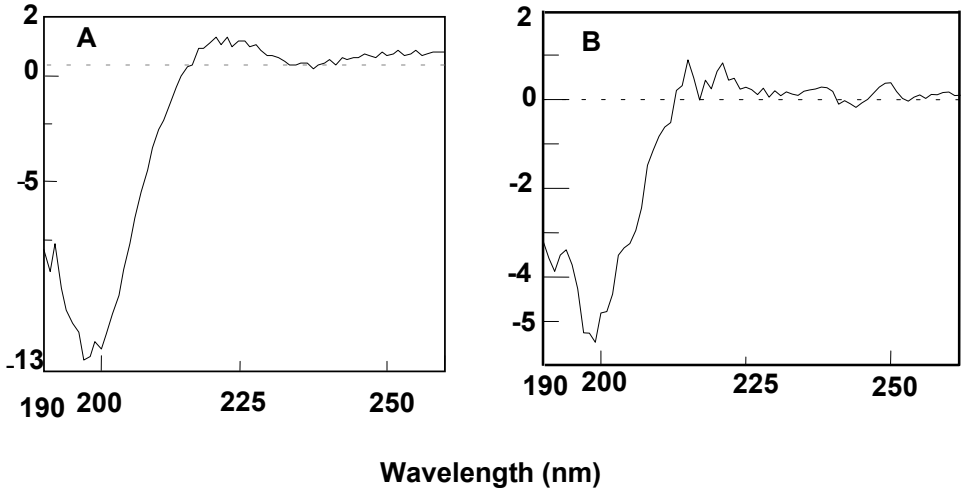

Figure. 5.15. CD spectral analysis of the peptide. (A**)** CD spectra of Aβ (1-16) dissolved in water showing random conformation. (B) CD spectra of Aβ (1-28) dissolved in water showing random conformation

Aβ (1-40) in the absence of metal ions (Fig. 5.16) caused open circular form. Mg^{2+} (Lane c, Fig. 5.15) and Ca^{2+} (Lane d, Fig. 5.16) significantly enhanced the nicking activity, however Zn^{2+} (Lane e, Fig. 5.16) exhibited protection against Aβ induced nicking in DNA.

Aβ (1-43) having the additional amino acid threonine at the C terminal end of the Aβ (1-42) is the primary constituent of SPs and cererbrovascular deposits in AD and Down's syndrome (Pike et al., 1993) showed DNA nicking activity only in the presence of Mg^{2+}(Fig. 5.17). At 5mM Mg^{2+} Aβ (1-43) resulted in complete disappearance of supercoiled band and subsequent appearance of open circular and linear form (Lane g, Fig. 5. 17).

Aβ (25-35) (the functional domain of Aβ required for both neurotrophic and neurotoxic effects, (Yankner et al., 1990)) unlike other peptides caused aggregation of the DNA (Fig. 5.18) and therefore DNA was not migrated in the agarose gel in the presence of Aβ (25-35). The aggregation of DNA was observed at the peptide concentrations above $50x10^{-6}$M (Lanes c and d, Fig. 5.18). As $50x10^{-6}$M did not cause any change in the sc DNA (Lane b, Fig. 5.18). It was reported by Ahn et al. (2000) that DNA could readily associated

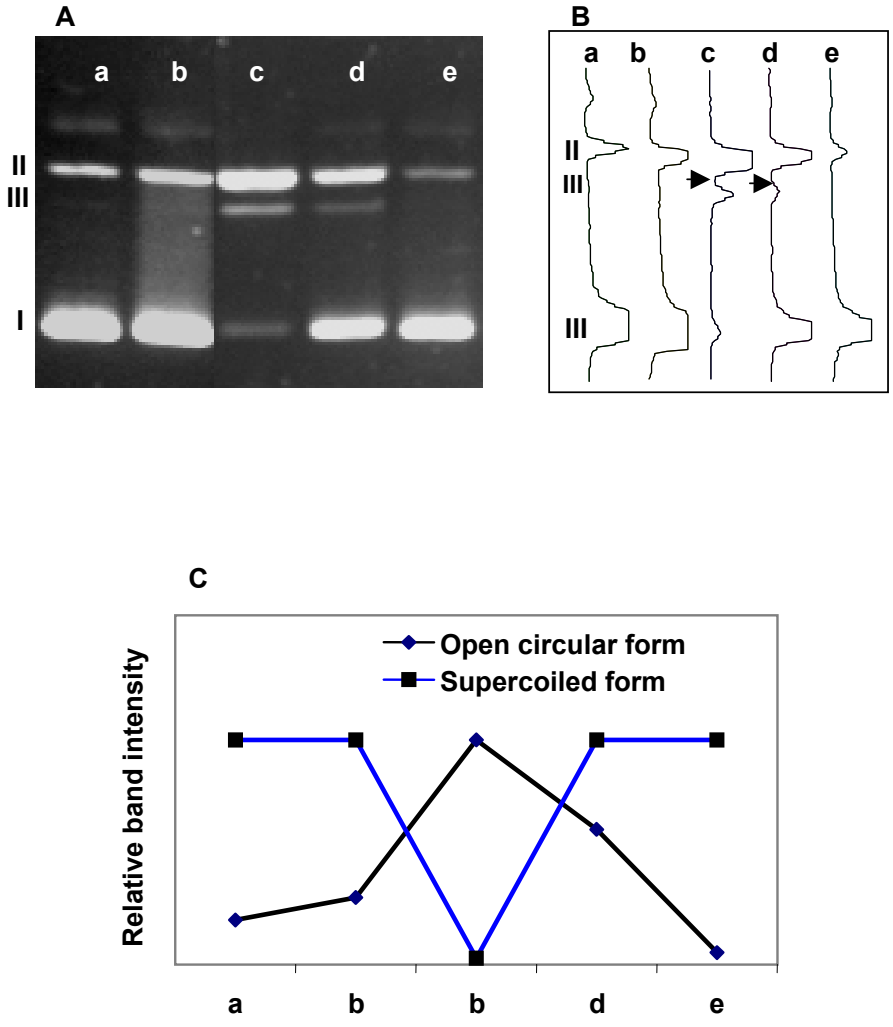

Figure. 5.16. Aβ (1-40) interaction with sc DNA. **(A)** Agarose gel electrophoresis (1.2%): (a) sc DNA alone (1μg); (b) sc DNA +Aβ (1-40) (75 x 10^{-6}); (c) sc DNA +Aβ (1-40) (75 x 10^{-6})+1mM $MgCl_2$; (d) sc DNA +Aβ (1-40) (75 x 10^{-6})+1mM $CaCl_2$ and (e) sc DNA +Aβ (1-40) (75 x 10^{-6}) +1mM $ZnCl_2$. **(B)** Peak representation of the DNA bands. Arrows indicate the linearised form of DNA (form III) induced by Aβ (1-40). **(C)** Densitometry of the above gel: correlation of relative amounts of supercoiled and open circular forms.

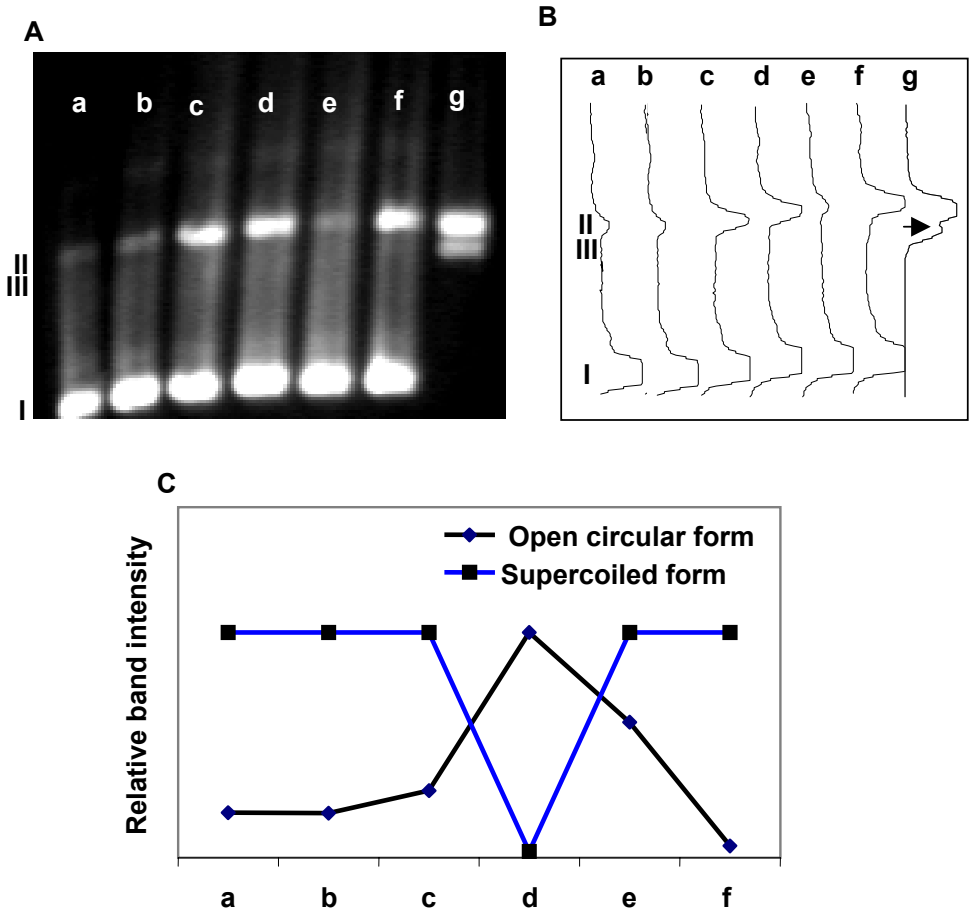

Figure. 5.17. Aβ (1-43) interaction with sc DNA. (A) Agarose gel electrophoresis: **(**a) sc DNA alone; (b) sc DNA +Aβ (1-43) (42 x 10^{-6}); (c) sc DNA +Aβ (1-43) (42 x 10^{-6})+1mM Mg^{2+}; (d) sc DNA +Aβ (1-43) (42 x 10^{-6})+5mM Mg^{2+}; (e) sc DNA +Aβ (1-43) (64 x 10^{-6}); (f) sc DNA +Aβ (1-43) (64 x 10^{-6})+1mM $MgCl_2$ and (g) sc DNA +Aβ (1-43) (64 x 10^{-6})+ 5mM $MgCl_2$. **(B)** Peak representation of the DNA bands. Arrow indicates the linearised form of DNA (form III) induced by Aβ (1-43). **(C)** Densitometry of the above gel: correlation of relative amounts of supercoiled (form I) and open circular (form II) forms.

with the aggregated forms of the Aβ (1-40) and Aβ (25-35) resulting in the shift in the electrophoretic mobility of DNA. They also reported that electrophoretic mobility of DNA was little influenced by the monomeric forms of Aβ (1-40) and Aβ (25-35). In our study we could get retardation of the DNA only by Aβ (25-35). We propose that the four N-terminal polar residues of Aβ (25-35) fragment which includes a charged lysine residue (the net charge is +1) might be responsible for strong binding of the DNA.

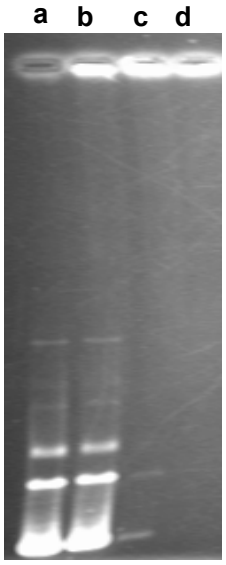

Figure. 5.18. Aggregation of sc DNA caused by Aβ (25-35): Agarose gel electrophoresis (1.2%) (a) sc DNA alone (1.0μg); (b) sc DNA +Aβ(25-35) ($10x10^{-6}$M); (c) sc DNA +Aβ(25-35) ($50x10^{-6}$M) and (d) sc DNA +Aβ(25-35) ($75x10^{-6}$M).

Ca, Mg and Zn were found to have no effect either on open circular form appearance or on linearisation of scDNA (Fig. 5. 19).

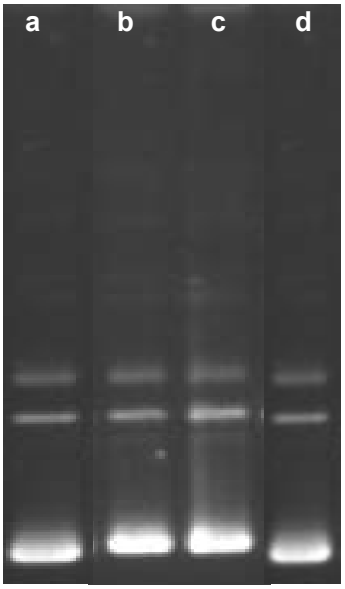

Figure. 5.19. Effect of Ca^{2+}, Mg^{2+}, and Zn^{2+} on sc DNA: Agarose gel electrophoresis (1.2%) (a) sc DNA alone (1.0 μg); (b) sc DNA +1mM $CaCl_2$; (c) sc DNA +1mM $MgCl_2$ and (d) sc DNA +1mM $ZnCl_2$.

5.2.6. Estimation of single strand beaks (SSB) induced by Aβ peptides

An estimate of single strand breaks caused by various Aβ peptides was made using the nick translation of *Ecoli* Polymerase (Klenow fragment). Figure. 20 shows the incorporation of ^{3} [H] -TMP into DNA samples when incubated with *E.Coli* DNA polymerase I in a nick translational assay. During the standardization of the assay conditions with a plasmid DNA (Cos T fragment of λ phage) having known number of single strand breaks, it is found an average of 1500 nucleotides are added at each of the 3'-OH group. From this it is inferred that each picomole of TMP incorporated is equivalent to 1.6×10^{9} 3' – OH groups or single strand breaks. The incorporation has been shown to be increased with increase in the length of the Aβ peptide except for Aβ (1-43). Thus the SSB in scDNA increased with increase in the peptide length (Table 1). Mg^{2+} increased the SSBs in case of all the peptides. Aβ (25-35) (Fig. 5.20) could induce strand breaks in this experiment though we could not observe any in the gel. In the presence of Mg, no strand breaks were observed and this may be due to the aggregation of DNA by Aβ (25-35) as revealed in the agarose gel (Fig. 5.20).

5.3. Discussion

Although the biological role of the amyloid β-peptide has yet to be determined it is thought to contribute to the progressive neuronal loss in AD. The mechanisms by which Aβ (1-42) associated toxicity occur in neurons in AD brain are under active investigation. Inspection of the Aβ sequence points out its amphipathic nature. The C-terminal third of the peptide (residues 29-40/42/43) is composed entirely of hydrophobic residues, while the N-terminal two-thirds (residues (1-28) is decidedly more polar, with both charged and hydrophobic residues. The eleven residue Aβ (25-35) fragment of amyloidogenic Aβ (1-40)/(1-42) retains activities of the full-length peptide, rapidly forming fibrillar aggregates (Harkany et al., 2000).

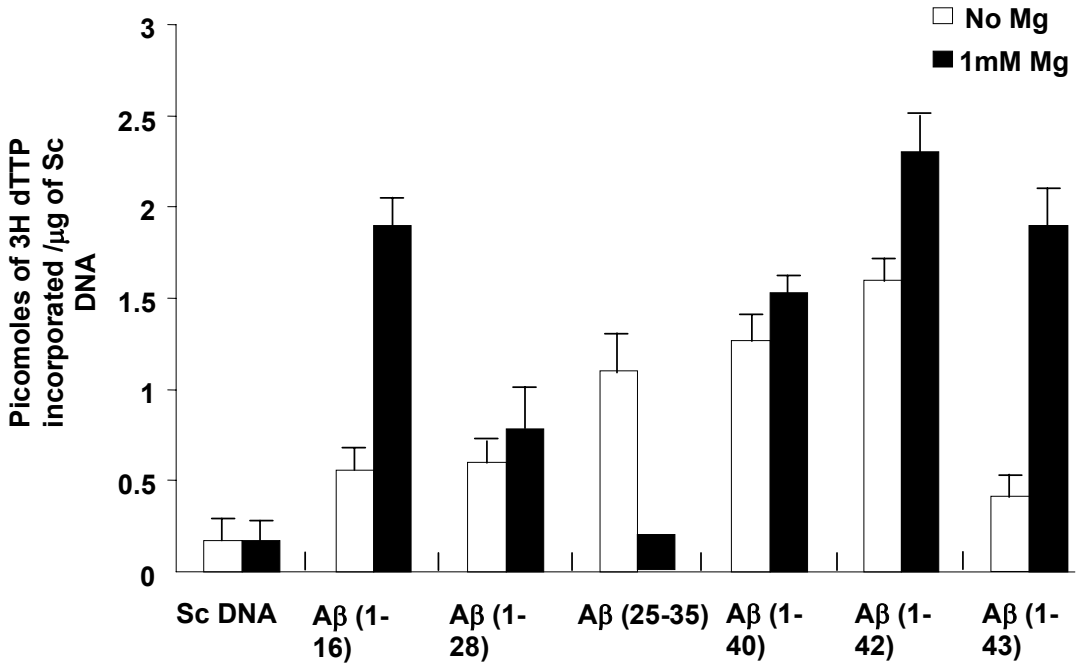

Figure. 5.20. Assessment of Single stranded breaks (SSB) in sc DNA induced by Aβ peptides through nick translation type incubation with E.coli DNA Polymerase I. Values are expressed as picomoles of 3H-TMP incorporated DNA per hour of incubation. For assay conditions see materials and methods. From standardized conditions each picomole of TMP incorporated would be equivalent to 1.6×10^9 single strand breaks in the DNA sample. Values are expressed as mean ±SD.

Sample	SSB/μg of Sc DNA	
	Without $MgCl_2$	With 1mM $MgCl_2$
sc DNA alone	2.7×10^8	2.7×10^8
sc DNA+ Aβ (1-16))	8.96×10^8	30.4×10^8
sc DNA + Aβ (1-28)	0.96×10^8	1.25×10^8
sc DNA + Aβ (25-35)	17.6×10^8	--------
sc DNA + Aβ (1-40)	20.3×10^8	24.5×10^8
sc DNA + Aβ (1-42)	26×10^8	37×10^8
sc DNA + Aβ (1-43)	0.48×10^8	43.2×10^8

Table. 5.1. DNA single strand breaks (SSBs) in sc DNA caused by Aβ peptides through Nick translational type of Incubation with E.coli DNA polymerase (Klenow fragment).

The four N-terminal polar residues of Aβ (25-35) fragment include a charged lysine residue (the net charge is +1), the C-terminus residues are predominantly hydrophobic. The hydrophobic segment corresponds to a part of the transmembrane domain of APP (Kang et al., 1987). The Aβ (1-42) and Aβ (25-35) are known to be neurotoxic peptides (Yankner et al., 1990). Purified Aβ, as well as many of its fragments, can be induced to form amorphous and/or fibrillary aggregates *in vitro* without the aid of other biological factors (Kirschner et al., 1987; Fraser et al., 1991; Malinchi et al., 1998). Several studies have shown that Aβ peptides can be neurotoxic but only when they form fibrillary aggregates (Yankner et al., 1990; Kowall et al., 1991). Aβ analogs and fragments in filamentous state adopt an antiparallel β-sheet conformation with two strands composed of residues 17-21 and 34-42 and suggest that the C-terminal part 17-42 of Aβ has high intrinsic amyloidogenic potential and contributes to the toxicity. However, Aβ (17-42) is not liberated from cells in an amyloidogenic form (Teplow, 1998) therefore it was suggested that one can not exclude that the N-terminal region 1-16 of Aβ is involved in the pathogenesis of AD in ways not appreciated from studies of fibril formation and toxicity of the C-terminal 17-42 Aβ peptide. Several groups have reported that synthetic β-peptides show either trophic or toxic response on neurons *in vitro* (Yankner et al., 1990). Studies also suggested that Aβ peptides may exert its toxic effect via activation of transcription factors (Baron et al., 1999).

Our results provided intriguing information on the Aβ induced DNA nicking property. Aβ (1-42) showed definite DNA nicking property in a concentration and time dependent manner. The DNA nicking is not only restricted to full length fragments (Aβ 42/40) but the smaller length peptides also showed nicking. However there exist subtle differences in their activity and are differentially modulated by metal ions Mg^{2+}, Ca^{2+} and Zn^{2+}. Nevertheless Aβ has been shown to be a metalloprotein that can bind transition metal ions such as Zn, Fe and Cu (Lovell et al., 1998). In our study Ca^{2+} and Mg^{2+} seem to be required for the DNA nicking activity as all the peptides showed enhance DNA nicking activity. Aβ (1-43) in particular showed

nicking activity only if Mg^{2+} is present. It was suggested that Mg^{2+} and Ca^{2+} (Mg and Ca are more abundant than Cu and Zn in brain samples) might participate in the resolubilization of Aβ (Cherny et al., 1999). Kuroda and Kawahara (1994) also reported that Ca and Mg did not promote aggregation of Aβ. In AD, there is a direct relationship between the aggregation state and the loss of Ca^{2+} homeostasis. Generally Aβ is thought to interfere with Calcium homeostasis by forming or activating Calcium channel as well as interfering with intra cellular calcium storage, resulting in the production of free radicals by calcium-sensitive enzymes (Holscher, 1998; Mattson et al., 1993). Nevertheless less is known about the interaction of Mg^{2+} and Ca^{2+} with Aβ.

Jang and Surh (2002) reported that Aβ (25-35) caused strand scission in phiX174 DNA only in the presence of ferrous iron. However our study yielded different results. Though agarose gel study of interaction of Aβ (25-35) showed total aggregation of DNA, Nick translation assay could identify the strand breaks caused by the Aβ (25-35). The strand breaks caused were in the absence of any metal. Further in the presence of Mg^{2+} no strand breaks were observed. It can be because of the hindrance of the polymerase to access the DNA because of the aggregation of DNA.

Zn^{2+} exhibited either protective (Aβ (1-40) and (1-42)) or no effect ((Aβ (1-16)). Zn^{2+} has been shown to be capable of causing deposition of amyloid, at least *in vitro* (Mantyh et al., 1993; Bush et al., 1994b). It was shown that Zn^{2+} can promote aggregation of Aβ (Mantyh et al., 1993). It has been shown that ionic interactions with zinc induce significant conformational changes in Aβ (Huang et al., 1997). Human Aβ (1-40) reported to have two specific binding sites for Zn^{2+} (Bush et al., 1994b). The obligatory zinc binding sequence has been mapped to the region 6-28 of Aβ (Bush et al., 1994). However, Zn showed enhancing effect only in case of Aβ (1-28). It has been reported that Aβ (1-16) undergoes a conformational change from random coil to some regular secondary structure in presence of zinc cations (Kozin et al., 2001). The authors also reported that Ca^{2+} and Mg^{2+} did not cause any conformational change in Aβ (1-16) as monitored by CD spectroscopy in the conditions where Zn^{2+} binding was observed. However, in our study we could

find enhanced DNA nicking activity of Aβ (1-16) in the presence of only Ca^{2+} and Mg^{2+} and not with Zn^{2+}.

The aging of the peptides is also shown to have implications in exacerbating the toxicity. In our study in the paradigm of Aβ (1-16) aging of the peptide has attained more toxicity in terms of DNA nicking activity. Aβ (1-16) has no intrinsic propensity to aggregate in physiological conditions, in contrast to the rest of the Aβ sequence and is highly soluble and monomeric in usual buffers in the 3.5-8.00 pH range. The case Aβ (1-16) has provided unambiguous example for aging associated toxicity. Our results are consistent with the observations of Pike et al. (1999 and 1993), which showed aged or aggregated β-peptide to be neurotoxic and fresh or monomeric β-peptide to be non-toxic (Pike et al., 1991; 1993). Nevertheless, our results also favor soluble species of Aβ rather than the fibrillar forms as more toxic. This has also been depicted clearly in the Aβ (1-42) time dependent study (thioflavine-T assay and corresponding DNA nicking activity and EM study) and involvement of metals. Aβ(1-42) showed more toxicity (DNA nicking) corresponding to the lower thioflavine-T binding and non-fibrillar form revealed by EM study. Thioflavine-T is more specific for fibrillar forms than aggregated and soluble forms (LeVine, 1993). The metal ions (Ca^{2+} and Mg^{2+}), which solubilize the Aβ have enhanced the DNA nicking activity and the metal ion Zn^{2+}, which promotes aggregation did not cause any effect. Based on the above results we conclude that some form of the soluble and stabilized conformation of the Aβ peptides are required for toxicity. Several lines of evidence have converged recently to demonstrate that soluble oligomers of Aβ, but not monomers or insoluble amyloid fibrils, may be responsible for synaptic dysfunction on the brains of AD patients and in AD animal models. Metastable intermediates in the formation of fibrils by synthetic Aβ- referred to as AD diffusible ligands (ADDLs) (Lambert et al., 1998) or protofibrils (Hartely et al., 1999) cause subtle injury to cultured neurons.

Further inhibition and enhancement of DNA nicking by ATA and metals respectively implicate the involvement of histidine residues. As early studies indicated that histidine residues were important for Aβ aggregation since the loss of histidine residues, such as in rat Aβ which contains three amino acid

substitutions (Arg-Gly, Tyr-Phe and His-Arg at positions 5,10 and 13 respectively) (Johnstone et al., 1991) or histidine modification, results in greatly diminished aggregation of Aβ by Cu (II), Zn (II) or Fe (III) (Atwood et al., 1998). Aβ binds transition metal ions via histidine residues (positions 6, 13, and 14) (Atwood et al., 1998; Syne et al., 2004). And also histidine is one of the strongest low molecular weight ligand for metal ions (Szczepanik et al., 2004). In blood serum, it constitutes one of the most common Cu (II) binding agents (Neumann and Sass-Kortsak, 1967). These results suggest that histidine residues are essential for metal-mediated assembly of Aβ. The mechanism of action of ATA in the inhibition of protein-nucleic acid complex formation also involves histidine residues (Gonzalez et al., 1980). Moreover recently it has been shown that oxidative DNA damage induced nuclear localization of Aβ(1-42) (Ohyagi et al., 2004). However the significance of its presence in the nucleus is not clear. Based on the above observations we propose that histidine residues are essential for the Aβ induced DNA nicking activity. The results evidenced a new toxic role of Aβ in terms of its DNA nicking activity and there by it alters the helicity of scDNA. Our finding of DNA nicking activity of Aβ peptides has biological significance in terms of causing DNA damage.

SUMMARY AND CONCLUSION

Alzheimer' disease (AD) is a complex neurodegenerative disorder with no definite etiological factor. It has complex pathology with multifaceted biochemical changes. AD involves parts of the brain that control thought, memory, and language and is characterized by selective primary neuronal and synaptic loss in the hippocampus and cerebral cortex that correlates clinically with dementia (**CHAPTER I**). The etiological factors of AD are not clearly known although unproven hypotheses have included genetics, head trauma, oxidative stress, infectious agents, and environmental factors including aluminium (Al) toxicity. The neuropathological characteristics include cortical and subcortical atrophy, formation of intraneuronal neurofibrillary tangles (NFT), deposition of amyloid beta peptide (Aβ) in neuritic plaques or senile plaques (SP), formation of neuropil threads, loss of synaptic function, oxidative stress, and apoptosis, leading to neuronal loss. These events are observed mostly in the hippocampal and cortical regions of AD brains. It is known that protein conformational change of protein plays important role in the pathology of AD and AD is some times termed as protein conformational disorder. Amyloid beta peptide has been hypothesized to have the central role in neurodegeneration. Aggregation of amyloid has been known to be associated with the disorder, which requires conformational transition from random coil to β-sheet conformation. However there are limited studies on the implications of altered genomic integrity in neurodegeneration in AD. Al and Tau have been shown to bind DNA and modulate the conformation. Therefore the present study focuses on genomic DNA conformational aspects and the role of amyloid peptides on DNA integrity with relevance to AD. To accomplish the above objectives, we utilized the brain tissue of AD affected and control from Netherlands brain bank. The study has the following three objectives: a) Structural integrity and stability of the genomic DNA isolated from the AD and control brain tissue, b) Interaction of amyloid peptides with the DNA and their implication in modulating the conformation and c) molecular mechanism of Aβ induced DNA nicking :a consequence of amyloid binding to DNA.

Genomic integrity has an essential role for the living organisms as any damage or structural change of the genome will affect the transcriptional fidelity, which ultimately affects the survival of the organism itself. The study (**CHAPTER III)** provided first evidence on alteration of DNA helicity in the hippocampus of AD brain. In AD, hippocampus is first and severely affected region, before other parts of the cortex and later, the frontal lobes too. Hippocampus region of brain appears to be absolutely necessary for making new memories. Memory is usually the first thing to start to falter in AD. The studies revealed that the genomic DNA, isolated from hippocampus showed Z- form of DNA rather than the usual right-handed B-form of DNA. This conformational alteration was observed only in the hippocampus and not in the superior frontal gyrus and parahippocampal gyrus. Moderately affected AD brain DNA revealed B-Z intermediate form and severely affected AD brain DNA showed Z-DNA form. Therefore the conformational change of DNA observed in the hippocampus was shown to be in a progression dependent fashion from B-DNA to B-Z-DNA to Z-DNA. DNA damage in terms of strand breaks were found to be more in the case of AD hippocampus compared to superior frontal gyrus and parahippocampal gyrus and also compared to the control brain DNA samples. It was reported that Z-DNA formation excludes nucleosome formation and could affect the placement of nucleosome as well as organization of chromosomes. The topological change in DNA, particularly the B-DNA to Z-DNA transition in the hippocampus, will have tremendous implications in the functional biology of the brain cells e.g. transcription, replication and recombination. It was also theoretically postulated that the guanine base present in DNA would be more susceptible to hydroxyl radical induced DNA damage if the conformation of DNA is Z –form rather than B or A forms because of greater exposure of nucleotide bases. Based on this DNA conformational change, we proposed an explanation to the unusual phenomenon like nucleosome mis-assembly, G* specific DNA oxidation, terminal differentiation and altered gene expression associated with AD brain. Our present finding on the presence of Z-DNA in AD brain supports the experimental findings on the presence

of GC* rich oxidized products in AD brain. The results lead to a hypothesis that the AD etiological factors like amyloid beta peptides, aluminium, tau etc might play an

important role in the in the DNA conformational alteration. Part of the work has been presented at 18th IUBMB (International Union of Biochemistry and Molecular Biology) held at Birmingham, UK (2000). The finding was cited in the Biochemist, Oct, 2000, page, 19.

Amyloid beta peptide a hallmark feature of senile plaques of AD has been shown to have putative neurotoxic role in causing neurodegeneration. Based on the previous results and to test the subsequent hypothesis we studied the interaction of DNA with amyloid peptides. There were few reports on the binding ability of aluminium and tau to DNA. However, there is no report to-date on Aβ binding to DNA. In the present study (**CHAPTER IV)**, we first time evidenced the binding of Aβ to scDNA. Our study provided unambiguous observation that amyloid peptides can bind to DNA. The study also reported the localization of the Aβ peptides at the nuclear region in the AD brain hippocampus tissue. The binding of full length Aβ peptide, Aβ (1-42) to DNA not only binds to scDNA but also is able to alter the conformation of DNA. An initial B→C transition was observed which gradually transformed into ψ-DNA, presumably reflecting a partial DNA collapse into a ψ-phase. ψ- DNA is shown to be structurally and immunologically closely related to Z-DNA family. The ψ-DNA conformation induced by Aβ (1-42) is structurally closer to Z-DNA, which was observed in severely affected AD brain. Whereas, the shorter length peptide Aβ (1-16) showed altered B-DNA conformation. Furthermore the Aβ peptides showed preferential binding to AT base pairs than to GC base pairs. The peptides showed preferential binding to AT rich sequences and caused B→A DNA transition. The binding of peptides to DNA was proposed to implicate the involvement of both hydrophobic and hydrophilic interactions of the peptide with DNA. These protein-DNA complex conformations (altered B-DNA, A-DNA, ψ- DNA, B-C-A complex) are energetically weak and are likely to go into Z-DNA conformation as reported in AD brain. Vast arrays of small scDNA packets have been found to be present in animal

and human cells and are known to be involved in gene expression. Our study also clearly shows that Aβ peptides relax the sc DNA partially. Thus the present study

hearsay for the first time that Aβ uncoils scDNA besides bringing about helicity changes. We propose that Aβ along with other etiological factors might modulate DNA topology in AD brain The results provided new understanding of Aβ in terms of its nuclear localization and DNA binding property. The work was presents at The 21st Annual Conference of The Hong Kong Society of Neurosciences, held at Hong Kong (2001).

The Aβ peptides are self aggregating and aggregating proteins are shown to exert the neurotoxicity and amyloid assembly into fibrillary structure is a defining characteristic of AD. The role of DNA damage in aging process has gained importance with increasing evidence of DNA damage in aging brain and age associated neurological disorders. Moreover Aβ was shown to cause to DNA fragmentation as part of its effect on inducing apoptosis. This chapter **(CHAPTER V**) provided fundamental contribution that Aβ peptides can cause DNA damage directly. The study revealed that Aβ peptides exhibited strand breaks in supercoiled DNA. The nicking property of Aβ has been shown to be in a concentration and time dependent manner. Our results also provided interesting observation that aging of the peptide is a major concern for the DNA damage directly. As the aged peptides acquired remarkable DNA nicking activity. The metal ions which solubilize the aggregated Aβ viz Ca and Mg are shown to enhance the Aβ induced DNA nicking. Several lines of evidence have converged recently to demonstrate that soluble oligomers of Aβ, but not monomers or insoluble amyloid fibrils, may be responsible for synaptic dysfunction on the brains of AD patients and in AD animal models. Zn^{2+}, which promotes aggregation of Aβ shown to either protect (in case of Aβ (1-40) and Aβ (1-42)) or showed no effect (in case of Aβ (1-16) and Aβ (1-28) on induced DNA nicking. Interestingly aurintricarboxylic acid (ATA) a nuclease inhibitor could prevent the DNA nicking induced by the Aβ. Further inhibition and enhancement of induced DNA nicking by ATA and metals respectively implicate the involvement of histidine

residues. Both ATA and metals function through binding to the histidine residues. ATA could also partially prevent Aβ induced DNA nicking activity in the presence of

metals. Based on the above observations we propose that histidine residues are essential for the Aβ induced DNA nicking activity. The results evidenced a new toxic role of Aβ in terms of its DNA nicking activity. Our finding of DNA nicking activity of Aβ peptides has biological significance in terms of causing DNA damage. Part of the work was presented at the ISN school, held at Avignon, France, 2004.

Significance: This study provided novel information on the genomic DNA topology and integrity in AD, which is shown to be altered. The study indicated an association of the altered conformation of the DNA to disease condition of AD. The study also evidenced that amyloid peptides can bind to DNA and lead to damage of the DNA. This has been a very intriguing observation, which exposed a new toxic role of the amyloid peptides. The study opens up new strategies in better understanding mechanism of neurodegeneration.

BIBLIOGRAPHY

1. Adamec A, Vonsattel JP, Nixon RA (1999) DNA strand breaks in Alzheimer's disease. *Brain Res*, 849, 67-77.

2. Ahn BW, Song DU, Jung YD, Chay KO, Chung MA, Yang SY, Shin BA (2000) Detection of β-Amyloid peptide aggregation using DNA electrophoresis. *Anal Biochem*, 284, 401-405.

3. Akama KT, Albanese C, Pestell RG, Van Eldik LJ (1998) Amyloid-beta peptide stimulates nitric oxide production in astrocytes through an NFkappaB-dependent mechanism. *Proc Natl Acad Sci (USA),* 95, 5795–5800.

4. Alzheimer A (1907) Uber eine eignartige erkrankung der birnrinde. *All Z Psychiat*, 64,146-148.

5. Ames BN, Cathcart R, Schwiers E, Hochstein P (1981) Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis. *Proc Natl Acad Sci (USA),* 78, 6858-6862.

6. Anderson AJ, Pike CJ, Cotman CW (1995) Differential induction of immediate early gene proteins in cultured neurons by β-amyloid (Abeta): association of c-Jun with Aβ-induced apoptosis. *J Neurochem*, 65, 1487-1498.

7. Anitha S, Rao KSJ, Latha KS, and Viswamitra MA (2002) First evidence to show the topological change of DNA from B-DNA to Z-DNA conformation in the hippocampus of Alzheimer's brain. *J Neuromol Med,* 2**,** 287–295.

8. Anitha S, Reuvin S, Rao KSJ, Latha KS and Viswamitra MA (2001) Alink between apoptotic DNAdamage and DNA topology in Alzheimer's disease brain: a hypothesis. *Alz Rep,* 4, 121–131.

9. Atwood CS, Huang X, Moir RD, Bacarra NM, Romano D, Tanzi RE Bush AI (1998) Dramatic aggregation of Alzheimer Aβ by Cu(II) is induced by conditions representing physiological acidosis. *J Biol Chem,* 273, 12817–12826.

10. Atwood CS, Huang X, Moir RD, Smith MA, Tanzi RE, Roher AE, et al (2001) Neuroinflammatory responses in the Alzheimer's disease brain promote the oxidative post-translational modification of amyloid deposits. In: Iqbal K, Sisodia SS, Winblad B, editors. Alzheimer's disease: advances in etiology, pathogenesis and therapeutics. Chichester, UK: Wiley, p. 341–61.

11. Augustinack J, Schneider A, Mandelkow E, Hyman B (2002) Specific tau phosphorylation sites correlate with severity of neuronal cytopathology in Alzheimer's disease. *Acta Neuropathol (Berl)*, 103, 26-35.

12. Bano S, Parihar MS (1997) Reduction of lipid peroxidation in different brain regions by a combination of α-tocopherol and ascorbic acid. *J Neural Transm*, 104, 1277-1286.

13. Barinaga M (1994) Death gives birth to the nervous system. But how? *Science*, 263, 754-756.

14. Baron DS, Gosset P, Nicole A, Sinet PM, Christen Y and Ceballos-Picot I (1999) Identification of β-amyloid Responsive Genes by RNA Differential Display: Early Introduction of a DNA Damage Inducible Gene, gadd45. *Experimental Neurology*, 158, 206-213.

15. Barrow CJ, Zagorski MG (1992) Solution structures of beta peptide and its constituent fragments: relation to amyloid deposition. *Science*, 253, 179-182.

16. Bauer WR, Crick FHC, White H (1980) Supercoiled DNA. *Sci Am,* 16**,** 243–245.

17. Behl A, Davis J, Cole GM, Shubert D (1992) Vitamin E protects nerve cells from amyloid-β protein toxicity. *Biochem Bipohys Res Commun,* 186, 944–950.

18. Behl C, Holsboer F (1999) The female sex hormone estrogen as a neuroprotectant. *Tredns Pharmacol Sci*, 20, 441-444.

19. Bertram L, Tanzi RE (2004) The current status of Alzheimer's disease genetics: what do we tell patients? *Pharmacol Res*, 50, 385-396.

20. Bhaskar MS, Subba Rao K (1994) Altered conformation and increased strand breaks in neuronal and astroglial DNA of aging rat brain. *Biochem Mol Biol Int*, 33, 377-384.

21. Binder LI, Frankfurter A, Rebhun LI (1985) The distribution of tau in the mammalian central nervous system. *J Cell Biol*, 101, 1371-1378.

22. Blessed G, Tomlinson BE, Roth M (1968) The association between quantitative measures of dementia and senile changes in the cerebral gray matter of elderly subjects. *BrJ Psychiatry,*114, 797.

23. Braak H, Braak E (1991) Neuropathological stageing of Alzheimer's-related changes. *Acta Neuropathol,* 82**,** 239–259.

24. Braak H, Braak E, Bohl J, Reintjes R (1996) Age, neurofibrillary changes, A beta-amyloid and the onset of Alzheimer's disease. *Neurosci Lett,* 210**,** 87–90.

25. Breslow JL, McPherson J, Nussbaum AL, Williams HW, Lofquist-Kahl F, Karathanasis SK, Zannis VI (1982) Identification and DNA sequence of a human apolipoprotein E cDNAclone. *J Biol Chem,* 257, 14, 639–14,641.

26. Brion JP, Flament-Durand J, Dustin P (1986) Alzheimer's disease and tau proteins. *Lancet*, 2, 1098.

27. Brody H (1955) Organization of the cerebral cortex. III. A study of aging in the human cerebral cortex. *J Comp Neurol*, 102, 511-6.

28. Burdick D, Soreghan B, Kwon M, Kosmoski J, Knauer M,. Henschen A, Yates J, Cotman C, Glabe C (1992) Assembly and aggregation properties of synthetic Alzheimer's A4/beta amyloid peptide analogs. *J Biol Chem,* 267, 546–554.

29. Busciglio J, Gabuzda DH, Matsudaira P, Yankner BA, (1993) Generation of beta-amyloid in the secretory pathway in neuronal and non neuronal cells. *Proc Natl Acad Sci* (*USA)*, 90, 2092-2096.

30. Busciglio J, Lorenzo A, Yeh J, Yankner BA (1995) β-Amyloid fibrils induces tau phosphorylation and loss of microtubule binding. *Neuron*, 14, 879-888.

31. Bush AI, Pettingell WH, Paradis M, Tanzi RE (1994a) Modulation of Aβ adhesiveness and secretase site cleavage by zinc. *J Biol Chem,* 269, 12152-12158.

32. Bush AI, Pettingell WH, Multhaup G, Paradis M, Vonsattel JP, Gusella JF, Beyreuther K, Masters CL, Tanzi RE, (1994b) Rapid induction of Alzheimer Abeta amyloid formation by zinc. *Science*, 265, 1464-1467.

33. Butterfield DA, Drake J, Pocernich C, Castenga A (2001) Evidence of oxidative damage in Alzheimer's disease brain; central role for amyloid beta-peptide. *Trends Mol Med,* 7, 548-554.

34. Butterfield DA, Martin L, Carney JM, Hensley K (1996) Aβ (25–35) peptide displays H_2O_2-like reactivity towards aqueous Fe^{2+}, nitroxide spin probes, and synaptosomal membrane proteins. *Life Sci,* 58, 217–228.

35. Byrne E (2002) Does mitochondrial chain dysfunction have a role in common neurodegenerative disorders? *J Clin Neurosci*, 29, 222-230.

36. Cacabelos R (2004) Genomic characterization of Alzheimer's disease and genotype-related phenotypic analysis of biological markers in dementia. *Pharmacogenomics*, 5, 1049-1115.

37. Campion D, Brice A, Hannequin D, Charbonnier F, Dubois B, Martin C, Michon A, Penet C, Bellis M, Calenda A, Martinez M, Agid Y, Clerget-Darpoux F, Frebourg T (1996) The French Alzheimer's disease study group: No founder effect in three novel Alzheimer's disease families with APP 717 Val-Ile mutation. *J Med Genet*, 33, 661-664.

38. Castellani RJ, Harris PL, Sayre LM, Fujii J, Taniguchi N, Vitek MP, Founds H, Atwood CS, Perry G, Smith MA (2001) Active glycation in neurofibrillary pathology of Alzheimer disease: N(epsilon) -(carv\boxymethyl) lysine and hexitol-lysine. *Free Radic Biol Med*, 31, 175-180.

39. Castenga A, Thongboonkerd V, Klein JB, Lynn B, Markesbery WR, Butterfield DA (2003) Proteomic identification of nitrated proteins in Alzheimer's disease brain. *J Neurochem*, 85, 1394-1401.

40. Cerna A, Caudrado A, Jouve N, Diaz de la Espina SM, De la Torre C (2004) Z-DNA, a new in situ marker for transcription. *Eur J Histochem*, 48, 49-56.

41. Champ PC, Maurice S, Vargason JM, Camp T, Ho PS (2004) Distribution of Z-DNA and nuclear factor I in human chromosome 22: a model for coupled transcriptional regulation. *Nucleic Acid Res*, 32, 6501-6510.

42. Champion CSKD (1995) (Ph.D thesis) Indian Institute of Science, Bangalore, India.

43. Champion CSKD, Rajan MT, Rao KSJ, Viswamitra MA (1998) Interaction of Co, Mn, Mg, and Al with d(GCCATGGC) and d(CCGGGCCCGG): a spectroscopic study. *Cell Mol Life Sci,* 54, 488–496.

44. Chandak GR, Sridevi MU, Vas CJ, Paniker DM, Sing L (2002), Apolipoprotein E and presenilin-1 allelic variation and Alzheimer's disease in India. *Hum Biol*, 74, 683-93.

45. Chaturvedi MM, Kanungo MS (1985) Analysis of conformation and function of the chromatin of the brain of young and old rats. *Mol Biol Rep*, 10, 215-219.

46. Chen SY, Harding JW, Barnes CD (1996) Neuropathology of synthetic beta –amyloid peptide analogs *in vivo. Brain Res*, 715, 44-50.

47. Cherny RA, Legg JT, McLean CA, Fairlie DP, Huang X, Atwood CS, Beyreuther K, Tanzi RE, Masters CL, Bush AI (1999) Aqueous dissolution of Alzheimer's Disease Amyloid deposits by biometal depletion. *J Bio Chem*, 274, 23223-23228.

48. Chui DH, Tanahashi H, Ozawa K, Ikeda S, Checler F, Ueda O, Suzuki H, Araki W, Inoue H, Shirotani K, Takahashi K, Gallyas F, Tabira T (1999) Transgenic mice with Alzheimer presenilin 1 mutations show accelerated neurodegeneration without amyloid plaque formation. *Nat Med*, 5, 560-564.

49. Citron M (2000) Secretases as targets for the treatment of Alzheimer's disease. *Mol Med Today*, 6, 392-397.

50. Clearly ML, Smith SD, Sklar J (1986) Cloning and structural analysis of cDNAs for bcl-2 and a hybrid cl-2/immunoglobulin transcript resulting from the t (14;18) translocation. *Cell*, 47, 19-28.

51. Clements A, Allsop D, Walsh DM, Williams CH (1996) Aggregation and metal-binding properties of mutant forms of the amyloid beta peptide of Alzheimer's disease. *J Neurochem*, 66**,** 740–747.

52. Cohen GM (1997) Caspases: the executioners of apoptosis. *Biochem J*, 326, 1-16.

53. Colurso GJ, Nilson JE, Vervoort LG (2003) Quantitative assessment of DNA fragmentation and beta-amyloid deposition in insular cortex and midfrontal gyrus from patients with Alzheimer's disease. *Life Sci*, 73, 1795-1803.

54. Cooke JR, McKie EA, Ward JM, Keshvraz-Moore E (2004) Impact of intrinsic DNA structure on processing of plasmids for gene therapy and DNA vaccines. *J Biotechnol*, 114, 239-254.

55. Corces VG, Manso R, DeLaTorre J, Avila J, Nasr A, Wiche G (1980) Effects of DNA on microtubule assembly. *Eur J Biochem*, 105, 7-16.

56. Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL, Pericak-Vance MA (1993) Gene dose of apolipoprotein E type e4 allele and the risk of Alzheimer's disease in late onset families. *Science*, 261, 921-923.

57. Cotman CW, Anderson AJ (1995) A potential role for apoptosis in neurodegeneration and Alzheimer's disease. *Mol Neurobiol*, 10, 19-45.

58. Cotman CW, Pike CJ, Copani A (1992) β-Amyloid neurotoxicity: a discussion of in vitro findings. *Neurobiol Aging*, 13, 587–590.

59. Cotman CW, Whittemore ER, Watt JA, Anderson AJ, Loo DT (1994) Possible role of apoptosis in Alzheimer's disease. *Ann N Y Acad Sci,* 15, 36-49.

60. Cotman CW, Su JH (1996) Mechanism of neuronal death in Alzheimer's disease. *Brain Pathol*, 6, 439-506.

61. Crapper DR, Quittkat S, De Boni U (1979) Altered chromatin conformation in Alzheimer's disease. *Brain,* 102, 483–495.

62. Crass P, Smith MA, Richey PL, Siedlak SL, Mulvihill P, Perry G (1995) Extracellular neurofibrillary tangles reflect neuronal loss and provide further evidence of extensive protein cross-linking in Alzheimer disease. *Acta Neuropathol*, 89, 291-295.

63. Cuajungco MP, Faget KY (2003) Zinc takes the center stage: its paradoxical role in Alzheimer's disease. *Brain Res Brain Res Rev*, 41, 44-56.

64. Cuajungco MP, Goldstein LE, Nunomura A, Smith MA, Lim JT, Atwood CS, Huang X, Farrag YW, Perry G and Bush AI (2000) Evidence that the Beta amyloid plaques of Alzheimer's disease represent the redox silencing and entombment of Aβ by Zinc. *J Biol Chem*, 275, 19439-19442.

65. Culvwenor JG, Henry A, Hartman T, Evin G, Galatis D, Friedhuber A, Jayasena UL, Underwood JR, Beyreuther K, Masters CL, Capai R (1998) Subcellular localization of the Alzheimer's disease amyloid precursor protein and derived polypeptide expressed in a recombinant yeast system. *Amyloid*, 5, 78-89.

66. D'Andrea MR, Nagele RG, Wang HY, Peterson PA, Lee DH (2001) Evidence that neurones accumulating amyloid can undergo lysis to form amyloid plaques in Alzheimer's disease. *Histopathology*, 38, 120-34.

67. Demeester N, Mertens C, Caster H, Goethals M, Vanderkerchhove J, Rosseneu M, Labeur C (2001) Comparsion of the aggregation properties, secondary structure and apoptotic effects of wild-type, Flemish and Dutch N-termianlly truncated amyloid beta peptides. *Eur J Neurosci*, 13, 2015-2024.

68. Deng GR, Wu R (1983) Termianl transferase: use in the tailing of DNA and for in vitro mutagenesis. *Methods Enzymol*, 100, 96-116.

69. Dikalov SI, Vitek MP, Maples KR, Mason RP (1999) Amyloid-β-peptides do not form peptide-derived free radicals spontaneously, but enhance metal-catalyzed oxidation of hydroxylamines to nitroxides. *J Biol Chem,* 274, 9392–9399.

70. Doraiswamy PM, Finefrock AE (2004) Metals in our minds: therapeutic implications for neurodegenerative disorders. *Lancet Neurol*, 3, 431-434.

71. Esposito L, Gan L, Yu GQ, Essrich C, Mucke L (2004) Intracellularly generated amyloid-beta peptide counteracts the antiapoptotic function of its precursor protein and primes proapoptotic pathways for activation by other insults in neuroblastoma cells. *J Neurochem*, 9, 1260-1274.

72. Farrer L, Cupples LA, van Duijin CM, Kurz A, Zimmer R, Muller U, Green RC, Clarke V, Schoffner J, Wallace D et al., (1995) Apolipoprotein E genotype in patients with Alzheimer's disease: implications for risk of dementia among relatives. *Ann Neurol*, 38, 797-808.

73. Flood JF, Morley JE and Roberts E (1991) Amnestic effects in mice of four synthetic peptides homologous to amyloid beta protein from patients with Alzheimer's disease. *Proc Natl Acad Sci (USA)*, 88, 3363-3366.

74. Forloni R, Chiesa S, Smiroldo L, Verga M, Salmona F, Tagliavini N, Angeretti (1993) Apoptosis-mediated neurotoxicity induced by chronic application of beta amyloid fragment 25–35. *Neuro Report*, 4, 523–526.

75. Frank RA, Galasko D, Hampel H, Hardy J, Leon MJ, Mehta PD, Rogers J, Siemers E, Trojanowski JQ (2003) Biological markers for therapeutic trials in Alzheimer's disease proceedings of the biological markers working group: NIA on neuroimaging in Alzheimer's disease. *Neurobiol Aging*, 24, 521-536.

76. Franskowski H, Missotten M, Fernandez PA, Martinou I, Michel P, Sadoul R, Martinou JC (1995) Function and expression of Bcl-x gene in the developing and adult nervous system, *Neuroreport*, 6, 1917-1921.

77. Fraser PE, Nguyen JT, Surewicz WK, Kirschner DA (1991) pH-dependent structural transitions of Alzheimer amyloid peptides. *Biophys J*, 60, 1190-1201.

78. Gabbita SP, Lovell MA, Markesbery WR (2001) Increased nuclear DNA oxidation in the brain in Alzheimer's disease. *J Neurochem*, 71, 2034-40.

79. Gagna CE, Lambert WC, Kuuo HR, Farnsworth PN (1997) Location of B-DNA and Z-DNA in terminally differentiating fiber cells in the adult lens. *J Histochem Cytochem*, 45, 1511-1521.

80. Gagna CE, Kuo HR, Lambert WC (1999) Terminal differentiation and left-handed Z- DNA: a review. *Cell Biol Int*, 23, 1–5.

81. Games D, Adams D, Alessandrini R, Barbour R, Berthelette P, Blackwell C Carr T, Clements J, Donaldson T, Gillespie F et al., (1995) Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. *Neuron*, 373, 523-527.

82. Garcia I, Martinou I, Tsujimoto Y, Martinou JC (1992) Prevention of programmed cell death of sympathetic neurons by the bcl-2 protooncogene. *Science*, 258, 302-304.

83. Garner MM, Felsenfeld G (1987) Effect of Z-DNA on nucleosome placement. *J Mol Biol,* 196, 581–590.

84. Gasparini L, Netzer WJ, Greengard P, Xu H (2002) Does insulin dysfunction play a role in Alzheimer's disease. *Trends Pharmacol Sci*, 23, 288-293.

85. Geddes JW, Anderson KJ, Cotman CW (1986) Senile plaques as aberrant sprout-stimulating structures. *Exp Neurol,* 94, 767–776.

86. Geula C, Wu CK, Saroff D, Lorenzo A, Yuan M, Yankner BA (1998) Aging renders the brain vulnerable to amyloid β-protein neurotoxicity. *Nature Med*, 4, 827-831.

87. Giri RK, Rajgopal V, Kalra VK (2004) Curcumin, the active constituent of turmeric inhibits amyloid peptide-induced cytochemokine gene expression and CCR5-mediated chemotaxis of THP-1 monocytes by modulating early growth response-1 transcription factor. *J Neurochem*, 91, 1199-1210.

88. Glenner GG, Wong CW (1984) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Comm*, 120, 885-890.

89. Goate A, Chartlier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L, Mount R, Newton P, Rooke K, Roques P, Talbot C, Pericak-Vance M, Roses A, Williamson R, Rossor M, Owen M, Hardy J (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature*, 349, 704-706.

90. Gomez-Isla T, Hollister R, West H, Mui S, Growdon JH, Petersen RC et al., (1998) Neuronal loss correlates with but exceeds neurofibrillary tangles in Alzheimer's disease. *Neurology*, 50,136-145.

91. Gonzalez GR, Haxo RS, and Schleich T (1980), Mechanism of Action of Polymeric Aurintricarboxylic Acid, a Potent Inhibitor of Protein-Nucleic Acid Interactions. *Biochemistry*, 19, 4299-4303.

92. Good PF, Werner P, Hsu A, Olanow CW, Perl DP (1996) Evidence of neuronal oxidative damage in Alzheimer's disease. *Am J Pathol*, 149, 21-28.

93. Gouras GK, Tsai J, Naslund J, Vincent B, Edgar M, Checler F, Greenfield JP, Haroutunian V, Buxbaum JF, Xu H, Greengard P and Relkin NR (2000) Intraneuronal Aβ (1-42) accumulation in Human Brain. *Am J Pathol*, 156, 15-20.

94. Graever MB, Kosel S, Egensperger R, Banati RB, Muler U, Bise K, Hoff P, Moller HJ, Fujisawa K, Mehraein P (1997) Rediscovery of the case described by Alois Alzheimer in 1911: historical, histological and molecular genetic analysis. *Neurogenetics*, 1, 73.

95. Grant SM, Ducatenzeiler A, Szyl M, Cuello AC (2000) Aβ immunoreactive material is present in all intracellular compartments in transfected neuronally differentiated, P19 expressing the human amyloid β-protein precursor. *J Alzheimer's Dis*, 2**,** 207–222.

96. Gray DM, Taylor TN, Lang D (1978) Dehydrated circular DNA: circular dichroism of molecules in ethanol solutions. *Biopolymers*, 17**,** 1–45.

97. Gray M, DRatliff RL, Vaughan MR (1992) Circular Dichroism Spectroscopy of DNA. *Methods Enzymol*, 211, 389–406.

98. Greenwood JA, Johnson GW, (1995) Localization and insitu phosphorylation state of nucleolar tau. *Exp Cell Res*, 220, 332-337.

99. Grundke-Iqbal I, Iqbal K, Quinlan M, Tung YC, Zaidid MS, Wisniewski HM (1986) Microtubule-associated protein tau. A compartment of Alzheimer paired helical filaments. *J Biol Chem*, 261, 6084-6089.

100. Grundke-Iqbal I, Vorbrodt AW, Iqbal K, Tung YC, Wang GP, Wisniewski HM (1988) Microtubule- associated polypeptide tau are altered in Alzheimer paired helical filaments. *Brain Res*, 464, 43-52.

101. Guillaume D, Bertrand P, Dea D, Davignon J, Poirier J (1996) Apolipoprotein E and low-density lipoprotein binding and internalization in primary cultures of rat astrocytes: isoform-specific alterations. *J Neurochem*, 66, 2410-8.

102. Gupta GS, Dhingra MM, Sharma RH (1983) Left-handed intercalated DNAdouble helix: rendezvous of ethidium and actinomycin D in the Z-helical conformation space. *J Biomol Struc Dyn*, 1, 97–113.

103. Haass C, Scholossmacher MG, Hung AY, Vigo-Pelfrey C, Mellon A, Ostaszewski BL, Lieberburg I, Koo EH, Schenk D, Teplow DB, Selkoe DJ (1992) Amyloid beta-peptide is produced by cultured cells during normal metabolism. *Nature*, 359, 322-325.

104. Halverson K, Fraser PE, Kirschner DA, Lansbury PT Jr (1990) Molecular determinants of amyloid deposition in Alzheimer's disease: conformational studies of synthetic beta-protein fragments. *Biochemistry*, 29, 2639-2644.

105. Hamada H, Kakunaga T (1982) Potential Z-DNA forming sequences are highly dispersed in the human genome. *Nature,* 298, 396–398.

106. Hanlon S, Brudno S, Wu TT, Wolf B (1975) Structural transitions of deoxyribonucleic acid in aqueous electrolyte solutions: reference spectra of conformational limits. *Biochemistry,* 14**,** 1648–1660.

107. Haque N, Tanaka T, Iqbal K, Grundke-Iqbal I (1999) Regulation of expression, phosphorylation and biological activity of tau during differentiation in SY5Y cells. *Brain Res*, 838, 69-77.

108. Hardy J (1997) Amyloid, the presenilins and Alzheimer's disease. *Trends Neurosci,* 20,154-159,

109. Hardy J, Allsop D (1991) Amyloid deposition as the central event in the aetiology of Alzheimer's disease. *TIPS*, 12, 383-388.

110. Hardy JA, Higgins GA (1992) Alzheimer's disease: the amyloid cascade hypothesis. *Science*, 256, 184-185.

111. Hardy JA, Selkoe DJ (2002) The Amyloid Hypothesis of Alzheimer's Disease: Progress and Problems on the Road to Therapeutics. *Science*, 297, 353-356.

112. Harkany T, Abraham I, Konaya C, Nyakas C, Zarandi M, Penke B Luiten PG (2000) Mechanism of beta-amyloid neurotoxicity: Perspectives of Pharmacotherapy. *Rev Neurosci,* 11, 329-382.

113. Harper JD, Lieber CM, Lansbury Jr PT (1997) Atomic microscopic imaging of seeded fibril formation and fibril branching by the Alzheimer's disease amyloid-beta protein, *Chem.Biol*, 4, 951-959.

114. Hartely DM, Walsh DM, Ye CP, Diehl T, Vasquez S, Vassilve PM, Teplow DB, Selkoe DJ (1999) Protofibrillar intermediates of amyloid beta-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. *J Neurosci,* 19, 8876.

115. Hasan R, Alam M, Ali. R (1995) Polyamine induced Z-conformation of calf thymus DNA. *FEBS Lett*, 36, 827–830.

116. Hegde ML, Anitha S, Latha KS, Musthak MS, Stein R, Ravid R, Jagannatha Rao KS (2004) First evidence for helical transitions in supercoiled DNA by Amyloid β peptide (1-42) and Aluminium: A new insight in understanding Alzheimer's disease. *J Mol Neurosci*, 22, 19-31.

117. Helpern JA, Jensen J, Lee SP, Falangola MF (2004) Quantitative assessment of Alzheimer's disease. *J Mol Neurosci*, 24, 45-48.

118. Henderson VW (1997) Estrogen, cognition, and a woman's risk of Alzheimer's disease. *Am J Med*, 103, 11S-18S.

119. Henderson VW (2004) Hormone therapy and Alzheimer's disease: benefit or harm? *Exper Opin Pharmacother*, 5, 389-406.

120. Hensley K, Carney J.M, Mattson MP, Aksenova M, Harris M, Wu JF, Floyd RA, Butterfield DA (1994) A model for beta-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer disease. *Proc Natl Acad Sci (USA),* 91, 3270–3274.

121. Herbert A, Rich A (1996) The biology of lefthanded Z-DNA. *J Biol Chem,* 71, 11, 595–11, 598.

122. Hock C, Konietzko U, Papassotiropolous A, Wallmer A strelfer J, Von Rotz RC, Davey G, Moritz E, Nitsch RM (2002) Generation of antibodies specific for β-amyloid by vaccination of patients with Alzheimer's disease. *Nature Med*, 8, 1270-1275.

123. Hockenbery DM (1995) bcl-2, a novel regulator of cell death. *Bioessays,* 17, 631-638.

124. Holscher C (1998) Possible causes of Alzheimer's disease: amyloid fragments, free radicals, and calcium homeostasis. *Neurobiol Dis*, 5, 129-141.

125. Hua Q, He RQ (2000) Human neuronal tau promoting the melting temperature of DNA. *Clin Sci Bull*, 45, 999-1001.

126. Hua Q and He RQ (2002a) Tau could protect DNA double helix structure. *Biochem Biophys Acta*, 36763, 1-7.

127. Hua Q, He RQ (2002b) Effect of phosphorylation and aggregation on tau binding to DNA. *Protein Pep Lett*, 9, 249-357.

128. Huang HM, Ou HC, Hsieh SJ (2000) Antioxidants prevent amyloid peptide-induced apoptosis and alteration of calcium homeostasis in cultured cortical neurons. *Life Sci,* 66**,** 1879–1892.

129. Huang X, Atwood CS, Hartshorn MA, Multhaup G, Goldstein LE, Scarpa RC, Cuajungco MP, Gray DN, Lim J, Moir RD, Tanzi RE, Bush AI (1999) The Aβ peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction. *Biochemistry,* 38, 7609–7616.

130. Huang X, Atwood CS, Moir RD, Hatshorn MA, Vonsatel JP, Tanzi RE and Bush AI (1997) Zinc induced Alzheimer's Abeta-40 aggregation is mediated by conformational factors. *J Biol Chem*, 272, 26464-26470.

131. Hutton M, Busfield F, Wragg M, Crook R, Perez-Tur J, Clark RF, Prihar G, Talbot C, Phillips H, Wright K, Baker M, Lendon C, Duff K, Martinez A, Houlden H, Nichols A, Karran E, Roberts G, Roques P, Rossor M, Venter JC, Adams MD, Cline RT, Phillips CA, Fuldner RA, Hardy J, Goate A (1996) Complete analysis of the presenilin 1 gene in early onset Alzheimer's disease. *NeuroReport,* 7, 801-805.

132. Hyman BT, West HL, Rebeck GW, Buldyrev SV, Mantegna RN, Ukleja M, Havlin S, Stanley HE (1995) Quantitative analysis of senile plaques in Alzheimer's disease: Observation of log-normal size distribution and molecular epidemiology of differences associated with ApoE genotype and trisomy 21 (Down syndrome). *Proc Natl Acad Sci (USA),* 92, 3586-3590.

133. Iqbal K, Alonso AC, Gong CX, Khatoon S, Singh TJ, Grundke-Iqbal I (1994) Mechanism of neurofibrillary degeneration in Alzheimer's disease. *Mol Neurobiol,* 9**,** 119–123.

134. Jacobsen MD, Weil M, Raff MC (1996) Role of Ced-3/ICE-family proteases in staurosporine-induced programmed cell death. *J Cell Biol*, 133, 1041-1051.

135. Jang JH, Surh YJ (2002) Beta-amyloid induces oxidative DNA damage and cell death through activation of c-Jun N terminal kinase. *Ann N Y Acad Sci*, 973, 228-236.

136. Jarrett JT, Lansbury Jr PT (1993) Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie?. *Cell,* 73, 1055–1058.

137. Jaruga P, Dizdaroglu M (1996) Repair of products of oxidative DNA base damage in human cells. *Nucl Acids Res,* 65, 2146-2156.

138. Johsstone EM, Chaney MO, Norris FH, Pascual R, Little SP (1991) Conservation of the sequence of the Alzheimer's disease amyloid peptide in dog, polar bear and five other mammals by corss-species polymerase chain reaction analysis. *Brain Res Mol Brain Res*, 10, 299-305.

139. Jorm AF, Van Duijn CM, Chandra V, Fratiglioni L, Graves AB, Heyman A, Kokmen E, Kondo K, Mortimer J, Rocca WA, Shalat SL, Soininen H, Hofman (1991) The Eurodem risk factors research group: Psychiatric history and related exposures as risk factors for Alzheimer's disease: a collaborative re-analysis of case-control studies. *Int J Epidemiol,* 20 (Suppl 2), S43-S47.

140. Kadioglu E, Sardas A, Aslan S, Isik E, East Karakaya A (2004) Detection of oxidative damage in lymphocytes of patients with Alzheimer's disease. *Biomarkers*, 9, 203-209.

141. Kang J, Lemaire H-G, Unterbeck A, Salbaum JM, Masters CL, Grzescnik K-H, Multhaup G, Beyreuther K, Muller-Hill B (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature,* 325, 733-736.

142. Karlik SJ, Eichhorn GL, Lewis PN, Crapper DR (1980) Interaction of aluminum species with deoxyribonucleic acid. *Biochemistry,* 19**,** 5991–6001.

143. Katzman R (2004) A neurologist's view of Alzheimer's disease and dementia. *Int Psychogeriatry*, 16, 259-273.

144. Kirschner DA, Abraham C, Selkoe DJ (1986) X-ray diffraction from intraneuronal paired helical filaments and extra-neuronal amyloid fibers in Alzheimer's disease indicates a cross beta conformation, *Proc Natl Acad Sci (USA)*, 84, 6953-6957.

145. Klysik J, Stirdivant SM, Larson JE, Hart PA, Wells RD (1981) Left handed DNAin restriction fragments and a recombinant plasmid. *Nature, 2*90, 672–677.

146. Kosik KS, Joachim CL, Selkoe DJ (1986) Microtubule-associated protein tau (tau) is a major antigenic component of paired helical filaments in Alzheimer disease. *Proc Natl Acad Sci (USA),* 83, 4044-40448.

147. Kowall N, McKee A, Yankner B, Beal M (1992), In vivo neurotoxicity of β-amyloid [β (1–40)] and the β(25–35) fragment. *Neurobiol Aging,* 13, 537–542.

148. Kowall NW, Beal MF, Busciglio J, Duffy LK, Yanker BA (1991) *Proc Natl Acad Sci (USA),* 88, 7247-7251.

149. Kowalska A (2004) The beta-amyloid cascade hypothesis: a sequence leading to neurodegeneration in Alzheimer's disease. *Neurol Neurochir Pol*, 38, 405-411.

150. Kozin SA, Zirah S, Rebuffat S, Hoa GHB, Debey P (2001) Zinc binding to Alzheimer's Aβ (1-16) peptide results in stable soluble complex. *Biochem Biophys Res Comm*, 285, 959-964.

151. Kril JJ, Hodges J, Halliday G (2004) Relationship between hippocampal volume and CA1 neuron loss in brains of humans with and without Alzheimer's disease. *Neurosci Lett*, 361, 9-12.

152. Kril JJ, Patel S, Harding AJ, Halliday GM (2002) Neurons from the hippocampus of Alzheimer's disease exceeds extracellular neurofibrillary tangle formation. *Acta Neuropathol (Berl)*, 103, 370-376.

153. Kuroda Y, Kawahara M (1994) Aggregation of amyloid beta-protein and its neurotoxicity: enhancement by aluminium and other metals. *Tohoku J Exp Med*, 174, 263-268.

154. Kurt MA, Davies DC, Kidd M (1997) Paired helical filament morphology varies with intracellular location in Alzheimer's disease brain. *Neurosci Lett*, 239, 41-44.

155. Kurtz DI, Russel AR, Sinex FM (1974) Multiple peaks in the derivative melting curve of chromatin from animals of varying age. *Mech Age Develop*, 3, 37-49.

156. Lacor PN, Buniel MC, Chang L, Fernandez SJ, Gong Y, Viola KL, Lambert MP, Velasco PT, Biogio EH, Finch CE, Krafft GA, Klein WL (2004) Synaptic targeting by Alzheimer's –related amyloid beta oligomers. *J Neurosci*, 24, 10191-10200.

157. Lahiri DK, Greig NH (2004) Lethal weapon: amyloid beta-peptide, role in the oxidative stress and neurodegeneration of Alzheimer's disease. *Neurobiol Aging*, 25, 581-587.

158. Lambert JC, Berr C, Pasquier F, Delacourte A, Frigard B, Cottel D, Perez-Tur J, Mouroux V, Mohr M, Cecyre D, Galasko D, Lendon C, Poirier J, Hardy J, Mann D, Amouyel P, Chartier-Harlin MC (1998) Pronounced impact of Th1/E47 cs mutation compare with-492 AT mutation on neural APOE gene expression and risk of developing Alzheimer's disease. *Hum Mol Genet*, 7, 1511-1516.

159. Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, Viola KL, Wals P, Zhang C,

160. Finch CE, Krafft GA, Klein GA, Klein WL (1998) Diffusible, nonfibrillar ligands derived from $A\beta_{1-42}$ are potent central nervous system neurotoxins. *Proc Natl Acad Sci (USA),* 95, 6448–6453.

161. Lamos ML, Walker GT, Krugh TR, Turner DI (1986) Fluorescence-detected Circular dichroism of ethidium bound to poly (dG-dC) and poly (dG-m5dC) under B- and Z-form conditions. *Biochemistry,* 25, 687–691.

162. Lannfelt L, Alexman K, Lilius L, Basun H (1995) Genetic counseling in a Swedish Alzheimer family with amyloid precursor protein mutation. *Am J Hum Genet*, 56, 332-335.

163. Lansbury PT Jr (1999) Evolution of amyloid: What normal protein folding may tell us about fibrillogenesis and disease. *Proc Natl Acad Sci (USA)*, 96, 3342-3344.

164. Larson EB, Kukull WA, Kartzman RL (1992) Cognitive impairment, dementia and Alzheimer's disease. *Annu Rev Public Health*, 13, 431-439.

165. Lassmann H, Bancher C, Breitschopf H, Wegiel J, Bobinski M, Jellinger K, Wisniewski HM (1995) Cell death in Alzheimer's disease evaluated by DNA fragmentation in situ. *Acta Neuropathol*, 89, 35-41.

166. Latha KS, Anitha S, Rao KSJ, Viswamitra MA (2002) Molecular understanding of aluminium induced topological changes in $(CCG)_{12}$ triplet repeats: relevance to neurological disorders. *Biochem Biophys Acta*, 1588, 56-64.

167. Lemaire HG, Salbaum JM, Multhaup G, Kang J, Bayney RM, Unterbeck A, Beyreuther K, Muller-Hill B (1989) The PreA4 (695) precursor protein of Alzheimer's disease A4 amyloid is encoded by 16 exons. *Nucleic Acids Res,* 17, 517-522,

168. Lendon CL, Ashall F, Goate AM (1997) Exploring the etiology of Alzheimer's disease using molecular genetics. *JAMA*, 277, 825-831.

169. Leslie RA (2002) Imaging Alzheimer's disease in vivo: not so implaqueable anymore. *Trends Neurosci*, 25, 232-233.

170. LeVine HI (1993) Thiflavine T interaction with synthetic Alzheimer's disease beta-amyloid peptides: detection of amyloid aggregation in solution. *Potein Sci*, 2, 404-410.

171. Levy-Lahad E, Wasco W, Poorkaj P, Romano DM, Oshima J, Pettigell WH, Yu C, Jondro PD, Schmidt SD, Wang K, Crowley AC, Fu Y-H, Guenette Sy, Galas D, Nemens E, Wijsman EM, Bird TD,

Schellenberg GD, Tanzi RE (1995a) Candidate gene for chromosome 1 familial Alzheimer's disease locus. *Science*, 269, 973-977,

172. Levy-Lahad E, Wijsman EM, Nemens E, Anderson L, Goddard KA, Weber JL, Bird TD, Schellenberg CD (1995b) A familial Alzheimer's disease locus on chromosome 1. *Science,* 269, 970–973.

173. Lewis PN, Lukiw WJ, DeBoni U, McLachlan DR (1981) Changes in chromatin structure assocaiated with Alzheimer's disease. *J Neurochem*, 37, 1193-1202.

174. Loo TD, Copani A, Pike CJ, Whittemore ER, Walencewicz AJ, Cotman CW (1993) Apoptosis is induced by β-amyloid in cultured central nervous system neurons. *Proc Natl Acad Sci (USA)*, 90, 7951-7955.

175. Loomis PA,, Howard TH, Castleberry RP, Binder LI (1990) Identification of nuclear tau isoforms in human neuroblastoma cells. *Proc Natl Acad Sci (USA)*, 87, 8422-8426.

176. Lorenzo A, Yankner BA (1996) Amyloid fibril toxicity in Alzheimer's disease and diabetes. *Ann N Y Acad Sci,* 777, 89–95.

177. Lovell MA, Robertson JD, Teesdale WJ, Campbell JL, Markesbery WR (1998) Copper, Iron and Zinc in Alzheimer's disease senile plaques. *J Neurol Sci*, 158, 47-52.

178. Lovestone S, Reynolds CH (1997) The phosphorylation of tau: a critical stage in neurodevelopmental and neurodegenerative process. *Neuroscience*, 78, 309-324.

179. Lu M, Kosik KS (2001) Competition for microtubule-binding with dual expression of tau missense and splice isoforms. *Mol Biol Cell*, 12, 171-184.

180. Lucassen PJ, Chung WC, Kamphorst W, Swaab DF (1997) DNA damage distribution in the human brain as shown by in situ end labeling; area-specific differences in aging and Alzheimer disease in the absence of apoptotic morphology. *J Neuropathol Exp Neurol*, 56, 887-900.

181. Lukiw WJ, Crapper McLachlan DR (1990) Chromatin structure and gene expression in Alzheimer's disese. *Brain Res Mol Brain Res*, 7, 227-233.

182. Lukiw WJ, Krishna B, Wong L, Kruck TPA, Bergeson C, McLachlan McLachlan DRC (1991) Nuclear compartmentalization of aluminium in Alzheimer's disease. *Neurolbiol Aging*, 13, 115-121.

183. Lyras L, Cairns NJ, Jenner A, Jenner P, Halliwell B (1997) An assessment of oxidative damage to proteins, lipids, and DNA in brain from patients with Alzheimer's disease. *J Neurochem,* 68**,** 2061-2069.

184. Maccioni RB, Cambiazo V (1995) Role of microtubule-associated proteins in the control of microtubule assembly. *Physiol Rev*, 75, 835-864.

185. Mahley RW (1988) Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science*, 240, 622-630.

186. Malinchik SB, Inouye H, Szumowski KE, Kirschner DA (1998) Structural analysis of Alzheimer's beta (1-40) amyloid: protofilament assembly of tubular fibrils. *Biophys J*, 74, 537-545.

187. Mann D, Yates P, Marcyniuk B, Ravindra C (1986) The topography of plaques and tangles in Down's syndrome patients of different ages. *J Neuropathol Appl Neurobiol*, 12, 447-57.

188. Mann DAM (1985) The pathological association between Down syndrome and Alzheimer's disease. *Mech Age Dev,* 43, 99-136.

189. Mantyh P, Ghilardi J, Rogers S, DeMaster E, Allen C, Stimson E, Maggio J (1993) Aluminium, iron or zinc ions promote aggregation of physiological concentration of beta amyloid peptide. *J Neurochem*, 61, 1171-1174.

190. Martin SJ, Green DR, Cotter TG (1994) Dicing with death: dissecting the components of the apoptosis machinery. *Trends Biochem Sci,* 19, 26-30.

191. Martyn CN, Barker DJ, Osmand C, Harris EC, Edwardson JA, Lacey RF (1989) Geographical relation between Alzheimer's disease and aluminum in drinking water. *Lancet*, 1, 59–62.

192. Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K (1985) Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc Natl Acad Sci (USA)*, 82, 4245-4249.

193. Mattson MP (1995) Untangling the pathophysiochemistry of beta-amyloid. *Nat Struct Biol*, 2, 926-8.

194. Mattson MP (2004) Pathways towards and away from Alzheimer's disease. *Nature*, 430, 631-639.

195. Mattson MP, Goodman Y (1995) Different amyloidogenic peptides share a similar mechanism of neurotoxicity involving reactive oxygen species and calcium. *Brain Res,* 676, 219-224.

196. Mattson MP, Tomaselli KJ, Rydel RE (1993) Calcium –destabilizing and neurodegenerative effects of aggregated beta-amyloid peptide are attenuated by basic FGF. *Brain Res*, 621, 35-49.

197. McGeer PL, McGeer EG (1995) The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. *Brain Res Brain Res Rev,* 2, 195-218.

198. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM (1984) Clinical diagnosis of Alzheimer's disease Report of the NINCDS-ADRDA work group under the auspices of Department of Health and Human Services Task Force on Alzheimer's disease. *Neurology,* 34, 939-944.

199. McLachlan DR, Bergeron C, Smith JE, Boomer D, Rifat SL (1996) Risk for neuropathologically confirmed Alzheimer's disease and residual aluminium in municipal drinking water employing weighted residential histories. *Neurology*, 46, 401–405.

200. McLachlan DR, Kruck TP, Lukiw WJ, Krishnan SS (1991) Would decreased aluminum ingestion reduce the incidence of Alzheimer's disease? *CMAJ*, 145, 793-804.

201. McLachlan DR, Lukiw WJ, Mizzen C, Percy ME, Somerville MJ, Sutherland MK, Wong L (1991) Anomalous gene expression in Alzheimer disease: cause or effect. *Can J Neurol Sci*, 18 (Suppl), 414-418.

202. McLachlan DR, Lukiw WJ, Wong L, Bergeron C, Bech-Hasen NT (1988) Selective messenger RNA reduction in Alzheimer's disease. *Brain Res*, 427, 255-261.

203. Mclaurin J, Franklin T, Chakrabarthy A, Fraser PE (1998) Phosphatidylinositol and inositol involvement in Alzheimer amyloid-beta growth and arrest. *J Mol Biol*, 278, 183-194.

204. Mecocci P, Beal MF, Cecchetti R, Polidori MC, Cherubini A, Chionne F, Avellini L, Ronano G, Senin U (1997) Mitochondrial membrane fluidity and oxidative damage to mitochondrial DNA in aged and AD human brain. *Mol Chem Neuropathol*, 31, 53-64.

205. Mecocci P, MacGarvey U, Beal MF (1994) Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. *Ann Neurol*, 36, 747-751.

206. Meda L, Cassatella MA, Szendrei GI, Otvos Jr L, Baron P, Villalba M, Ferrari D, Rossi F (1995) Activation of microglial cells by beta-amyloid protein and interferon-gamma. *Nature*, 374, 647-650.

207. Melvin T, Cunniffe SM, O'Neill P, Parker AW, Roldan-Arjona T (1998) Guanine is the target for direct ionisation damage in DNA, as detected using excision enzymes. *Nucleic Acids Res*, 26, 4935-4942.

208. Mendoza-Ramirez JL, Beltran-Parrazal L, Verdugo-Diaz L, Morgado-Valle C, Drucker-Colin R (1995) Delay in manifestations of aging by grafting NGF cultured chromaffin cells in adulthood. *Neurobiol Aging*, 16, 907-916.

209. Michak V, Maurizot MS, Charlier M (1995) Calculation of Hydroxy Radical Attack of Different Forms of DNA. *J Biomol Struct Dyn,* 13, 565–575.

210. Miller FD, Dixon GH, Rather JB, van de Sande JH (1985) Assembly and charaterisation of nucleosomal cores on B-vs.Z-form DNA. *Biocheimstry*, 24, 102-109.

211. Minn A, Boise L, Thomson C (1996) Bcl-$x_{(s)}$ antagonizes the protective effects of Bcl- $x_{(L)}$. *J Biol Chem*, 271, 6306-6312.

212. Minn AJ, Rudin CM, Boise LH, Thomson CB (1995) Expression of bcl-xL can confer a multidrug resistance phenotype. *Blood*, 86, 1903-1910.

213. Mirra S, Hart MN, Terry RD (1993) Making the diagnosis of Alzheimer's disease, a primer for practicing pathology pathologists. *Arch Pathol Lab Med,* 117, 132–144.

214. Mirra SS (1997) The CERAD neuropathology protocol and consensus recommendations for the postmortem diagnosis of Alzheimer's disease: a commentary. *Neurobiol Aging*, 18 (Suppl.), S91–94.

215. Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, Vogel FS, Hughes JP, van Belle G, Berg L (1991) Participating CERAD neuropathologists The Consortium to Establish a Registry for Alzheimer's disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurology*, 41, 479-486.

216. Mochizuki A, Tamaoka A, Shimohata A, Komatsuzaki Y, Shoji S (2000) Abeta42-positive non-pyramidal neurons around amyloid plaques in Alzheimer's disease. *Lancet*, 355, 42-43.

217. Morocz M, Kalam J, Juhasz A Sinko I, McGlynn AP, Downes CS, Janka Z, Rasko I (2002) Elevated levels of oxidative damage in lymphocytes from patients with Alzheimer's disease. *Neurobiol Aging*, 23, 47-53.

218. Mortimer J, Van Duijn CM, Chandra V, Fratiglioni L, Graves AB, Heyman A, Jorm AF, Kokmen E, Kondo K, Rocca WA, Shalat SL, Soininen H, Hofman A (1991) for the Eurodem risk factors research group: Head trauma as a risk factor for Alzheimer's disease: a collaborative re-analysis of case-control studies. *Int J Epidemiol*, 20(Suppl 2), S28-S35.

219. Mosbaugh DW, Linn S (1982) Characterization of the action of *E. Coli* DNA polymerase I as incisions produced by repair endo deoxyribonucleases. *J Biol Chem*, 257, 575-583.

220. Mullaart E, Boerrigter ME, Ravid R, Swaab DF, Vijg J (1989) Increased levels of DNA breaks in cerebral cortex of Alzheimer's disease patients. *Neurobiol Aging*, 11, 169-173.

221. Naiki H, Higuchi K, Hosokawa M, Takeda T (1989) Fluorometric determination of Amyloid Fibrils in *vitro* using the fluorescent dye, Thioflavine T. *Anal Biochem*, 177, 244-249.

222. Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J (2000) Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by β-amyloid. *Nature,* 403, 98–103.

223. Naslund J, Haroutanian V, Mohs R, Davis KL, Davies P, Greengard P, Buxdaum JP (2000) Correlation between elevated levels of amyloid β peptide in the brain and cognitive decline. *Am Med Assoc*, 283, 1571.

224. Neumann PZ, Sass-Kortsak A (1967) The state of copper in human serum: evidence for an amino acid-bound fraction. *J Clin Invest,* 46, 646-658.

225. Nicholson DW, Thornberry NA (1997) Caspases: killer proteases. *Trends Biochem Sci*, 22, 299-306.

226. Nielsen SA, Ravid R, Kamphorst W, Orgensen OS (2003) Apoliprotein E e4 in an autopsy series of various dementing disorders. *J Alzheimers Dis,* 5**,** 119–125.

227. Nunomura A, Perry G, Pappolla M.A, Wade R, Hirai K, Chiba S, Smith MA (1999) RNA oxidation is a prominent feature of vulnerable neurons in Alzheimer's disease. *J Neurosci,* 19, 1959–1964.

228. Nunomura A, Perry G, Pappolla MA, Freidland RP, Hirai K, Chiba S, Smith MA (2000) Neuronal oxidative stress precedes amyloid-beta deposition in Down syndrome, *J Neuropathol Exp Neurol*, 59, 1011-1017.

229. Ohyagi Y, Asahara H, Chui DH, Tsuruta Y, Sakae N, Miyoshi K, Yamada T, Kikuchi H, Taniwaki T, Murali H, Ikezoe K, Furuya H, Kawarabayashi T, Shoji M, Checler F, Iwaki T, Makifuchi T, Takeda K, Kira JI, Tabira T (2004) Intracellular Abeta 42 activates p53 promoter: a pathway to neurodegeneration in Alzheimer's disease. *FASEB J*, 17.

230. Olsson A, Vanderstichele H, Anderson N, De Meyer G, Wallin A, Holmberg B, Rosengreen L, Vanmechelen E, Blenow K (2004) Simultaneous measurement of Abeta (1-42), total tau and phosphorylated tau (Thr 181) in cerebrospinal fluid by the xMAP technology. *Clin Chem*, 24.

231. Oltvai ZN, Milliman CL, Korsmeyer SJ (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*, 74, 609-619.

232. Pappolla M, Bozner P, Soto C, Shao H, Robakis NK, Zagorski M, Frangione B, Ghiso J (1998) Inhibition of Alzheimer beta-fibrillogenesis. *J Biol Chem*, 273, 7185-7188.

233. Pappolla MA, Chyan YJ, Poeggeller B, Bozner P, Ghiso J, LeDoux SP, Wilson GL (1999) Alzheimer beta protein mediated oxidative damage to mitochondrial DNA: Prevention by mealtonin. *J Pineal Res*, 27, 226-229.

234. Parihar MS, Hemnani T (2003) Phenolic antioxidants attenuate hippocampal neuronal cell damage against kainic acid induced neurotoxicity. *J Biosci*, 28, 21-128.

235. Parihar MS, Hemnani T (2004) Experimental excitoxicity provokes oxidative damage in mice brain and attenuation by exatract of Asparagus racemosus. *J Neural transm*, 111, 1-12.

236. Pericak-Vance MA, Bebout JL, Gaskell PC, Yamaoka LA, Hung WY, Alberts MJ, Walker AP, Bartlett RJ, Haynes CS, Welsh KA, Earl NL, Heyman A, Clark CM, Roses AD (1991) Linkage studies in familial Alzheimer's disease: evidence for chromosome 19 linkage. *Am J Hum Genet*, *48,* 1038-1050.

237. Perry G, Siedlak SL, Richey P, Kawai M, Cras P, Kalaria RN, Galloway PG, Scardina JM, Cordell B, Greenberg BD, et al. (1991) Association of heparan sulfate proteoglycan with the neurofibrillary tangles of Alzheimer's disease. *J Neurosci,* 11, 3679-3683.

238. Pike CJ, Burdick D, Walencewicz AJ, Glabe CG and Cotman CW (1991). *Brain Res*, 563, 311-314.

239. Pike CJ, Burdick D, Walencewicz AJ, Glabe CG, Cotman CW (1993) Neurodegeneration induced by beta-amyloid peptides in vitro: the role of peptide assembly state. *J Neurosci,* 13, 1676-87.

240. Pike CJ, Cummings BJ, Cotman CW (1992) β-amyloid induces neuritic dystrophy in vitro: similarities with Alzheimer pathology. *Neuroreport*, 3, 769–772.

241. Pike CJ, Walencewicz AJ, Glabe CG, Cotman CW (1991) Aggregation-related toxicity of synthetic beta-amyloid protein in hippocampal cultures. *Eur J Pharmacol*, 207, 367–368.

242. Pike CJ, Walencewicz-Wasserman AJ, Kosmoski J, Cribbs DH, Glabe CG, Cotman CW (1995) Structure–activity analyses of beta-amyloid peptides: contributions of the beta 25–35 region to aggregation and neurotoxicity. *J Neurochem,* 64 253–265.

243. Pohl FM, Jovin TM (1972) Salt-induced co-operative conformational change of a synthetic DNA: equilibrium and kinetic studies with poly (dG-dC). *J Mol Biol,* 67, 375–396.

244. Prasad KN, Hovland AR, Cole WC, Prasad KC, Naheini P, Edwards-Prasad J, Andretta CP (2000) Multiple antioxidants in the prevention and treatment of Alzheimer disease: analysis of biological rationale. *Clin Neuropharmacol*, 23, 2-13.

245. Pratico D, Uryu K, Leight S, Trojanoswdi JQ, Lee VM (2001) Increased lipid peroxidation precedes amyloid plaque formation in an animal model of Alzheimer amyloidoses. *J Neurosci,* 21, 4183-4187.

246. Rachakonda V, Pan TH, Le WD (2004) Biomarkers of neurodegenerative disorders: how good are they? *Cell Res*, 14, 347-358.

247. Rajan MT, Champion CSKD, Vishnuvardhan D, Rao KSJ, Viswamitra MA (1996) Interaction of Co, Mn, Mg and Al with d(GCGTACGC): a spectroscopic study. *Mol Biol Rep,* 22**,** 47–52.

248. Rao KSJ, Anitha S, Latha KS (2000) Aluminium induced neurodegeneration in hippocampus of aged rabbits mimics Alzheimer's disease. *Alz Rep,* 3**,** 83–88.

249. Rao KSJ, Divaker S (1993) Spectroscopic studies of Al-DNA interactions. *Bull Environ Contam Toxicol*, 50**,** 92–99.

250. Rao KSJ, Letada P, Haverstick DM, Herman MM, Savory J (1998) Modifications to the in situ TUNEL method for detection of apoptosis in paraffin-embedded tissue section. *Ann Clin Lab Sci,* 28**,** 131–137.

251. Rao KSJ, Rao BS, Vishnuvardhan D, Prasad KVS (1993) Alteration in superhelical state of DNA by aluminium. *Biochem Biophys Acta*, 1172**,** 17–20.

252. Rao KSJ, Rao RV, Shanmugavelu P, Menon RB (1999) Trace elemental inter-relationships in Alzheimer's brain: A new hypothesis. *Alz. Rep,* 2, 241–246.

253. Rapport SI (2002) Hydrogen magnetic resonance spectroscopy in Alzheimer's disease. *Lancet Neurol*,1, 82.

254. Rapport SI, Pettingrew KD, Schapiro MB (1991) Discordance and condcordance of dementia of the Alzheimrer's type (DAT) in monozygotic twin indicate heritable and sporadic forms of Alzheimer's disease. *Neurology*, 41, 1549-1553.

255. Ravid R, Swaab DF, Kamphorst W, Salehi A (1998) Brain banking in aging and dementia research—the Amsterdam experience, in *Progress in Alzheimer's and Parkinson's disease,* Fisher A, Yorshida M, Hanin,I, eds., Plenum Press, New York, 277–286.

256. Ravid R, Swaab DF, Van Zwieten EJ, Salehi A (1995) Controls are what makes a brain bank go round, in *Neuropathological Diagnostic Criteria for Brain Banking,Biomedical and Health Research,* Vol. 10, Cruz-Sanchez FF, Cuzner ML, Ravid R, eds., IOS Press, Amsterdam, The Netherlands, pp. 4–13.

257. Ravid R, Van Zwieten EJ, Swaab DF (1992) Brain banking and the human hypothalamus -factors to match for, pitfalls and potentials. *Prog Brain Res,* 93**,** 83–95.

258. Ravid R, Winblad B (1993) Brain banking in Alzheimer's disease: factors to match for, pitfalls and potentials, in *Alzheimer's Disease: Advances in Clinicaland Basic Research,* Corain B, Iqbal K, Nicolini M, Winblad B, Wisniewsky H, Zatta P, eds., John Wiley and Sons, NY, pp. 213–218.

259. Rebeck GW, Reiter JS, Strickland DK, Hyman BT (1993) Apolipoprotein E in sporadic Alzheimer's disease: allelic variation and receptor interactions. *Neuron*, 11, 575-80.

260. Reed JC (1995) Bcl-2: Prevention of apoptosis as a mechanism of drug resistance. *Hematol Oncol Clin North Am*, 9, 451-473.

261. Reich Z, Levin ZS, Gutman SB, Arad T, Minsky A (1994) Supercoiling-regulated liquid-crystalline packaging of topologically-constrained, nucleodsomefree DNA molecules. *Biochemistry,* 33**,** 14177–14184.

262. Reisberg B (1998) Functional assessment staging (FAST). *Psychopharmacol Bul,* 24, 653.

263. Reisberg B, Ferris SH, Del Leon MJ, Crook T (1982) The global deterioration scale for assessment of primary degenerative dementia. *Am J Psychol,* 139**,** 1136–1139.

264. Reiter RJ (1997) Oxidative process and antioxidation in Alzheimer's disease. *Free Radic Biol Med*, 9, 526-533.

265. Ricciarelli R, d'Abramo C, Massone S, Marinari U, Prozato M, Tabaton M (2004) Microanalysis in Alzheimer's disease and normal aging. *IUBMB Life*, 56, 349-54.

266. Rich A (1993) DNA comes in many forms*(Duplex; triple-stranded DNA; quadruplex DNA; Z-DNA; zuotin; syn and anti conformations; telomers). *Gene,* 135, 99–109.

267. Rogaev EI, Sherrington R, Rogaeva EA, Levesque G, Ikeda M, Liang Y, Chi H, Lin C, Holman K, Tsuda T, Mar L, Sorbi S, Nacmias B, Poacentini S, Amaducci L, Chumakov L, Cohen D, Lannfelt L, Fraser PE, Rommens JM, St Gerorge-Hyslop (1995) Familial Alzheimer's disease in kindreds with missense mutations in a gene on

chromosome 1 related to the Alzheimer's disease type 3 gene. *Nature*, 376, 775-778.

268. Rogaev EI, Sherrington R, Wu C, Levesque G, Liang Y, Rogaeva EA, Ikeda M, Holman K, Lin C, Lukiw WJ, deJong PJ, Fraser PE, Rommens JM, St George Hyslop P (1997) Analysis of the 5' sequence, genomic structure, and alternative splicing of the Presenilin-1 gene (PSEN1) associated with early onset Alzheimer's disease. *Genomics,* 40, 415–424.

269. Rogers J, Morrison JH (1985) Quantitative morphology and regional and laminar distributions of senile plaques in Alzheimer's disease. *J Neurosci,* 5, 2801-2808.

270. Rottkamp CA, Nunomura A, Hirai K, Sayre LM, Perry G, Smith MA (2000) Will antioxidants fulfill their expectations for the treatment of Alzheimer disease? *Mech Ageing Dev,* 116, 169-179.

271. Saenger W (1983) Principles of nucleic acid structure: Springer-Verlag. New York.

272. Salomon AR, Marcinowski KJ, Friedland RP, Zagorski MG (1996) Nicotine inhibit amyloid formation by the beta-peptide. *Biochemisitry*, 35, 13568-13578.

273. Saunders AM, Schmader K, Breitner JC, Benson MD, Brown WT, Goldfarb L, Goldgaber D, Manwaring MG, Szymanski MH, McCown N, Dole KC, Schmechel DE, Strittmatter WJ, Pericak-Vance MA, Roses AD (1993) Apolipoprotein E e4 allele distributions in late-onset Alzheimer`s disease and in other amyloid-forming diseases. *Lancet*, 342, 710-711,

274. Savory J, Rao KSJ, Huang Y, Letada PR, Herman MM (1999) Age related changes in Bcl-2 and Bax ratio, oxidative stress, redox active iron, and apoptosis associated with aluminium induced neurodegeneration: increased susceptibility with aging. *Neurotoxicology,* 20**,** 805–818.

275. Sayre LM, Perry G, Harris PLR, Liu Y, Schubert KA, Smith MA, (2000) In situ oxidative catalysis by neurofibrillary tangles and senile plaques in Alzheimer's disease: a central role for bound transition metals. *J Neurochem,* 74, 270–279.

276. Scatchard G (1949) The attraction of proteins for small molecules and ions. *Ann N Y Acad Sci*, 51, 660-672.

277. Schenk D (2003) Amyloid-β immunotherapy for Alzheimer's disease: The end of the beginning. *Nature Rev Neurosci,* 3, 824-828.

278. Schroth GP, Chou PJ, Ho PSJ (1992) Mapping Z-DNA in the human genome. Computeraided mapping reveals a nonrandom distribution

of potential Z-DNA-forming sequences in human genes. *J Biol Chem,* 267, 11846–11855.

279. Schuff N, Capizzano AA, Du AT (2002) Selective reduction of N-acetylaspartate in medial temporal and parietal lobes in AD. *Neurology*, 58, 928-935.

280. Schuurmans Stekhoven JH, Renkawek K, Otte-Holler I, Stols A (1990) Exogenous aluminum accumulates in the lysosomes of cultured rat cortical neurons. *Neurosci Lett,* 119, 71–74.

281. Seherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, Chi H, Lin C, Li G, Holman K, Tsuda T, Mar L, Foncin J.-F, Bruni AC, Montesi MP, Sorbi S, Rainero L, Pinessi L, Nee L, Chumakov L, Pollen D, Brookers A, Sanseau P, Polinsky RJ, Wasco W, Da Silva HAR, Haines JL, Pericak-Vance MA, Tanzi RE, Roses AD, Fraser PE, Rommens J, St George-Hyslop P (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature*, 375, 754-760.

282. Selkoe DJ (1989) Biochemistry of altered brain proteins in Alzheimer's disease. *Ann Rev Neurosci,* 12, 463-490.

283. Selkoe DJ (1991) The molecular pathology of Alzheimer's disease. *Neuron*, 6, 487-498.

284. Selkoe DJ (1996) Amyloid beta-protein and the genetics of Alzheimer's disease. *J Biol Chem,* 271**,** 18295–18298.

285. Selkoe DJ (1997) Alzheimer's disease: genotypes phenotypes and treatment. *Science*, 275, 630-631.

286. Selkoe DJ (2001) Alzheimer's disease: genes, proteins and therapy. *Physiol Rev*, 81, 741-766.

287. Selkoe DJ, Polisny MB, Joachim CL, Vickers EA, Lee G, Fritz LC, Oltersdorf T (1988) β-amyloid precursor protein of Alzheimer disease occurs as 110- to 135-kilodalton membrane-associated proteins in neural and nonneural tissues. *Proc Natl Acad Sci* (*USA),* 85, 7341-7345,

288. Shafer RH, Brown SC, Delbarre A, Wade D (1984) Binding of ethidium and bis(methidium) spermine to Z DNA by intercalation. *Nucleic Acids Res,* 12, 4679–4690.

289. Shin YA, Eichhorn GL (1984) Formation of psi(+) and psi (–) DNA. *Biopolymers,* 23**,** 325–335.

290. Shoji M, Golde TE, Ghiso J, Cheung TT, Estus S, Shaffer LM, Cai X.-D, Mc Kay DM, Tinter R, Frangione B, Younkin SG (1992) Production of the Alzheimer amyloid beta protein by normal proteolytic processing. *Science*, 258, 126-129.

291. Shoji M, Kawarabayashi T, Mastubara E, Ikeda M, Ishiguro K, Harigaya Y, Okamoto K (2000) Distibution of amyloid beta protein precursor in Alzheimer's disease brain. *Psychiatry Clin Neurosci*, 54, 45-54.

292. Sinden RR (1994) *Left-handed Z-DNA*. In: DNA Structure and Function. New York, Academic Press, p. 24.

293. Sisodia SS (1992) Secretion of the beta-amyloid precursor protein. *Ann N Y Acad Sci,* 674, 53–57.

294. Smale G, Nichols NR, Brady DR Finch CE, Horton WE (1995) Evidence for apoptotic cell in Alzheimer's disease. *Exp Neurol*, 133, 225-230.

295. Smith MA (1998) Alzheimer disease. *Int Rev Neurobiol*, 42, 1-54

296. Smith MA, Harris PLR, Sayre LM, Perry G (1997) Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. *Proc Natl Acad Sci (USA),* 94, 9866–9868.

297. Smith MA, Perry G, Richey PL (1996) Oxidative damage in Alzheimer's. *Nature*, 382, 120-121.

298. Smith MA, Perry G, Richey PL, Sayre LM, Anderson VE, Beal MF, Kowall N (1996) Oxidative damage in Alzheimer's. *Nature,* 382, 120–121.

299. Smith MA, Sayre LM, Monnier VM, Perry G (1995) Radical ageing in Alzheimer's disease. *Trends Neuosci*, 18, 172-176.

300. Smith MA, Richey PL, Taneda S, Kutty RK, Sayre LM, Monnier VM, Perry G (1994) Advanced Maillard re action products are associated with Alzheimer disease pathology. *Proc Natl Acad Sci (USA),* 91, 5710–5714.

301. Snowdon DA, Greiner LH, Mortimer JA, Riley KP, Greiner PA, Markesbury WR (1997) Brain infarction and the clinical expression of Alzheimer disease. The Nun study. *JAMA*, 277, 813-817.

302. Soyer-Gobillard M-O, Geraud M-L, Coulaud D, Barry M, Theveny. B, Revet B (1990) Location of B and Z-DNA in the chromosomes of a primitive eukaryote dinoflagellate. *J Cell Biol,* 111, 293–308.

303. Spina MB, Squinto SP, Miller J, Lindsay RM (1992) Human BDNF protects dopaminergic neurons against 6-OH and MPP^+ toxicity: involvement of the glutathione syatem. *J Neurochem,* 59, 99-105.

304. Stadelmann C, Bruck W, Bancher C, Jellinger K, Lassmann H (1998) Alzheimer's disease: DNA fragmentation indicates increased neuronal vulnerability, but not apoptosis. *J Neuropathol Exp Neurol,* 57,456-64.

305. Steenken S, Jovanovic SV, Candeias LP, Reynisson J (2001) Is "frank" DNA-strand breakage via the guanine radical thermodynamically and sterically possible? Chemistry. 7, 2829-33.

306. Stellar H (1995) Mechanisms and genes of cellular suicide. *Science,* 267, 1445-1449.

307. Strittmatter WJ, Roses AD (1995) Apolipoprotein E and Alzheimer disease. *Proc Natl Acad Sci (USA),* 92, 4725-4727.

308. Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS, Roses AD (1993) Apolipoprotein E:high avidity binding to b-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer's disease. *Proc Natl Acad Sci (USA),* 90, 1977-1981,

309. Su J, Satou T, Anderson A, Cotman C (1996) Upregulation of Bcl-2 is associated with neuronal damage. *Neuroreport*, 7, 437-440.

310. Su JH, Cummings BJ, Cotman CW (1994) Early phosphorylation of tau in Alzheimer's disease occurs at Ser 202 and is preferentially located within neurites. *Neuroreport*, 84, 265-272.

311. Sugaya K, Reeves M, McKinney M (1997) Topographic association between DNA fragmentation and Alzheimer's disease neuropathology in the hippocampus. *Neurochem Int,* 31, 275-281.

312. Suh D, Sheardy RD, Charles J B (1991) Unusual binding of Ethidium to a Deoxyoligonucleotide containing a B-Z junction. *Biochemistry,* 30, 8722–8726.

313. Syme CD, Nadal RC, Rigby SE, Viles JH (2004) Copper binding to the amyloid-beta (Abeta) peptide associated with Alzheimer's disease: folding, coordination, geometry, pH dependence, stoichiometry and affinity of Abeta (1-28): insights from a range of complementary spectroscopic techniques. *J Biol Chem*, 279, 18169-18277.

314. Szczepanik W, Swiatek M, Skala J, Jezowska-Bojczuk M (2004) ATP, histidine or magnesium ions can protect DNA against sisomicin-induced damage, following stray Cu(II) binding. *Archiv Biochem Biophy*, 431, 88-94.

315. Takeuchi H, Hanamura N, Harada I (1994) Structural specificity of peptides in Z-DNA formation and energetics of the peptide-induced B-Z transition of poly (dG-m5dC). *J Mol Biol,* 23, 6610–6617.

316. Tanzi RE, Kovacs DM, Kim TW, Moir RD, Guentte SY, Wasco W (1996) The gene defects responsible for familial Alzheimer's disease. *Neurobiol Dis*, 3, 159-168.

317. Tanzi RE, Vaula G, Romano DM, Mortilla M, Huang TL, Tupler RG, Wasco W, Hyman BT, Haines JL, Jenkins BJ, Kalaitsidaki M, Warren

AC, McInnis MC, Antonarakis SE, Karlinsky H, Percy ME, Connor L, Crowden J, Crapper-McLachlan DR, Gusella JF, St George-Hyslop PH (1992) Assessment of amyloid b-protein precursor gene mutations in a large set of familial and sporadic Alzheimer's disease cases. *Am J Hum Genet,* 51, 273-282.

318. Teller J.K, Russo C, DeBusk L.M, Angelini G, Zaccheo D, Dagna-Bricarelli F, Scartezzini P, Bertolini S, Mann DM, Jabaton M, Gambettip P (1996) Presence of soluble amyloid-beta peptide precedes amyloid plaque formation in Down's syndrome. *Nat Med,* 1, 93–95.

319. Teplow DB (1998) Structural and Kinetic features of amyloid beta-protein fibrillogenesis. *Amyloid,* 5, 121-142.

320. Terry RD (1994) Neuropathological changes in Alzheimer disease. *Prog Brain Res*, 101, 383-390.

321. Terry RD, Katzman R (1983) Senile dementia of the Alzheimer type. *Ann Neurol,* 14, 497-506.

322. Thomas TJ, Bloomfield VA (1985) Toroidal condensation of Z DNA and identification an intermediate in the B to Z transition of poly (dG-m5dC) X poly (dG-m5dC). *Biochemistry*, 24, 731-719.

323. Thomas TJ, Thomas T (1989) Direct evidence for the presence of left handed conformation in a supramolecular assembly of polypeptides. *Nucleic Acids Res,* 17**,** 3795–3810.

324. Trumbore CN, Myers YN, Hyde CK, Hudson RD, Rhodes CN, Masselink JK (1994) Metal-ion assisted, radiation-induced conversion of B- to Z-DNA in poly (dG-dC) and two natural DNA. *Int J Radiat Biol,* 66, 479–483.

325. Turnbull S, Tabner BJ, El-Agnaf OMA, Twyman LJ, Allsop D (2001) New evidence that the Alzheimer β-amyloid peptide does not spontaneously form free radicals: an ESR study using a series of spin-traps. *Free Radic Biol Med,* 30, 1154–1162.

326. Ugolini G, Cattaneo A, Novak M (1997) Co-localization of truncated tau and DNA fragmentation in Alzheimer's disease neurones. *Neuroreport,* 8, 3709-3712.

327. Van Duijn CM, Clayton D, Chandra V, Fratiglioni L, Graves AB, Heyman A, Jorm AF, Kokmen E, Kondo K, Mortimer J, Rocca WA, Shalat Sl, Soininen H, Hofman A (1991) for the Eurodem risk factors research group: Familial aggregation of Alzheimer's disease and related disorders: a collaborative re-analysis of case-control studies. *Int J Epidemiol* , 20(Suppl 2), S13-S20,

328. Van Hoesen GW, Augustinack JC, Dierking J, Redman SJ, Thangavel R (2000) The Parahippocampal Gyrus in Alzheimer's

disease: Clinical and Preclinical Neuroantomical Correlates. *Ann NYAcad Sci,* 911, 254-275.

329. van Leeuwen FW, Burbach JP, Hol EM (1998) RNA: a first example of molecular misreading in Alzheimer's disease. *Trends Neurosci,* 21, 331-335.

330. Vitek MP, Bhattacharya K, Glendening JM, Stopa E, Vlassara H, Bucala R, Manogue K, Cerami A (1994) Advanced glycation end products contribute to amyloidosis in Alzheimer's disease. *Proc Natl Acad Sci (USA),* 91, 4766-4770.

331. Walker GT, Stonyl P, MKrugh TR (1985) Ehidium Binding to Left-Handed (Z) DNAs results in regions of right-handed DNA at the intercalation site. *Biochemistry,* 24, 7462–7471.

332. Walsh DM, Hartley DM, Kusumoto Y, Fezoui Y, condron MM, Lomakin A, Benedek GB, Selkoe DJ, Teplow DB (1999) Amyloid beta-protein fibrillogenesis structure and biological activity of protofibrillar intermediates. *J Biol Chem,* 274, 25945-25952.

333. Walsh DM, Selkoe DJ (2004) Oligomers on the brain: the emerging role of soluble protein aggregates in neurodegeneration. *Protein Pept Lett*, 11, 213-228.

334. Wang A H, Quigley GJ, Kolpak FJ, Crawford JL, vanBoom JH, vander Marel G, Rich A (1979) Molecular structure of a left-handed double helical DNA fragment at atomic resolution. *Nature,* 282, 680–686.

335. Wang A H, Quigley GJ, Koplak FJ, vander Marel G, vanBoom JH, Rich A (1981) Left-handed double helical DNA: variations in the backbone conformation. *Science,* 211, 171–176.

336. Weisgraber KH, Mahley RW (1996) Human apolipoprotein E: the Alzheimer's disease connection. *FASEB J,* 10, 1485-94.

337. Wertin MA, Turner RS, Pleasure SJ, Golde TE, Younkin SG, Throjanowski JQ and Lee V-MY (1993) Human neurons derived from a teratocarcinoma cell line expressed solely the 695 amino acid amyloid precurosr protein and produce intracellular β-amyloid or A4 peptide. *Proc Natl Acad Sci (USA),* 90, 9513-9517.

338. Westermark P (1997) Classification of amyloid fibril proteins and their precursors: an ongoing discussion. *Amyloid*, 4, 216-218.

339. Wholrab F, Wells R D (1987) Enzymatic probes for left-handed Z-DNA. *Gene Amplif Anal,* 5, 247–256.

340. Wild-Bode C, Yamazaki T, Capell A, Leimer U, Steiner H, Ihara Y and Hass C (1994) Intracellular generation and accumulation of amyloid beta-peptide terminating at amino acid 42. *J Biol Chem*, 272, 16085-16088.

341. Wilson CA, Doms RW, Lee VM (1999), Intracellular APP processing and A beta production in Alzheimer disease. *J Neuropath Exp Neurol,* 58, 787-794.

342. Winblad, B, Wisniewsky H, Zatta P, eds., John Wiley and Sons, NY, pp. 213–218.

343. Wisniewski K, Wisniewski H, Wen G (1985) Occurrence of neuropathological changes and dementia of Alzheimer's disease in Down's syndrome. *Ann Neurol*, 17, 277-282.

344. Wolfe MS (2002) Therapeutic strategies for Alzheimer's disease. *Nature Rev Drug Discov,* 1, 859-866.

345. Xu H, Gouras GK, Greenfield JP, Vincent B, Naslund J, Mazzarelli L, Fried G, Jonanvic JN, seeger M, Relkin NR, Liao F, Checler F, Barbaum JD, Chait BT, Thinakaran G, Sisodia SS, Greengard P, Gandy S (1998) estrogen reduces neuronal generation of Alzheimer's β-amyloid peptides. *Nature Med,* 4, 447-451.

346. Yang Y, Geldmacher DS, Herrup K (2001) DNA replication precedes neuronal cell death in Alzheimer's Disease. *J Neurosci,* 21, 2661–2668.

347. Yang Y, Mufson EJ, Herrup K (2003) DNA replication precedes neuronal cell death in Alzheimer's Disease. *J Neurosci,* 23, 2557.

348. Yankner BA (1996) New clues to Alzheimer's disease: unraveling the roles of amyloid and tau. *Nat Med*, 2, 850-852.

349. Yankner BA, Duffey LK, Kirshner DA (1990), Neurotrphic and neurotoxic effects of amyloid β protein: reversal by tachykinin neuropeptides. *Science*, 250, 279-282.

350. Zuidam NJ, Barenholz Y, Minsky A (1999) Chiral DNApackaging in DNA-cationic liposome assemblies. *FEBS Lett,* 457**,** 419–422.

PUBLICATIONS

Jagannatha Rao KS, **Anitha S**, Latha KS (2000) Aluminium induced neurodegeneration in hippocampus of aged rabbits mimics Alzheimer's disease. ***Alz Rep***, 3(2), 83-88.

Anitha S, Reuven Stein, Jagannatha Rao KS., Latha KS and Viswamithra MA (2001) A link between apoptotic DNA damage and DNA topology in Alzheimer's disease brain: A hypothesis. ***Alz Rep***, 4(3), 121-131.

Latha KS, **Anitha S**, Liora Lindenboim, Reuven Stein, Jagannatha Rao KS, Viswamithra MA (2001) The Relevance of DNA Structural Dynamics to Neurological Disorders: A new thought. ***Biotech International***, 13(6), 20-24.

Latha KS, **Anitha S***,* Rao KSJ, Geetha Bali, Eswaran KRK (**2001**) Aluminium induced racemization of aspartate and glutamate in hippocampal region of aged rabbit brain: relevance to Alzheimer's disease. **Alz Rep**, 4, 197-204.

Latha K.S, **S.Anitha**, Rao K.S.J and Viswamitra M.A (**2002)** Molecular understanding of aluminium induced topological changes in $(CCG)_{12}$ triplet repeats: relevance to neurological disorders. **Biochem. Biophys. Acta**, 1588, 56-64.

Anitha S, Jagannatha KS, Latha KS and Viswamitra MA (**2002**) First evidence to show the topological change of DNA from B-DNA to Z-DNA conformation in the hippocampus of Alzheimer's brain**. J Neuromolecular Med**, 2, 287-295.

Anitha S, Jagannatha Rao KS (2002) The complexcity of Al-DNA interactions: Relevance to Alzheimer's and other neurological disorders. Struct Bond, 104, pages 79-98.

Hegde ML*, **Anitha S ***, Latha KS, Musthak MS, Stein R, Ravid R, Jagannatha Rao KS (2003) First evidence for helical transitions in supercoiled DNA by Amyloid β peptide (1-42) and Aluminium: A new insight in understanding Alzheimer's disease. ***J Mol Neurosci*****,** 22,67-79**. (* Shared First Authorship**).

Hegde ML, Anitha S,and Jagannatha KS (2004) Are Monomer-Oligomer Aggregates of Amyloidogenic Peptides Toxic Species in Neurodegeneration. ***Neuro Biol Aging*** (**Abstract**) P1-261, 25, S2, 170.

Veer Bala, **Anitha S**, Hegde ML**,** Zecca L, Garruto RM, Ravid R, Stein R, Shankar S, Shanmugavelu P, Rao KSJ (2005) Aluminum in Alzheimer's disease: Are we at a cross road. ***Cell Mol Life Sci****,* 62, 1-16.

CHAPTERS IN BOOKS:

1. Anitha S, Shanmugavelu P, Valeswara-Rao G, Shankar SK, Rani B. Menon, Rao RV, Jagannatha Rao KS, Zecca L (2002) Chapter 16: Molecular Understanding of Aluminum Bio-inorganic Chemistry in relevance to the Pathology of Alzheimer's Disease. In: ACS Symposium series on Group 13 Chemistry: From Fundamentals to Applications (Eds: Shapiro PJ, Atwood DA) Vol 822, pages: 228-245.

Rao KSJ, Rao RV, Rukmini RP, **Anitha S**, Zecca L (2004) Trace elemental homeostasis in human brain and its relevance to Alzheimer's disease, in: Toxicity of metals, (Oxford Pub)