STUDIES ON METAL-DNA INTERACTIONS INDUCED DNA CONFORMATIONS IN RELEVANCE TO NEUROLOGICAL DISORDERS

A Thesis submitted to the

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in

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by

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November 2006

... ...Dedicated to my

beloved parents,

my wife & daughter

DECLARATION

I hereby declare that the work reported in this thesis entitled "Studies on Metal –DNA interactions induced DNA conformations in relevance to neurological disorders" is entirely original and was carried out by me under the supervision of Dr. K.S. Jagannatha Rao, Scientist, Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore, India.

I further declare that the contents of this thesis have not been the basis for the award of any degree, diploma, fellowship, associateship or any other similar title of any University or Institute.

Mohammed Shafiul Mustak. B.

November ….., …….

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CERTIFICATE

This is to certify that the Ph.D thesis entitled **"Studies on Metal-DNA interaction induced DNA conformations in relevance to neurological disorders"** submitted by **Sri. Mohammed Shafiul Mustak** to the Mysore University for the award of the degree of **Doctor of Philosophy** in Biochemistry is the result of bona fide research work carried out by him in the Department of Biochemistry and Nutrition, C.F.T.R.I, Mysore under my guidance and direct supervision.

I further certify that this thesis or part thereof has not previously formed the basis for the award of any degree, diploma, and associateship of any other University or Institution.

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Mohammed Shafiul Mustak

CONTENTS

Publications

Abbreviations

A^0	**Anstrong**
AD	**Alzheimer's disease**
Al	**Aluminum**
ALS	**amylotrophic lateral sclerosis**
APOE	**Apolipoprotein, E**
APP	**amyloid precursor protein**
As	**Arsenic**
ATP	**adenosine tri phosphate**
Aβ peptide	**amyloid beta peptide**
BBB	**blood brain barrier**
bp	**base pair**
BPD	**Bipolar disorder**
Br	**Bromine**
Ca	**Calcium**
Cd	**Cadmium**
CD	**circular dichroism spectroscopy**
CNS	**central nervous system**
Co	**Cobalt**
Cr	**Chromium**
Cs	**Cesium**
CSF	**cerebrospinal fluid**
ctDNA	**calf thymus DNA**
Cu	**Copper**
dATP	**deoxy adenine triphosphate**
DCT 1	**divalent cation transporter**
dCTP	**deoxy cytosine triphosphate**
DFO	**desferrioxamine mesylate**
dGTP	**deoxy guanine triphosphate**
DSB	**double strand breaks**

dTTP	**deoxy thymine triphosphate**
EDTA	**Ethylenediamine tetra acetic acid**
EGCG	**Epigallocatechin gallate**
EM	**electron microscopy**
ER	**endoplasmic reticulum**
EtBr	**ethidium bromide**
Fe	**Iron**
FG	**Feralex G**
Fr	**Francium**
FTIR	**Fourier transformed infrared spectroscopy**
G	**Guanine**
Ga	**Gallium**
GSH	**glutathione**
H'-NMR	**Proton nuclear magnetic resonance**
H_2O_2	**hydrogen peroxide**
H_2SO_4	**sulphuric acid**
HEPES	**4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid**
Hg	**Mercury**
HPLC	**high performance liquid chromatography**
ICH	**intracerebral hemorrhage**
ICPAES	**inductively coupled plasma atomic emission spectrometer**
In	**Indium**
IRE	**Iron regulatory elements**
IRP	**Iron regulatory protein**
K	**Potassium**
LBs	**Lewy bodies**
Li	**Lithium**
LNs	**Lewy neuritis**
Maltolate	**3-hydroxy-2-methyl-4H-pyran-4-one**
MCI	**mild cognitive impairment**
MCT	**monocarboxylate transporter**

Mg	**Magnesium**
Mn	**Manganese**
Mo	**Molibdenum**
MQ	**millique**
MTP	**mitochondrial permeability transition pore**
N	**Nitrogen**
Na	**Sodium**
NADH	**Nicotanimide adenine dinucleotide**
NFTs	**neurofibrillary tangles**
Ni	**Nickel**
OH*	**hydroxyl radical**
P	**Phosphorous**
Pb	**Lead**
PD	**Parkinson's disease**
PET	**Positron emission tomography**
PHFs	**paired helical filaments**
PMI	**postmortem interval**
PP2A	**protein phosphorylase 2A**
ppb	**parts per billion**
ppm	**parts per million**
Rb	**Rubidium**
RNAse	**Ribonuclease A**
ROS	**reactive oxygen species**
rpm	**rotation per minute**
RT	**room temperature**
S	**Sulphur**
scDNA	**supercoiled DNA**
SDS	**sodium dodecyl sulphate**
SEM	**scanning electron microscope**
Si	**Silicon**
SOD	**superoxide dismutase**

SPs	**senile plaques**
Sr	**Strontium**
SSB	**single strand breaks**
TA	**Tris acetate**
TEM	**transmission electron microscope**
Tf	**transferring**
Tf-Fe	**diferric iron**
TfR	**transferrin receptor**
Tl	**Tallium**
t_m	**melting temperature**
TUNEL	**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling**
V	**Vanadium**
Zn	**Zinc**

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Title of thesis:

STUDIES ON METAL-DNA INTERACTION INDUCED DNA CONFORMATIONS IN RELEVANCE TO NEUROLOGICAL DISORDERS

Candidate: **Mohammed Shafiul Mustak. B**

Trace metal ions play a very important role in biological processes. Trace elements are sub divided into three main categories: i) Essential, like Fe, Cu, Se, Zn, Mn, Cr, Mo, Co and I; ii) Beneficial but not currently thought to be essential including F, V, Br, and Li ; iii) Toxic metals like Pb, Cd, Hg and Al. Trace metal homeostasis is essential for normal biological functions and any change in homeostasis leads to pathophysiology of cells.

Metal imbalances are reported to be one of the etiological factors of neurological diseases like Bipolar Disorders (BPD), Alzheimer's disease (AD) and Parkinson's disease (PD). Moreover, metals like Aluminium (Al) and iron (Fe) are found to be increased in specific regions of neurodegenerative brain of AD and PD. These two metals play a pivotal role in neurotoxicity and neurodegeneration. They cause neurofibrillary tangles formation (NFTs), amyloid beta and α-synuclein aggregation. Further, Al in conjunction with Fe promotes oxidative stress and apoptosis. Thus Fe, Al plays a significant role in neuronal cell death.

DNA dynamics is an essential parameter for normal neuronal cell function. Metal ions interact with DNA, might show significant changes in structure and function. Al and Fe are reported to cause cellular toxicity through their interaction with DNA. DNA damage causes genomic instability and cell death. Moreover nuclear localization of Al and Fe has been observed in AD and PD brain. Recent reports from our lab showed the

presence of Z-DNA in hippocampus of AD brain. It was also hypothesized that environmental factors like metals (Al, Fe), oxidative stress, and trace metal homeostasis imbalance might be responsible for the DNA topological changes seen in AD brain. However, there are no studies on Al, Fe in modulating DNA topology and stability and its relevance to neuronal cell death.

The present study is focused to understand the Al, Fe interaction with DNA, trace metal homeostasis in serum and brain samples of bipolar disorders, genomic DNA conformation and stability of bipolar depression brain.

The objectives of the present investigation are

1. To study trace metal homeostasis in serum and brain samples of bipolar disorder and to develop trace metals interrelation pattern.
2. To study Al-Fe-DNA interactions *in vitro* with reference to supercoiled DNA, linearized DNA and genomic DNA.
3. To study the conformation and stability in genomic DNA of bipolar depression brain.

The research work is carried towards achieving these objectives makes the subject matter of the thesis. The thesis is divided in to five chapters.

Chapter 1. General Introduction

This chapter begins with a general account of metals like Al and Fe in neurological diseases like AD, PD and BPD followed by an overview of current literature. The neurotoxic role of Al and Fe in AD and PD has been discussed in detail. The role of these metals in inducing the oxidative stress and apoptosis also discussed. This chapter highlights the importance of the DNA, supercoiled DNA, metal DNA interactions and gene environmental interaction in neurological diseases. The chapter also highlights the scope of metal chelation therapy and the focus of the present study. We also provided a hypothesis on the metal toxicity in neurodegeneration.

Chapter 2. Trace elements levels in serum and brain of BPD and control groups:

Bipolar disorder (BPD) or manic-depressive illness is one of the two major psychiatric disorders characterized by recurrent depressive and manic episodes. BPD affects about 1% of the population and causes severe neuropsychological impairments. The illness is implicated in functional impairment and represents an important risk factor for suicide. Pivotal biochemical alterations underlying neuropsychiatric disorders are unknown. There are limited reports on changes in trace elemental levels in the bipolar disorder. It has shown the elevation of Vanadium (V) and Molybdenum (Mo) in serum samples of bipolar mood disorder subjects. Also an increase in Al and Br levels in the serum samples of Li-treated patients has also been reported. Moreover, most of the available information is limited to few selected elements and there is no study, which examines inter-elemental relationships with regard to three different types of BPD.

The aim of this study is to examine the levels of eleven elements namely Na, K, S, P, Fe, Mn, Ca, Mg, Zn, Cu and Al in serum samples of bipolar patients type I, II, V and compare them with controls, and also to understand the element homeostasis through element-to-element interrelationship in bipolar disorder. The present study also analyzed few trace metals like Cu, Zn, Al and Fe in the hippocampus and frontal cortex region of BPD brain. Further, this study proposes a hypothesis explaining the possible relevance of trace elemental homeostatic imbalance in serum of BPD to possible effects on the brain.

The results showed a definite pattern of variation among certain elements in three types of BPD compared to control. The comparative account of trace elements between the control group and bipolar group patients' serum samples revealed the following trends: a) in the bipolar I patients, Na, K, P, Cu, Al and Mn are elevated significantly b) in bipolar II hypomania-Na, Mn, and Al are increased significantly, c) in bipolar II patients suffering from depression-Na, K, Cu, and Al are increased significantly and d) in bipolar V depressive patients Na, Mg, P, Cu and Al are increased significantly.

But elements namely S, Fe, and Zn levels are decreased significantly in all three bipolar types. Further, bipolar I (manic) have higher concentrations of Cu, Al, and Mn than bipolar II hypomania. The total elemental concentration (µmole/mL) is higher in the bipolar groups serum compared to the control group indicating possible imbalance in elemental homeostasis. The data also revealed a clear imbalance in the elemental interrelationship in all three types of BPD serum compared to control as shown by the direct and inverse correlations. These results suggest that a definite disturbance in the elemental homeostasis occurs during the development of BPD. In this perspective, the present study provided a comprehensive database on concentrations of 11 elements (majority of essential and few important toxic elements) in BPD serum in comparison with control groups. And also level of Al, Zn, Cu and Fe in the hippocampus and frontal cortex region of BPD are analyzed. The result showed that Al. Fe and Cu are higher in concentration in frontal cortex but not in hippocampus where as Zn is reduced in both the brain region of BPD. Further, we proposed a model that increased Al levels in serum of BPD is likely to alter the trace elemental homeostasis pool in the brain elemental homeostasis. We also suggested that irrespective of elements being primary risk factors or consequences of disease mechanism, a change in an individual metal ion will upset the elemental homeostasis pool resulting in a significant imbalance in elemental levels and charge distribution pattern in the biological system.

Chapter 3A. Aluminium-DNA interactions

Al is one of the strongly suspected etiological factors in neurodegenerative disorders like AD, PD and Guam Parkinsonism/amylotrophic lateral sclerosis. Recently, our lab for the first time, provided evidence for the presence of left-handed rigid Z-DNA in hippocampus region of severely affected AD brain. It has been hypothesized that one of the etiological factors like Al might be playing a role in right to left handed helical change associated with AD. Studies from our laboratory and others showed that Al

preferentially binds to DNA and also showed the strong binding to AT*-specific oligomers and induced conformational transition from Z to A in GC*-rich oligomers.

To investigate the above mentioned hypothesis experimentally, the present study is undertaken to examine the interaction of Al with supercoiled plasmid DNA (scDNA) and genomic calf thymus DNA (ctDNA). Plasmid scDNA as model system makes an interesting study, in view of the observation that vast arrays of small scDNA packets have been found in animal and human cells and known to be involved in gene expression and these superhelical packets are analogous to plasmid DNA supercoiling. Hence the results can be correlated to human brain genomic DNA to provide an insight into possible role of Al in the progression of AD pathology with reference to DNA topology.

We demonstrated in the present study, the toxic role of Al. Firstly, We showed that Al not only binds to DNA but also able to alter the conformation of the DNA. Secondly, Al also caused DNA nicking by breaking the double strand breaks and altered the stability of the DNA. It is observed that Al induced helical transitions from (B to A, B to C or BCA mixed) in scDNA. BCA mixed DNA conformation is energetically weak and is likely to go into Z-DNA conformation. Further, Al induced helical transition from B to Z in case of genomic ctDNA. This may be biological important as Z-DNA was observed in AD brain. The present study also showed that only Al is capable of inducing the DNA conformation, while other elements (group 13/IIIA) such as Ga, In and Tl are able to destabilize the DNA but could not induce any conformational change.

Chapter 3B. Iron-DNA interactions

Iron plays a specific role in the central nervous system (CNS). It is involved in myelin formation as well as in the synthesis of several neurotransmitters and in generation of GABAergic activity. Iron is needed as a cofactor for many enzymes that are involved in the normal function of neuronal tissue such as the non-haem iron enzyme ‘tyrosine hydroxylase’ which is essential for dopamine synthesis and as a cofactor for the

production of norepinephrine and serotonin. However, Fe overload has been implicated as one of the causes of neuronal death and can lead to vast range of disorders of the CNS. Abnormally high levels of Fe in specific brain regions related to PD and AD where neurons undergo degeneration have been reported.

Iron is a potentially toxic molecule because it contributes to the enhanced generation of Reactive Oxygen Species (ROS), and causes oxidative damage to biomolecules like DNA, proteins and lipids and thus suspected to play a major role in AD. The present study detected the high level of Fe in the frontal cortex of BPD depression brain. Further, a recent report has shown that Fe is localized in the chromatin region of the neurons in AD brain. We also hypothesized that metals like Al and Fe^{2+} might play important role in right-to-left DNA handedness change associated with AD

In this perspective, we tried to understand the effects of iron cations (Fe^{2+}, Fe^{3+} ions) on the conformation, stability and strand breaks on DNA by in vitro studies. Further, the possible effects of Fe^{2+} ions on linearized scDNA have been also examined. The experiment is not based on free radical generation but via Fenton reaction.

From our study, we observed the differential effect of Fe^{2+} and Fe^{3+}on the DNA conformation and its stability. The present finding showed that Fe^{2+} not only binds to scDNA but is also able to alter the conformation of DNA from normal B to C. Importantly the study showed that for the conformation of DNA to occur, Fe in the reduced state (II) is essential since Fe in the oxidized state (III) lacks such ability to alter the transition. Hence Fe^{3+} could not induce any structural change in DNA. A previous study from our lab showed that spermine could convert C-DNA to Ψ-DNA. It is known that Ψ-DNA, an ordered, twisted, tight-packing arrangement of the double helix is structurally and immunologically closely related to the left handed Z-DNA family. This indicates that DNA topological changes induced by Fe^{2+} ions could contribute to the DNA conformational changes as seen in AD brain.

The present study also analyzed the effect of Fe^{2+} ions on the linearized scDNA with a cohesive end. Interestingly, we observed that Fe^{2+} can circularize the sticky ends of the restricted fragments of the scDNA. This study therefore showed for the first time the role played by Fe^{2+} in the circularization of linearized scDNA. The chapter also discussed the possible mechanism of Fe induced DNA conformation and damage.

Chapter 4. DNA stability and damage in BPD brain

More recently remarkable progress has been made in identifying changes in the brain related to pathophysiology of BPD. One of the consequences of oxidative stress in BPD is apoptosis and/or necrosis and recent preliminary study showed that apoptosis is associated with etiology of BPD.

Moreover no studies to date have presented quantitative evidence for the presence of neurodegeneration in major psychiatric disorders. However, recent study evidenced decreased DNA fragmentation in BPD anterior cingulated cortex brain region. Further, DNA fragmentations have been shown to associate with neurodegenerative disorder like PD. More recently, reported the presence of DNA fragmentation in some region of PD brain. Further, it is shown that presence of an altered DNA conformation in hippocampus of AD brain. However such information on topology and stability of DNA is lacking in BPD depression brain and no study has been done on DNA fragmentation with respect to different region of BPD brains compared to control brains.

In this perspective, we studied the stability, integrity and topology of DNA isolated from ten clinically and postmortem psychiatrically confirmed BPD depressive cases and ten age-matched controls. Genomic DNA is isolated from ten regions in the human brain, namely parietal lobe, temporal lobe, occipital lobe, hippocampus, thalamus, cerebellum, hypothalamus, medulla, pons, and frontal cortex collected at post-mortem from cases of BPD and controls and are analyzed for single and double strand breaks in DNA, and their conformations and topology.

The results showed that DNA from thalamus, pons, medulla, temporal lobe, cerebellum and frontal cortex are damaged accumulating significantly higher number of single and double strand breaks compared to control subjects. However, the complex brain region hippocampus did not show any DNA fragmentation in both control and BPD brains. We have also showed classical apoptotic DNA laddering pattern in some regions of BPD brains like thalamus, pons, medulla and frontal cortex evidencing the presence of apoptotic cell death in BPD depressive patients in these brain regions. Circular Dichroism studies showed that DNA conformation is altered in frontal cortex, cerebellum, pons and parietal lobe of BPD. However, both control and BPD hippocampus DNA showed normal right handed helical B-DNA conformation. To our knowledge to date, this is the first study that showed the presence of apoptotic DNA fragmentation and genomic instability and conformation in region specific BPD brains compared to control.

The thesis ends with a comprehensive Summary and Conclusions of the present study.

In a nut shell the study provides the following significant contributions

1. The present study has generated a comprehensive data on the levels of 11 elements (both essential and toxic) in serum samples of three different types of BPD affected patients and showed that there is a definite disturbance in inter-element homeostasis pattern in three types of BPD serum. We also analyzed the four trace metal level in two human brain region of BPD and showed high concentrations of Al, Fe and Cu in frontal cortex and no change in Zn level. (Chapter 2).
2. We have provided evidence for the role of Aluminium and iron in the alteration of DNA topology, stability and DNA damage. Al induces a BCA –mixed and Z-DNA conformations in case of scDNA and ctDNA respectively; however, other group 13 metals could not induce any conformational change. It is interesting to observe that

Al has property of nicking the DNA by double strand breaks and damages the DNA. (Chapter 3A).

3. Iron in the form of Fe^{2+} could induce the conformational change from B-DNA to C-DNA in supercoiled and calf thymus DNA. However, the Fe^{3+} did not bring any conformational change to the normal DNA. We also observed that Fe^{2+} has ability to nick the DNA by single strand breaks and not by double strand breaks. The present study also observed that Fe^{2+} has ability to circularize the linearized scDNA (Chapter 3B). These have been very intriguing observations which exposed a new toxic role of Al and Fe. The observations lead to a new debate on whether direct Al and Fe induced DNA damage and conformation change in vitro has any role to play in DNA conformation observed in AD brain and also DNA fragmentation observed in BPD brain and hence open new avenues of research in this direction .
4. The study provided a new data on DNA stability; integrity and topology of genomic DNA isolated from ten different brain regions from BPD affected human brain samples. We also evidenced the presence of apoptosis some regions of brain of BPD.

The present study insights that the trace metal homeostasis is altered in the BPD serum and brain compare to respective controls. Also mapped the mechanism of Al, Fe induced DNA topological changes. Further, the DNA topology and stability is altered in BPD brain regions except hippocampus. The significance of the results is discussed in detail in the thesis.

Introduction

Metal ions play a vital role in various biological processes. They are critical in many of the enzymatic and metabolic reactions. Several metals such as Na, K, Mg, Ca and P occur in large concentrations in organisms and serve as structural components of tissue and constituents of body fluids [Mertz, 1981]. A second set of metals are known as 'trace elements' have been defined as those elements occurring in the human body but constituting <0.01% of the body weight [O'Connor, 2001].

Trace elements may be sub divided into three main categories: i) Essential, like Fe, Cu, Se, Zn, Mn, Cr, Mo, Co and I; ii) Beneficial but not currently thought to be essential including F, V, Br, and Li ; iii) Toxic metals like Pb, Cd, Hg and Al [Xiu, 1996; O'Connor, 2001]. The simplest definition of an essential element is that it is an element absolutely required for the maintenance of life. Its absence results in severe malfunction of the organism or death. Essential trace elements such as Fe, Zn, Cu, Mn, I, Cr, Se, Mo and Co are required at optimum level for metabolic function in human. They serve a variety of functions like catalytic, structural and regulatory activities in which they interact with macromolecules such as enzymes, DNA, pro-hormones, pre-secretory granules, and biological membranes [Aggett, 1985; Hediger, 1997].

However, there are several important considerations when dealing with trace elements, for instance at higher concentrations any trace element has the potential to be toxic whereas at low concentration it may also lead to many pathological consequences. Hence, optimum biological levels of trace elements are required for numerous metabolic and cellular functions in the human body. Zn, for example, which is the second most prevalent trace element in the body and most abundant transition metal in the brain

[Weiss *et al*., 2000; Koh, 2001], on excessive vesicular release is thought to play a key role in neuronal cell death following ischemia [Choi and Khol, 1998]. Fe is an essential component of electron transport proteins, oxygen carrying proteins, and many enzymes. If Fe is found in excess, it is highly toxic to the organism. For instance, free Fe in the presence of hydrogen peroxide, catalyses the production of hydroxyl radicals by the Fenton type reaction. These hydroxyl radicals are extremely reactive and are known to cause devastating damage to biomolecule including peroxidation of lipids, proteins and DNA strand breakages [Halliwell and Gutteridge, 1984]. Thus imbalances in the optimum level of trace elements such as Fe, Cu, Zn, and Al may adversely affect biological processes and are associated with many neurological diseases [Strong and Garruto, 1994; Garruto *et al*., 1993; Lai *et al*., 2002; Richardson, 2004; Zecca *et al*., 2004; Gotz *et al*., 2004].

The list of essential elements is expanding rapidly however its classification is summarized in Table 1. Elements like Al, Pd and Hg are not included in the classification of essential trace elements because they are not essential, rather they are associated with toxic effects to the cells of an organism [Connor, 2001].

Table 1. Classification of essential elements.

1. Bulk structural Elements: H, C, N, O, P, and S.
2. Macro elements: Na, K, Mg, Ca, Cl-, PO_4^{2-} , SO_4^{2-}
3. Trace elements: Fe, Zn, Cu, Mn, Mo, Co, Cr, V, Ni, Se, I, F,

A number of general chemical parameters which influence the selection of metal ions by living cells have been reported [Williams and De Silva, 1978]. These include charge-type, ionic radius, and donor atom of ligand, preferential co-ordination geometry,

kinetic controls and the chemical reactivity of metal ions in solution. Metals such as Fe and Cu have another property of readily changing their oxidation states so that they can also serve as catalytic electron carriers. They produce oxidized substrates which then can fit into a variety of metabolic cycles. In eukaryotes the utilization of oxygen inevitably involves the production of reactive toxic oxygen products, particularly O_2^- and H_2O_2. Thus for the survival of aerobic cells there must be a compliment of intra cellular enzymes, whose major activity is to convert O_2^- and H_2O_2 into non –toxic forms of oxygen, O_2, or H_2O a shown in equations (1.1 to 1.3).

$$2O_2^- + 2H^+ \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2 \qquad (1.1)$$

$$H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \qquad (1.2)$$

$$AH_2 + H_2O_2 \xrightarrow{\text{peroxidase}} 2H_2O + A \qquad (1.3)$$

1. 1. Trace Metals in Brain

Brain is a specialized organ that concentrates metal ions. Trace metals such as Zn, Mn and Fe are necessary for the growth and function of the brain. They usually serve as the function of metalloproteins in neurons and glial cells, while a portion of trace metals exists in the presynaptic vesicles and may be released with neurotransmitters [Takeda, 2004] and if found in excess contributes to toxic effect. Therefore, homeostasis of trace metals in the brain is important for the brain function and also prevention of brain diseases. In terms of total concentration, the brain has enough of these metal ions in its tissue to damage or dysregulate numerous proteins and metabolic systems. For example, the concentration of Zn^{2+} that is released during neurotransmission is 300μM, which is more than sufficient to be neurotoxic in neuronal cell culture [Frederickson, 1989]. Brain

must have efficient homeostatic mechanisms and buffers in place to prevent the abnormal discompartmentalization of metal ions. The blood brain barrier (BBB) is relatively impermeable to fluctuating levels of plasma metal ions. It is known that several of the metal regulatory transport systems are energy dependent, e.g. the Wilson's disease Cu-ATPase. Therefore, damage to the BBB or energy compromise in the brain, are two characteristics of several neurodegenerative disorders which could perturb metal levels and lead to deranged protein behavior and altered DNA structure and DNA damage. The present work is focused on Al and Fe toxicity and its relevance to neuropathological diseases.

1.1.1. Metals in normal brain

Substantial information is available on trace metal distribution in normal brains [Rajan *et al*., 1997; Zecca *et al*., 1994]. Concentration of Al is known to be high (58 - 196µg/g wet weight) and vary greatly in different brain regions of the normal human brain [Rajan *et al*., 1997]. Xu *et al*. (1992) reported that 6.2 to 9.8µg/g(dry mass basis) of Al are present in human brain. Evidences from clinical and animal model studies demonstrate that brain Al content increases with age, suggesting increased exposure with age or decreased ability to remove Al from the brain with age [Markesbery *et al*., 1981; 1984]. Similar to Al; the concentration of Fe in various regions of the normal brain varies greatly. Rajan *et al*. (1997) showed that concentration of Fe showed great variation in its levels in different regions of normal brain ranging from 16 – 59µg/g wet weight.

Rajan *et al*. (1997) reported that the distribution of trace elements was not similar in different regions of the brain and their concentrations varied significantly in different regions. They reported that K is more abundant in most of the regions followed by Na,

and P while Cd was found to be lowest. Based on the concentrations, they classified analyzed elements into four sets. First set of elements consisted of Na, K and P and their concentration ranged from 1.17 to 4.4mg/g. Second set of elements were Ca, Mg, and Al and their level ranged from 58 to 196µg/g. Fe, Zn, Si and Cu formed third set and their concentration varied from 3.5 to 59.5µg/g in all region of the brain. Fourth set consisted of As, Ni, Pd, Cd, Cr and their concentration were less and found to be within a limit of 0.015 – 2.64µg/g.

1.1.2. Metals in Alzheimer's disease brain

Alzheimer's disease (AD), the cause of one of the most common types of dementia, is a brain disorder affecting the central nervous system. It is a chronic progressive disease characterized by memory loss and deficits in one or more of the following cognitive domain: aphasia (language disturbance), agnosia (failure to recognize people or objects in presence of intact sensory function), apraxia (inability to perform motors acts in presence of intact motor system), or executive function (plan, organize, sequence action or form abstraction) [Brown *et al*., 2005]. Pathologically AD is characterized by the formation of two main protein aggregates: senile plaques and neurofibrillary tangles, which are involved in the process leading to progressive neuronal degeneration and death. Neurodegeneration in AD is a pathologic condition of cells rather than an accelerated way of aging. The aggregates namely neurofibrillary tangles (NFTs) and the senile plaques (SPs) have been implicated in the pathogenesis of AD [Maccioni *et al*., 2001]. The SPs are generated by a deposition of fibrils of the beta-amyloid peptide (Aβ), a fragment derived from the proteolytic processing of the amyloid precursor protein (APP) [Selkoe, 1994]. Tau protein is the major component of paired helical filaments (PHFs),

which form a compact filamentous network described as NFTs. Beside these AD brain has 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 brain.

Trace metal distribution in AD brain is available in literature [Rao *et al*., 1999a; 1999b]. Number of studies also showed that Al, Fe, Cu, Ca and Zn levels are altered in neurodegenerative disorders like AD [Kawahara, 2005; Maret, 2005; Ong and Farooqui, 2005; Huang *et al*., 2004; Perry *et al*., 2002]. Metals like Cu, Zn, Fe including Al found to be high level in the neocortical regions which is more prone to AD pathology [Hartter and Barnea, 1988a; 1988b] and also found to be high in cerebral Aβ deposits [Lovell *et al*., 1998; Suh *et al*., 2000]. Crapper *et al*. (1973) reported elevated Al (9.0 – 11.0µg/g dry weight) concentrations in the cortex, mesial and temporal cortex of the AD brain. It is interesting to note from the above studies that Al tends to accumulate more in the cortex and hippocampus which are known to be affected in AD brains [McDermott *et al.,* 1979]. The distribution of Al at the sub cellular level is more selective to lipofuscin, cytosolic, mitochondrial, lysosomal and nuclear compartments [Stekhoven *et al*., 1990]. Lukiw *et al.* (1991) have shown increased amounts of Al in chromatin and also detected a high concentration of Al (885.4µg/g DNA) in DNA isolated from the neuronal nuclei of AD.

The investigations from Good *et al*. (1992) and Bouras *et al*. (1997) reported that Al and Fe are in high concentration in AD brain. Another study by Deibel *et al*. (1996) showed that Cu level found to be significantly decreased whereas Fe and Zn were increased in regions of amygdala and hippocampus of AD brain region than normal brain region. The concentration of Fe in hippocampus and frontal cortex found 501 and 240 µmole/g respectively in AD brain which is higher than level detected in normal brain

[Rao *et al*., 1999a]. Cu, Zn, and Fe are essential for normal central nervous system (CNS) development and function. However, imbalances, either excess or deficiency, can result in neuronal apoptosis [Levenson, 2005].

1. 1. 3. Metals in Parkinson's disease brain

Parkinson's disease (PD) is the second most common neurodegenerative disease after AD affecting approximately 1% of the human population over age of 65 suffers from this disorder. PD is a slowly progressive neurodegenerative disease that affects the dopaminergic neurons in the substantia nigra, a small area of cells in the mid-brain. Gradual degeneration of these cells causes a reduction in dopamine production. This, in turn, can produce one or more of the clinical signs of PD: resting tremor; generalized slowness of movement (bradykinesia); stiffness of limbs (rigidity); gait or balance problems (postural dysfunction).The decline in cognitive function, affective disorders and depression are particularly common in PD patient [Weintraub and Stern, 2005].

Pathological features of PD include cytosolic filamentous inclusions known as Lewy bodies and Lewy neurites [Forno, 1996] in some surviving nigral dopaminergic neurons. The major fibrillar material of Lewy bodies (LBs) and Lewy neurites (LNs)was shown to be the presynaptic protein α-synuclein [Spillantini, 1997, 1998]. The exact etiology of PD is unknown, but study has shown that, except in extremely rare cases, there is no direct genetic basis for this disease [Tanner, 1989]. Other studies have implicated some environmental factors such as metals in the PD origin [Gorell *et al*., 1999a; 1999b].

The possible involvement of heavy metals in the etiology of PD follows primarily from the results of epidemiological studies reported in Michigan (1986-1988) [Rybicki *et*

al., 1993]. An another epidemiological study (1987-1989) of Valleyfield, southern Quebec, Canada, established that an increased risk for PD is associated with occupational exposure to the three metals Mn, Fe, and Al especially when the duration of exposure is longer than 30 years [Zayed *et al*., 1990]. Although a number of genetic polymorphisms are linked to PD [Checkoway *et al*., 1998] it is likely that the majority of cases of PD are not inherited but related to environmental factor [Tanner *et al*., 1999].

Trace metal distribution in PD brains are available [Dexter *et al*. 1991; 1989; Zecca *et al*., 1994]. Number of studies showed that Al, Fe, Cu, Ca and Zn levels are altered in PD [Kawahara, 2005; Maret, 2005; Ong and Farooqui, 2005; Huang *et al*., 2004; Perry et al., 2002]. The considerable increase in total Fe, Zn, and Al content were observed in substantia nigra of PD postmortem brain [Hirsch *et al*., 1991; Dexter *et al*., 1991; Riederer, 1989]. Moreover, analysis of Lewy bodies in the parkinsonian substantia nigra revealed high levels of Fe and the presence of Al [Hirsch *et al*., 1991; Dexter *et al*., 1987; 1989]. The study showed that Zn homeostasis which is important metal for brain normal function is altered in PD and AD and many neurological disease brains [Mocchegiani et *al*., 2005; Mekmouche *et al*., 2005]. Uitti *et al*., (1989) have shown that Fe and Zn concentrations are increased in the substantia nigra, lateral putamen and caudate nucleus of the PD brain.

1. 1. 4. Metals in Bipolar disorder

Bipolar disorder (BPD) previously termed as ‘manic-depressive illness’ or affective disorder is a relatively common, recurrent and chronic psychiatric disorder in which patients experience episodes of depression, usually with intervening periods of relative mood stability. The illness is characterized by episodes of mania, depression, or

mixed states (simultaneously occurring manic and depressive symptoms). The World Health Organisation 2002 report states that of all the neuropsychiatric conditions, BPD is the fourth cause of disability worldwide. Often beginning in adolescence or early adulthood, BPD has a profound negative effect on interpersonal, social, family and vocational outcomes and is a risk factor for substance abuse and suicide [Cassidy *et al*., 2001; Jamison, 2000; Maj *et al*., 2002].

BPD is one of the neuropsychiatric disorders and the exact cause of this disorder has not been elucidated, but there are likely to be multiple contributors to the pathoetiology of the disorder. But evidences suggest that imbalance in metal homeostasis and oxidative stress may play causative role in developing BPD [Srinivasan, 1984; Siwek *et al*., 2005a and 2005b; Frey *et al*., 2006a and 2006b]. Siwek *et al*. (2005a) reported that Mg levels were low and Cu levels were high in bipolar disorders and the alterations of Mg and Cu level were related to variety of neuropsychiatric disorders.

There are numerous studies linking free radical damage formed by Fe, Cu and Mn with neuropsychiatric illness [Lohr and Browning, 1995]. Yung, (1984) described about the neuropsychiatric aspects of alkali metal such as Li, Na, K, Rb, Cs and Fr deficiencies and excesses (intoxications). Na and K are two essential alkali metals whereas Li is used as therapeutic agent in BPD. Rb has been investigated for its antidepressant effect in a group of psychiatric disorders and Cs plays a role in carcinogenesis and depressive illness. A number of other studies have implicated several areas of the brain and have focused attention towards abnormalities in the intra-cellular processes of brain function, such as cell receptors and neurotransmitter effects. Most recently, studies have explored the possibility of neural degeneration as a potential

common final pathway in the disorder [Monkul *et al*., 2005; Brahmbilla *et al*., 2005].

There are limited studies on trace elemental levels in serum of three types of BPD (Type I, Type II and Type V) and moreover, there is no study on inter-elemental relationship with regards to three types of BPD compared to control groups. In the present study we propose to analyse the eleven elemental levels and their interrelationship in three types of BPD and compare them to control by using Inductively Coupled Plasma Atomic Emission Spectrometry (ICPAES).

1. 2. Neurotoxicity Studies

1. 2. 1. Role of Al

Al is the most prevalent and third most abundant metal in the earth crust. It is available to human through drinking water, Al vessels, Al foils used in food packing and higher levels of Al in food and beverages such as tea [Rao and Rao, 1995; Steel, 1911; Rao, 1994]. It is also present in higher quantity in certain drugs such as antacids and also common flocculent used in water treatment plants, open the possibilities of considerable contamination in drinking water. The uptake of Al across the GI tract and transport to cells are similar to Fe uptake and transport or alternatively the passage of Al between the gap junctions of the enterocytes, perhaps as Al citrate [Crichton *et al*., 2002]. Once Al reaches serum, it can be transported bound to transferrin (Tf) or also by albumin and low molecular ligands like citrate. However, the Tf – Al complex will be able to enter the cell via the Tf – transferrin receptor (TfR) pathway similar to Fe uptake to cells [Fig. 1]. But still exactly how Al could exit from the endosome into the cytosol remains unknown, since reduction step is once again required prior to transport across the endosomal membrane.

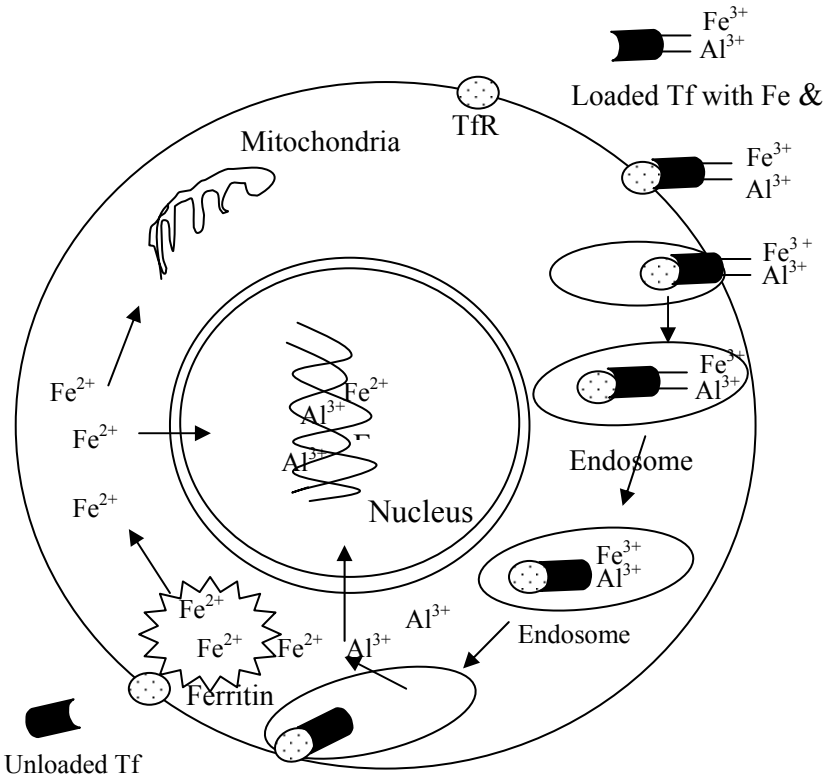


Figure. 1 Entry of Fe and Al into brain cells by transferrin (Tf) and transferrin receptor (TfR) pathway.

Brain Al homeostasis is achieved both by its transport out of the brain [Ackley and Yokel, 1998] and by its uptake into neurons and glial cells [Campbell *et al*., 1999]. Despite Al having low permeability at BBB [Birchall and Chappel, 1988] Al enters the brain by a mechanism which is not yet known. Three routes have been proposed by which Al could enter the brain from systemic circulation: BBB, nasal olfactory pathway and cerebrospinal fluid (CSF) [Yokel *et al*., 1999; Perl and Good, 1987]. More rapid exchange is possible through the BBB as many carriers of Al have been identified at the BBB. Tf mediated transport of Al has been suggested to be one of the mechanisms [Roskams and Connor, 1990]. Another important carrier for brain Al influx may be monocarboxylate transporter (MCT), a proton co-transporter which is located at both the luminal and abluminal surfaces of the BBB [Gerhart *et al*., 1997]. Other studies suggest

that some form of membrane defect may permit the excessive influx of Al to enter the brain [Perl and Good, 1987; Prasad, 1982].

Al has never been shown to have any definite biological function. Many scientific studies have brought to light the potential toxicity of Al in experimental animal models and in humans under different clinical conditions [Spafforth J, 1921; McLaughlin *et al*., 1962]. The pioneering studies on Al neurotoxicity in experimental animals were initially described in 1886 by Siem and Dollken [Dolken, 1897]. Most of the understanding of Al toxicity in humans was established as a result of studies of disorders experienced by dialysis patients when the dialysis fluid contained Al at or above 0.55μmole/L. In those patients Al accumulated in various tissues, including kidney, liver, bone and heart [Kerr and Ward, 1988; Seibert and Wells, 1929] giving rise to pathological conditions such as (i) dialysis encephalopathy that can lead to dementia and death and (ii) dialysis osteomalacic osteodystrophy.

1. 2. 2. Al and NFTs

NFTs are the most consistent post-mortem characteristics of AD, consisting of phosphorylated fibrillary proteins aggregated within the neuronal cytoplasm. They are composed of PHFs of tau proteins. Tau is bound by microtubules in healthy neurons and is essential to the form and function of neuronal cytoskeleton. It consists of a family of polypeptides of molecular mass in the range of 45 – 70kDa. It promotes assembly of microtubules and contributes to their stability, thus constituting a major component of the neuronal axoplasmic transport system. Tau contains multiple phosphorylation sites and in vivo its function can be controlled by phosphorylation and dephopshorelation reactions [Madhav *et al*., 1996].

Al is thought to be involved in the formation of PHFs and it is the constituent part of NFTs [Shin *et al*., 1994; Savory and Garruto, 1998]. Al-ATP, like glutamate, stimulates glutamate receptor activity and leads to an increased level of neuronal tau [Esclaire *et al*., 1997]. Stimulation by Al-ATP results in high concentration of tau that is not bound by microtubules, and self – assembly of free tau leads to formation of PHFs. So, it is clear that Al plays a role both in the abnormal expression of tau and in the intraneuronal deposition in NFTs. Studies also showed that Al exposure induces the hyperphosphorylation of tau [Guy *et al*., 1991], neurofilament protein in cultured human neuroblastoma cells [shea *et al*., 1995] and Al injected rabbits [Troncoso *et al*., 1986]. In synaptosome preparations, Al causes tau hyperphosphorylation by inhibiting the activity of protein phosphorelase 2A (PP2A) [Yamamoto *et al*., 1990], the main phophorylase in brain that normally removes phosphates from tau [Gong *et al*., 2000]. Similarly AD hyperphophorylated tau accumulates as a result of depressed PP2A synthesis and inhibition of PP2A activity [Gong *et al*., 2000; Vogelsberg - Ragaglia *et al*., 2001]. Furthermore, Al induces aggregation of PHF – tau by covalent binding. This has been shown by circular dichroism (CD) spectroscopy [Madhav *et al*., 1996]. Studies by Shin *et al*. (1994) showed that Al stabilizes the PHF – tau aggregates. Hence insolubility of partially purified PHF from AD tissue implies that the filaments in human neuron contain covalent bonds that cross link the individual filaments into rigid polymer [Selkoe *et al*., 1982]. This cross linking agent is likely Al given that it binds covalently to PHF-tau [Madhav *et al*., 1996] and it is present in human neurons and induces the aggregation of tau protein.

However, the increased content of Al in NFTs has been established [Perl *et al*., 1980; Lovell *et al*., 1993] to prove the association of Al toxicity in the evolution of Alzheimer's dementia. Al concentrations of 15-80ppm were detected in NFTs [Shin *et al*., 1994; Savory and Garruto, 1998; Esclaire *et al*., 1997; Good *et al*., 1992]. Although the neurotoxic effects of Al leading to NFTs in neurons is fairly well established, the etiological role in initiation and progression to dementing pathology, recaputulating the classical pathology of AD, is doubtful. Al hypothesis in AD came to light following discovery of Klatzo *et al*. (1965) and Terry and Pena, (1965) that injection of Al salts into rabbit brain lead to the formation of NFTs which appeared similar to the NFTs of AD at the optical microscopy level. However NFTs in rabbit are relatively less compact, rich in neurofilament protein and made up of 12nm straight filaments [Wisneiwski and Sofer, 1979], unlike the irreversible nature of NFTs, ultra structurally made up of PHFs and rich in phosphorylated tau and polymerized ubiquitin. NFTs in AD and Al-induced encephalopathies reflect an anomaly of neurofilament processing, inefficient degradation by protease, enhanced and aberrant phosphorylation and protein misfolding.

1. 2. 3. Al and Aβ/α-synuclein

Aβ, which is the major component of SPs in the brain of AD patients has intrinsic tendency to form insoluble aggregates. Aβ peptide (1-42) derived from a larger transmembrane protein, APP. Al, a non transition metal is also known to enhance the processing of APP. Studies of Clauberg and Joshi, (1993) showed that Al accumulated in AD brain accelerates the generation of Aβ due to the faulty proteolysis of normal APP. It has been shown that APP has a domain homologous to bovine pancreatic trypsin inhibitor and the activity of serine protease inhibitors are inhibited by Al. Thus Al is indirectly

involved in activating serine proteases such as α-chymotrypsin, enhancing the processing of APP and leading to accumulation of Aβ and plaque formation [Clauberg and Joshi, 1993].

A large number of studies have focused on the structure, aggregation properties and neurotoxicity of Aβ and their roles in AD [Barrow *et al*., 1992; Lorenzo and Yanker, 1994; Ikezu *et al*., 2003]. Al a constituent of SPs influences the aggregation and toxicity of Aβ [Tokutake *et al*., 1995; Evans and Harrington, 1998]. It has been supported by many studies like neurofibrillary lesions in experimental Al induced encephalomyelopathy and AD share immunoreactivity for APP, Aβ, α 1–chymotrypsin and ubiquitin –protein conjugates [Huang *et al*., 1997]. In addition, an increased burden of amyloid plaques was observed in patients with renal failure, which involves accumulation of Al in the brain [Duchen and Jacobs, 1984]. In physiological buffers , Ca, Co, Cu, Mn, Mg, Na, or K at 10mM concentration had no effect on the rate of Aβ aggregation but same concentrations of Al, Fe and Zn strongly promoted aggregation (a rate enhancement of 100 -1000fold) [Mantyth *et al*., 1993; Kawahara *et al*., 1994].

On the other hand, α-synuclein, a small (14 kDa), highly conserved presynaptic protein of unknown function was shown to be a major fibrillar component of LBs and LNs and implicated in the pathogenesis of PD [Spillantini et al., 1997, 1998; Jakes *et al*., 1994]. Structurally, purified α-synuclein belongs to the rapidly growing family of natively unfolded proteins, which have little or no ordered structure under physiological conditions [Uversky *et al*., 2001a; Eliezer *et al*., 2001]. Uversky *et al*. (2001b) studied the effect of Al on α-synuclein fibrillation and the effect of protein concentration on Al-induced folding, aggregation, and fibrillation of α-synuclein. They concluded that Al

induces structural transformations in natively un-folded α-synuclein leading to an increased propensity to aggregate and/or form fibrils. Al competes effectively with Na for the α-synuclein cation-binding sites supports the importance of Al toxicity.

1. 2. 4. Al and oxidative stress

Oxidative stress is one of the critical features in the pathogenesis of AD. Evidene of oxidative damage has been demonstrated in brain tissue from AD patients by Smith *et al*.(1996). Al in being, non-redox status still has a significant pro-oxidant activity in part because it is strong Lewis acid. There is evidence that Al complexes with the superoxide anion, forms Al superoxide anion, a more potent oxidant than superoxide anion on its own [Exley, 2004; Kong *et al*., 1992]. Further, Al is an activator of superoxide dismutase (SOD) and an inhibitor of catalase, which will result in increase in superoxide radical and readily converted to H_2O_2, further the breakdown of H_2O_2 to H_2O and O_2 by catalase is slowed down [Rao *et al*., 1999a]. Excess accumulation of H_2O_2 further leads to the production of OH* radicals which in turn damage various proteins, DNA and membrane lipids [Bondy and Kistein, 1996; Ferretti *et al*., 2003]. Gutteridge et al. (1985) reported that Al enhances membrane oxidative damage. At acidic pH, Al salts accelerate peroxidation of membrane lipids in the presence of Fe^{2+}. Also Al alters membranes in a way that promotes free radical initiated by Fe. Al binds avidly to negatively charged phosphate groups on membrane phospholipids [Deleers *et al*., 1985], causing fatty acids to cluster and pack into acidic micro domains that produce a local increase in membrane rigidity [Oteiza *et al*., 1993]. Under these conditions, Al enhances ferrous iron (Fe^{2+}) – initiated lipid peroxidation in a dose-and time dependent manner [Oteiza, 1994; Verstraeten *et al*., 1997], promoting the formation of H_2O_2 and OH* radicals that

contribute to an oxidizing environment.

Further, Al is considered one of the contributing factors to oxidative stress as it generates ROS through Fe [Gutteridge *et al*., 1985; Zatta *et al*., 2002]. ROS have been observed in rat glial and neuronal cells after treatment with Al. The level of ROS generated by neuronal predominant cells was 1.5 or 1.8 times higher than that of each of the untreated control cells. The ROS level in the neuronal predominant cells was 2.5 times higher than that of glial predominant cell [Mashiko *et al*., 1991]. In another separate study on rats, Al-induced pro-oxidative effects were demonstrated in liver and brain (cortex, hippocampus and cerebellum) tissues by examining oxidative stress markers- glutathione transferase, reduced glutathione, SOD, glutathione reductase, glutathione peroxidase [Esparza *et al*., 2003]. Hence both in vitro and in vivo studies indicate that Al is a definite promoter of oxidative stress.

Savory *et al*. (1999) proposed that aging is an important factor in the susceptibility of neurons to oxidative stress and to subsequent apoptosis. Aged rabbits treated with Al-maltolate exhibit intense intranueronal silver positivity indicative of the formation of oxidative stress. These changes occur in the CAI region of the hippocampus, as well as in the cerebral cortical areas and it was also found that apoptosis was co-localized with oxidative stress in the same neurons. In contrast to the aged rabbits, young animals treated similarly with Al exhibit much less oxidative stress with no apoptosis and negative Bax staining. These findings strongly support the key role of Al in oxidative damage in the process of neurodegeneration and increased vulnerability to Al-induced injury in the aged animals.

1. 2. 5. Al and apoptosis

Apoptosis is one of the mechanisms contributing to neuronal cell loss in AD [Cotman *et al*., 1996]. Neurons in susceptible regions (cortex and hippocampus) of the AD brain show evidence of DNA damage, nuclear apoptotic bodies and chromatin condensation. Ample studies have shown that Al induces cell death similar to that of AD [Savory *et al*., 1999; Rao *et al*., 2000; Ghribi *et al*., 2001; Savory *et al*., 2003]. Al leads to the alteration of the Bcl-2: Bax ratio in the brain (anti-apoptotic: proapoptotic)[Ghribi *et al*., 2001]. This alteration is an important indication in the development of extensive apoptosis. Apoptosis under mitochondrial control has been implicated in the neuronal death process and involves the release of cytochrome c into the cytoplasm and initiation of the apoptosis cascade. However a growing body of evidence suggests an active role for the endoplasmic reticulum (ER) in regulating apoptosis, either independent of mitochondria, or in concern with mitochondrial initiated pathway. A study on New Zealand White rabbits an animal system relevant to study of human neurodegenerative diseases in that it reflects many of the histological and biochemical changes associated with AD, has shown that Al induces neuronal apoptosis by its effect on the functioning of both the ER and mitochondria[Ghribi *et al*., 2001]. At the ER level, Al leads to the activation of caspase 12, which in turn can activate effector caspase-3. Al which is a stress-inducing agent in ER, has been shown to activate the expression of various genes, such as those coding for the transcription factors gadd 153, important in growth arrest and DNA damage induction and NF-kB, which initiates apoptosis. Stress in mitochondria leads to opening of the mitochondria permeability transition pore (MTP), release of cytochrome c, activation of caspase-9 and of caspase-3[Ghribi *et al*., 2001]. Stress in both

mitochondria and ER leads to down regulation of the anti-apoptotic proein Bcl-2, increase in the level of the pro-apoptotic Bax, and activation of the effector of apoptosis, caspase-3. Stress in the ER may also lead to the perturbation of the Ca^{2+} stores and incorrect folding of proteins, resulting in an increase in cytosolic Ca^{2+} concentration. Subsequent to this increase in cytosolic Ca^{2+} levels, mitochondrial Ca^{2+} is also observed when Al inhibits Na^{+}/Ca^{2+} exchange by accumulating in neurons following cell depolarization [Szutowicz *et al*., 1998]. Since mitochondria have limited capacity for storing Ca^{2+}, when this capacity exceeded, the excess of $Ca^{2+}$is released back into cytosol, a consequence of which is the opening of the MTP and release of cytochrome c- an apoptogenic factor. Cytochrome c complexes to Apaf-1 in cytoplasm and activate initiator caspase-9, which inturn activates the effector caspase-3 leading to apoptosis.

Therefore these proteins and proteases in the apoptotic pathway are directly or indirectly affected by Al [Savory *et al*., 2003]. Further, Su *et al*. (1997) evidenced apoptosis during neuronal DNA damage as an early event preceding NFT formation in AD. Subsequent to this study, Kitamura *et al*. (1998) provided evidence of alteration in the Bcl-2: Bax ratio, supporting the similar role played by Al in AD.

To summarize Al toxicity, it has a role in NFT formation, Aβ aggregation; it inhibits PP2A activity, resulting in the hyperphosphorylation of tau. Al's properties, in conjunction with Fe, promote an oxidizing environment. Al forms covalent bonds with phosphate groups on PHF – tau and in this oxidative environment, induces PHF-tau aggregates forming PHFs that are resistant to in vivo proteolysis. Al alters Bcl-2:Bax ratio inducing apoptosis. Thus Al is reported to play a multiple role in altering the normal physiological function of nervous system in AD.

1. 3. Role of iron in brain

Fe belongs to a group of elements known as transition metals. It is an essential element for the survival of almost all types of cells and organisms that live in an oxygen rich environment. The brain which only weighs 2% of the total body weight accounts for 25% of the oxygen consumption, making the brain the highest oxygen consuming organ in the body. Consequently, oxygen transport and associated metabolic activities are relatively high in the brain and place a high demand on Fe transport and utilization within the brain. Fe exist in two redox states , ferrous (Fe^{2+}) and ferric (Fe^{3+}) and it can readily convert from one to the other by either losing or gaining an electron. It is this property of Fe that makes it useful in all of those metabolic reactions that require a loss or gain of electrons. This same property however, makes Fe toxic by generating free radicals when it is present in excess and not protein bound. Genetic and biochemical manipulation of Fe have indicated that Fe has a pivotal role in neurotoxicity and neurodegenaration in animal models of neurodegenerative disease.

The metabolic processes take place in the brain at a relatively high level. Fe level is also require by oligodendrocytes the cell responsible for the myelin formation and its maintenance [Connor *et al*., 1990; Dwork *et al*., 1988; LeVine and Macklin, 1990]. The synthesis of neural transmitters is also Fe dependent. Fe is needed as a cofactor for the production of neurotransmitters dopamine, which is affected in PD patients while norepinephrine and serotonin which play a significant role in some of the mood disorders [Beard *et al*., 1993; Hill, 1985; Youdim, 1990]. Moreover the presence of HFE mutation in AD strongly supports the idea that Fe imbalances in the brain contributes to AD, and its prevalence indicates that it could be an important risk factor for AD [Moalem *et al*.,

2000]. The significance of the Fe for brain function is also reflected by the presence of receptors for Tf (Fe transport protein) on brain capillary endothelial cells.

The brain is unique among organs of the body with regard to Fe metabolism. First, it resides behind a vascular barrier i.e. BBB which limits its access to plasma Fe. There is a transport mechanism in the BBB that moves Fe across the endothelial cells and into the brain [Burdo and Connor, 2003]. However, little is known about the regulation of transport mechanism. Insight into this transport mechanism could be crucial for understanding how an excess of Fe accumulate in the brain of many neurodegenerative diseases.

Transferrin –transferrin receptor (Tf-TfR) pathway might be a major route of Fe transport to brain [Fig 1]. Evidence shows that most cells take up Fe from diferric transferrin (Tf-Fe_2) or transferrin bound Fe by the way of the TfR mediated endocytosis from blood into the cerebral endothelial cells and transferring - to- cell cycle [Bradbury, 1997; Andrews, 2000]. This process includes several steps: binding, endocytosis, acidification and dissociation. After binding and internalization of the Tf-Fe_2 –TfR complex, the Fe is released from transferrin (Tf) in an acidic, endosomal compartment, and is transported across the endosomal membrane into the cytosol by the divalent cation transporter DCT1 previously referred as Nramp2 [Fleming et al., 1997; Gunshin et al., 1997; Andrews,1999]. It either passes into the mitochondria, to supply Fe for haem and Fe-sulphur cluster biosynthesis, or is stored in the cytosolic Fe-storage protein ferritin accounting for 1/3 to 3/4 of brain Fe [Hallgren, 1958; Octave *et al*., 1983]. The Fe-free transferring (apotransferrin) is transported, bound to TfR, back to the plasma membrane, where it is released into the circulation to undergo further rounds of Fe mobilization and

delivery. Thus cellular Fe homeostasis in mammalian cells is maintained by the coordinated regulated expression of the TfR and the Fe storage protein ferrittin [Klausner, 1988]. The iron regulatory proteins (IRP1 and IRP2) are the central regulator of Fe homeostasis system. In most cells, changes in Fe status (Fe overload or depletion) lead to compensating changes in the IRP/iron regulatory elements (IRE) system of translational control of Fe homeostasis [DeRusso et al., 1995; Walden, 1989].IRPs thus represent a critical site in the pathway for maintaining cellular Fe homeostasis that could become dysfunctional in neurodegenerative disorder. In animal studies it is clearly established that the presence of Al in the brain will increase Fe content. It is supported by recent studies in murine erythroleukemia cell lines [Yamanaka *et al*., 1999; Ward *et al*., 2001] have indicated that IRP-2 may be stabilized by Al thereby preventing its degradation and resulting elevated transferring receptor biosynthesis and suppression or blocking of ferritin production.

In summary, brain is unique among organs because of its non-uniformity of Fe and Al distribution both regionally and cellularly and methods of uptake. It is well known that both elements have some similar properties. They are carried by the same serum proteins. Studies suggested that the Fe and Al carried by tranferrin may together with other factors modulate the absorption and cellular uptake of Al [Cannata *et al*., 1991a]. But in turn some studies claim that Al overload may reduce the gastrointestinal absorption and cellular intake of Fe [Cannata *et al*., 1991b] and needs further investigation in this direction.

1. 3. 1. Fe and NFTs

Similar to Al; Fe is also reported to accumulate in neurons with NFTs of AD brain [Yamamoto *et al*., 2002; Shin *et al*., 2003]. They also demonstrated that Fe in the oxidized state (III) is only capable to induce aggregation of soluble PHF tau leading to the formation of NFTs in AD brain. However, the resulting aggregate is less stable than the Al induced aggregate (Yamamoto *et al*., 2002).

1. 3. 2. Interrelation between Fe and Aβ/α-synuclein/neuromelanin

Fe is increased in the brain particularly in cells that are associated with neuritic plaques and has been investigated extensively [Levine *et al*., 2004]. Fe might have a direct impact on plaque formation through its effects on APP processing [Rogers *et al*., 2002]. Further more the ability of α-secretase to cleave APP can be modulated by Fe [Bodovitz *et al*., 1995]. Fe promotes both depositions of Aβ and oxidative stress, which is associated with plaques [Mantyh *et al*., 1993; Rottkamp *et al*., 2001], although some have argued that by binding Fe, Aβ might, infact protect the surrounding neurons from oxidative stress [Perry *et al*., 2002].

Fe contributes to the protein aggregation, including the aggregation of α-synuclein [Ostrerova-Golts *et al*., 2000; Uversky *et al*., 2001b]. The studies from Hashimoto *et al*. (1999) indicate that Fe catalysed oxidative reactions mediated by cytochrome c / hydrogen peroxide might be crucially involved in promoting α synuclein aggregation. In other study showed that in the presence Fe and free radical generators such as dopamine or hydrogen peroxide, BE-M17 human neuroblastoma cells that over express wild-type, A53T or A30P α-synuclein produce intra cellular aggregates that contain α-synuclein and ubiquitin [Ostrerova-Golts *et al*., 2000].

Large amounts of Fe are sequestered in neuromelanin granules in the dopaminergic neurons of the substantia nigra and the noradrenergic neurons of the locus coeruleus [Zecca *et al*., 1996]. Neuromelanin is complex molecule, the structure of which is arranged as a multilayer system, where each layer is polymer that is composed of melanic groups bound to aliphatic and peptide chains. This melanic group contains benzothiazine and dihydroxyindole units (ratio 1:3) and dihydroxyindole units are responsible for the strong chelating ability for Fe compared to other metals [Zecca *et al*., 1996; Wakamatzu *et al*., 2003]. Neuromelanin is synthesized by the oxidation of excess cytosolic catechols that are not accumulated in synaptic vesicle by vesicular monoamine transporter-2[Sulzer *et al*., 2000]. Neuromelanin binds Fe avidly, forming stable octahedral complexes that contain high-spin oxy-hydroxide Fe (III) clusters [Zecca *et al*., 2003]. It is also reported that synthetic dopamine-melanin and neuromelanin both bind Fe to a high degree and in saturation – dependent manner [Zecca *et al*., 2004].

An increase in redox-active Fe can also be associated with neuromelanin occurs in substantia nigra neurons in PD. This increase is higher in patients that have the most severe neuronal loss, and it is not found in the immediate vicinity of melanized neurons, providing further evidence for a central role for neuromelanin in modulating Fe reactivity [Faucheux *et al*., 2003]. Various methods like electron paramagnetic resonance spectroscopy and total reflection X-ray fluorescence spectroscopy studies [Zecca *et al*., 1996; Jellinger *et al*., 1992] have provided evidence for the accumulation of Fe in pigmented neurons – in particular, in the form of a neuromelanin–Fe complex [Gotz *et al*., 2004]. The neuromelanin-Fe complex activates microglia in vitro leading to the release of neurotoxic compounds such as tumour necrosis factor-α, interleukin-6, and

nitric oxide. In PD, although different toxic or genetic mechanism can initiate neuronal damage in the substatia nigra, the neuromelanin that is released by dying neurons can induce release of neurotoxic microglial factors, potentally leading to aggravation of neurodegeneration [Wilms *et al*., 2003].

1. 3. 3. Fe and oxidative stress

The accumulation of Fe in AD brain plays major role in oxidative damage in AD [Smith *et al*., 1997]. Fe is in the labile ionic pool (free form) can participate in the Fenton reaction with H_2O_2 to generate the reactive OH*. It is suggested that this reaction will not take place unless GSH peroxidase, the main enzyme that is responsible for breaking down the hydrogen peroxide in the brain is inactive [Bharath *et al*., 2002]. The study showed that reduction of GSH (the rate limiting co-factor of GSH peroxidase) which occurs in PD [Riederer *et al*., 1989] and is response to increase Fe accumulation and reactive hydroxyl radical generation might be taken up the evidence for the presence of free Fe. Indeed it is the free form of Fe that initiates depletion of cellular GSH [Bharath *et al*., 2002]. If cells are depleted of GSH the release of Fe is accompanied by oxidative stress. GSH depletion by antimetabolites does not induce ferritin bound Fe release or accumulation. Therefore, the fate of the cell in which Fe release and accumulation occurs seems to depend on intracellular GSH.

The mechanism by which neurotoxins increase Fe concentrations is not known, but it must be associated with an alteration in the brain Fe uptake process, as normal circumstances serum Fe does not cross the BBB. As we mentioned earlier the consequence of increased cellular Fe level is due to degradation of IRP2 by UBIQUITINS pathway [Mandel *et al*., 2004] or by Al [Yamanaka *et al*., 1999]. Recent

studies showed that neurotoxins kainite increases the expression of DMT1 in hippocampus of the mouse [Wang *et al*., 2003]. Interestingly, the substantia nigra of the PD brain is characterized by the significant increase in the ferritin and in parallel, significantly lower glutathione content is present [Riederer *et al*., 1989]. Finally, it was shown that unilateral injection of $FeCl_3$ into the substantia nigra of adult rats resulted in a substantial selective decrease of striatal dopamine (95%). As a result, dopamine-related behavioral responses were significantly impaired in the Fe-treated rats. This supports the assumption that Fe initiates dopaminergic neurodegeneration in PD [Youdim *et al*., 1991]. Fe contributes to the enhanced generation of ROS and increases oxidative stress and protein aggregation, including the aggregation of α-synuclein in PD [Ostrerva Golts *et al*., 2000; Uversky *et al*., 2001b].

1. 3. 4. Iron and apoptosis

The studies of Hashimoto *et al*. (1999) indicate that Fe-catalyzed oxidative reactions mediated by cytochrome c/H_2O_2 might be crucially invoved in promoting α-synuclein aggregation. Fe also promotes the Aβ aggregation and induce oxidative stress [Zecca *et al*., 2004]. Such aggregates disturb the cytosolic environment and intract with vesicles and their dopamine transporters and intraneuronal mitochondria, and these disturbances might result in activation of cell-death cascades. The Fe complex with neuromelanin activates microglia in vitro, leading to the release of neurotoxic compounds such as tumour necrosis factor-α, interleukin-6 and nitric oxide [Zecca *et al*., 2004]. Hence we hypothesis that increase level of Al and Fe might leads to oxidative DNA damage, apoptosis, DNA structural alteration and neuronal cell death [Fig. 2].

1. 3. 5. Chelation therapy of metals

The chelation therapy could be a viable neuroprotective approach for treating PD, AD and other neurological disorders that are associated with abnormal metals homeostasis such as Al and Fe in the brain [Barnham *et al*., 2004]. Limited in vitro and neuroprotective studies so far carried out seem to support chelation therapy because it has potential to prevent Fe induced ROS, oxidative stress and aggregation of α-synuclein and Aβ [Zecca *et al*., 2004]. The Fe and Al intoxication are amenable to treatment by in vivo chelation. For example, hemochromatos is the condition where high load of Fe, in such condition preferred treatment will be by Fe chelation with trivalent metal ion chelator desferrioxamine mesylate (DFO), only chelator tested extensively and approved by health authorities [Kruck and Burrow, 2002]. Studies of Kruck and Burrow, (2002) support DFO promoted Al and Fe chelation as valid treatment for the sporadic form of AD, unfortunately the use of DFO as a medicine has several draw back. First it is expensive to produce and poor penetration across BBB. Second, it can only be administered systemically, and long term therapy many patients develop noxious side effects [Kruck *et al*., 1990].

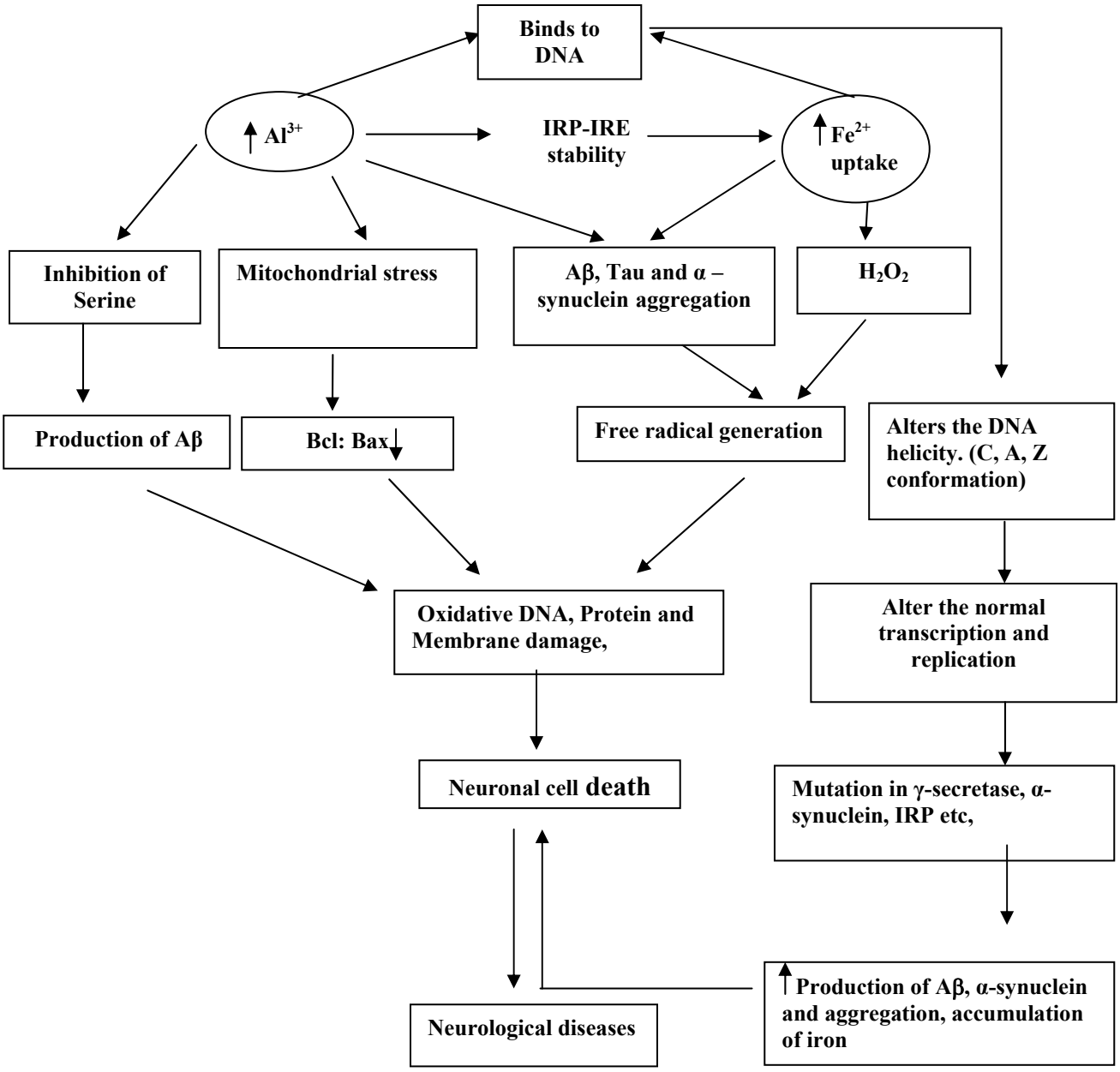


Figure 2. Consequence of Al and Fe toxicity in brain which might leads to neuronal cell death. Increased concentration of Al leads to stability of IRP –IRE iron regulatory protein there by accumulation of iron in the neuronal cell. They induce oxidative DNA, protein and lipid damage through generation of free radicals, Aβ and α-synuclein aggregation. We hypothesis that these metals can directly bind to DNA and alters the secondary structure of DNA similar to seen in AD hippocampus and impair the transcription and replication processes which result in neurological disease.

The amyloid cascade hypothesis states that the dementia of AD is caused in part by the toxic accumulation of Aβ in extracellular amyloid -containing neuritic plaques and as accumulates of neuronal cytoplasmic Aβ oligomers [Rosenberg, 2000]. On the basis of this hypothesis innovative strategy for reducing Aβ synthesis and accumulation are needed. Ritchie and colleagues [Richiie *et al*., 2003] present a novel strategy for reducing Aβ neurotoxicity by attenuation of Aβ-metal ion interactions. Studies showed that Aβ accumulation or aggregation toxicity is influenced by Zn, Cu, Fe, and Al [House *et al*., 2004]. They demonstrated these peptide aggregates caused by Al and Fe can be dissolved in the presence of both DFO and EDTA (Ethylenediamine tetra acetic acid). These metals are enriched in Aβ deposits and their removal results in the solubilization of Aβ. Cherny *et al*., (2001) reported that the antibiotic clioquinol which is Cu-Zn chelator increases the solubilization of Aβ in the AD plaque from postmortem human brain tissue, reduces H_2O_2 generation from Aβ and result in a significant decrease in brain Aβ deposition in a transgenic mouse model of AD.

Clioquinol is an 8-hydroxyquinoline derivative, and it has been suggested as a possible option for treatment of AD [Ibach *et al*., 2005]. As earlier mentioned the significant dyshomeostasis of Cu, Fe, and Zn has been detected and the mismanagement of these metals induces Aβ precipitation and neurotoxicity [Finefrock *et al*., 2003; Cherny *et al*., 2001]. Clioquinol can crosses BBB and has greater affinity for Zn and Cu ions that for Ca and Mg ions [Regland *et al*., 2001; Di vaira *et al*., 2004]. Thus Clioquinol represents a potential therapeutic route that may not only inhibit Aβ neurotoxicity, but may also reverse the accumulation of neocortical Aβ induced by chelating Cu and Zn ions. Unfortunately, however clioquinol is highly toxic.

Non-toxic lipophilic brain permeable Fe chelators offer potential therapeutic benefits for progressive neurodegenerative diseases. Compounds such as VK-28 [Youdim *et al*., 2004] and the bifunctional Fe chelators HLA-20 and M30, which possess the propargylamine monoamine oxidase inhibitory and neuroprotective moiety of the P-therapeutic drug N-propargyl-1-(R) aminoindan (Rasagiline, Teva Pharmaceutical Industries Ltd), offer a potential therapeutic solution to the neurotoxicity that is induced by divalent metal misregulation [Zecca *et al*., 2004]. Priliminary studies in animals have not shown any interference with neurotransmitter metabolism or nicotanimide adenine dinucleotide (NADH) oxidoreductase (complex I) activity, but the use of Fe chelators must be carefully controlled to avoid toxic effects [Dexter *et al*., 1999].

One possible non toxic approach for treating AD and PD with metal chelators could make use of the polyphenols EGCG and curcumin (a constituent of turmeric). Both compounds have anti oxidant, Fe chelating and anti-inflammatory activities. These are neuroprotective in animal models of PD and AD; also regulate the processing of APP through a non-amyloidogenic pathway [Mandel *et al*., 2004; Baum and Ng, 2004].

Nuclei appear to be one directed target for Al (III) mediated dysfunction due in part to their high content of phosphorelated nucleic acids, nucleotides and nucleoproteins. Al preferentially binds to within the nuclear compartment particularly heterochromatin [Crapper *et al*., 1980] and DNA phosphate and bases [Karlik *et al*., 1980]. Thus the design of chelation therapies dealing with the removal of Al and Fe from this genetic compartment therefore represents an attractive strategy to alleviate the development and /or progression of CNS dysfunction. Kruck *et al*. (2004) showed that combination of ascorbate+Feralex-G (FG) (2-deoxy -2-(N-carbamoylmethyl-[N'-

2'-methyl-3'-hydroxypyrid-4'-one])-D-glucopyronose) affective particularly removing Al from the nuclear matrix human brain cell nuclei and they also suggested that chelator carrying cis-hydroxy ketone groups such as FG are particularly suited to the removal of Al from the complex biological system. They also suggest mechanism whereby small chelating molecules may penetrate the nucleus, bind Al (III), diffuse to regions accessible by the larger FG molecules and transfer their Al (III) to FG. Another study by Shin *et al*.(2003) demonstrated that chemical chelator FG is capable of removing Al and Fe bound from hyper phosphorelated tau of neurofibrillary tangles of major constituents of the lesion of AD.Thus FG is potential clinical usefulness as a medicine in the Al/Fe chelation therapy. Studies by Kruck *et al*. (2002) showed that FG can able to chelate Fe/Al ions from biological samples.

1. 4. DNA

DNA, the primary genetic material of most organisms is usually visualized as double helix in which two chains of complimentary nucleotides (the sub units whose sequence constitute the genetic message) wind around a straight common axis. Double stranded DNA molecules have many different secondary structure termed A,B, C, Z, etc., The native conformation of DNA in solution is B-DNA right handed double helix, base pair stacking the 0.34nm apart, helix repeats every 10bp and its pitch is 3.4nm. B- form of DNA is very stable conformation and this is because of hydrogen bonds between complimentary purine and pyramidine, while polar atom in the sugar-phosphate back bone form charged phosphate group are all situated exterior surface of the helix and are free to interact with electrostatically with cations such as Mg^{2+} and base pairs hydrophobic and wander waals forces for stacking, contribute the stability of the DNA.

The inter wind chains create major groove and minor groove. Most molecular interaction between ligand and DNA occurs in major groove.

Natural DNA exhibits structural polymorphism. In addition to the various polymorphs of Watson-Crick double helices, a range of altered DNA structures encompassing left handed Z- helices, cruciform, heteronymous DNA, bent DNA, parallel DNA etc. have been shown to exist under defined conditions [Bramachari *et al*., 1989].

1. 4. 1. Supercoiled DNA.

Supercoiling is a wide spread phenomenon, characterizing many of the medically and biologically most interesting DNA's. DNA double helix can wind in space to form a new helix of higher order, in which case said to be supercoiled. It appears that a large proportion of known DNA's exhibit some form of supercoiling in at least one stage of their life cycle. An entire class of DNA tumor viruses, including the polyomas, the human papilloma (wart) viruses, contains such DNA, the DNA of mitochondria of human and other animal cells are supercoiled. Animal cells have also been found to contain a vast array of very small supercoiled DNA(scDNA) molecules whose function has not been determined. It is particularly striking that the majority of the known smaller genomes (set of genes) fall in this category, including genetic factor for fertility and drug resistance [Bauer *et al*., 1980]. An appreciation of this structural feature and its consequences is therefore essential to a complete understanding of the biology of DNA. Affecting DNA's size and shapes, supercoiling takes a variety of forms. For example, in the chromatin of higher organism the DNA wound around a core of protein to form a left handed solenoid super helix. Because of its biological importance, DNA supercoiling has often been studied with bacterial plasmids or other circular DNA molecules. The

understanding the mechanism of supercoiling and the consequence of this structural features of DNA is very important.

DNA supercoiling plays important role in the regulation of various cellular processes via specific and non specific DNA-protein interaction. Natural negative supercoilling often modulates the interactions and hence the efficiency /specificity/stability of the complexes [Cherny and Jovin, 2001]. The supercoiling of closed circular DNA is specified by the linking number difference, ΔLk, a measure of the deviation from the helical chain of relaxed double helix. The linking number, a topological variant is the sum of two parameters which describe the topology of DNA in terms of twist and writhe components [Vologodskii and Cozzarelli, 1994]. Twist and writhe are very sensitive to environmental conditions, such as temperature, and the nature and concentration of mono-and divalent cations.

Microscopy techniques, i.e. transmission electron microscopy (TEM) and scanning force microscopy (SFM), provide powerful means for the analysis of scDNA and its complexes with specifically and /non specifically bound ligands and their tertiary structure [Cherny and Jovin, 2001]. Other techniques like circular dichroism, agarose gel electrophoresis also used to study the secondary structure of scDNA. Tight supercoiling may have a great impact on many cell processes for instance, recombination, transcription, DNA compaction etc.[Bednar *et al*., 1994] and be very important in the mediation of non sequence specific interaction of small ligands with DNA such as polycations [Arscott *et al*., 1990].

1. 4. 2. Modulation in DNA helicity and damage in AD/PD

As we discussed above, the B-DNA double helix as inherently stable, but it is not. It is prone to spontaneous breakdown, attack by exogenous and endogenous agents and errors in replication. Among the damaging agents are free radicals or ROS arising during normal respiration, which cause strand breaks and oxidized bases. Extensive information is available on the role of point mutations, abnormal proteins (Aβ, presenilin, phosphorelated aggregated tau) and various environmental factors (Al, head injury etc.) are involved in AD (Rao *et al*., 1998; Abraham *et al*., 1990; Terry 1994). Oxidative DNA damage has long been considered a likely cause of neurological disorders, partly because of the abundance of its products, notably 8-oxoguanine, in human cell DNA. Also increasing evidence suggests that oxidative damage is associated with normal aging and several neurodegenerative diseases. Mild Cognitive Impairment (MCI), the phase between normal aging and early dementia, is a common problem in the elderly with many subjects going on to develop AD. Recently, Wang *et al*. (2006) quantified multiple oxidized bases in nuclear and mitochondrial DNA isolated from frontal, parietal and temporal lobes and cerebellum of short post-mortem interval autopsies of eight amnestic patients with MCI and six age-matched control subjects using gas chromatography/mass spectrometry with selective ion monitoring. They found statistically significant elevations ($p<0.05$) of 8-hydroxyguanine, a widely studied biomarker of DNA damage, in MCI nuclear DNA from frontal and temporal lobe and in mitochondrial DNA from the temporal lobe compared with age-matched control subjects and levels of 8-hydroxyadenine and 4,6-diamino-5-formamidopyrimidine were significantly elevated in nuclear DNA from all three neocortical regions in MCI. These results suggest that

oxidative damage to nuclear and mitochondrial DNA occurs in the earliest detectable phase of AD and may play a meaningful role in the pathogenesis of this disease [Wang *et al*., 2006].

DNA damage plays important role in genomic instability and cell death. Recently Lyras *et al*. (1997) showed level of oxidative damaged cytosine and guanine based namely 5-hydroxy cytosine and 8-hydroxy guanine was significantly higher in AD brain. It was postulated that Guanine base present in DNA might be more susceptible to OH* induced DNA damage with the base exposed in Z-DNA conformation. Recently, Nakamura, (2006) showed that two hallmarks of DNA oxidation i.e. 8-OHdG and apurinic/apyrimidinic abasic (AP) sites was in increased in the brain after intracerebral hemorrhage (ICH). These were analyzed by immunohistochemistry, PANT and TUNEL labeling and this study suggested that Fe-induced oxidation causes DNA damage in brain after ICH and that Fe is a therapeutic target for ICH.

Recent evidence implicates Cu^{2+} and Fe^{3+} in the overproduction of free radicals as a possible causal factor in the death of nigral cells associated to PD. Fe deposits selectively involving neuromelanin in substantia nigra neurons of PD patients have been found; in addition, contents of nigral Cu are decreased, while its concentration in CSF is raised. Increased oxidation markers and impaired mitochondrial electron transport mechanisms appear to be closely related to interactions between Fe and neuromelanin, which result in accumulation of Fe and a continuous production of cytotoxic species leading to neuronal death [Jellinger, 1999].

DNA damage plays very important role in the genomic instability and neuronal cell death. Studies have shown that DNA fragmentation occur through oxidative damage

[Cotman and Su, 1996; Sugaya *et al*., 1997], which leads to point mutations and changes in the integrity of chromatin structure [Crapper *et al*. 1979; Lukiw *et al*., 1989]. Studies on DNA damage in AD brain showed that the levels of oxidatively damaged cytosine and guanine bases, namely 5- hydroxycytosine and 8-hydroxyguanine were significantly higher in AD brain than in normal brain [Lyras *et al*., 1997]. It was theoretically postulated hat the guanine base present in DNA would be more susceptible to OH* induced DNA damage if the conformation of DNA is Z-rather than B or A because of greater exposure of bases [Michak *et al*., 1995]. Our previous finding on the presence of Z-DNA in AD brain supports the finding of the presence of GC* (gaunsine and cytosine) rich oxidized products in AD brain [Lyras *et al*., 1997].

Oxidative stress in PD could result from several mechanisms, such as generation of H_2O_2 by normal and abnormal metabolism of dopamine, increased amount of redox active metals ions, particularly Fe [Graham, 1984; Zecca *et al*., 2001]. Studies have shown that the level of nucleoside, 8- hydroxyl-2'-deoxyguanosine (8-OHdG), a product of free radical attack on DNA were generally increased and differentially distributed in PD brains [Alam *et al*., 1997]. Several studies have reported presence of DNA fragemtation in substatia nigra in PD [Kingsbury *et al*., 1998; Tatton *et al*., 1998; Tatton and Olanov, 1997]. Recent study of Hegde *et al*. (2006) showed that DNA from mid brain of PD had both single and double strand breaks. They also reported the state of conformation and topology of DNA in the PD brain regions. Earlier, our lab has shown an altered DNA conformation in hippocanmpus of AD brains [Anitha *et al*., 2002]. However similar information on topology, conformations, single strand and double strand breaks are lacking in BPD depression brain.

1. 4. 3. Metal - DNA interaction

The important role played by metals ions in nucleic acid processes has been well investigated during last two decades. It is well known that all the reactions in which nucleic acids generally participate in biological systems are mediated by metal ions. For instance the absolute requirement of divalent metal ions like Mg, Mn as cofactor for DNA polymerase has been long known. Earlier it was discovered that transition metal ions such as Fe and Cu were components of tobacco mosaic virus DNA and that they are strongly attached to complexing sites in the nucleic acid. Metal ions are known to be involved in the stabilization of DNA strands and in the catalytic activity of DNA polymerases [Marzilli *et al*., 1980]. Number of divalent cations like Cu, Zn, and Cd bring about complete renaturation of thermally denatured DNA.

There are several ways in which trace metals can interact with DNA. Nucleic acid contains large amounts of firmly bound metal ions. The binding of some metals may alter DNA polymerase activity, increasing the incorporation of genetic errors.For example, some elements are thought to interfere with the bindings of Mg, which is an essential for the interaction between DNA and DNA polymerase but some elements can substitute for Zn in Zn finger proteins, affecting their function [Kasprzak, 1995; Sagripanto, 1999].

The important factor which underlies the interactions of metal with DNA is their selective coordination. DNA presents a large number of potentially reactive sites or metal binding sites: oxygen atoms of the phosphodiester linkages, the heterocyclic ring nitrogen atoms, and the exocyclic carbonyl groups of the purine and pyrimidine bases. Early investigations on the metal binding sites on nucleotides and polynucleotides were mostly carried out by spectral methods and the measurement of physical properties like

viscosity, melting point, hyperchromicity. The melting point (t_m) or the mean temperature of transition of double helical form of nucleic acids into random disordered structure was taken as measure of stability of the molecules. An increase in t_m has been attributed to stabilization the double helical structure of DNA by metal binding to phosphate. A decrease in t_m is considered as being due to the metal binding to the electron donor groups on the bases which would disrupt the hydrogen bonds holding the bases together resulting destabilization of the double helical structure of DNA. It was shown that metal binding to phosphates stabilize the DNA helix, while those binding to base can unwind and rewind the double helix in a reversible manner resembling the steps in the biological replication and transcription of DNA [Eichhorn, 1962; 1965]. Eichhorn, (1965) also demonstrated that different metals have different influence on the conformation of DNA. This fact is dramatically illustrated by the opposite effects of Mg^{2+} and Cu^{2+} ions on the melting curves of DNA. Mg^{2+} ions increase t_m as expected for a metal ion that stabilizes the double helix; more energy is required to destroy the helix in the presence of the stabilizing Mg^{2+}. Cu^{2+} ions on the other hand, decrease the t_m of the DNA. Such a decrease indicates that less energy is required to 'melt' the DNA double helix and that the Cu^{2+} ions therefore destabilize the DNA. The reason for the difference in behavior of the two metal ions with respect to DNA can be explained by difference in binding tendency. Mg^{2+} binds to phosphate, stabilizing the ordered structure by counter ion effect. Cu^{2+} ions bind not only to phosphate; but to base as well and binding to base competes with the hydrogen-bonding of the double helix and therefore helps to destroy it. Other studies showed that lower concentration of Al enhanced the t_m of the DNA but higher concentration did not alter the t_m of the DNA [Champion *et al*., 1998]. Rajan *et al*. (1997)

reported that Al did not alter the t_m of the DNA sequence d(GCGTACGC) at both lower and higher metal/DNA ratios. Duguid *et al*., (1995) also studied the effect of divalent metals namely Sr, Mg, Ca, Mn, Co, Ni and Cd on thermal denature of DNA. But there are no studies on effect of Fe on thermal denature of DNA.

Transition metals such as Mn and Co at higher concentration have been found to be mutagenic and carcinogenic and change handedness of their DNA [Richardson *et al*., 1981; Beckman *et al*., 1985; Champion *et al*., 1998]. Metal Al is reported to be associated with neurological disorders [Crapper *et al*., 1976] and causes cellular toxicity through its interaction with DNA in cells [Crapper *et al*., 1980; Rao *et al*., 1993]. Few data are available on the effect of metal ions on the melting behavior of genomic DNA, poly (A-T) and poly (G-C) oligomers [Karlik *et al*., 1989; Eichhorn and Shin, 1968]. Recently studies showed that metals like Cu can cleave the double strand DNA in presene of H_2O_2 [Biver *et al*., 2006; An *et al*., 2006]. Pedreno *et al*.(2005) reported that polyamines such as spermine under certain condition facilitate the interaction of Cu and Fe with DNA leading to the formation of ROS in close proximity to DNA.

Upon interaction with metal ions, the DNA molecule shows significant changes in structure, conformation. However many of the lesions that have been observed on exposure of DNA to trace elements such as DNA strand breaks, cross linking and base modification by some indirect means, such as occurrence of Fenton - type reactions. Recently, it has been reported the presence of left handed Z-DNA in the hippocampus region of severely affected AD brain [Anitha et al., 2002]. The topology change in DNA particularly the B to Z transition will have tremendous implications in the functional biology of the brain cells causing abnormality in transcription, replication, and

recombination [Van Berkum *et al*., 1986]. It was hypothesized that environmental factors such as Al, Fe, oxidative stress, and trace metal homeostasis imbalance might be few causes for the DNA topological change in AD brain [Anitha *et al*., 2002]. Moreover the nuclear localization of Al and Fe has been observed in AD brain [Crapper *et al*., 1980, Perl and Brody, 1980; Smith *et al*., 1997; Meneghini, 1997]. We suggest that accumulation of metal ions such as Fe, Al and their co localization in the nuclear region might be responsible for bringing about transition in DNA conformation. The recent finding of nuclear localization of Fe and Al in the neurons of AD brain offers a new prospective for further study of interaction of these metals with DNA in relevance to DNA helicity.

The binding of metal ions to DNA is greatly influenced by the physicochemical properties of metal ions as well as the base sequence of the DNA. There are many studies in the literature demonstrating Fe induced DNA damage through Fenton reaction [Cragg *et al*., 1998]. Studies showed that Cu ions can change the conformation of B-DNA to A–DNA or Z-form [Kornilova *et al*., 1994; Andrushchenko *et al*., 2003]. The effect of metals like Mg, Ca, Cu and Pb on calf thymus DNA (ctDNA) in aqueous solution are studied with relevance to cation binding mode, DNA condensation, and DNA denaturation as well as DNA conformation features [Tajmir-Riahi *et al*., 1993; Ahmad *et al*., 2003]. Recently Quameur et al. (2005) reported on the comparative study on binding of Fe^{2+} and Fe^{3+} on the major and minor groove of the DNA. However, there are limited studies on Al and Fe induced DNA conformation and its effects on DNA stability and DNA damage.

The third chapter provides the Al and Fe interactions with DNA and their conformation relevance to AD.

1. 4. 4. Gene – Environmental interaction in neurodegenerative disease

It is now considered that AD and PD are possibly result of a multifactorial process involving genetic and environmental components [Heininger, 2000; Grant *et al*., 2002; Zatta *et al*., 2003; Tanner *et al*., 1999; Checkoway *et al*., 1998; Spillantini, 1998]. It has been thought that a genetic susceptibility to Al loading and clearance may increase one's risk not only to AD but also to developmental problems associated with Al loading in infants and children, even those with normal renal function [Golub and Domingo, 1997]. Recent progress in genetic analysis has made it possible to identify three genes that cause familial AD, which are the genes for APP on chromosome 21, presenilin 1 on chromosome 14 and presenilin 2 on chromosome I [Gupta *et al*., 2005]. In addition, the ε4 allele of the ApoE gene present on chromosome 19 is found to be an important risk factor for senile and presenile AD. The genetic components account for only a part of AD cases [Van Broeckhoven, 1995] suggesting that interaction of environmental risk factors and genes could be a possible hallmark in the etiology of AD. Gauthier *et al*. (2000) reported a significant association between water Al exposure and the development of AD. Recently a genetic variant of transferrin, namely TfC2, was identified which may be involved in the aberrant transport of Al and found high frequency in patients with AD compared with non-demented control [Van Landeghem *et al*., 1998]. It has also been shown that early onset of AD is observed in patients with both ApoE4 and TfC2 alleles [Van Rensburg *et al*., 2000]. Studies also reported that Al uptake occurs more in neurons of cortex and hippocampus, which are rich in Tf receptors [Gupta *et al*., 2005]. Further

work in this direction is needed to validate the Al-ApoE4-TfC2 interrelationships. Fosmire *et al*. (1993) suggested that there are genetic differences in the permeability of the BBB and differential loading and toxicity of Al. Matsuzaki *et al*. (2004) reported that chronic Al exposure has accelerated PS2V(the aberrant splicing isoform generated by skipping exon 5 of thr PS-2 gene) production through hypoxia mechanism and also reported HMGA Ia, a mediator of PS2V production induced by Al as well as by hypoxia. These studies provide an insight into the cross-talk between Al, ApoE4, PS2V and TfC2, showing that Al has a potential role in familial AD as well as an independent part to play in sporadic AD.

1. 5. THE FOCUS OF INVESTIGATION

From above literature it is evident that trace elemental imbalances are involved in etiology of neurological diseases like AD, PD and BPD. Till now studies were concentrated on single elements and no studies related to inter-elemental relations and their correlations in BPD. The present study we have emphasized on the analysis of 11 elemental levels and inter elemental relationship in the serum of three types of bipolar disorders compared to control. We also assessed level of Al, Cu, Zn and Fe in the different region of postmortem brain samples of bipolar depressed patients by using ICPAES. The present findings on the trace elemental analysis in three types of BPD and its significant results are discussed in chapter two. We hypothesized that increase in metals like Al, Cu in serum can induce an imbalance in trace elemental homeostasis level of BPD. Accumulation of Al in the brain would result in neurotoxic effect and may lead to neurodegeneration.

Numerous reports are based on the two generic reactions of relevance to neurodegenerative disease.

1. Metal + Protein ⟶ Protein aggregation.

2. Metal catalysed protein oxidation leading to protein damage and denaturation.

But our focus is on

1. Metal + DNA ⟶ alter the conformation, damage and stability of the DNA double helix.

Here we emphasize on metals like Al and Fe because they are found deposited in AD brain and also co-localised in DNA of AD brain. Having revealed the presence of left handed Z- DNA in the hippocampus of AD brain through previous studies, we hypothesized that Al or Fe in AD brain cells may be the reason for the helicity change of the DNA. The metal DNA interaction studies were well established with regard to metal induced free radical damage to DNA. However, there are few studies where DNA conformation change, damage and stabilty by Al and Fe metal is highlighted. Hence, we have analysed interaction of Al and Fe with super coiled DNA and linear DNA with various techniques to support our hypothesis. Fe^{2+} could change the supercoiled DNA from B to C conformation, while Fe^{3+} could show strong binding only. The most significant evidence was that Fe^{2+} could circularize the linearised DNA and is revealed by electron microscopic studies. Further Al was found to induce linear form from scDNA. The observation and their significance to neurodegenerative disorder is presented in chapter III A and IIIB of this thesis.

To observe conformational change, stability and damage of genomic DNA in different regions of postmortem human brain bipolar depression and control brain

regions. Recently, apoptosis has been reported to be one of the etiological factors for BPD. There are no reports on DNA damage and its stability regards to secondary structure of genomic DNA in BPD-depression human brain. Hence we isolated genomic DNA from ten human brain region of the BPD-depressed (N=10) and compared with age matched control brains and found that few brain regions are affected and showed apoptotic DNA fragmentation which was hither to unknown. The findings and their significance are discussed in chapter four. These new and interesting results are likely to provide a new dimension in understanding the metal toxicity in neurodegeneration

1. 5. 1. Objectives

1. To study trace elemental levels in serum and brain samples of bipolar disorder and to develop trace metals interrelation pattern.
2. To study Al-Fe-DNA interactions *in vitro* with reference to supercoiled DNA, linearized DNA and genomic DNA.
3. To study the conformation and stability in genomic DNA of bipolar depression brain.

1. 6. References

Abraham CR, Bem Meir A, Tempst P. Facile and sensitive assay for monitoring proteolytic activities with defined specifities: studies on amyloid beta-proteinprocessing in Alzheimer disease. Pept Res. 1990; 3:211-215.

Ackley DC, Yokel RA. Alu minum transport out of brain extra cellular fluid is proton dependent and inhibited by mersalyl acid, suggesting mediation by the monocarboxilate transporter (MCTI). Toxicology. 1998; 127: 59-67.

Aggett PJ. Physiology and metabolism of essential trace elements: an outline. Clin Endocrinol Metab. 1985; 14: 513 – 543.

Ahmed R, Arakawa H, Tajmir-Riahi HA. A comparative study of DNA complexation with Mg(II) and Ca(II) in aqous solution: major and minor grooves bindings. Biophys J. 2003; 84: 2460-2466.

Alam ZI, Jenner A, Daniel SE, Lees AJ, Cairns N, Marsden CD, Jenner P, Halliwell B. Oxidative DNA damage in the parkinsonian brain: an apparent selective increase in 8-hydroxyguanine levels in substantia nigra. J Neurochem. 1997; 69:1196-1203.

An Y, Liu SD, Deng SY, Ji LN, Mao ZW. Cleavage of double-strand DNA by linear and triangular trinuclear copper complexes. J Inorg Biochem. 2006; 100:1586-1593.

Andrews NC. Iron homeostasis: insight from genetics and animal models. Nature Rev. Genet. 2000; 1: 208 -217.

Andrews NC. The iron transporter DMT1. Int. J. Biochem. Cell. Biol. 1999; 31: 991-994.

Andrushchenko V, Van de Sande JH, Wieser H. Vibrational circular dichroism and IR absorption of DNA complexes with Cu^{2+} ions. Biopolymers.2003; 72:374-390.

Anitha S, Rao KSJ, Latha KS, Viswamitra MA. First evidence to show the topological change of DNA from B-DNA to Z-DNA Conformation in the hippocampus of Alzheimer's Brain. Neuro Molecular Medicine 2002; 2: 289-297.

Arscott PG, Li AZ, Bloomfield VA. Condensation of DNA by trivalent cations. 1. Effects of DNA length and topology on the size and shape of condensed particles. Biopolymers. 1990; 30:619-630.

Barnham KJ, Masters CL, Bush AI. Neurodegenerative diseases and oxidative stress. Nat Rev Drug Discov. 2004; 3: 205-214.

Barrow CJ, Yasuda A, Kenny PTM, Zagorski MG. Solution conformations and aggregational properties of synthetic amyloid beta-peptide of Alzheimer's disease. J. Mol. Biol. 1992; 225; 1075 -1093.

Bauer WR, Crick FH, White JH. Supercoiled DNA. Sci Am. 1980; 243: 100-113.

Baum L, Ng A. Curcumin interaction with copper and iron suggests one possible mechanism of action in Alzheimer's disease animal models. J. Alzheimer's Dis. 2004; 6: 367 – 377.

Beard JL, Connor JR, Jones BC. Iron in the brain. Nutr. Rev. 1993; 51: 157 – 170.

Beckman RA, Mildvam AS, Loeb LA. On the fidelity of DNA replication: manganese mutagenesis in vitro. Biochemistry. 1985; 24: 5810-5815.

Bednar J, Furrer P, Stsiask A, Dubochet J, Egelman EH, Bates AD. The twist, writhe and over all shape of supercoiled DNA change during counterion-induced transition from a loosely to a tightly interwound superhelix. Possible implications for DNA structure in vivo. J Mol. Biol. 1994; 235: 825 – 847.

Bharath S, Hsu M, Kaur D, Rajagopalan S, Andersen JK. Glutathione, iron and Parkinson's disease. Biochem. Pharmacol. 2002; 64: 1037 – 1048.

Birchall JD, Chappel JS. Aluminium, Chemical physiology and Alzheimer's disease. Lancet, 1988; 1008-1010.

Biver T, Lombardi D, Secco F, Tine MR, Venturini M, Bencini A, Bianchi A, Valtancoli B. Kinetic and equilibrium studies on the polyazamacrocycle neotetren: metal-complex formation and DNA interaction, Dalton Trans. 2006; 12: 1524-1533.

Bodovitz S, Falduto MT, Frail DE, Klein WL. Iron levels modulate α-secretase cleavage of amyloid precursor protein. J Neurochem. 1995; 64: 307 – 315.

Bondy SC, Kirstein S. The promotion of iron induced generation of ROS in nerve tissue by aluminum. Mol. Chem. Neuropathol. 1996; 27:185-194.

Bouras C, Glannakopoulos P, Good PF, Hsu A, Hof PR, Perl DP. A laser microprobe mass analysis of brain aluminum and iron in dementia pugilistica: comparison with Alzheimer's disease. Eur Neurol. 1997; 38: 53-58.

Bradbury MW. Transport of iron in the blood-brain-cerebrospinal fluid system. J Neurochem. 1997; 69:443-454.

Brahmachari SK, Mishra RK, Bagga R, Ramesh N. DNA duplex with the potential to change handedness after every half a turn. Nucleic Acids Res. 1989; 17:7273– 7281.

Brambilla P, Glahn DC, Balestrieri M, Soares JC. Magnetic resonance findings in bipolar disorder. Psychiatr Clin North Am. 2005; 28: 443-467.

Brown RC, Lockwood AH, Sonawane BR. Neurodegenerative Disease: An Overview of Environmental Risk Factors, Environ Health Perspect. 2005; 113:1250-1256.

Burdo JR, Connor JR. Brain iron uptake and homeostatic mechanisms: an overview. BioMetals. 2003; 16: 63-75.

Bush AI. The metallobiology of Alzheimer's disease. Trends Neurosci. 2003; 26: 207-214.

Campbell A, Prasad KN, Bondy SC. Aluminum induced oxidative events in cell lines: glioma are more responsive the neuroblastoma. Free Radical Biol. Med. 1999; 26: 1166 – 1171.

Cannata JB, Fernanez-Soto I, Fernandez-Menendez MJ, Fernandez-Martin JL, McGregor SJ, Brock JH, Halls D. Role of iron metabolism in absorption and cellular uptake of aluminum, Kidney Int. 1991a; 39: 799 – 803.

Cannata JB, Fernanez-Soto I, Fernandez-Menendez MJ, Fernandez-Martin JL, McGregor SJ, Brock JH, Halls D. Iron uptake in aluminum overload: in vivo and in vitro studies. Nephrol Dial Transplant. 1991b; 6: 637 -642.

Cassidy F, Ahearn EP, Carroll BJ. Substance abuse in bipolar disorder. Bipolar Disord. 2001; 3: 181-188.

Champion CS, Kumar D, Rajan T, Rao KSJ, Viswamitra MA. Interaction of Co, Mn, Mg and Al with d(GCCCATGGC) and d(CCGGCCCGG): a spectroscopic study. Cell. Mol. Life.Sci. 1998; 54: 488-496.

Chekoway H, Farin FM, Costa-Mallen P, Kirchner SC, Costa LG. Genetic polymorphisms in Parkinson's disease. Neurotoxicology. 1998; 19: 635-643.

Cherny DI, Jovin TM. Electron and Scanning Force Microscopy Studies of Alterations in Supercoiled DNA Tertiary Structure. J. Mol. Biol. 2001; 313: 295 – 307.

Cherny RA, Atwood CS, Xilinas ME, Gray DN, Jones WD, McLean CA, Barnham KJ, Volitakis I, Fraser FW, Kim Y, Huang X, Golstein LE, Moir RD, Lim JT, Beyeruther K, Zheng H, Tanzi RE, Masters CL, Bush AI. Treatment with a copper-zinc chelator markedly and rapidly inhibits beta-amyloid accumulation in Alzheimer's disease transgenicmice. Neuron. 2001; 30: 665 - 676.

Choi DW, Koh JY. Zinc and brain injury. Annu Rev Neurosci. 1998; 21: 347 -375.
Clauberg M, Joshi JG. Regulation of serine protease activity by Al: Implications for Alzheimer's disease. Proc. Natl. Acad. Sci. USA 1993; 90: 1009 -1012.

Connor JR, Menzies SL, Burdo JR, Boyer PJ. Iron and iron management proteins in neurobiology. Pediatr Neurol. 2001; 25: 118 – 129.

Connor JR, Menzies SL, St Martin SM, Mufson EJ. Cellular distribution of transferring, ferritin, and iron in normal and aged human brains. J. Neurosci. Res. 1990; 27: 595- 611.

Cotman CW, Su JH. Mechanism of neuronal death in Alzheimer's disease. Brain Pathol. 1996; 6: 69-72.

Cragg L, Hebbel RP, Miller W, Solovey A, Selbey S, Enright H. The iron chelator L1 potentiates oxidative DNA damage in iron-loaded liver cells. Blood. 1998; 92: 632 – 638.

Crapper DR, Krishnan SS, Dalton AJ. Brain aluminum distribution in Alzheimer's disease and experimental neurofibrillary degeneration. Science, 1973; 180: 511- 513.

Crapper DR, Krishnan SS, Quittkat S. Aluminum, neurofibrillary degeneration, and Alzheimer's disease. Brain. 1976; 99: 67 – 80.

Crapper DR, Quittkat S, de Boni U. Altered chromatin conformation in Alzheimer's disease.Brain. 1979; 102:483-495.

Crapper DR, Quittkat S, Krishnana SS, Dalton AJ, DeBoni U: Intracellular Aluminum content in Alzheimer's disease, dialysis encephalopathy and experimental aluminum encephalopathy. Acta .Neuropathology 1980; 50: 19-24.

Crichton RR, Florence A, Ward RJ. Aluminum and iron in the brain-prospects for chelation. Coord Chem Rev. 2002; 2: 365 -371.

Crichton, RR. Inorganic Biochemistry of iron Metabolism: From Molecular Mechanisms to Clinical Consequences, John Wiley and Sons, Chichester, 2001.

Dedman DJ, Treffry A, Candy JM, Taylor GAA, Morris CM, Bloxham CA, Perry RH, Edwardson JA, Harrison PM. Iron and aluminum in relation to brain ferritin in normal individuals and Alzheimer's disease and chronic renal-dialysis patients. Biochem J. 1992; 287: 509 – 514.

Deibel MA, Ehmann WD, Markesbery WR. Copper, iron, and zinc imbalances in severly degenerated brain regions in Alzheimer's disease: possible relation to oxidative stress. J. Neurol. Sci., 1996; 143: 137 -142.

Dellers M, Servias JP, Wulfert E, Micromolar concentrations of Al^{3+} induce phase separation, aggregation and dye release in phophatidylserine-containing lipid vesicles. Biochim Biophys Acta. 1985; 813: 195 – 200.

DeRusso PA, Philipott CC, Iwai K, Mostowski HS, Klausner RD, Rouault TA, Expression of a constitutive mutant of iron regulatory protein1 abolishes iron homeostasis in mammalian cells. J. Biol.Chem. 1995; 270: 15451 – 15454.

Dexter DT, Carayon A, Agid FJ, Agid Y, Wells FR, Daniel Se, Lee AJ, Jenner P, Marsden CD. Alterations in the levels of iron, ferritin and other trace metals in Parkinson's disease and other neurodegenerative diseases affecting the basal ganglia. Brain 1991; 114: 1953- 1975.

Dexter DT, Ward RJ, Florence A, Jenner P, Crichton RR. Effects of desferrithiocin and its derivatives on peripheral iron and striatal dopamine 5-hydroxytryptamine metabolism in the ferrocene-loaded rat. Biochem. Pharmacol. 1999; 58. 151-155.

Dexter DT, Wells FR, Agid F, Agid Y, Lees AJ, Jenner P, Marsden CD. Increased nigral iron content in postmortem parkinsonian brains. Lancet. 1987; 2: 1219 -1220.

Dexter DT, Wells FR, Lees AJ, Agid F, Agid Y, Jenner P, Marsden CD. Increased nigral iron content and alterations in other metal ions occurring in brain in Parkinson's disease. J Neurochem. 1989; 52:1830-1836.

Di Vaira M, Bazzicalupi C, Orioli P, Messori L, Bruni B, Zatta P. Clioquionol, a drug for Alzheimer's disease specifically interfering with brain metal metabolism: structural characterization of its zinc (II) and copper (II) complexes. Inorg Chem. 2004; 43: 3795 – 3797.

Dolken V. Uber die Wirkkung des Aluminiums mit besonderer Berucksichtigung der durch das Aluminium verursachten Lasionenim Zentralnervensystem. Arch. Exper.Pathol. Pharmacol. 1897; 98 -120.

Duchen LW, Jacobs JM. Nutritional deficiencies and metabolic disorders. In: Adams JH, Corsellis JAN, Duchen LW (Eds), Greenfield's Neurpopathology. Wiley, NewYork, 1984; pp 595 – 610.

Duguid JG, Bloomfield VA, Benevides JM, Thomas GJ Jr. Aggregation of melted DNA by divalent metal ion-mediated cross-linking, Biophys J. 1995; 69:2623-2632.

Dwork AJ, Schon EA, Herbert J. Nonidentical distribution of transferring and ferric iron in human brains. Neuroscience 1988; 27: 333-345.

Eichhorn GL, Clark P. Interaction of metal ions with polynucleotides and related compounds. V. the unwinding and rewinding of DNA strands under the influence of Copper (II) ions. Proc Natl Acad Sci USA. 1965; 53: 586-593.

Eichhorn GL, Shin YAJ. Interaction of metal ions with polynucleotides and related compounds. XII. The relative effect of various metal ions on DNA helicity. J Am Chem Soc. 1968; 90: 7323 – 7329.

Eichhorn GL. Metal ions as stabilizers or destabilizers of the deoxyriboucleic acid structure. Nature. 1962; 194: 474- 475.

Eliezer D, Kutluay E, Bussell R Jr, Browne G. Conformational properties of alpha-synuclein in its free and lipid-associated states. J Mol Biol. 2001; 307:1061-1073

Esclaire F, Lesort M, Blanchard C, Hugon J. Glutamate toxicity enhances tau gene expression in neuronal cultures. J.Neurosci. Res. 1997; 49: 309 -318.

Esparza JL, Gomez M, Romeu M, Mulero M, Sanchez DJ, Mallol J, Domingo JL. Aluminum-induced pro-oxidant effects in rats: protective role of exogenous melatonin. J Pineal Res. 2003; 35:32-39.

Evans P, Harrington C. Aluminosilicate participate and beta-amyloid in vitro interaction: a model of Alzheimer plaque formation. Biochem. Soc. Trans. 1998; 26: S251.

Exley C. The pro-oxidant activity of aluminum. Free Radic Biol Med. 2004; 36: 380 – 387.

Faucheux BA, Martin ME, Beaumont C, Hauw JJ, Agid Y, Hirsch EC. Neuromelanin associated redox-active iron is increased in the substantia nigra of patients with Parkinson's disease. J Neurochem. 2003; 86: 1142-1148.

Ferretti G, Marchionni C, Bacchetti T, Galeazzi T, Dousset N. Effect of aluminum on lipid peroxidation of human high density lipoproteins, Free Rad. Res. 2003; 37:515-521.

Finefrock AE, Bush AI, Doraiswamy PM. Current status of metals as therapeutic targets in Alzheimer's disease. J Am Geriatr Soc. 2003; 51: 1143 – 1148.

Fleming MD, Trenor CC 3rd, Su MA, Foernler D, Beier DR, Dietrich WF, Andrews NC. Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. Nat Genet. 1997; 16: 383-386.

Forno LS. Neuropathology of Parkinson's disease. J Neuropathol Exp Neurol. 1996; 55: 259-272.

Fosmire GJ, Focht SJ, McClearn GE. Genetic influences on tissue deposition of aluminum in mice. Biol Trace Elem Res. 1993; 37: 115-121.

Frederickson CJ. Neurobiology of zinc and zinc – containing neurons. Int Rev Neurobiol. 1989; 31: 145-328.

Frey BN, Andreazza AC, Kunz M, Gomes FA, Quevedo J, Salvador M, Goncalves CA, Kapczinski F. Increased oxidative stress and DNA damage in bipolar disorder: A twin-case report. Prog Neuropsychopharmacol Biol Psychiatry. 2006b. Epub ahead of print.

Frey BN, Valvassori SS, Gomes KM, Martins MR, Dal-Pizzol F, Kapczinski F, Quevedo J. Increased oxidative stress in submitochondrial particles after chronic amphetamine exposure. Brain Res. 2006a; 1097:224-229.

Garruto, RM., Flaten, TP, Wakayama I. Natural and experimental models of environmentally induced neurodegenaration: Implications for Alzheimer's disease. In: Corain B, Winsneiwski H and P. Zatta P.(Eds), Alzheimer's disease: Advances in Clinical and Basic Research, John Wiley and Sons Ltd, New York, 1993, pp.257 – 266.

Gauthier E, Fortier I, Courchesne F, Pepin P, Mortimer J, Gauvreau D. Aluminum forms in drinking water and risk of Alzheimer's disease. Environ. Res. 2000; 84: 234-246.

Gerhart DZ, Enerson BE, Zhdankina OY, Leino RL, Drenes LR. Expression of monocarboxylate transporter MCT 1 by brain endothelium and glia in adult and suckling rats. Am.J. Physiol. 1997; 273: E207-213.

Ghribi O, DeWitt DA, Forbes MS, Herman MM, Savory J. Co-involvement of mitochondria and endoplasmic reticulum in regulation of apoptosis: changes in cytochrome c, Bcl-2 and Bax in the hippocampus of aluminum-treated rabbits. Brain Res. 2001; 903:66-73.

Golub MS, Domingo JL. What we know and what we need to know about developmental aluminum toxicity. In: Yokel RA, Golub MS (Eds), Research issues in Aluminum Toxicity. Taylor and Francis, Washington, DC, 1997; pp 585-597.

Gong CX, Lidsky T, Wegiel J, Zuck L, Grundke-Iqbal I. Phosphorylation of microtubule associated protein tau is regulated by protein phophatase 2A in mammalian brain. Implications for neurofibrillary degeneration in Alzheimer's disease. J. Biol Chem 2000; 275: 5535 -5544.

Good PF, Perl DP, Bierer LM, Schmeidler J. Selective accumulation of aluminium and iron in NFT of Alzheimer's disease; a laser microprobe (LAMMA) study. Ann Neurol. 1992; 31: 286-292.

Gorell JM, Johnson CC, Rybicki BA, Peterson EL, Kortsha GX, Brown GG, Richardson RJ. Occupational exposure to manganese, copper, lead, iron, mercury and zinc and the risk of Parkinson's disease, Neurotoxicology. 1999b; 20: 239- 247.

Gorell JM, Rybicki BA, Cole Johnson C, Peterson EL. Occupational metal exposures and the risk of Parkinson's disease, Neuroepidemiology. 1999a; 18: 303 – 308.

Gotz ME, Double K, Gerlach M, Youdim MB, Riederer P.The relevance of iron in the pathogenesis of Parkinson's disease. Ann N Y Acad Sci. 2004; 1012: 193-208.

Graham DG. Catecholamine toxicity: a proposal for the molecular pathogenesis of manganese neurotoxicity and Parkinson's disease. Neurotoxicology. 1984; 5:83-95.

Grant WB, Campbell A, Itzhaki RF, Savory J. The significance of environmental factors in the etiology of Alzheimer's disease. J Alzheimer's Dis. 2002; 4:179-189.

Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, Hediger MA. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature. 1997; 388: 482 -488.

Gupta VB, Anitha S, Hegde ML, Zecca L, Garruto RM, Ravid R, Shankar SK, Stein R, Shanmugavelu P, Jagannatha Rao KS. Aluminium in Alzheimer's disease: are we still at a crossroad? Cell Mol Life Sci. 2005; 62:143-158.

Gutteridge JMC, Quinlan GJ, Clark I, Halliwell B. Aluminum salts accelerate peroxidation of membrane lipids stimulated by iron salts. Biochim Biophys Acta. 1985; 835: 441-447.

Guy SP, Jones D, Mann DMA, Itzhaki RF. Human neuroblastoma cells treated with aluminum express an epitope associated with Alzheimer's disease neurofibrillary tangles. Neurosci Lett. 1991; 121:166-168.

Hallgren B. The effect of age on the non haemin iron in the human brain. J. Neurochem. 1958; 3: 41-51.

Halliwell B, Gutteridge JM. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem. J. 1984; 219: 1-14.

Hartter DE, Barnea A. Brain tissue accumulates copper by two liand-dependent saurable processes. J.Biol.Chem.1988a; 263: 799-805.

Hartter DE, Barnea A. Evidence of release of copper in the brain: depolarization-induced release of newly taken up copper, Synapse. 1988b; 2: 412-415.

Hashimoto M, Takeda A, Hsu LJ, Takenouchi T, Masliah E. Role of cytochrome c as a stimulator of alpha-synuclein aggregation in Lewy body disease. J Biol Chem. 1999; 274: 28849-2852.

Hediger MA. Membrane permeability. The diversity of transmembrane transport processes. Curr Opin. Cell Biol. 1997; 9: 543 - 546.

Hegde ML, Gupta VB, Anitha M, Harikrishna T, Shankar SK, Muthane U, Subba Rao K, Jagannatha Rao KS. Studies on genomic DNA topology and stability in brain regions of Parkinson's disease. Arch Biochem Biophys. 2006; 449:143-156.

Heininger K. A unifying hypothesis of Alzhiemr's disease. III. Risk factors. Hum. Psychopharmacol. 2000; 15:1-70.

Hill JM. Iron concentration reduced in ventral pallidum, globuspallidus, and substancia nigra by gaba-transaminase inhibitor, gama-vinyl GABA. Brain Res. 1985; 342: 18-25.

Hirsch EC, Brandel JP, Galle P, Javoy-Agid F, Agid Y.Iron and aluminum increase in the substantia nigra of patients with Parkinson's disease: an X- ray microanalysis. J Neurochem. 1991; 56: 446-451.

House E, Collingwood J, Khan A, Korchazkina O, Berthon G, Exley C. Aluminium, iron, zinc and copper influence the in vitro formation of amyloid fibrils of Abeta42 in a manner which may have consequences for metal chelation therapy in Alzheimer's disease. J Alzheimers Dis. 2004; 6: 291-301.

Huang X, Moir RD, Tanzi RE, Bush AI, Rogers JT. Redox-active metals, oxidative stress, and Alzheimer's disease pathology. Ann N Y Acad Sci. 2004; 1012: 153-163.

Huang Y, Herman MM, Liu J, KAtsetos CD, Wills MR, Savory J. Neurofibrillary lesions in experimental aluminum-induced encephalopathy and Alzheimer's disease share immunoreactivity for amyloid precursor protein, A beta, alpha 1-antichymotrypsin and ubiquitin-protein conjugates. Brain Res. 1997; 771: 213-220.

Ibach B, Haen E, Marienhagen J, hajak G. Clioquinol treatment in familiar early onset of Alzheimer's disease: a case report. Pharmacopsychiatry. 2005; 38: 178-179.

Ikezu T, Luo X, Weber GA, Zhao J, McCabe L, Buescher JL. Amyloid precursor protin-processing products affect mononuclear phagocyte activation: pathways for sAPP-and Abeta-mediated neurotoxicty. J Neurochem. 2003; 85: 925 -934.

Jakes R, Spillantini MG, Goedert M. Identification of two distinct synucleins from human brain. FEBS Lett. 1994; 345: 27-32.

Jamison KR. Suicide and bipolar disorder.J Clin Psychiatry. 2000; 61 Suppl 9: 47-51.

Jellinger K, Kienzl E, Rumpelmair G, Riederer P, Stachelberger H, Ben-Shachar D, Youdim MB. Iron-melanin complex in substatia nigra of parkinsonian brains: an x-ray microanalysis. J Neurochem. 1992; 59: 1168-1171.

Jellinger K, Paulus W, Grundke-Iqbal I, Riederer P, Youdim MB. Brain iron and ferritin in Parkinson's and Alzheimer's diseases. J Neural Transm Park Dis Dement Sect. 1990; 2: 327-340.

Jellinger K. The role of iron in neurodegeneration: prospects for pharmacotherapy of Parkinson's disease. Drugs Aging. 1999; 14: 115-140.

Karlik SJ, Chong AA, Eichhorn GL, De Boni U. Reversible toroidal compaction of DNA by aluminum. Neurotoxicology. 1989; 10: 167 – 176.

Karlik SJ, Eichorn Gl, Lewis PN, Crapper DR. Interaction of Aluminum species with DNA. Biochemistry. 1980; 19: 5991-5998,.

Kasprzak KS. Possible role of oxidative damage in metal-induced carcinogenesis. Cancer Inves. 1995; 13. 411-430.

Kausner RD. From receptors to gene – insight from molecular iron metabolism. Clin. Res. 1988; 36: 494-500.

Kawahara M, Muramoto K, Kobayashi K, Mori H, Kuroda Y. Aluminum promotes the aggregation of Alzheimer's amyloid β-protein in vitro. Biochem. Biophys.Res. Commun. 1994; 198; 531-535.

Kawahara M. Effects of aluminum on the nervous system and its possible link with neurodegenerative diseases. J Alzheimer's Dis. 2005; 8:171-182.

Kerr DNS, Ward MK. Metal ion in biological system. In: Sigel H, Sigel A (Eds), Aluminum and Its role in Biology, Marcel Dekker, New York, 1988, pp.217-258.

Kingsbury AE, Mardsen CD, Foster OJ. DNA fragmentation in human substantia nigra: apoptosis or perimortem effect? Mov Disord. 1998; 13:877-884.

Kitamura Y, Shimohama S, Kamoshima W, Ota T, Matsuoka Y, Nomura Y, Smith MA, Perry G, Whitehouse PJ, Taniguchi T. Alterarion of proteins regulating apoptosis, Bcl-2, Bcl-x, Bax, Bak, Bad, ICH-1 and CPP32, in Alzheimer's disease. Brain Res. 1998; 780: 260-269.

Klatzo I, Wisnieewski HM, Streicher E. Experimental production of neurofibrillary degeneration. J. Neuropath. Exp. Neurol. 1965; 24: 187 – 199.

Koh JY. Zinc and disease of the brain. Mol.Neurobiol. 2001; 24: 99 -106

Kong S, Liochev S, Fridovich I. Aluminum (III) facilitates the oxidation of NADPH by the superoxide anion. Free Radic Biol Med. 1992; 13: 79-81.

Kornilova SV, Kapinos LE, Tomkova A, Mishkovskii P, Blagoilu P, Bol'bukh TV. Study of interaction of DNA with copper ions using IR- and Raman spectroscopy, Biofizika. 1994; 39:423-436.

Kruck TP, Burrow TE. Synthesis of feralex a novel aluminium/iron chelating compound. J Inorg. Biochem. 2002; 88: 19–24.

Kruck TP, Cui JG, Percy ME, Lukiw WJ. Molecular shuttle chelation: the use of ascorbate, desferrioxamine and Feralex-G in combination to remove nuclear bound aluminum. 2004; 3: 443-459.

Kruck TP, Fisher EA, McLachlan DR. Suppression of deferoxamine mesylate treatment-induced side effects by coadministration of isoniazid in a patient with Alzheimer's disease subject to aluminum removal by ionspecific chelation. Clin Pharmacol Ther. 1990; 48: 439- 446.

Kruck TP. Aluminium - Alzheimer's link? Nature.1993; 363: 119.

Lai BC, Marison SA, Teshke K, Tsui JK. Occupational and environmental risk factors for Parkinson's disease. Parkinsonism Rela Disord 2002; 8: 297 - 309.

Levenson CW. Trace metal regulation of neuronal apoptosis: from genes to behavior.Physiol Behav. 2005; 86: 399-406.

Levine S, Connor J, Schipper H. Redox-active metals in neurological disorders. Ann. NY Acad. Sci. 2004; 1012.

LeVine SM, Macklin WB. Iron-enriched oligodendrocytes: A reexamination of their spatial distribution. J. Neurosci. Res. 1990; 26: 508 – 512.

Lohr JB, Browning JA. Free radical involvement in neuropsychiatric illnesses, Psychopharmacol Bull. 1995; 31: 159 -165.

Lorenzo A, Yanker BA. Beta – amyloid neurotoxicity requires fibril formation and is inhibited by congo red. Proc. Natl. Acad. Sci. USA. 1994; 91: 12243 – 12247.

Lovell MA, Ehmann WD, Marksbery WR. Laser microprobe analysis of brain aluminum in Alzheimer's disease. Ann. Neurol. 1993; 33:36-42.

Lovell MA, Robertson JD, Teesdale WJ, Campbell JL, Markesbery WR. Copper, iron and zinc in Alzheimer's disease senile plaques. J Neurol Sci. 1998; 158: 47-52.

Lukiw WJ, Krishnan B, Wong L, Kruck TPA, Bergeson C, McLAchlan DRC. Nuclear compartmentalization of aluminum in Alzheimer's disease. Neurobiol. Aging. 1991; 13: 115-121.

Lukiw WJ, Kruck TP, McLachlan DR. Linker histone-DNA complexes: enhanced stability in the presence of aluminum lactate and implications for Alzheimer's disease. FEBS Lett. 1989; 253:59-62.

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

Maccioni RB, Munoz JP, Barbeito L. The molecular bases of Alzheimer's disease and other neurodegenerative disorders. Arch Med Res. 2001; 32: 367-81.

Madhav TR, Vatsala S, Ramakrishna T, Ramesh J, Easwaran KRK. Preservation of native conformation during aluminum-induced aggregation of tau protein. Neuroreport. 1996; 7: 1072 – 1076.

Maj M, Pirozzi R, Magliano L, Bartoli L. The prognostic significance of "switching" in patients with bipolar disorder: a 10-year prospective follow-up study. Am J Psychiatry. 2002; 159: 1711-1717.

Mandel S, Maor G, Youdim MB. Iron and alpha-synuclein in the substantia nigra of MPTP-treated mice: effect of neuroprotective drugs R-apomorphine and green tea polyphenol(-)-epigallocatechin-3-gallate. J Mol Neurosci. 2004b; 24: 401- 416

Mandel S, Weinreb O, Amit T, Youdim MB. Cell signaling pathways in the neuroprotective actions of the green tea polyphenol (-)-epigallocatechin-3-gallate: implications for neurodegenerative diseases. J Neurochem. 2004a; 88: 1555-1569.

Mantyh PW, Ghilardi JR, Rogers S, DeMAster E, Allen CJ, Stimson ER, Maggio JE. Aluminum, iron, and zinc ions promote aggregation of physiological concentrations of beta-amyloid peptide. J Neurochem. 1993; 61:1171-1174.

Maret W. Imbalance between pro-oxidant and pro-antioxidant functions of zinc in disease. J Alzheimers Dis. 2005; 8: 161-170.

Markesbery WR, Ehman WD, Alauddin M, Hossain TIM, Brain trace element concentrations in aging. Neurobiol.Aging. 1984; 5: 19-28.

Markesbery WR, Ehman WD, Hossain TIM, Alauddin M, Goodin DT. Instrumental neutron activation analysis of brain aluminum in Alzheimer disease and aging. Ann Neurol. 1981; 10: 511-516.

Marzilli L.G, Kistenmacher TJ, Eichhorn GL. Nucleic acid-metal ion interactions, John Wiley and Sons, New York, 1980, pp179 – 250.

Mashiko S, Suzuki N, Koga S, Nakano M, Goto T, Ashino T, Mizumoto I, Inaba H. Measurement of rate constants for quenching singlet oxygen with a Cypridina uciferin analog (2-methyl-6-[p-methoxyphenyl]-3,7-dihydroimidazo[1,2-a]pyrazin-3-one) and sodium azide. J Biolumin Chemilumin. 1991; 6: 69-72.

Matsuzaki S, Manabe T, Katayama T, Nishikawa A, Yanagita T, Okuda H, Yasuda Y, Miyata S, Meshitsuka S, Tohyama M. Metals accelerate production of the aberrant splicing isoform of the presenilin-2. J Neurochem. 2004; 88:1345-1351.

McDermott JR, Smith AI, Iqbal K, Wisniewski HM. Brain aluminum in aging and Alzheimer's disease. Neurology. 1979; 29: 809 – 814.

McLaughlin AIG, Kazantzis G, King E, Teare D, Porter RJ, Owen R. Pulmonary fibrosis and encephalopathy associated with the inhalation of aluminium dust. Br. J. Ind. Med. 1962; 19: 253-263.

Mekmouche Y, Coppel Y,Hochgrafe K, Guilloreau L, Talmard C, Mazarguil H, Faller P, Characterization of the ZnII binding to the peptide amyloid-beta1-16 linked to Alzheimer's disease. Chembiochem. 2005; 6: 1663-1671.

Meneghini R. Iron homeostasis, oxidative stress, and DNA damage. Free Radical Biol. Med. 1997; 23: 783 – 792.

Mertz W. The essential trace elements. Science. 1981; 213: 1332 – 1338.

Michak V, Maurizot MS, Charlier M. Calculation of hydroxyl radical attack of different forms of DNA. J Biomol Struct Dynam. 1995; 13:565-575.

Moalem S, Perey ME, Andrews DF, Kruck TP, Wong S, Dalton AJ, Mehta P, Fedor B, Warren AC. Are hereditary hemochromatosis mutations involved in Alzheimer disease? Am J Med Genet. 2000; 93: 58-66.

Mocchegiani E, Bertoni-Freddari C, Marcellini F, Malavolta M. Brain, aging and neurodegeneration: role of zinc ion availability.Prog Neurobiol. 2005; 75: 367-390.

Monkul ES, Malhi GS, Soares JC.Anatomical MRI abnormalities in bipolar disorder:do they exist and do they progress? Aus NZ J Psychiatry.2005; 39:222-226.

Nakamura T, Keep RF, Nagao S, Hoff JT. Iron-induced oxidative brain injury after experimental. Acta Neurochir Suppl. 2006; 96:194 -198.

O'Connor JM. Trace elements and DNA damage. Biochem Soc Trans.2001; 29: 354 -357.

Octave JN, Schneider YJ, Trouet A, Crichton RR. Iron uptake and utilization by mammalian cells. I. Cellular uptake of transferrin and iron. Trends Biochem. Sci. 1983; 8: 217 – 221.

Ong WY, Farooqui AA. Iron, neuroinflammation, and Alzheimer's disease .J Alzheimers Dis. 2005; 8:183-200.

Ostrerova-Golts N, Petrucelli L, Hardy J, Lee JM, Farer M, Wolozin B. The A53T alpha-synuclein mutation increases iron-dependent aggregation and toxicity. J Neurosci. 2000; 20: 6048-6054.

Oteiza PI, A mechanism for the stimulatory effect of aluminum on iron – induced lipid peroxidation. Arch Biochem Biophys. 1994; 308: 374 – 379.

Oteiza PI, Fraga CG, Keen CL. Aluminum has both oxidant and antioxidant effect in mouse brain membranes. Arch Biochem Biophys. 1993; 300: 517- 521.

Pedreno E, Lopez-Contreras AJ, Cremades A, Penafiel R. Protecting or promoting effects of spermine on DNA strand breakage induced by iron and copper ions as a function of metal concentration, J Inorg Biochem. 2005; 99: 2074 -2080.

Perl DP, Brody AR. Alzheimer's disease: X-ray spectrometric evidence of aluminum accumulation in neurofibrillary tangle-bearing neurons. Science.1980; 18:208:297-299.

Perl DP, Good PF. Uptake of aluminum into central nervous system along nasal-olfactory pathways. Lancet, 1987; i: 1028.

Perry G, Sayre LM, Atwood CS, Castellani RJ, Cash AD, Rottkamp CA, Smith MA. The role of iron and copper in the etiology of neurodegenerative disorders: therapeutic implications. CNS Drugs. 2002; 16: 339-352.

Prasad AS. Clinical and biochemical spectrum of zinc deficiency in human subjects.In: Clinical, Biochemichal and Nutritional Aspects of Trace Elements. New York, 1982, pp 46.

Quameur AA, Arakawa H, Ahmad R, Naoul M, Tajmir-Riahi HA. A comparative study of Fe (II) and Fe (III) interactions with DNA duplexes: mjor and minor grooves bindings, DNA Cell Biol. 2005; 24:394-401.

Rajan MT, KSJ, Mamatha MB, Rao RV, Shanmugavelu P, Menon RB, Pavithran MV. Quantification of trace elements in normal human brain by Inductively Coupled Plasma Atomic Emission Spectrometry. J Neurol Sci. 1997; 146: 153 – 667.

Rao KSJ, Anitha S, Latha KS. Aluminum induced neurodegenration in the hippocampus of aged rabbit mimics Alzheimer's disease, Alz rep. 2000; 3: 83-88.

Rao KSJ, Rao BS, Vishnuvardhan RD, Parasad KVS. Alteration of superhelical state of DNA by aluminum. Biochem. Biophys. Acta. 1993; 1172: 17 – 20.

Rao KSJ, Rao RV, Shanmugavelu P, Menon RB. Trace elements in Alzheimer's brain: A new hypothesis. Alz Rep. 1999a; 2: 241-246.

Rao KSJ, Rao VG. Aluminum leaching from utensils-a kinetic study. Int. J. Food Sci. Nutr. 1995; 46: 31-38.

Rao KSJ, Shanmugavelu P, Shankar SK, Rukmani Devi RP, Rao RV, Pande S, Menon RB. Trace elements in the cerebrospinal fluid in Alzheimer's disease. Alz Rep 1999b; 2: 333-338.

Rao KSJ. Aluminum content in tea leaves and in differently prepared tea infusions. Nahrung. 1994; 5: 533-537.

Regland B, Lehmann W, Abedini I, Blennow K, Jonsson M, Karlsson I, Sjogren M, Wallin A, Xilinas M, Gottfries CG. Treatment of Alzheimer's disease with clioquinol. Dement Geriatr Cogn Disord. 2001; 12: 408- 414.

Richardson CL, Verma J, Schulman GE, Shipp K, Grant AD. Metal mutagens and carcinogens effectively displace acridine orange from DNA as measured by fluorescence polarization. Environ. Mutagen. 1981; 3: 545-548.

Richardson DR. Noval chelators for central nervous system disorders that involve alteration in the metabolism of iron and other metal ions. Ann NY Acad Sci 2004; 1012: 326 – 341.

Richiie CW, Bush AI, Mackinnon A, Macfarlane S, Mastwyk M, MacGregor L, Kiers L, Cherny R, Li QX, Tammer A, Carrington D, MAyros C, Volitakis I, Xilinas M, Ames D, Davis S, Beyreuther K, Tanzi RE, Masters CL. Metal – protein attenuation with iodochlorohydroxyquin(clioquinol) targeting Aβ amyloid dposition and toxicity in Alzheimer disease: a pilot phase 2 clinical trial. Arch Neurol.2003; 60:1685-1691.

Riederer P, Sofic E, Rausch WD, Schmidt B, Reynolds GP, Jellinger K, Youdim MB. Transition metals, ferritin, glutathione, and ascorbic acid in parkinsonian brains. J Neurochem. 1989; 52: 515-520.

Roberts NB, Clough A, Bellia JP, Kim JY. Increased absorption of aluminum from a normal dietary intake in dementia. J. Inorg. Biochem. 1998; 69: 171 – 176.

Rogers JT, Randall JD, Cahill CM, Eder PS, Huang X, Gunshin H, Leiter L, McPhee J, Sarang SS, Utsuki T, Greig NH, Lahiri DK, Tanzi RE, Bush AL, Giordano T, Gullans SR. An iron-responsive element type II in the 5'-untranslated region of the Alzheimer's amyloid precursor protein transcript. J Biol Chem. 2002; 77: 45518 – 45528.

Rosenberg RN. The molecular and genetic basis of AD: the end of the beginning: the 2000 Wartenberg Lecture. Neurology. 2000; 54: 2045 – 2054.

Roskams AJ and Connor JR. Aluminum access to the brain: a role for transferring and its receptor. Proc. Natl. Acad. Sci. USA. 1990; 87: 9024 – 9027.

Rottkamp CA, Raina AK, Zhu X, Gaier E, Bush AI, Atwood CS, Chevion M, Perry G, Smith MA. Redox-active iron mediates amyloid-beta toxicity. Free Radic Biol Med. 2001; 30: 447-450.

Rybicki BA, Johnson CC, Uman J, Gorell JM. Parkinson's disease mortality and the industrial use of heavy metals in Michigan. Mov Disord. 1993; 8: 87 – 92.

Sagripanti Jl. DNA damage mediated by metal ions with special reference to copper and iron. Metal Ions Biol. Syst. 1999; 36: 179 – 209.

Savory J, Exley C, Forbes WF, Huang Y, Joshi JG, Kruck T, McLachlan DR, Wakayama I. Can the controversy of the role of aluminum in Alzheimer's disease be resolved? What are the suggested approaches to this controversy and methodological issues to be considered? J Toxicol Environ Health. 1996; 48: 615 - 635.

Savory J, Garruto RM. Aluminum, Tau protein and Alzheimer's disease: an important link? Nutrition. 1998; 14: 313 – 314.

Savory J, Herman MM, Ghribi O. Intracellular mechanisms underlying aluminum-induced apoptosis in rabbit brain. J Inorg Biochem. 2003; 97:151-154.

Savory J, Rao JK, Huang Y, Letada PR, Herman MM.Age-related hippocampal changes in Bcl-2:Bax ratio, oxidative stress, redox-active iron and apoptosis associated with aluminum-induced neurodegeneration: increased susceptibility with aging. Neurotoxicology. 1999; 20: 805-817.

Seibert FB, Wells HG. The effect of aluminum on mammalian blood and tissues. Am. J. Pathol. 1929; 8: 230 – 262.

Selkoe DJ, Ihara Y, Salazar FJ. Alzheimer's disease: Insolubility of partially purified paired helical filaments in sodium dodecyl sulfate and urea. Science.1982; 215: 1243 -1245.

Selkoe DJ. Cell biology of the amyloid β-protein precursor and the mechanism of Alzheimer's disease. Annu Rev Cell Biol. 1994; 10: 373-403.

Shea TB, Beerman ML, Nixon RA. Aluminum treatment of intact neuroblastoma cells alters neurofilament subunit phosphorelation. Mol. Chem Neuropathol. 1995; 26: 1-14.

Shin RW, Kruck TP, Murayama H, Kitamoto T. A novel trivalent cation chelator Feralex dissociates binding of aluminum and iron associated with hyperphosphorelated tau of Alzheimer's disease. Brain Res. 2003; 961: 139 – 146.

Shin RW, Lee VMY, Trojanowski JQ. Aluminum modifies the properties of Alzheimer's disease PHF tau protein in vivo and in vitro.J.Neuroscience. 1994; 14: 7221-7233.

Siwek M, Wrobel A, Dudek D,Nowak G, Zieba A,. The role of copper and magnesium in the pathogenesis and treatment of affective disorders, Psychiatr Pol. 2005a; 39: 911-920.

Siwek MS, Wrobel A, Dudek D, Nowak G, Zieba A. The role of zinc in the pathogenesis and treatment of affective disorders, Psychiatr Pol. 2005b; 39: 899-909.

Smith MA, Harris PL, Sayre LM, Perry G. Iron accumulation in Alzheimer's disease is a source of redox-generated radicals. Proc Natl Acad Sci. U S A. 1997; 94: 9866-9868.

Smith MA, Perry G, Richey PL, Sayre LM, Anderson VE, Beal MF, Kowall N. Oxidative damage in Alzheimer's, Nature. 1996; 382: 120-121.

Spafforth J. Case of aluminum poisoning. Lancet. 1921; i: 1301.

Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M. alpha-synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. Proc Natl Acad Sci. USA. 1998; 95: 6469-6473.

Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. Nature. 1997; 388: 839 -840.

Srinivasan DP. Trace elements in psychiatric illness. Br J Hosp Med. 1984; 32:77-79.

Steel M. On the absorption of aluminum from aluminized food. Am. J. Physiol. 1911; 28: 94-102.

Stekhoven SJH, Renkawek K, Otte-Holler I, Stols A. Exogenous aluminium accumulate in the lysosomes of cultured rat cortical neurons. Neurosci. Lett. 1990; 119: 71 -74.

Strong, MJ, Garruto R.M. Experimental paradigms of motorneuron degeneration, In: Woodruff ML, Nonneman AJ (Eds), Toxin–induced models of neurological disorders. Plenum Press, New York, 1994, pp 39-88.

Sugaya K, Reeves M, McKinney M. Topographic associations between DNA fragmentation and Alzheimer's disease neuropathology in the hippocampus. Neurochem Int. 1997; 31:275-281.

Suh SW, Jensen KB, Jensen MS, Silva DS, Kesslak PJ, Danscher G, Frederickson CJ. Histochemically-reactive zinc in amyloid plaques, angiopathy, and degenerating neurons of Alzheimer's diseased brains. Brain Res. 2000; 852: 274-278.

Sulzer D, Bogulavsky J, Larsen KE, Behr G, Karatekin E, Kleinman MH, Turro N, Krantz D, Edwards RH, Greene LA, Zecca L. Neuromelanin biosynthesis is driven by excess cytosolic catecholamines not accumulated by synaptic vesicles. Proc. Natl Acad. Sci. USA. 2000; 97: 11869 – 11874.

Szutowicz A, Bielarczyk H, Kisielevski Y, Jankowska A, Madziar B, Tomaszewicz M. Effects of aluminum and calcium on acetyl-CoA metabolism in rat brain mitochondria. J Neurochem. 1998; 71: 2447-2453.

Tajmir-Riahi HA, Naoui M, Ahmad R. The effect of Cu2+ and Pb2+ on the solution structure of calf thymus DNA: DNA condensation and denaturation studied by Fourier transform ir difference spectroscopy. Biopolymers. 1993; 33:1819-1827.

Takeda A. Essential trace metals and brain function. Yakugaku Zasshi. 2004; 124: 577-585.

Tanner CM, Ottman R, Goldman SM, Ellenberg J, Chan P, Mayeux R, Langston JW. Parkinson disease in twins: an etiologic study. JAMA. 1999; 281: 341 – 346.

Tanner CM. The role of environmental toxins in the etiology of Parkinson's disease. Trends Neurosci. 1989; 12: 49 -54.

Tatton NA, Maclean-Fraser A, Tatton WG, Perl DP, Olanow CW. A fluorescent double-labeling method to detect and confirm apoptotic nuclei in Parkinson's disease. Ann Neurol. 1998; 44:S142-148.

Tatton WG, Olanow CW. Apoptosis in neurodegenerative diseases: the role of mitochondria. Biochim Biophys Acta. 1999; 1410:195-213.

Terry RD, Pena C. Experimental production of neurofibrillary degeneration. J Neuropath. Exp. Neurol. 1965; 24: 200-210.

Terry RD. Neuropathological changes in Alzheimer's disease. Prog Brain Res. 1994; 101:383-390.

Tokutake S, Nagase H, Morisaki S, Oyanagi S. aluminumdetected in senile plaques and neurofibrillay tangles is contained in lipofuscin granules with silicon, probably as aluminosilictae. Neurosci. Lett. 1995; 185: 99 – 102.

Troncoso JC, Sternberger NH, Sternberger LA, HoffmanPN, Price DL. Immunocytochemical studies of neurofilament antigens in the neurofibrillary pathology induced by aluminum. Brain Res. 1986; 364: 295-300.

Uitti RJ, Rajput AH, Rozdilsky B, Bickis M, Wollin T, Yuen WK. Regional metal concentrations in Parkinson's disease, other chronic neurological disease, and control brains. Can J Neuol Sci. 1989; 16: 310- 314.

Uversky VN, Li J, Fink AL. Evidence for a partially folded intermediate in alpha-synuclein fibril formation. J Biol Chem. 2001a; 276:10737-10744.

Uversky VN, Li J, Fink AL. Metal-triggered structural transformations, aggregation, and fibrillation of human α-synuclein. A possible molecular link between Parkinson's disease and heavy metal exposure. J Biol Chem. 2001b; 276: 44284- 44296.

Van Broeckhoven C. Molecular genetics of Alzheimer's disease: Identification of genes and gene mutations. Eur. Neurol.1995; 35: 8-19.

Van Landeghhem GF, Sikstrom C, Beckman LE. Adolfsson R, Beckman L. Transferrin C2, metal binding in Alzheimer's disease. Neuroreport. 1998; 9:177-179.

Van Rensburg SJ, Potocnik FC, De Villiers JN, Kotze MJ, Taljaard JJ. Earlier age of onset of Alzheimer's disease in patients with both the transferrin C2 and polipoprotein E-epsilon 4 alleles. Ann N Y Acad Sci. 2000; 903: 200-203.

VanBerkum MF, Wong I, Lewis PN, Crapper DR. Total and poly (A) RNA yields duringan aluminum encephalopathy in rabbit brains. Neurochem. Res. 1986; 11: 1347 – 1359.

Verstraeten SV, Golub MS, Keen CL, Oteiza PI. Myelin is preferential target of aluminum–mediated oxidative damage. Arch Biochem Biophys. 1997; 344: 289 – 294.

Vogelsberg-Ragaglia V, Schuck T, Trojnowski JQ, Lee VM-Y. PP2A mRNA expression is quantitively decreased in Alzheimer's disease hippocampus. Exp Neurol. 2001; 168: 402-412.

Vologodskii AV, Cozzarelli NR. Conformational and thermodynamic properties of supercoiled DNA. Annu. Rev. Biophys. Biomol. Struct. 1994; 23: 609 – 643.

Walden WE, Patino MM, Gaffield L. Purification of a specific repressor of ferritin mRNA translation from rabbit liver. J Biol Chem. 1989; 264: 13765 – 13769.

Wang J, Markesbery WR, Lovell MA. Increased oxidative damage in nuclear and mitochondrial DNA in mild cognitive impairment. J Neurochem. 2006; 96:825-832

Wang XS, Ong WY, Connor JR. Quinacrine attenuates increases in divalent metal transporter-1 and iron levels in the rat hippocampus, after kainate-induced neuronal injury. Neuroscience. 2003; 120:21-29.

Ward RJ, Zhang Y, Crichton RR. Aluminum toxicity and iron homeostasis, J.Inorg. Biochem. 2001; 87: 9-14.

Weintraub D, Stern MB. Psychiatric complications in Parkinson disease, Am J Geriatr Psychiatry. 2005; 13: 844-851.

Weiss JH, Sensi SL, Koh JY. Zn (2+): a novel ionic mediator of neuronal injury in brain disease. Trends Pharmacol Sci. 2000; 21, 395-401.

Williams RJP, De Silva JJRF. New Trends in Bioinorganic Chemistry, Academic Press, New York, 1978.

Wilms H, Rosenstiel P, Sievers J, Deuschl G, Zecca L, Lucius R. Activation of microglia by human neuromelanin is NF-kappaB dependent and involves p38 mitogen-activated protein kinase: implications for Parkinson's disease. FASEB J. 2003; 17: 500-502.

Wisniewski HM, Sofer D. Neurofibrillary pathology: current status and research perspectives.Mech. Aging Dev. 1979; 1: 119-142.

Xiu YM. Trace elements in health and diseases. Biomed Environ Sci. 1996; 2-3:130-136.

Xu N, Majidi V, Ehmann WD, Markesbery WR. Determination of aluminium in human brain by electro thermal atomic absorption spectrometry. Anal. At. Spectrom. 1992; 7: 749 -751.

Yamamoto A, Shin RW, Hasegawa K, Naiki H, Sato H, Yoshimasu F, Kitamoto T. Iron (III) induces aggregation of hyperphosphorelated tau and its reduction to iron (II) reverses the aggregation: implications in the formation of neutrofibrillary tangles of Alzheimer's disease. J Neurochem. 2002; 82:1137-1147.

Yamamoto H, Saitoh Y, Yasugawa S, Miyamoto E. Dephosphorelation of tau factor by protein phophotase 2A in synaptomal cytosol fractions, and inhibition by aluminum. J. Neurochem. 1990; 55: 683-690.

Yamanaka K, Minato N, Iwai K. Stabilization of iron regulatory protein 2, IRP2, by aluminum. FEBS Lett. 1999; 462: 216-220.

Yokel RA, Allen DD, Ackley DC, The distribution of Al into and out of the brain. J. Inorg. Biochem. 1999; 76: 127-132.

Youdim MB, Ben-Shachar D, Riederer P. Iron in brain function and dysfunction with emphasis on Parkinson's disease. Eur Neurol. 1991; 31 Suppl 1, 34-40.

Youdim MB, Stephenson G, Ben Shachar D. Ironing iron out in Parkinson's disease and other neurodegenerative diseases with iron chelators: a lesson from 6-hydroxydopamine and iron chelators, desferal and VK-28.Ann N Y Acad Sci. 2004; 1012: 306 – 325.

Youdim MBH. In: Dobbing J (Eds).Neuropharmacological and neurobiochemical aspects of iron deficiency: Brain, behavior, and iron in the infant diet. Springer-Verlag, London, 1990, pp 83-106.

Youdim MBH. Neuropharmacological and neurobiochemical aspects of iron deficiency. In: Dobbing J (Eds). Brain, behavior, and iron in the infant diet. Springer-Verlag, London, 1990, pp 83-106.

Yung CY. Neuropsychiatric manifestations of alkali metal deficiency and excess, Pharmacol Biochem Behav. 1984; 21:71-75.

Zatta P, Kiss T, Suwalsky M, Berthon G. Aluminum as a promoter of cellular oxidation. Coord. Chem. Rev. 2002; 228: 271-284.

Zatta P, Lucchiini R, Rensburg SJ, Taylor A. The role of metals in neurodegenerative processes: aluminum, manganese and zinc. Brain Res. Bull. 2003; 62:15-28.

Zayed J, Ducic S, Campanella G, Panisset JC, Andre P, Masson H, Roy M. Environmental factors in the etiology of Parkinson's disease, Can J Neurol Sci. 1990; 17: 286 – 291.

Zecca L, Gallorini M, Schunemann V, Trautwein AX, Gerlach M, Riederer P, Vezzoni P, Tampellini D. Iron, neuromelanin and ferritin content in the substantia nigra of normal subjects at different ages: consequences for iron storage and neurodegenerative processes.J Neurochem. 2001; 76:1766-1773.

Zecca L, Moussa B, Youdim H, Riederer P, Connor JR, Crichton RR. Iron, Brain Ageing and Neurodegenerative disorders. Nat Rev Neurosci. 2004; 5: 863- 873.

Zecca L, Pietra R, Goj C, Mecacci C, Radice D, Sabbioni E. Iron and other metals in neuromelanin, substaia Nigra and Putamen of human brain. J Neurchem. 1994; 62: 1097-1101.

Zecca L, Shima T, Stroppolo A, Goj C, Battiston GA, Gerbasi R, SarnaT, Swartz HM. Interaction of neuromelanin and iron in substatia nigra and other areas of human brain. Neuroscience. 1996; 73: 407-415.

Zecca L, Zucca FA, Wilms H, Sulzer D. Neuromelanin of the substatia nigra: a neuronal black hole with protective and toxic characteristics. Trends Neurosci. 2003; 26: 578-580.

2. 1. Introduction

Macro and trace elements play a dual role in the biological processes through their interaction with biomolecules. They regulate a number of cellular metabolic reactions, while a few of them act as etiological agents in many environmentally induced neurological disorders [Strong and Garruto, 1994]. Trace elements are required at optimum concentration for proper functioning of the human biological system. Their deficiency would cause various metabolic disorders while elevated levels are toxic [Mertz, 1995]. Elements like Al, Pb, and Cd are highly toxic even in trace amounts and have been reported to be associated with neurological disorders found in man [Crapper Mc Lachlan and DeBoni, 1980; Carrington et al., 1993]. Other studies have shown that trace elements are involved in neuropsychiatric illness [Linter et al., 1985]. Toxic metals like Pb and Cd are increased in depressives and schizophrenics but reduced in manic patients [Stanley and Wakwe, 2002] and Cu, Zn, and Cs deficiencies are seen in women affected by chronic depression [Hansen et al., 1983; Ali et al., 1985]. It has been found that the levels of Mg and Zn are decreased in serum samples of schizophrenia and dementia patients [Srinivasan et al., 1982].

Although pivotal biochemical alterations underlying neuropsychiatric disorders are unknown, changes in trace elements play important roles in the bipolar disorder. For instances V an essential element has been implicated as a causative factor for bipolar mood disorder, and elevation of V and Mo has been reported in serum samples of bipolar mood disorder [Naylor and Smith, 1981; Naylor et al., 1985]. Br is significantly elevated in serum and hair of bipolar patients on Li treatment [Campbell et al., 1986]. A rise in Al and Br levels in the serum samples of Li-treated patients has also been reported

[Campbell et al., 1988; Harvey et al., 1992]. Li is said to alter the levels of elements such as Ca and Mg in serum samples during treatment [Christiansen et al., 1978].

Apart from these data we have limited information on the concentrations of trace elements in serum of individuals with BPD [Naylor et al., 1985]. Moreover, there are no inter-elemental complexity studies pertaining to trace elemental levels in serum samples of three types of BPD. Though the above-mentioned studies have focused on the individual elements, no attempt has been made to understand inter-elemental relationship and trace elemental homeostasis in BPD.

The aim of this study is to examine the levels of eleven elements namely Na, K, S, P, Fe, Mn, Ca, Mg, Zn, Cu and Al in serum samples of bipolar patients type I, II, V and compare them with controls, and also to understand the element homeostasis through element-to-element interrelationship in bipolar disorder. Further, also analysed the few trace metals (Fe, Cu, Al and Zn) in hippocampus and cortical regions of BPD depressive and control human brain regions. The present paper proposes a hypothesis explaining the possible relevance of trace elemental homeostatic imbalance in serum of BPD to possible effects on the brain.

2. 2. PATIENTS AND METHODS

2. 2. 1. Patients

The levels of eleven elements in serum samples of bipolar I (n=40), bipolar II hypomania (n=25), bipolar II depression (n= 27) and bipolar V depression (n=25) were assessed in comparison with a control group (n=25). Blood samples of three types of BPD patients were collected from the Department of Psychiatry, JSS Medical College hospital, Mysore, India. The BPDs are widely classified as bipolar I,

II, III, IV, V and VI [Young and Klerman, 1992]. Blood samples of those patients meeting the diagnostic criteria of manic episode of BPD-I, hypomania, depression of BPD type II and depressives of BPD type V were collected. During sample collection, no patients who meet the diagnostic criteria of the other bipolar disorders, namely III, IV and VI were found. Blood was drawn before medication. The age and sex of the patients and the controls are shown in Table 1. Further this study analysed the few important trace metal like Fe, Cu, Al and Zn from two regions of depressive BPD and control human brain. The description of BPD depressive and control human are brains are given in chapter 4. The age, sex and postmortem intervals of patients and contro group are shown in table 1 from chapter 4.

2. 2. 2. Clinical diagnostic criteria applied for BPD groups

The bipolar patients were clinically assessed and diagnosed according to the D.S.M. IV criteria [American Psychiatric Association, 1994] and Young and Klerman (1992). The BPD type I, BPD type II hypomanic, BPD type II depressives and BPD type V depressives are studied [Fig 1]. The essential feature of bipolar I disorder is a clinical course that is characterized by the occurrence of one or more manic episodes with mixed or major depressive episodes in the past. The essential feature of bipolar II disorder is a clinical course that is characterized by the occurrence of one or more major depressive episodes accompanied by at least one hypomanic episode and no manic episodes. The bipolar II patients were divided into two categories depending on whether they were suffering from hypomania, or from depression. The hypomanic episode is not severe enough to cause marked impairment in social or occupational functioning, or to necessitate hospitalization, and there are no psychotic features. The essential feature of

bipolar V disorder is characterized by the occurrence of one or more episodes of depression without hypomania or mania and with a significant family history of BPD is a well recognized condition [Young and Klerman, 1992].

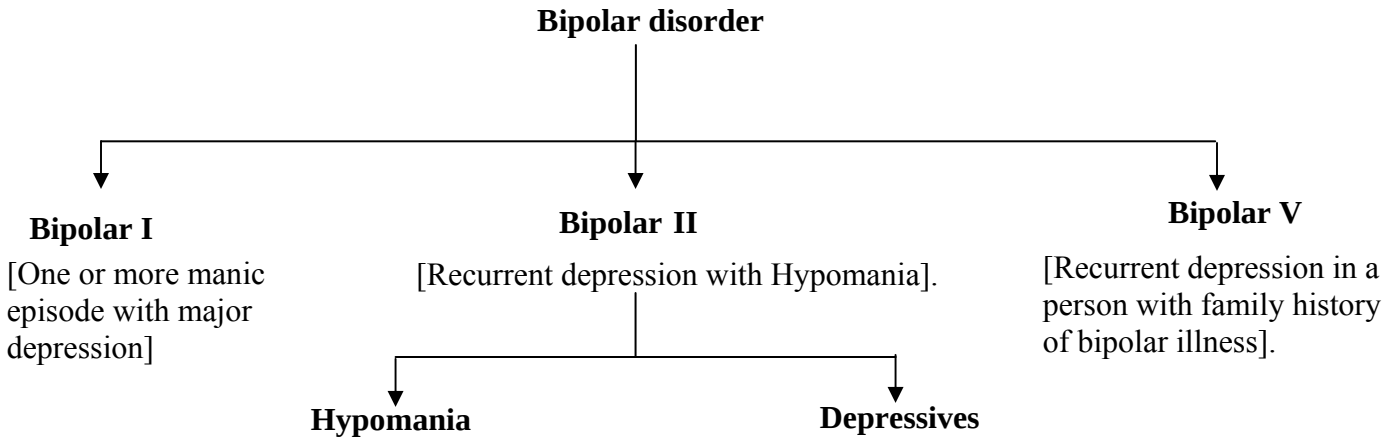


Figure 1. The schematic representation of three different types of bipolar patients undertaken for the current study i.e., BPD I, BPD II and BPD V. BPD II has once again classified into BPD II hypomanic, BPD II depressives depending on the diagnosis at the time of sample collection.

Table 1. Age (shown in range and as mean with standard deviation) and gender of the bipolar patients and control subjects.

	BPD-I	BPD-II hypomanic	BPD-II depressives	BPD- V depressives	Controls
Number	40	25	27	25	25
Age					
Mean ± SD	29.5 ± 6.9	27.3 ± 5.8	31.7 ± 7.6	33.9 ± 8.6	27± 8.1
Range	22.6 – 36.4	21.5 – 33.1	24 – 39	24 - 42	21.9 – 35.1
Gender					
Men	27 (67.5 %)	18 (72%)	13 (48%)	15 (60%)	17(68%)
Women	13 (32.5%)	7 (38%)	14 (52%)	10 (40%)	8 (32%)

2. 2. 3. Control groups

Individuals with no known psychiatric problems served as the control group. The control group comprised 25 "healthy" volunteers having neither significant medical illness nor under medications for at least three months' duration at the time of blood collection. Both controls and bipolar group were assessed based on DSM IV criteria [American Psychiatric Association, 1994].

2. 2. 4. Exclusion Criteria adopted

The following exclusion criteria were applied to the bipolar patients and controls [Fell and Burns, 1981; Triplett, 1985]: (a) ethanol intake higher than 80g/day in the last six months, (b) previous history of chronic hepatopathy or disease causing malabsorption, (c) previous history of severe systemic disease, (d) a typical dietary habits (diets constituted exclusively by one type of food stuff, such as vegetables,

fruits, meat, or others, special diets because of religious reason etc., (e) previous blood transfusions, anemia and polycythemia, (f) intake of supplements of Fe, Cu, Al, Zn or chelating agents, (g) acute infectious disorders, traumatisms or surgery in the last six months, and (i) hemolytic anemia.

2. 2. 5. Ethical issues

Ethical approval for collecting blood and brain samples of bipolar patients and controls were obtained from the Research Ethical Committee of J.S.S. Medical College Hospital, Mysore, India. A written consent was obtained from the patients/guardians prior to the collection of blood samples.

2. 2. 6. Precaution to avoid cross contamination during sample collection and storage

Venous blood sample (10mL) was collected from each bipolar patient/control and serum was separated by centrifugation. Blood collection was done and the serum was separated in a dust free room. The serum was frozen at -20°C and protected from exposure to light until analysis. All the tubes used were polypropylene in nature and no glass material was used to prevent possible metal contamination. All the precautions were taken in accordance with NCCLS criteria [National committee for Clinical Laboratory Standards Approved guidelines, 1997] to eliminate metal contamination while collecting and storing the samples.

2. 2. 7. Trace metal analysis in control and BPD depressive human brain

Ten BPD depressive and respective control human brain samples were collected from the JSS medical college, Myosre. Brain regions such as hippocampus and cortical regions are isolated by neuropathologist under laminar flow hood in dust free

environment using titanium nitride coated instruments. Gloves were powder free and nitric acid pre-treated. Isolated regions were kept under deep freezer -80^{o}C for further metal analysis. Only polyethene material is used in the samples collection and preparation. Samples processing and analysis are done in dust free environment. Ultarpure grade with 18 mega ohm resistance water are used for the analysis.

Sample digestion:

1. Samples are wet weighed in micro balance in dust free environment for ICPAES.
2. Samples are digested in nitric acid using Teflon lined pressure digesting bombs at 140^{o}C in a oven for 4 hrs.
3. The resulting solution in nitric acid are evaporated under low temperature of 50-60^{o}C to near dryness and then ripurtinated with high purity water to final volume (0.1M HNO3) and analyzed by ICPAES.

2. 2. 8. Instrumentation and Elemental Analysis

Elemental analysis was carried out using Inductively Coupled Plasma–Atomic Emission Spectrometry (ICP-AES) model JOBIN YVON 38 sequential analyzer. The elements measured were Na, K, S, Ca, Mg, P, Cu, Fe, Zn, Mn and Al. All dilutions were made with ultra pure milliQ water (18 MΩ-cm Resistance) in dust-free environment. The optimization of ICP-AES was carried out by line selection and detection limits for each element. The validation of the analysis was tested by analyzing serum matrix match multi element synthetic standard and certified standard reference material (Bovine liver 1577a) obtained from National Bureau of Standards, USA[Rajan et al., 1997]. The lines were selected for each element in such a way that interference from the other elements was

minimum. The wavelength used and the detection limit of the elements are summarized in Table 2.

2. 2. 9. Statistical analysis of data

The concentrations of elements were expressed as μmole/mL with mean, standard deviation and p-values (test of significance). The mole percentage (elemental concentration in mole percentage=elemental concentration (μmole/mL)x100/total elemental concentration (μmole/mL) of analyzed elements in each sample) was calculated for the analyzed elements and the relative distribution of these elements based on mole percentage was computed. Mole percentage calculations are essential for understanding the relative distribution of each element in relation to other elements in biological matrix and they also help to normalize the data of different samples in order to arrive at clear inter-element relationship [Rao et al., 1999a]. Element-to-element ratios and correlations were calculated based on mole percentage in order to arrive at possible elemental inter-relationship (direct or inverse) both in control and bipolar patients' serum. The relative charge distribution in terms of single (Na, K), double (Mg, Cu, Zn, Ca) and triple (Al, Fe) charged ions distribution were calculated using the formula:

Sum of concentration of a particular element in μmole per mL x Z x Avogadro's number x 10^{-6} ; where Z is 1,2,3 for single, double, triple charged ions respectively.

All statistical calculations such as inter-relations, correlation coefficients and t-tests were carried out using statistical software packages.

Table 2. Wavelengths used and the detection limits of the elements analyzed.

Element	*Wavelength (nm)*	*Detection limit**	
		μg/mL	μmole/mL
Na	588.995	0.03	0.00130
K	766.49	0.06	0.00153
S	182.98	0.05	0.00156
P	213.618	0.05	0.00162
Ca	393.366	0.002	0.00005
Mg	279.806	0.001	0.00004
Cu	224.7	0.002	0.00003
Zn	213.856	0.002	0.00003
Fe	259.94	0.005	0.00009
Al	396.152	0.002	0.00007
Mn	257.61	0.001	0.00002
Si	251.611	0.08	0.00285

*Detection limit (μg/mL) for each element was calculated by running a multi-element standard solution containing 500ng/mL of each of the above-cited elements.

2. 3. Results

2. 3. 1. Elemental concentration

Trace elemental concentration (μmole/mL) for the control group and bipolar groups (I, II, V) were tabulated (Table 4). The comparative account of trace elements between the control group and bipolar group patients' serum samples revealed the following trends: a) in the bipolar I patients, Na, K, P, Cu, Al and Mn were elevated significantly ($p<0.001$), b) in bipolar II hypomania, Na, Mn, and Al were increased significantly ($p<0.01$), c) in bipolar II patients suffering from depression, Na, K, Cu, and Al were increased significantly ($p<0.001$) and d) in bipolar V depressive patients Na, Mg, P, Cu and Al were increased significantly ($p<0.001$). But elements namely S, Fe, and Zn

levels were decreased significantly ($p<0.001$) in all three bipolar types. Further, bipolar I (manic) have higher concentrations of Cu, Al, and Mn than bipolar II hypomania. The total elemental concentration (μmole/mL) was higher ($p<0.04$) in the bipolar groups serum compared to the control group indicating possible imbalance in elemental homeostasis. The data generated in the present study on elemental concentration in control human subjects were compared with the reference elemental values in serum from the 'Handbook on metals in clinical and analytical chemistry [Christensen and Kristiansen, 1994] and also from Muniz et al., (1999); Iyengar, (1989) in Table 3.

2. 3. 2. Trace metal concentration in Brain

The distribution of trace metals and their concentrations in two different regions of the BPD depressive human and age matched control brain is shown in Table 3. The present study the concentration of Al, Fe and Cu are increased significantly ($p<0.002$) but Zn level is reduced significantly ($p<0.01$) in frontal cortex. However, the levels of Al, Fe, Cu and Zn were not changed significantly in hippocampus of BPD depressive brain compared to control subjects.

Table 3. Trace metals concentrations in two different regions of humn BPD depressive and control brains. (Concentration in μg/g of wet weight of tissue) mean ± SD of ten brains.

Brain regions	Trace metals	Control subjects (N=10)	BPD depressive (N=10)
Frontal cortex	Cu	4 .0 ± 1.7	8.0 ± 1.5*
	Al	58 0± 20	113 ± 30*
	Fe	50.8 ± 13.6	75 ± 15.5*
	Zn	7.5 ± 0.5	4.5 ± 0.4**
Hippocampus	Cu	4.0 ± 1.0	4.5 ± 1.5
	Al	45 ± 16	47 ± 14
	Fe	26.6 ± 7.6	24.6 ± 8
	Zn	6.5 ± 0.5	5.5 ± 0.2

Statistically significant *< p< 0.002; **p< 0.01

Table 4. Trace element concentrations (μmole/mL) in serum samples of control and BPD.

Elements	Reference Values#	Control (N=25)	BPD type I (N=40)	% Change	BPD type II				BPD type V (N=30)	%Change
					Hypomania (N=25)	%Change	Depression (N=25)	%Change		
Na	134.78	133.53 ±7.5	153.57±17.9*	15	156.11±6.5***	17	150.83±9.5***	13	142.17±10***	6.47
K	4.09	3.27 ± 0.8	4.28 ± 1.44**	31	4.09±0.4	25	4.37±0.7**	33.6	3.73 ±0.31	4.06
S	NA	36.63 ± 3.6	31.79± 5.5*	-13	34.40±2.8*	-6	33.86±3.1*	-8	31.37±4.51**	-14
Ca	2.20	2.26 ±0.3	2.37± 0.36	5	2.56±0.1	13	2.46±0.2	9	2.42±0.15	7.07
Mg	0.82	0.93 ±0.1	1.08± 0.14	16	1.04±0.1	12	1.03±0.1	11	1.03±0.09***	10.7
P	3.55	3.16± 0.4	3.77± 0.67*	19	3.45±0.4	9	3.78±0.6	20	3.91±0.61**	23.7
Fe	0.021	0.02±0.01	0.0195± 0.01***	-15	0.020±0.01	-18	0.018±0.01***	-22	0.0153±0.01**	-35
Cu	0.016	0.013±0.004	0.023± 0.01*	77	0.020±0.002	54	0.020±0.01***	54	0.020±0.01**	53.8
Zn	0.013	0.0086±0.001	0.008± 0.002***	-45	0.0088±0.003	2	0.0077±0.001**	-11	0.0071±0.002**	-17
Al	0.00018	0.00050±0.001	0.0040± 0.001*	578	0.0010±0.0003**	100	0.0012±0.001*	140	0.0012±0.0004**	140
Mn	NA	0.00031±0.0001	0.002±0.001*	486	0.0009±0.00004*	190	0.00029±0.0001	-7	0.0003±0.0001	-3
Total		179.82	196.91		201.71		196.38		184.68	

NA = Not available.

Statistically significant $^{*}p<0.0001$; $^{**}p<0.001$; $^{***}p<0.01$

Reference values from handbook on metals in clinical and analytical chemistry. (Christensen and Kristiansen, 1994; Muniz et al., 1999)

Reference values for Na and K are from G.V. Iyengar "Elemental Analysis of Biological Systems. 1989.

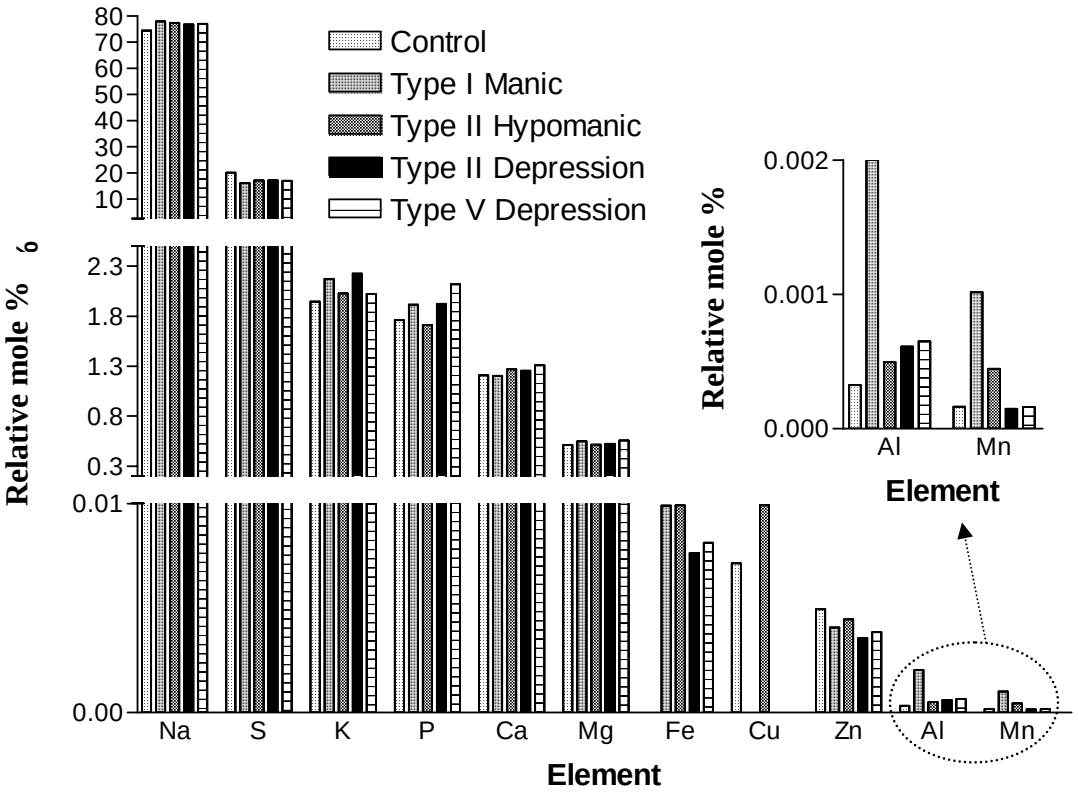


Figure 2. Comparison of relative mole percentage of elements in control and type I type II and type V - BPD human serum samples

2. 3. 2. Relative Mole percentage and Mole percentage ratios

Relative mole percentage was calculated in order to normalize the concentration levels (μmole/mL) of each element in a sample of total elements analyzed. The relative distribution is represented [Fig. 2]. The mole percentage data indicates that Fe, S and Zn levels were higher in control group serum compared to bipolar groups' serum. The levels of Na, K, Cu, P, Al and Mn were increased in bipolar I. The levels of Na, Al and Mn

increased in bipolar II hypomanic and Na, K, Cu and Al are increased in bipolar II depression. The levels of Mg, P, Cu, Al levels were increased in bipolar V. Further, the levels of Cu, Al, and Mn were high in bipolar I (manic) compared to bipolar II and V. However, the levels of Fe were decreased in all three bipolar types. The data are expressed in terms of element-to-element mole percentage in control, bipolar I, bipolar II, bipolar V serum samples (Table 5). These ratios may reflect the inter-relationship of elements in biological system. The element- to-element mole ratios namely, Na/Fe, K/Fe, Ca/Fe, Mg/Fe, Cu/Fe, and Cu/Zn were increased in serum samples of all three types of bipolar groups. Al/Fe, Al/Zn, ratios were increased in bipolar I (Manic patients) serum samples. The ratio of Al/Fe and Al/Zn was higher in bipolar I (mania) compared to others. The ratio of Al/Mn is lower in bipolar II (hypomania) compared to control. We observe a pattern in element-to-element displacement in BPDs compared to control.

Table 5. Element to element mole percentage ratio in serum samples of control, BPD-I, BPD-II and BPD- V.

Element Ratio	*Normal*	*BPD-I (Manic)*	*BPD-II (Hypomanic)*	*BPD-II (Depressives)*	BPD-V (Depressives)
Na/Fe	5674	7782	7310	8374	*9172*
K/Fe	139	213	200	242	*241*
Ca/Fe	96	120	120	136	*156*
Mg/Fe	39	55	49	57	*66*
Cu/Fe	0.55	1.17	0.82	1.13	*1.28*
Cu/Zn	1.52	2.83	2.02	2.62	*2.78*
Al/Fe	0.021	0.20	0.05	0.07	*0.08*
Al/Zn	0.057	0.49	0.12	0.15	*0.16*
Al/Mn	1.61	2.21	1.19	4.07	*3.98*

2. 3. 3. Inter Elemental Correlations: An insight into elemental homeostasis

Correlation coefficients were calculated using the concentration micromoles of elements of control group and bipolar groups. The results showed a distinct pattern of

direct and inverse correlation (Table 6). The direct relation (confidence level 95–99.7%) is observed between K vs (S, Ca, Mg, Fe, Cu), S vs (Ca, Mg, Fe), Ca Vs (Mg, Fe), and Mg vs Fe in serum samples of control group. The inverse correlation (confidence level 95%-99.7%) is also observed between Na vs (S, Cu, K, Ca, Mg, Fe), K vs (P, Zn), and P vs (Fe, Zn) in serum samples of control group. In bipolar I serum samples direct correlation (confidence level 95%-99.7%) is seen between K vs Zn, S vs Ca, Mg vs (Cu, Zn), P vs Fe and inverse correlation observed between Na vs (S, K, Ca) and K vs Al. In the serum samples of bipolar II hypomania direct correlation (95%-99.7% confidence level) is observed between Ca vs (Mg, Fe), P vs (Fe, Mn) and inverse correlation is seen between Na vs (S, Mn), K vs Al, S vs (Ca, Mg, Al), Ca vs Zn, Fe vs Zn, whereas the direct correlation (95% -99.7% confidence level) is observed between Ca vs (Mg, Fe), Fe vs Zn and an inverse correlation is seen between Na vs S, S vs Al in the serum samples of bipolar II depression. In the serum samples of bipolar V direct correlation is observed (95%-99.7% confidence level) between Na vs Cu, K vs (Ca, Cu), Ca vs (Mg, Fe), Mg vs Fe,P vs Mn, and inverse relation is seen between Na vs S, K vs S, S vs (Mg, Cu), Ca vs Al, P vs Zn, Cu vs (Al, Zn). Overall few correlation coefficients differ from bipolar groups to control group. The direct correlation is seen between K vs Zn and P vs Fe in serum samples of bipolar I, but in control group serum samples K vs Zn and P vs Fe show an inverse relation. In bipolar II hypomania direct relation is observed between P vs Fe whereas in control serum it is in inverse relation. In bipolar V depressives Na vs Cu shows direct correlation while in control it is an inverse relation. Inverse relation is observed between K vs S and S vs Mg in serum samples of bipolar V but it shows direct relation in the control group.

Table 6. Comparison of inter-elemental relations between controls, BPD types I, II and V.

Correlation between elements		Correlation co-efficient				
1	2	Control (n=25)	BPD I (n=40)	BPD II Hypomanic (n=25)	BPD II depression (n=25)	BPD V (n=30)
Na	S	-0.97*	-0.95*	-0.97*	-0.96*	-0.97*
Na	Cu	-0.46**	NS	NS	NS	+0.57**
Na	Mn	NS	NS	-0.66*	NS	NS
Na	K	-0.70*	-0.32**	NS	NS	NS
Na	Ca	-0.63*	-0.37**	NS	NS	NS
Na	Zn	NS	-0.31**	NS	NS	NS
Na	Mg	-0.52**	NS	NS	NS	+0.36**
Na	Fe	-0.59**	NS	NS	NS	NS
K	S	+0.53**	NS	NS	NS	-0.39**
K	Ca	+0.58**	NS	NS	NS	+0.50**
K	Mg	+0.52**	NS	NS	NS	NS
K	P	-0.46**	NS	NS	NS	NS
K	Fe	+0.65*	NS	NS	NS	NS
K	Cu	+0.70*	NS	NS	NS	+0.43**
K	Zn	-0.46**	+0.42**	NS	NS	NS
K	Al	NS	-0.46**	-0.54**	NS	NS
S	Ca	+0.54**	+0.33**	NS	NS	NS
S	Mg	+0.40**	NS	NS	NS	-0.43**
S	Fe	+0.52**	NS	NS	NS	NS
S	Cu	NS	NS	NS	NS	-0.63*
S	Al	NS	NS	-0.50**	-0.45**	+0.33
S	Mn	NS	NS	+0.58**	NS	NS
Ca	Mg	+0.77*	NS	+0.40**	+0.42**	+0.54**
Ca	Fe	+0.74*	NS	+0.43**	+0.57**	+0.54**
Ca	Al	NS	NS	NS	NS	-0.48**
Ca	Zn	NS	NS	-0.66*	NS	NS
Mg	Fe	+0.43**	NS	NS	NS	+0.55**
Mg	Cu	NS	+0.49**	NS	NS	NS
Mg	Zn	NS	+0.33**	NS	NS	NS
P	Fe	-0.61*	+0.39**	+0.54**	NS	NS
P	Zn	NS	NS	NS	NS	-0.49**
P	Mn	NS	NS	+0.57**	NS	+0.61*
Cu	Al	NS	NS	NS	NS	-0.62*
Cu	Zn	NS	NS	NS	NS	-0.39**
Fe	Zn	NS	NS	-0.81*	+0.38**	NS

Note: Confidence levels: *>99.7%, **>95%, the expected correlation co-efficient for sample size of 25 are 0.613 and 0.396 respectively. Positive and negative signs indicate direct and inverse correlation respectively.
NS = Not significant; BPD = bipolar disorder.

2. 3. 4. Charge distribution

The relative percentage charge distribution in control; bipolar I, bipolar II hypomania, bipolar type II depression and bipolar V were computed (Fig.3). The triple charged ions were decreased in bipolar II and bipolar V. The single charged ions (Na, and K) and double charged ions (Mg, Ca, Zn and Cu) increased marginally in bipolar I and II while there was no change in bipolar V. Fe charge has been used as +3 in bipolar serum samples as we do not have evidence either from literature or our own data that Fe exits as Fe^{2+} in serum, unlike in the brain [Rao et al., 1999a].

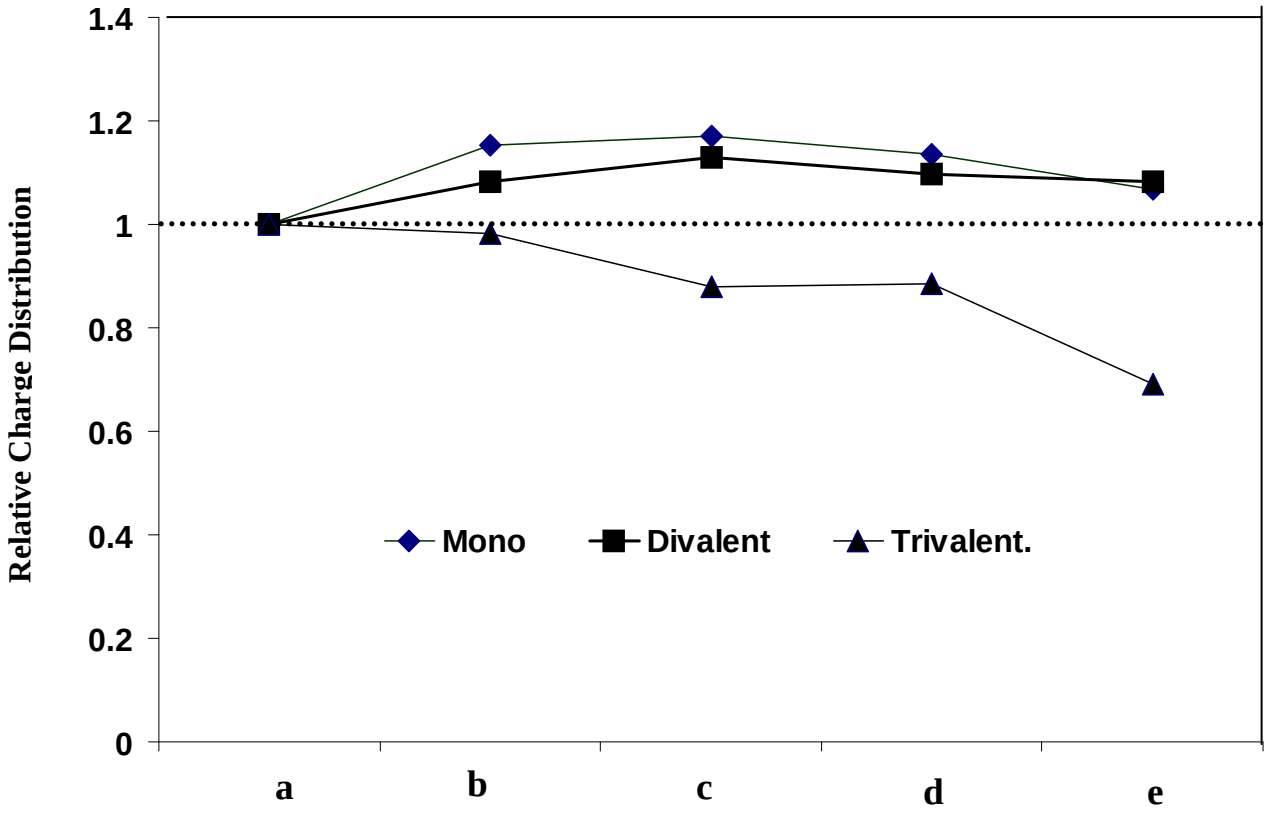


Figure 3. The relative charge distribution in bipolar disorders with respect to control; a: Control; b: BPD-I; c: BPD- II hypomanic; d: BPD- II depressives; e BPD- V depressives. The dotted line represents the relative charge distribution for control subjects.

2. 4. Discussion

Macro and trace elements are significant either as causative or therapeutic factors in neurological and neuropsychiatric disorders [Linter et al., 1985]. The first breakthrough providing evidence of the therapeutic potential of trace elements in neuropsychiatry came with the use of Li in manic-depressive psychosis [Campbell et al., 1988; Harvey et al., 1992]. A similar therapeutic role has been proposed for Rb in an affective illness [Fieve and Jamison, 1982]. V and Al have been implicated as causative factors in bipolar mood disorder and dementia respectively [Naylor and Smith, 1987; Alfrey et al., 1976]. Studies show that like Li, Mg also has therapeutic potential; it damages Ca^{2+}-PKC-related neurotransmission and Na-K-ATPase enzymes implicated in the pathophysiology of depression [Merck, 2002].

The data generated in the present study on elemental concentration in control human subjects matched with the reference elemental values in serum [Christensen and Kristiansen, 1994; Muniz et al., 1999; Iyengar, 1989]. However the same cannot be said of Al. The normal value for Al in serum sample appears to be an open question. Though it is agreed that Al level should be less than 0.005µgram/mL in control human serum, in many studies the values of around 0.010-0.020µgram/mL has been reported. The Al level in our present investigation for control human serum was 0.016µg/mL and it is well within the range of earlier reported values.

Our results show increased Cu level in three types of BPDs (I, II and V). Naylor et al., (1985) also showed a significant increase of Cu in the serum of manic patients. However, they observed no significant change in Na, S, Mg, Ca, Mn and Al in manic-depressive patients, while our results indicate a significant increase in Na, Mn and Al in

manic and hypomanic patients and no change in Mn levels in case of bipolar II depression and bipolar V. Our results also demonstrate decreased Zn levels and increased Cu levels in three types of bipolar groups. Other studies have reported a similar trend in schizophrenic and major depressive patients [Alias, 1972; Pfeiffer and Ilier et al., 1972; Olatunbosun et al., 1975; Diebel and Ehmann, 1996]. Cu is an essential element for the activity of a number of physiologically important enzymes such as cytochrome C oxidase, dopamine-beta-hydroxylase, and Cu/Zn superoxide dismutase, which is critical in scavenging reactive oxygen species. Malfunctions related to any of these enzymes due to lack of Cu may contribute to several neurological symptoms and diseases [Strausak, et al., 2001]. In addition, high concentrations of Cu also influence the generation of reactive oxygen species leading to Cu-mediated oxidative stress which will affect several intracellular alterations and contribute to the induction of cell death pathway [Carri et al., 2003].

Our results show higher levels of Al and lower levels of Fe in the serum of three bipolar types compared to control group. Interestingly, Van Rensburg *et al*. (2001) have shown Al to be significantly high and Fe to be low in serum of patients suffering from chronic fatigue syndrome which affects cognition and influences neurological abnormalities. The low levels of Fe in serum that we observed could indicate high levels of the same in the brain, as has been suggested earlier [Rajan *et al*., 1997; Rao *et al*., 1999a; 1999b]. The brain, unlike other organs, requires a constant supply of Fe and diminished supply could result in neurological and cognitive dysfunction [Thompson et al., 2001]. Conversely, the brain has also developed mechanisms to maintain a constant supply, resulting in the accumulation of Fe, which may lead to iron-induced oxidative

stress and loss of metal homeostasis, so characteristic of neurodegenerative disorders. Our data indicate high levels of Mn in bipolar I manic and II hypomanic. Mn toxicity has been shown to be associated with mitochondrial dysfunction and DNA fragmentation in rat primary striatal neurons [Malecki, 2001].

In the present investigation we observed that total concentrations of trace elements are relatively higher in the bipolar groups than the control group, indicating imbalance in the trace elemental homeostasis. We believe that elevated Al level causes imbalance in trace elemental homeostasis pool resulting in an increase in the levels of Na, K, P, Cu and Mn and decrease in the levels of Fe, S, and Zn in the serum of the bipolar groups. The effect of an increase or decrease in a single element concentration is not restricted to this element alone, but the total element distribution pattern in the system will be affected. Thus interdependency in the concentration of certain elements to maintain homeostasis of trace elements pool which is apparent from our results seems to be crucial in a biological system.

Most of the studies conducted so far were primarily limited to trace elemental level only in neuropsychiatric disorders, and no attempt has thus far been made to understand the element-to-element interrelationship in BPDs. This report is to our knowledge, the first that shows the inter-elemental relations among elements in bipolar groups serum samples in comparison to a control group. From our analysis of the trace elemental concentration and element-to-element interrelationship in three types of bipolar patients in comparison with the control group, a definite correlation pattern is observed. Further, our data expressing element-to-element mole percentage ratios in control, bipolar

I, bipolar II, bipolar V serum samples provide an insight into the interrelationship of elements in biological system as have also been shown earlier [Rajan *et al*., 1997].

Our results from the element-to-element ratio and correlation patterns indicate that there is a definite imbalance in the trace elemental homeostasis and element-to-element inter-relationship pattern in serum of bipolar groups compared to controls. The relative percentage charge pattern of bipolar group is different from the control group. It has been shown that element-to-element mole ratio provides clues to the possible inter-relationships of metals in the neurobiological system [Rajan *et al*., 1997; Rao *et al*., 1999a; Van Rensburg *et al*., 2001]. The high Cu/Zn ratios in BPDs that we observed may result in systemic oxidant burden as shown earlier [Mazetti *et al*., 1998]. However, more significantly, Johnson (2001) has demonstrated that the developing fetus of pregnant women who have low Zn and high Cu might experience major difficulties in the early development of the brain, which may later manifest into mental illness.

Based on our new findings and earlier literature sources, we developed a hypothetical model to explain the possible relevance of macro and trace element homeostatic imbalance in serum of BPD to possible effects in brain (Fig 4). We propose two pathways: pathway I propose that increased Al levels in serum of BPD is likely to alter the trace elemental homeostasis pool. We suggest that irrespective of elements being primary risk factors or consequences of disease mechanism, a change in an individual metal ion will upset the elemental homeostasis pool resulting in a significant imbalance in elemental levels and charge distribution pattern in the biological system.

The element-to-element mole ratio of Al/Fe and Al/Zn for instance is increased because of the high concentration of Al present and it alters other elemental levels.

Elevated Al may disturb metal homeostasis in serum by increasing the paramagnetic oxidant elements like Cu and Mn while decreasing Zn, an antioxidant metal required for the production of CuZn-SOD and Zn-thionein which are essential to prevent oxidative damage. Elevated Al is found to increase superoxide dismutase (SOD) activity to protect the cell from oxidative damage. Kuloglu *et al*., (2002) showed that SOD activity levels are higher in BPD serum with the presence of lipid peroxidation also being reported. Increased Al may therefore be one of the reasons for high SOD activity in BPD. Elevated redox metals Cu and Mn levels in serum may catalyze the conversion of H_2O_2 to potent hydroxyl radical, which could lead to oxidative damage.

In pathway II, the elements in the serum possibly reflect the brain elemental homeostasis. Previous studies have shown that Al and Fe are decreased in cerebrospinal fluid (CSF) while they are elevated in the brain [Van Rensburg et al., 2001]. Al is known to be co-transported with Fe-transferrin complex in neurological disorders [Rao et al., 1999a]. In a normal brain Fe and Al compete for transport across the blood brain barrier [Rajan et al., 1997] with Al crossing the blood brain barrier with the help of ferritin. Al not only promotes Fe-mediated oxidative stress by inhibiting catalase activity in the brain, but also causes mitochondrial dysfunction leading to oxidative stress and neuron dysfunction [Savory et al., 1999]. It has also been implicated in the degeneration of cholinergic terminals in cortex and hippocampus [Platt et al., 2001]. The present result also detected high level of Fe, Al, and Cu in the frontal cortex of the BPD depressive brain compared to control. However the Zn levels were observed to be low in this region of the BDP depressive compared to human control brains. This may be one of the reasons for the presence of high oxidative stress in bipolar disorders.

These pathways suggest that trace elemental imbalances in BPD serum may cause imbalances in trace elemental levels in the brain which may lead to oxidative stress and damage biomolecules like DNA, lipids, and proteins. This may be the reason for alteration in the neurotransmitter receptors and the levels of secondary messenger in the BPD brain. Thus imbalance of the elemental homeostasis may play a role in etiology of BPD. Certain metals such as Zn, Al and Pb have been implicated in the aggregation of α-synuclein, a crucial protein in PD [Yamin *et al*., 2003], while others such as Cu, Fe and Zn are involved in the precipitation and aggregation of beta-amyloid peptide in AD [Bush, 2003; Kawahara, 2001]. Similar metal-ion-biomolecular related mechanisms could underlie the etiology of other neurological disorders like BPD. It is an agreed upon fact that a combination of environmental metal pollution and oxidative stress play an important role in triggering neurodegeneration [Rao and Balachandran, 2002].

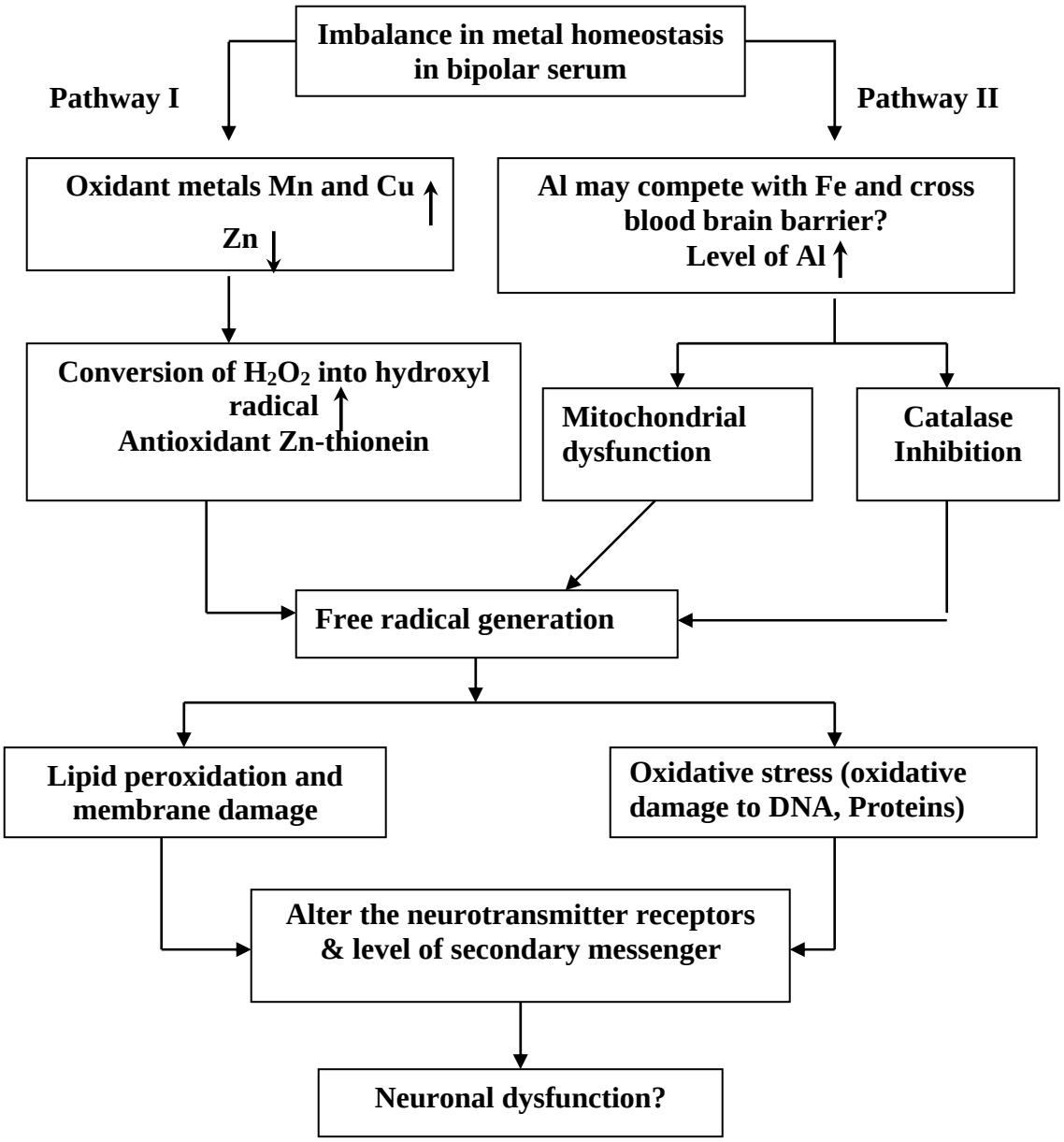


Figure 4. Possible pathways for neuronal dysfunction associated with bipolar disorders due to imbalance in metal homeostasis. The illustration advocates imbalance in trace elemental homeostasis leading to oxidative stress as prime risk factor for behavioral and neuronal change seen in neurological disorders like bipolar disorder.

Note: The upward and downward arrow marks indicating increasing and decreasing trend

In this scenario, our data suggest that elemental homeostatic imbalance results in the imbalance of biochemical events and oxidative stress in BPD, which may later manifest into neurodegeneration. This has been shown by Campbell et al. (2001) who suggest that redox active metals may be involved in neurodegeneration. This hypothesis is supported by other studies: PET scanning [Bier et al., 2002] showed that depression might cause neurodegeneration, while presence of neuritic pathology has been confirmed in patients having BPD [Buhl and Bojsen-Moler, 1988]. On the other hand, Damadzic *et al*., (2002) report no neuritic pathology in the entorhinal cortex, subiculum and hippocampus in middle-aged adults with schizophrenia, BPD or unipolar depression. Neuritic pathology may be the final onset phase of neurodegeneration, with the initial phases being neuropsychiatric phenomena involving biochemical and brain function alterations. We have an evidence of human brain DNA fragmentation in BPD (in chapter IV), but clearly more studies are required to conclusively link neuropsychiatry to neurodegeneration.

2. 5. References

Alfrey AC, LeGendre GR, Kaehny WD. The dialysis encephalopathy syndrome possible aluminium intoxication. N Engl J Med. 1976; 294: 184 -188.

Ali SA, Peet M, Ward NI. Blood levels of vanadium, cesium and other elements in depressive patients. J Affec. Disrd. 1985; 9: 187-191.

Alias AG. Serum ceruloplasmin in schizophrenia: significant increase in acute cases especially in catatonia. Biol Psychiatry. 1972; 4: 231-238.

American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders, 4th Ed. Washington, DC, American Psychiatric Association, 1994.

Bier JC, Fery P, Elincx S, Claes T, Ventura M, Goldman S. Depression may mimic the clinical and metabolic patterns of degenerative disease. Alz Rep. 2002; 5: 41- 44.

Buhl L, Bojsen-Moler M. Frequency of Alzheimer's disease in a postmortem study of psychiatric patients. Dan Med Bull. 1988; 35: 288-290.

Bush AI. The metallobiology of Alzheimer's disease. Trends Neurosci. 2003; 26: 207-214.

Campbell A, Smith MA, Sayre LM, Bondy SC, Perry G. Mechanisms by which metals promote events connected to neurodegenerative diseases. Brain Res Bull. 2001; 55:125-132.

Campbell CA, Peet M, Ward NI. Vanadium and Other Trace Elements in Patients Taking Lithium. Biol Psychiatry. 1988; 24: 775-781.

Campbell CA, Ward NI, Peet M. Increased bromide levels in serum and hair during lithium treatment. J Affec Disrd. 1986; 11: 161-164.

Carri MT, Ferri A, Cozzolino M, Calabrese L, Rotilio G. Neurodegeneration in amyotrophic lateral sclerosis: the role of oxidative stress and altered homeostasis of metals. Brain Res Bull. 2003; 61: 365-374.

Carrington CD, Sheehan DM, Bolger PM. Hazard assessment of lead. Food Addit. Contamin. 1993; 10: 325-335.

Christensen JM, Kristiansen J. Lead. In: Seiler HG, Siegel A, Sigel H, (Eds), Hand book on metals in clinical and analytical chemistry. Marcel Dekker, New York, 1994, pp 217-667.

Christiansen C, Baastrup PC, Lindgreen P, Transbol I. Endocrine effects of Lithium: II 'primary' hyperparathyroidism. Acta Endocrinol. 1978; 88: 528-534.

Crapper Mc Lachlan DR, DeBoni U. Aluminium in human brain disease-An overview. Neurotoxicology. 1980; 1: 3-16.

Damadzic R, Shuangshti S, Giblen G, Herman MM. Neuritic pathology is lacking in the entorhinal cortex, subiculum and hippocampus in middle-aged adults with schizophrenia, bipolar disorder or unipolar depression. Acta Neuropathol. 2002; 103: 488-494.

Diebel MA, Ehmann WD, Markesbery WR. Copper, iron and zinc imbalances severely degenerated brain regions in Alzheimer's disease: possible relation to oxidative stress. J Neurol Sci. 1996; 143: 137-142.

Fell GS, Burns RR. Zinc and other trace elements. Adv Parent Nutr. 1981; 16: 241-261.

Fieve RR, Jamison KR. Rubidium. Overview and clinical perspectives. Mod Probl Pharmacopsychiatry. 1982; 18:145-163.

Garruto RM, Flaten TP, Wakayama I. Natural and experimental models of environmentally induced neurodegeneration: Implication for Alzheimer's disease. In: Corain B, Wisneiwski H, Zatta P (Eds), Alzheimer's disease: Advances in Clinical and Basic research. John Wiley and Sons Ltd, 1993, pp257-266.

Hansen CR, Malecha M, Mackenzie TB, Kroll J. Copper and Zinc deficiencies in association with depression and neurological findings. Biol Psychiatry. 1983; 18: 395- 401.

Harvey NS, Jarratt J, Ward NI. Trace elements and the Electroencephalogram during long-term lithium treatment. Br J Psychiatry. 1992; 160: 654-658.

Iyengar GV. Elemental Analysis of Biological system, CRC press, Boca Raton. 1989; pp 242.

Johnson S. Micronutrient accumulation and depletion in schizophrenia, epilepsy, autism and Parkinson disease? Med Hypotheses. 2001; 56: 641-645.

Kawahara M, Kato M, Kuroda Y. Effects of aluminium on the neurotoxicity of primary cultured neurons and on the aggregation of beta-amyloid protein. Brain Res Bull. 2001; 55: 211-217.

Kuloglu M, Ustundag B, Atmaca M, Canatan H, Tezcan AE, Cinkilinc N. Lipid peroxidation and antioxidant enzyme levels in patients with schizophrenia and bipolar disorder. Cell Biochem Funct. 2002; 20: 171-175.

Linter CM. Neuropsychiatric aspects of trace elements. Br J Hosp Med. 1985; 34: 361-365.

Malecki EA. Manganese toxicity is associated with mitochondrial dysfunction and DNA fragmentation in rat primary striatal neurons. Brain Res Bull 2001; 55: 225-228.

Mazetti A, Pierdomenico SD, Costantini F, Romano F, De Cesare D, Cuccurullo F, Imbastaro T, Riario-sforza G, Di Giacomo F, Zuliai G, Fellin R. Copper/Zinc ratio and systemic oxidant load: effect of aging and aging –related degenerative diseases. Free Radical Biol Med. 1998; 25: 676-681.

Mertz W. Risk assessment of essential trace elements: New approaches to setting recommended dietary allowances and safety limits. Nutrit Rev. 1995; 53: 179 – 185.

Muniz CS, Marchante-Gayon JM, Alonso JIG, Sanz–Medl A. Multi elemental trace analysis of human serum by double focusing ICP-MS. J Anal Atomic Spectr. 1999; 14: 193-198.

Murck H. Magnesium and affective disorders. Nutr Neurosci. 2002; 5: 375-389.
National committee for Clinical Laboratory Standards Approved guidelines.

*Control of pre-analytical variation in trace element determination.*1997; 17: 1-30.
Naylor GJ, Smith AH. Vanadium a possible etiological factor in manic-depressive illness. Psycho Med. 1981; 11: 249- 256.

Naylor GJ, Smith AHW, Bryce-Smith D, Ward NI. Trace elements in Manic Depressive Psychosis. J Affec Disrd. 1985; 8: 131-136.

Olatunbosun DA, Akindele BK, Adadevoh BK, Asuni T. Serum Copper in schizophrenia in Nigerians. Br J Psychiatry. 1975; 127: 119-121.

Pfeiffer CC, Ilier UA. Study of Zinc deficiency and copper excess in the schizophrenias. Int.Rev. Neurobiol. 1972; 1: 141-165.

Platt B, Fiddler G, Riedel G, Henderson Z. Aluminium toxicity in the rat brain: histochemical and immunocytochemical evidence. Brain Res Bull. 2001; 55: 257-267.

Rajan MT, Rao KSJ, Mamatha BM, Rao RV, Shanmugavelu, P Menon RB, Pavithran MV. Quantification of trace elements in normal human brain by inductively coupled plasma atomic emission spectrometry. J Neurol Sc. 1997; 146: 153-166.

Rao AV, Balachandran B. Role of oxidative stress and antioxidants in neurodegenerative diseases. Nutr Neurosci. 2002; 5:291-309.

Rao AV, Balachandran B. Role of oxidative stress and antioxidants in neurodegenerative diseases. Nutr Neurosci. 2002; 5: 291-309.

Rao KSJ, Rao RV, Shanmugavelu P, Menon RB. Trace elements in Alzheimer's disease brain: A new hypothesis. Alz Rep. 1999a; 2: 241-246.

Rao KSJ, Shanmuga velu P, Shankar SK, Rukmani Devi RP, Rao RV, Pande S, Menon RB. Trace elements in the cerebrospinal fluid in Alzheimer's disease. Alz Rep. 1999b; 2: 4 - 9.

Savory J, Rao KSJ, Huang Y, Letada PR, Herman MM. Age- Related Hippocampal Changes in Bcl-2: Bax Ratio, Oxidative Stress, Redox-Active Iron and Apoptosis Associated with Aluminum-Induced Neurodegeneration: Increased Susceptibility with Aging. Neurotoxicology. 1999; 20: 805-817.

Srinivasan DP, Marr S, Wareing RA, Birch NJ. Magnesium, zinc and copper in acute psychiatric patients. Mag Bull. 1982; 4: 45-48.

Stanley PC, Wakwe VC. Toxic trace metals in the mentally ill patients. Niger Postgrad Med J. 2002; 9: 199-204.

Strausak D, Mercer JF, Dieter HH, Stremmel W, Multhaup G. Copper in disorders with neurological symptoms: Alzheimer's, Menkes, and Wilson diseases. Brain Res Bull. 2001; 55:175-185.

Strong MJ, Garruto RM. Experimental paradigms of motor neuron degeneration. In: Woodruff ML, Nonneman AJ (Eds), Toxin-induced models of neurological disorders. Plenum Press, New York, 1994, pp39-88.

Thompson KJ, Shoham S, Connor JR. Iron and neurodegenerative disorders. . Brain Res Bull. 2001; 55:155-164.

Triplett WC. Clinical aspects of Zinc, Copper, manganese, chromium and selenium metabolism. Nutr Int. 1985; 1: 60-67.

Van Rensburg SJ, Potocnik FC, Kiss T, Hugo F, van Zijl P, Mansvelt E, Carstens ME, Theodorou P, Hurly PR, Emsley RA, Taljaard JJ. Serum concentrations of some metals and steroids in patients with chronic fatigue syndrome with reference to neurological and cognitive abnormalities**.** Brain Res Bull. 2001; 55: 319-325.

Yamin G, Glaser CB, Uversky VN, Fink AL. Certain metals trigger fibrillation of methionine-oxidized alpha-synuclein. J Biol Chem. 2003; 278: 27630-27635.

Young RC, Klerman GL. Mania in late life: focus on age at onset. Am J Psychiatry. 1992; 149: 867-876.

3A 1. Introduction

Aluminium (Al) is the third most abundant element in earth's crust. It is not an essential trace element for human and animals system and has never been demonstrated to have any definite biological function, suggesting that Al possesses properties which are incompatible with fundamental life processes. It is usually excluded from normal biochemical and metabolic processes because of its low solubility and chemical speciation, namely silicates, phosphates and oxides, which render Al physiologically unviable. Abnormal uptake and accumulation of Al in human has been linked to a number of diseases including vitamin- D- resistant osteomalacia, iron adequate microcytic anemia and dialysis dementia [Corain *et al*., 1996] and also various neurodegenerative disorders like Alzheimer's disease, Parkinson's diseases and amyotrophic lateral sclerosis [Perl *et al*., 1982; Garruto *et al*., 1986; Savory *et al*., 1996; Rao *et al*., 1999; Gupta *et al.,* 2005].

AD is one of the devastating progressive neurodegenerative disorders with complex multiple etiologies. The suspected etiology of AD includes genetic factors, infectious agents, and environmental toxins. Recent studies on experimental animals as well as circumstantial human evidences suggest that Al neurotoxicity might be one of the suspected factors causing neuronal cell death in neurodegenerative disorders [Alfrey, 1989; Martin *et al*., 1989; Rao *et al*., 1998; Ghiribi *et al.,* 2002a and 2002b; Savory *et al.,* 2003, Griffioen *et al.,* 2004; Walton, 2006]. Although linking of Al to AD is debated, the relationship between Al neurotoxicity and AD is not ruled out [Gupta *et al.,* 2005]. Recently our lab demonstrated a left handed Z-DNA conformation in severely affected AD brain hippocampus cells and moderately affected AD exhibited B-Z intermediate DNA conformation; whereas DNA exhibited B-DNA conformation in hippocampus

region of normal brain [Anitha *et al*., 2002]. Based on these findings, it was hypothesised that metals like Al and Fe might play a pivotal role in modulating DNA topology in AD brain [Anitha *et al.,* 2002]. An evidence for the nuclear localization of Al in AD brain had been well established [Crapper *et al.,* 1980; Perl and Brody, 1980; Lukiw *et al*., 1992; Walton *et al.,* 2006]. Al was found to accumulate in nuclear chromatin of cortical and hippocampal neurons as well as inside the abnormal protein aggregates present in dystrophic neurons [Perl and Brody, 1980] and in senile plaques in AD [Edwardson *et al*., 1991; Candy *et al.,* 1986; Exley, 2005]. Based on this evidence for nuclear localization of Al, it may be presumed that Al might play a role in modulating DNA topology and possibly contribute to the B to Z helical transition associated with AD [Anitha *et al.,* 2002]. Studies have shown that Al preferentially binds to heterochromatin [Crapper *et al*., 1980], DNA phosphates and bases [Karlik *et al*., 1980; De Boni *et al*.,1974; Crapper *et al*.,1978; Wen and Wisneiwski, 1985; Wedrychowski *et al*., 1986]. Previous studies showed that high concentration of Al in the cell decrease the rate of DNA synthesis, increase the errors in DNA replication [Berlyne *et al*., 1972] and found to inhibit hormone induced chromosome puffing[Sanderson *et al*., 1982]. It was also shown that Al potentially inhibits both neuronal RNA polymerases A and B [Sarkander *et al*., 1983] and alters the messenger RNA pool size [VanBerkum *et al*., 1986]. McLachlan *et al.* (1983) showed that Al also inhibits the DNA associated enzymes namely polymerase. Lukiw *et al*. (1987) reported that Al presence in the chromatin region affects the affinity of histone-DNA interactions. Moreover various studies have been carried out to understand the interaction of Al with DNA sequence specific oligomers under physiological pH. Studies show that Al binds strongly to B-DNA [Karlik *et al*., 1980;

Rajan *et al*., 1996; Champion *et al*., 1998]. Our lab had reported for the first time that Al binds strongly to AT* rich sequence d(GCCCATGGC) with no structural transition [Rajan *et al*., 1996] whereas it causes Z to A helical transition in GC* rich oligomers d(CCGGGCCCGG) [Champion *et al*., 1998]. Other studies had shown that Al caused conformation transition from B to Z in CCG repeats $(CCG)_{12}$ [Latha *et al*., 2002]. All the studies showed that Al may have the potential to alter the genetic information leading to malfunctioning nerve cells and impairment of brain function.

To investigate the above mentioned hypothesis experimentally, this study undertook to examine the interaction of Al with supercoiled plasmid DNA (scDNA) and genomic calf thymus DNA (ctDNA). Plasmid scDNA as model system makes an interesting study, in view of the observation that vast arrays of small scDNA packets have been found in animal and human cells and known to be involved in gene expression [Bauer *et al.,* 1980]. Negative supercoiling in the 2686bp E coli plasmid pUC19 is comparable in linking number (Lk(0) = 258) and superhelical density (sigma = -0.05) to the moderate supercoiling exhibited by many eukaryotic chromosomal DNA's in vivo. These superhelical packets are analogous to plasmid DNA supercoiling. Hence the results can be correlated to human brain genomic DNA to provide an insight into explaining the possible role of Al in the progression of AD pathology with reference to DNA topology. In the present work, Al $(maltol)_3$ is used to study the binding aspects with scDNA and ctDNA and its effect on DNA conformation, stability and strand breaks using various methods like CD spectroscopy, agarose gel studies, nick translation studies, melting profile studies, electron microscopy studies, fluorescence studies etc. Al-maltol was used because it overcomes the speciation chemistry of Al.

3A. 2. Materials and Methods

3A. 2. 1. Materials

pUC 19 scDNA (cesium chloride – purified, 90% supercoiled structure), Calf thymus DNA(ctDNA) and agarose were purchased from Genei, India. Chloroquine, ethidium bromide (EtBr), HEPES, Tris(hydroxymethyl) aminomethane (Tris) were purchased from sigma (USA). pUC 19 scDNA was purified using Qiagen kits (Qiagen, Germany). Linearized DNA fragments were prepared according to the following procedure. Samples of (5-10μg) pUC 19 scDNA were cut with the restriction enzyme of interest (New England Biolabs, USA) until the completion as judged by EM observation and scDNA were purified using Qiagen kits (Qiagen, Germany).

E. Coli DNA Polymerase I and Terminal deoxynucleotidyl transferase enzymes were purchased from Amersham chemicals, UK. ^{3}[H]- TTP (Sp. Act.40Ci/mmol) was purchased from Amersham radiochemicals, UK. ^{3}[H] Thymidine (17Ci/mmol) was bought from Bhaba Atomic Research Center, Bombay, India. dATP, dTTP, dCTP, dGTP, Aluminum Chloride hexahydrate and maltol were purchased from Sigma (USA). $GaCl_3$, $InCl_3$ and $TlCl_3$, were from Merck (Germany) .Stock solution of Al-maltol, $GaCl_3$, $InCl_3$ and $TlCl_3$, were prepared freshly in autoclaved MQ water. All other chemicals used were of analytical grade and were purchased from local chemical companies. Water used for preparing the stock solutions and washing the vials was MQ water.

3A. 2. 2. Speciation chemistry of Al

The chemistry of Al is fairly simple as it exhibits as Al^{3+} the only one-oxidation state in the biological system. Al cannot undergo oxidation-reduction chemistry in biological system. The Al^{3+} ion is the hardest of the trivalent metal ions with an ionic radius of 0.50A^{o} which is smaller than other commonly encountered trivalent metal ions. Trivalent boron has an ionic radius of 0.23A^{o} but does not form positive ions in aqueous solution. The acidity of an aquous metal ion is measured by its affinity for the OH^- ion. The acidity of the metal ion is controlled by size, charge, and also by covalence of the metal– oxygen bond [Martell *et al.,* 1996]. Al^{3+} which is the smallest metal ion and has a strong tendency to hydrolyze in aqueous solution, but is the least strongly hydrolyzed of the trivalent metal ions of group 13 (IIIA) such as Ga^{3+}, In^{3+}, and Tl^{3+}.

The information on speciation of Al is crucial in determining Al-toxicity. Most studies on Al toxicity have not paid sufficient attention to metal speciation chemistry. The toxicity of Al depends on free Al and its various species are a function of the pH range (3.0-11.0) [Rao and Rao, 1992]. Martin (1986) indicated that at physiological pH 7.0 – 7.5, Al predominantly exists as $Al(OH)^{3+}$, at lower PH value (<6.0), Al (3+) exists as an octahedral hexahydrate, Al $(H_2O)_6^{3+}$, usually abbreviated as Al^{3+}. As the solution becomes less acidic, Al $(H_2O)^{3+}$, undergoes successive deprotonation to yield $Al(OH)^{2+}$, $Al(OH)^{2+}$ and soluble $Al(OH)_3$, with a decreasing and variable number of water molecules. At neutral solution it gives $Al(OH)_3$ precipitates that redissolve owing to formation of tetrahedral aluminates $Al(OH)^{4-}$, the primary soluble Al^{3+} species at pH >8.0. The hydrolysis of hydrated Al^{3+} produces several hydrated species such as Al^{3+},

$Al(OH)^{2+}$, $Al(OH)_2^{+}$, $Al(OH)_3$, $Al(OH)^{4-}$. Thus it clear that Al^{3+} exists as a polynuclear species.

Apart from a very few recent studies, toxicological work has been carried out by starting from a variety of Al^{3+} (inorganic) salts or complexes in which the counter ion was either the conjugated base of a strong acid (Cl^-, So_4^{2-}, NO^{3-}) (type I) or a weak α hydroxocarboxylic acid (lactate, citrate, tartarate) (Type II). The type I salts undergo extensive hydrolysis in H_2O giving rise to acidic solution which after neutralization do unavoidably produce $Al(OH)_3$. The same occurs for type II toxin, when analytical concentrations are in millimolar range [Corain *et al*., 1996]. While interaction of Al complexes with the DNA the affinity of each one of the Al species varies, due to their different electrical charges. For example $Al(OH)_3$, $Al(OH)^{4-}$ are not attracted to the back bone PO_2 due to their neutral and negative charges respectively. Whatever ligand may be present, understanding the state of Al^{3+}in any aqueous system demands awareness of the species that Al^{3+} forms with components of water at different pH values. Hence preparing of the Al stock solution for use in biological studies has to give important to Al speciation.

The strong hydrolytic tendency of the Al^{3+} ion and the very limited solubility of $Al(OH)_3$ make it rather difficult to prepare Al-stock solutions. $Al(maltol)_3$ can by pass the solubility and Al speciation problem as function of pH. The lack of speciation chemistry of Al^{3+} in experiments might have occurred in the contradictory results reported earlier [Meiri *et al*., 1993].

3A. 2. 3. Al-Maltolate Synthesis

Understanding the toxicity of Al is governed by the speciation chemistry of Al over pH range. To overcome this hydrolytic chemistry problem, in the present study, a highly soluble Al-maltolate [$(Al\text{-}maltol)_3$] was used. Al-maltolate was prepared in our laboratory from aluminum chloride hexahydrate and maltolate (3-hydroxy-2-methyl-4H-pyran-4-one), following the method of Finnegan *et al.* (1986).The concentrated aqueous solutions of maltol and aluminum chloride hexahydrate was mixed in the ratio of 3:1 and the pH was adjusted to 8.3 by drop by drop addition of 10N NaOH. The mixture was heated for few minutes by keeping at 65^{o}C produce a finely divided precipitate. The solution was cooled; white crystals filtered and then washed with methanol for several times and allowed to dry overnight in vacuum desiccators. Al-maltol is highly soluble and hydrolytically stable from pH 2.0 to 12.0. It overcomes the hydrolytic speciation chemistry problem of inorganic Al. This complex of Al can deliver a predicted amount of free aqueous Al by 100 fold at a physiological pH compared to any other inorganic or organic Al complex and has been used as a model compound for the understanding molecular effects of Al in biological system [Rao *et al.,* 1998]. The application of this compound in DNA conformational studies will overcome Karlick *et al*. (1980) complex explanation on Al speciation – DNA interactions. Most of the researchers have used inorganic salts of Al where the concentration of soluble free Al at pH 7.0 will be 50μM in a 10mM solution. This means free Al concentration is 10^{-12}M only and the rest of Al is in $Al(OH)_3$ precipitate form. The concentration of free Al which was used in the present study satisfies the observations of Martin (1986).

3A. 2. 4. Methods

3A. 2. 4. 1. CD Spectroscopy - CD spectra were recorded on a Jasco J 500 Spectropolarimeter at 25^{o}C with 2mm cell length. ScDNA and ct DNA were titrated with different concentrations of Al-maltol (5 x 10^{-7} to 1 x 10^{-3} M), $GaCl_3$ (2.5 x 10^{-4} to 1 x 10^{-3} M), $InCl_3$ (2.5 x 10^{-4} to 1x10^{-3}M), $TlCl_3$ (2.5x10^{-4} to 1x10^{-3} M). CD spectra (200-300nm) were recorded for each concentration in 1mM HEPES buffer (pH 7.4). The CD signals of scDNA were monitored in the presence of chloroquine to examine the effect of uncoiling the DNA on CD signals. The spectra were buffer subtracted and four scans were averaged (the spectra at each concentration represent an average of four recordings). DNA conformations were characterized using the guidelines of Gray *et al*., (1978; 1992), Hanlon *et al*., (1975; 1978).

3A. 2. 4. 2. Agarose Gel electrophoresis - Integrity of the scDNA was studied by 1% agarose gel electrophoresis. The DNA samples used (scDNA, linearized scDNA, and scDNA + Al-maltol complexes) were loaded in 1% agarose gels in TA buffer [40mM Tris-acetate (pH 8.0)] and electrophoresed at 4V/cm at RT for 8-10 hours. DNA concentration loaded in all lanes was 1μg. The sample scDNA was incubated with Al-maltol (1x10^{-3}M) for 45min at RT. In other experiments we also studied the effects of $GaCl_3$, $InCl_3$ and $TlCl_3$ on the integrity of the scDNA with the same condition and concentration. The gels were stained with EtBr and photographed using the gel documentation.

3A. 2. 4. 3. Fluorescence Measurements (EtBr binding Studies) - The EtBr binding patterns to scDNA(1μg), scDNA+(1x10^{-3}M)Al-maltol, scDNA+(1x10^{-3}M)$GaCl_3$, scDNA+(1x10^{-3}M)$InCl_3$, and scDNA + (1x10^{-3}M) $TlCl_3$ complexes are analyzed by

mixing 1:1 (w/w) DNA/EtBr before measuring fluorescence emission. The DNA:EtBr solutions were excited at 525nm and the emission was monitored at 600nm with 10mm path-length quartz cuvettes using a Hitachi F-2000 Fluorescence Spectrophotometer.

The average amount of EtBr bound to base pair of scDNA (1µg), linearized scDNA(1µg),scDNA+($1x10^{-3}$M) Al- maltol are also calculated using the Scatchard plots of 'r' vs r/Cf' . The fluorescence was measured using a constant amount of DNA and DNA metal complexes with increasing EtBr against the blank containing no DNA. [Suh *et al*., 1991; Chatterjee and Rao, 1994].

The concentration of bound EtBr in 1.0mL dye-DNA mixture (Cb') was calculated using the equation:

$Cb' = Co'[(F-F_o)/Vx\ F_o)]$

Where Co=EtBr (pmoles) present in the dye – DNA mixture,

F= observed fluorescence at any point of dye-DNA mixture,

F_o= observed fluorescence of dye,

V= experimentally derived value, ratio of bound EtBr/free EtBr at saturation point.

The concentration of free dye(Cf') was then calculated by the relation

Cf'=Co′-Cb′

Where Cf', Co′ and Cb′ were expressed in pmoles. The amount of bound EtBr/base pair 'r' was calculated by

r = Cb′ (pmoles)/DNA conc.(pmolesof base pair).

A plot was made for r vs r/Cf and the point where the straight line intersects the axis r was defined as the maximum amount of dye bound per base pairs(n),where Cf=Cf'x10^{15} M.

3A. 2. 4. 4. Melting Profile - The melting profile curves for scDNA (1µg), scDNA + (1x10^{-3}M) Al-maltol, scDNA + (1x10^{-3}M) $GaCl_3$, scDNA + (1x10^{-3}M) $InCl_3$, scDNA + (1x10^{-3}M)$TlCl_3$ complexes are performed (1^oC/min,28-95^oC) using a Amersham ultraspec 4300 pro UV/Visible Spectrophotometer with thermostat. Melting temperature(t_m) values were determined graphically from hyperchromicity versus temperature plots. The precision in t_m values estimated in triplicate was ± 0.5^oC. We also studied the t_m of ctDNA(1µg), ctDNA + (1x10^{-3}M) Al-maltol, ctDNA + (1x10^{-3}M) $GaCl_3$, ctDNA + (1x10^{-3}M) $InCl_3$, and ctDNA + (1x10^{-3}M) $TlCl_3$. All these experiments are carried out in 1mM HEPES buffer (pH 7.4).

3A. 2. 4. 5. Estimation of Single/double strand breaks.

Single strand breaks (SSB) - SSBs in scDNA, Single strand circular DNA (sscDNA) and double strand circular DNA (dscDNA) induced by Al-maltol was determined through a nick translation type of reaction using E.Coli DNA Polymerase I (pol I.). 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. It is essentially a nick translation type of a reaction and if one of the deoxynucleotide triphosphates is labelled, then the incorporation of radioactivity into substrate DNA would be proportional to the number of single strand breaks present in the DNA sample. The assay mixture consisted in a total reaction volume of 50µl: 40mM Tris-Cl (pH 8.0), 1mM β-Mercaptoethanol,

7.5mM $MgCl_2$, 4mM ATP, 100uM each of dATP, dCTP, and dGTP and 25uM dTTP, 1uCi of ^{3}[H] TTP and 0.2µg of scDNA (from Al-maltol-DNA reaction mixture) and 1 unit of E. Coli DNA polymerase I. Incubation was at 37^{o}C for 20 minutes. During the standardization of the assay, it was noticed that about 1500 total nucleotides are added to each 3'-OH groups (SSB). Assuming one fourth of these are ^{3}[H]-TMP, each Pico mole of ^{3}[H]-TMP incorporated into DNA was calculated to be equivalent to $1.6x10^9$ SSB.

Double Strand Breaks (DSB) - Terminal deoxynucleotidyl transferase catalyzes the addition of deoxynucleotides to the 3' termini of DNA and does not need direction from template strand. Although single stranded DNA is the preferred substrate, 3' ends of duplex DNA also serve as substrates. We have used similar conditions to incubate DNA with Terminal transferase as in the case of E. coli polymerase I assay. The incorporation of the ^{3}[H]-dTTP into DNA would be proportional to the number of double strand breaks in the DNA. The reaction mixture consisted for terminal transferase enzyme assay consisted of 100mM Na Cacodylate buffer (pH 7.0), 1mM $CoCl_2$, 0.2mM DTT, 1uCi of ^{3}[H]-dTTP, and 1 unit of the enzyme in a total volume of 50µl. Incubation was at 37^{0}C for 45 minutes. Each femtomole of TMP incorporated is equivalent to 1.2×10^7 3' ends or half that number minus one double strand breaks.

For measuring the radioactivity of incorporated ^{3}[H]-dTTP in Liquid Scintillation Counter: After incubation of DNA which is treated by Al-maltol at RT with E.coli polymerase I / terminal transferase, the reaction was stopped by adding 1 ml 10% TCA containing 10mM Na pyrophosphate. 100ug each of ctDNA and BSA were added to aid in the precipitation of DNA which acts as carrier molecule. Pellet was separated after centrifugation at 12000 rpm for 15 seconds. Dissolved the pellet in 400ul NaOH (0.2 N)

by vortexing. Reprecipitate the DNA in 10% TCA containing 10mM Na pyrophosphate, which was separated by centrifugation at 6000 rpm for 5 minutes. The precipitate was dissolved in 1 ml 5%TCA, filtered through Glass Fibre Filter (GF 6). Then the filter was washed with 5% TCA and 95% ethanol 6 times each. Filters were dried at room temperature for ~12 hrs. Filter was then inserted in bottles containing 5ml BRAJI'S mixture and disintegration per minute (DPM) in liquid scintillation counter are recorded.

3A. 2. 4. 6. Transmission electron microscopy - 1μg of scDNA, scDNA+ Al-maltol complex, were aliquoted for 10μl containing 10mM Tris-Cl (pH 7.4) buffer containing 50mM NaCl and incubated for 45min at room temperature. pUC19 scDNA were subjected to EcoR I, restriction enzyme digest to produce linearised DNA with cohesive ends. A drop (6-8μl) of each aliquot was withdrawn from the incubation mixture and was placed onto the surface of a glow-discharged carbon film mounted on an EM grid. Carbon films, 3 to 4-nm thick, were glow-discharged in the presence of pentylamine vapor (residual pressure ~150millitorr (1Torr ≈ 133.322 Pa), discharge current 2-3mA, duration of discharge 30 seconds) as described elsewhere [Dubochet *et al*., 1971]. The adsorption continued for 1-2 min, after which the grids were rinsed with few drops of 2% (w/v) aqueous uranyl acetate, blotted with a filter paper, and dried. The samples were examined with a Philips CM12 electron microscope in a tilted dark field mode at a magnification of 28,000-35,000x. The negatives were scanned with an agfa DuoScan T2500 scanner (agfa, Germany) at 600-1200 dots per inch. Micrographs were measured using Image software (NIH) modified for Windows. For printing, images were flattened using a high pass filter with a radius of 250 pixels and subsequently adjusted for contrast/brightness using Adobe PhotoShop™.

3A. 3. Results

3A. 3. 1. CD studies on DNA helicity

In Fig.1a, CD spectra of scDNA showed B-DNA conformation with a characteristic positive peak at 275nm and negative peak at 245nm. Addition of increasing concentration of Al-maltol (from 5x 10^{-7} to 1x10^{-3}M) to the DNA caused a decrease in the positive band at 275nm with no concomitant change in the magnitude of the negative peak at 245nm (Fig 1b-d). In addition, there was a several fold increase in the magnitude of the negative peak at 210nm. This characteristic decrease in the positive peak at 275nm, without change in the magnitude of the negative peak at 245nm belongs to C–DNA conformation. However, there is presence of deep, narrow negative peak at 210nm which is associated with A-DNA [Gray et al., 1978]. In view of this, the spectrum reflects the properties of a B-C-A mixed conformation. A possible simultaneous helical transition of B to C and B to A could have been formed leading to a complex conformation.

Further, ctDNA was treated with Al-maltol the changes in DNA helicity were monitored by CD spectroscopy. CD spectra analysis of ctDNA has shown classical B-DNA conformation (Fig. 2a). Addition of increasing concentration of Al-maltol (from 5x 10^{-7} to 1x10^{-4}M) to the ctDNA caused significal change in positive and negative peaks at 275 and 245nm respectively. But on addition of higher concentrations (from 1x10^{-3} to 2x10^{-3}M) DNA attained the conformation with characteristic features of two negative peaks at 245 and 295nm and a positive peak at 260nm (Fig.2c and d). This type of characteristic CD spectra reveals Z-DNA conformation. This is a new finding. The treatment of desferoximine (10^{-5}), Al chelating drug, on ctDNA–Al complex (Fig. 2d) could

not reverse the spectral characteristic back to the B-conformation from Z-conformation. This indicated that Al formed stable Z-DNA conformation.

3A. 3. 2. Gallium Chloride, Indium Chloride and Thallium Chloride does not affect sc DNA and ctDNA conformation

To examine whether the effect of Al on scDNA conformation is specific or a more general effect of metals that belong to the same group 13 in periodic table, the effect of gallium(Ga), indium(In) and thallium(Tl) metals having essentially a similar chemical behavior in aqueous solution as Al were studied. The ionic radii of Al, Ga, In, Tl are $0.57A^{o}$, $0.62A^{o}$, $0.92A^{o}$, $1.05A^{o}$ respectively. We compared the effects of $GaCl_3$, $InCl_3$, and $TlCl_3$ on scDNA and ctDNA to those of Al-maltol. The results showed that in contrast to Al – maltol, $GaCl_3$, $InCl_3$, and $TlCl_3$ did not alter the conformation of both scDNA (Fig.3A-3C) and ctDNA (Fig. 4A-4C), but showed strong binding to DNA. However, higher concentrations Ga, In and Tl caused aggregation of scDNA and ctDNA as observed by the zeroing or flattening of the CD signal (Fig.3and 4).

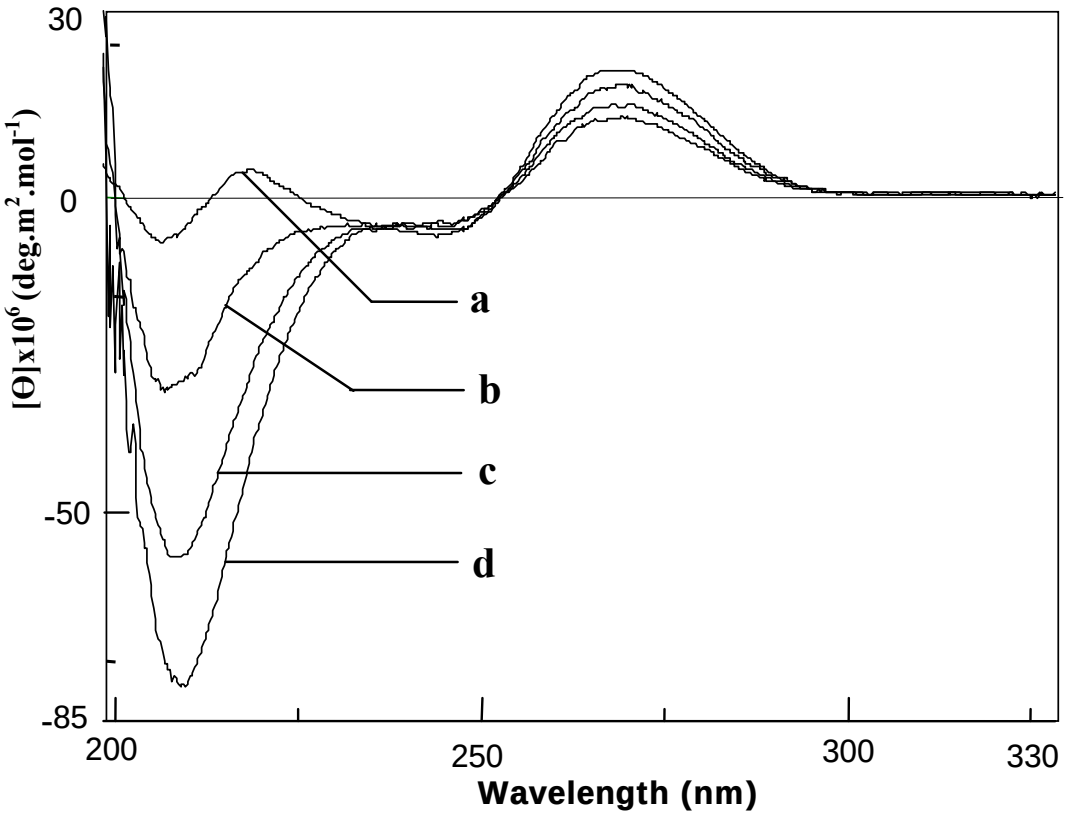


Figure 1. Circular dichroism spectra of Al interaction with scDNA: Effect of Al-maltol binding on conformation of pUC 19 supercoiled DNA (scDNA) in 10^{-4}M Hepes buffer (pH 7.0). scDNA was titrated with increasing concentrations of Al-maltol and the changes in the molar ellipticities were monitored at 25^0C in a 2-mm path length cell. The scDNA alone showed B-DNA conformation (a) On addition of increasing concentrations of Al-maltol (b, $1x10^{-6}$M Al; c, $1x10^{-4}$M Al; d, $5x10^{-3}$M Al) the native B-DNA conformation was transformed into a complex B-C-A mixed conformation.

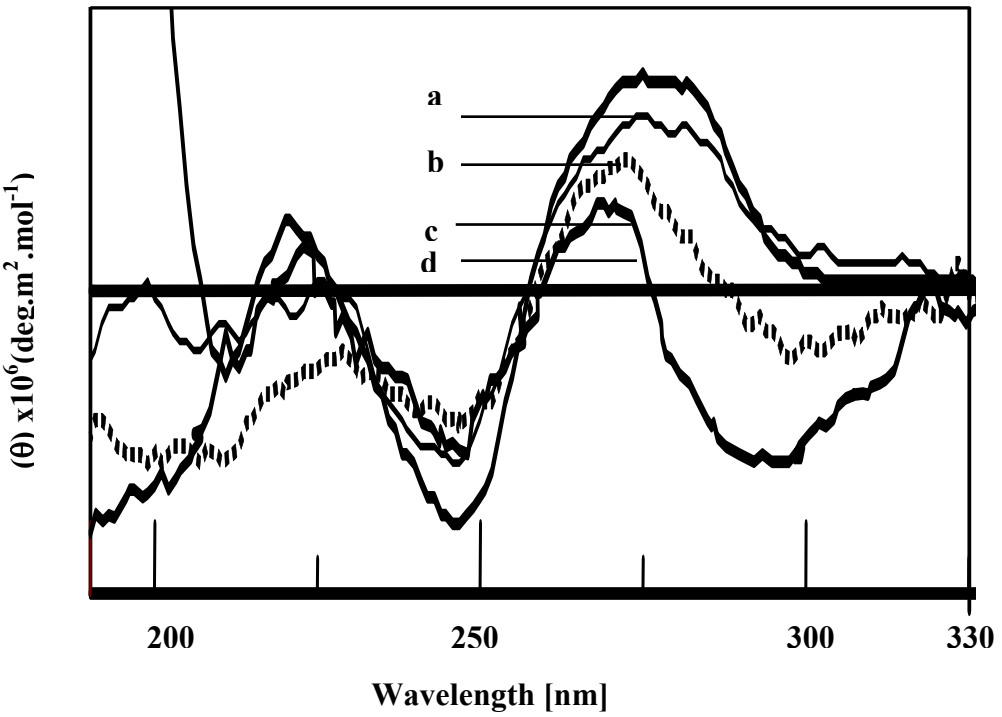


Figure 2. Circular dichroism spectra of Al interaction with ctDNA. Effect of Al-maltol binding on conformation of calf thymus DNA (ctDNA) in 10^{-4} M Hepes buffer (pH 7.0). ctDNA was titrated against increasing concentrations of Al-maltol and the changes in the molar ellipticities were monitored at 25^0C in a 2-mm path length cell. The ctDNA alone showed B-DNA conformation (a) On addition of increasing concentrations of Al-maltol (b, $1x10^{-4}$M Al; c, $1x10^{-3}$M Al; d, $2x10^{-3}$M Al) the native B-DNA conformation was transformed into a Z –DNA conformation.

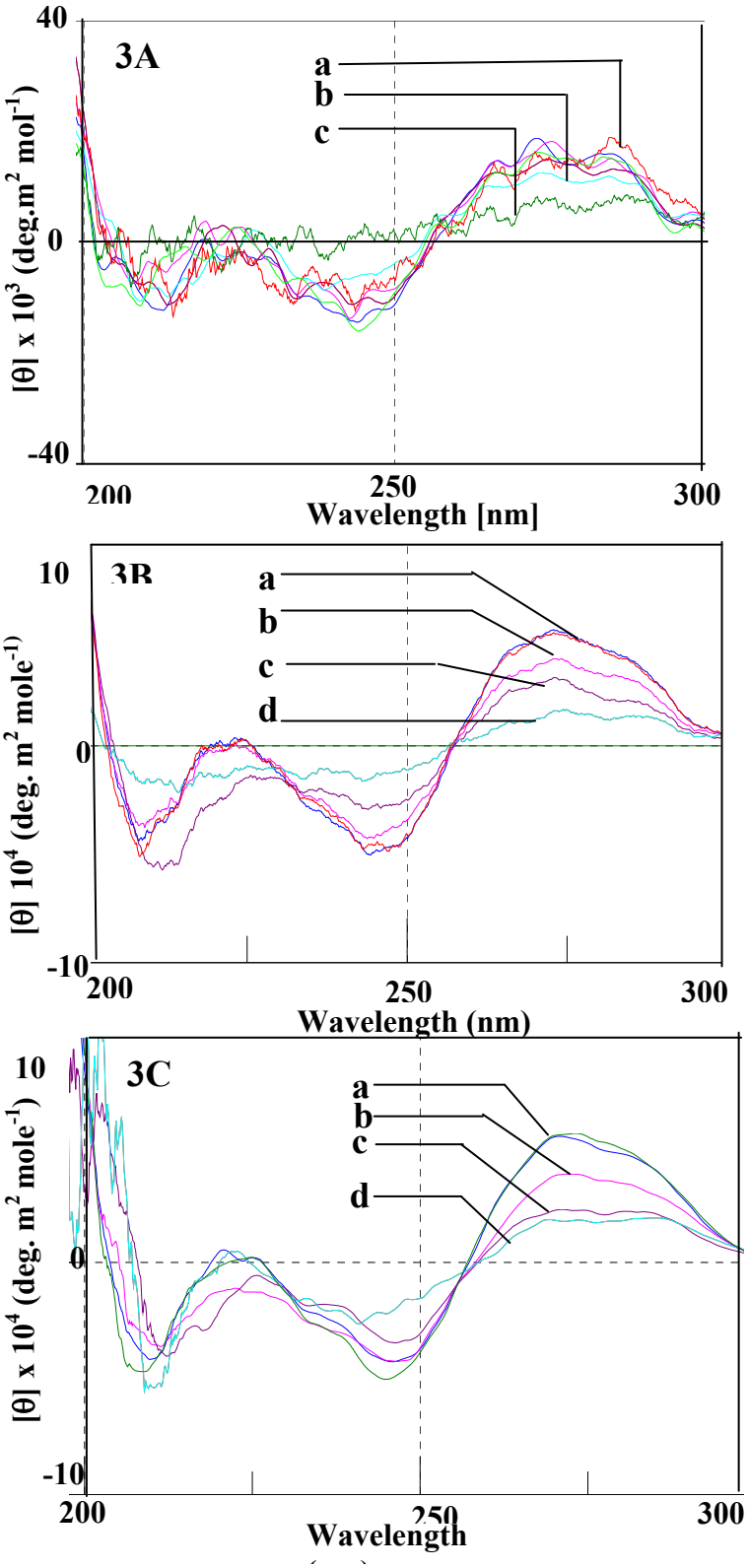


Figure 3. Circular dichroism spectra of Ga (3A), In (3B) and Tl (3C) interaction with scDNA The scDNA alone showed B-DNA conformation (a) On addition of increasing concentrations of Ga, In and Tl Chlorides (b, $1x10^{-6}$M; c, $1x10^{-4}$M ; d, $1x10^{-3}$M) the B-DNA conformation was not transformed into any other conformation but it showed lowering of the both peaks at 245nm and 275nm. The conditions for the experiments are the same described in the fig 1.

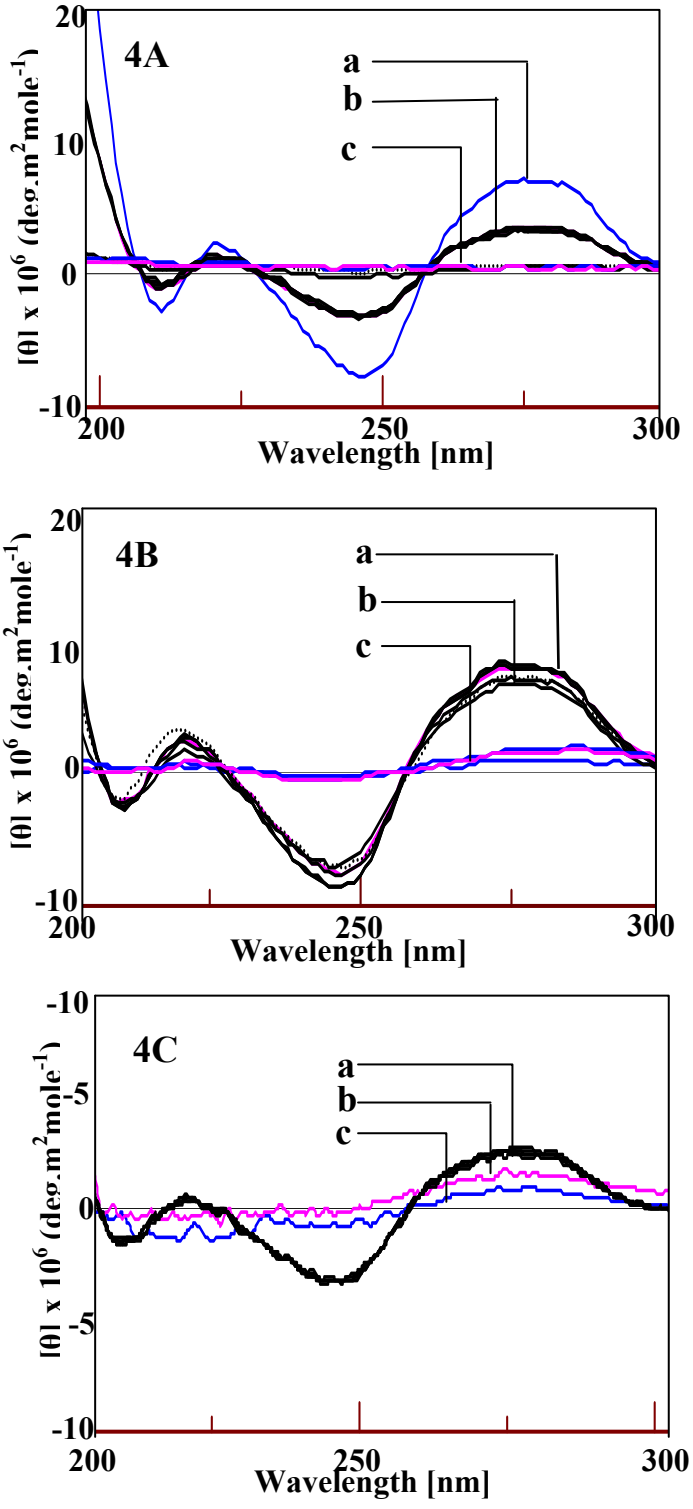


Figure 4. Circular dichroism spectra of Ga(4A), In(4B) and Tl(4C) interaction with ctDNA: a) ctDNA alone and $0.25x10^{-3}$ to $0.5x10^{-3}$M; b) $0.75x10^{-3}$ to $1x10^{-3}$M; c) $>1x10^{-3}$ M. conformation was not transformed into any other conformation but it showed lowering of the both peaks at 245nm and 275nm. The conditions for the experiments are the same described in the fig 1.

3A. 3. 3. Agarose Gel Electrophoresis Studies- Integrity of the scDNA was studied by 1% agarose gel electrophoresis (Fig 5).In figure 5a showed supercoiled form, 5b linear form; 5c Al-maltol causes more linear DNA over open circular form. The effect of other 13 groups metals like Ga, In and Tl on scDNA were studied (Fig.6). Results showed that Ga and In (lane B and C) causes smear to DNA and precipitated DNA.

3A. 3. 4. EtBr Binding Studies

The quantification of the integrity of scDNA changed by Al-maltol and other 13 group metals was studied by measuring EtBr fluorescence intensity at 1:1(w/w) DNA/EtBr concentrations (Fig.7). EtBr fluorescence of scDNA(1µg) and scDNA+ (1mM)Al-maltol complex are 22.25 (Fig.7a), 14.72 (Fig. 7b) respectively. On the other hand, other 13 group metals showed less EtBr intensity and the values are 7.02 for scDNA– ($1x10^{-3}$M)$GaCl_3$ complex (7c), 7.11 for scDNA – ($1x10^{-3}$M) $InCl_3$ complex (7d), and 2.9 for scDNA–($1x10^{-3}$M) $TlCl_3$ complex (7e). The scatchard's plot analysis of the values of the EtBr binding to the scDNA indicated that the average number EtBr molecule bound per base pairs 'r' was equal to 0.17 for scDNA alone (Fig.8), 0.48 for the scDNA + Al complex(Fig 8) and 0.27 for the linearized scDNA by EcoRI (Fig. 8). It was of interest to determine the binding of EtBr to linearised scDNA by EcoRI for comparison purpose. The result showed that our 'n' value 0.27 was closer to the value of 0.25 proposed earlier studies [Chatterjee and Rao, 1994]. The result also showed that scDNA has low EtBr binding compared to full length linear DNA. In another experiment, EtBr intensity for the ctDNA alone and ctDNA with other 13 group metal complex was studied (Fig. 9). The intensity value for the ctDNA alone and ctDNA + Al-maltol complex are found to be 106 and 35.29. The EtBr intensity for ctDNA+Ga complex,

ctDNA+In complex, ctDNA+Tl complex are 60, 40 and 55 respectively. The low value for ctDNA +Al complex supports the Z-DNA conformation, because Z-DNA binds less EtBr molecules. But reduction in EtBr intensity for other 13 group metal may be attributed to DNA strand breaks and precipitation.

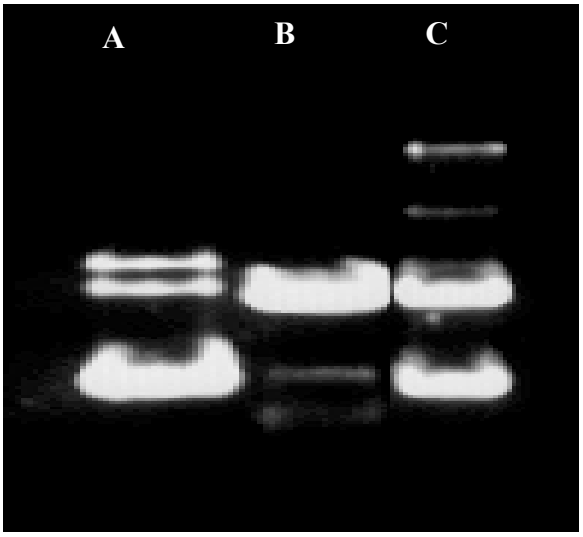


Figure 5. Effect of Al-maltol on scDNA mobility in 1% agarose gel. scDNA was incubated in the absence and presence of Al-maltol for 45minutes and then studied using 1%agarose gel elecrophpresis. scDNA alone (cesium chloride purified) showed >90% superhelicity (lane A). EcoR I digested scDNA linerized form (lane B), Al-maltol ($1x10^{-3}$ M) linearize the scDNA form II (lane C).DNA concentration loaded in all lanes was 1µg.

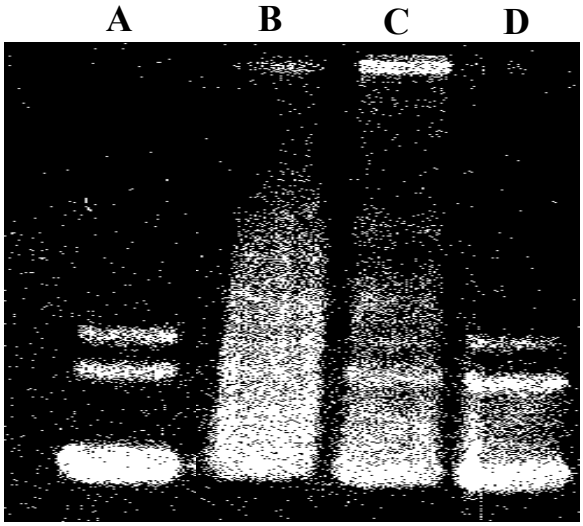


Figure 6. Effect of $GaCl_3$, $InCl_3$ and $TiCl_3$ on scDNA mobility in 1% agarose gel. scDNA was incubated in the absence and presence of $GaCl_3$, $InCl_3$ and $TiCl_3$ then was separated by 1%agarose gel electrophoresis. scDNA alone (cesium chloride purified) showed >90%superhelicity (lane A).$GaCl_3$ ($1x10^{-3}$M) induced damage to scDNA (lane B), $InCl_3$ ($1x10^{-3}$ M) induced damage and aggregation of scDNA (lane C).$TlCl_3$ ($1x10^{-3}$M) on scDNA showed not much damage (lane D). DNA concentration loaded in all lanes was 1µg.

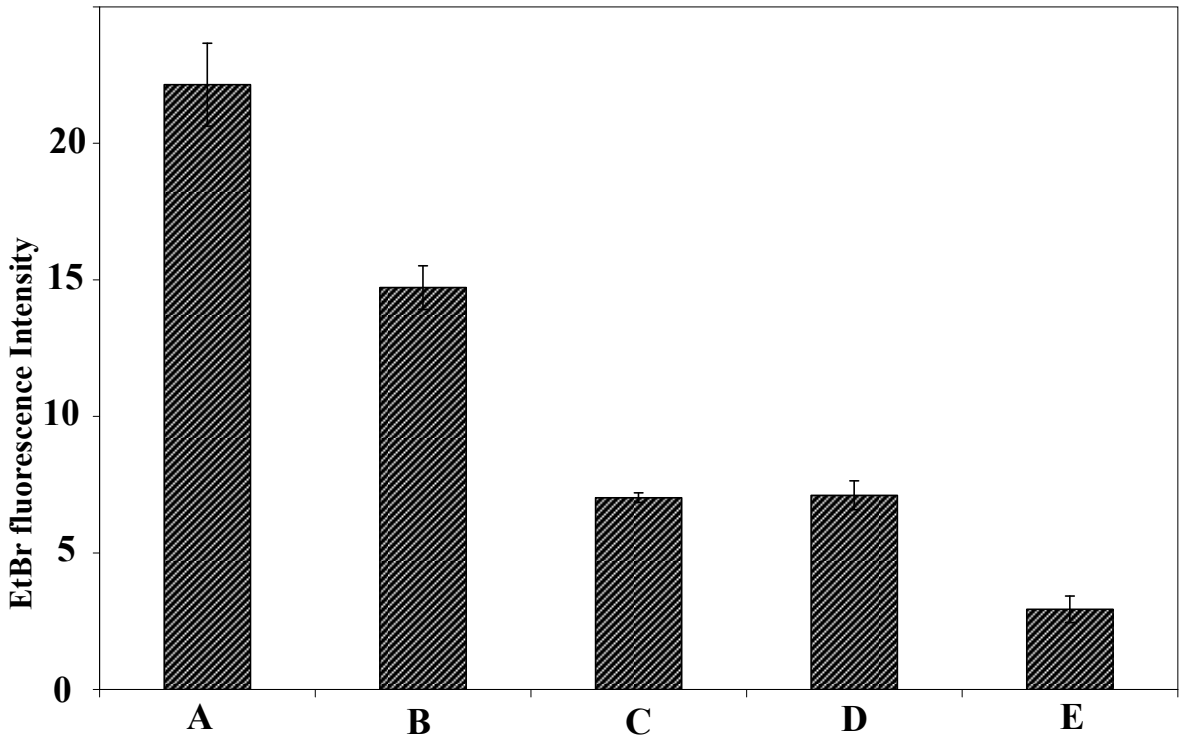


Figure 7. EtBr binding pattern. Equal concentrations (w/w) of scDNA and EtBr were used as models to study the effect of EtBr intensity on Al-maltol, $GaCl_3$, $InCl_3$ and $TlCl_3$. EtBr intensity also provides information on DNA conformation. The uncoiling of scDNA was quantified by measuring the EtBr fluorescence intensity of 1:1(w/w) DNA/EtBr solutions. The solutions were excited at 535 nm and emission monitored at 600 nm. The emission intensity values for scDNA, scDNA + Al, scDNA + Ga, scDNA + In, scDNA + Tl, were 22.15 (A); 14.72 (B); 7.02 (C); 7.11(D); 2.94 (E) respectively.

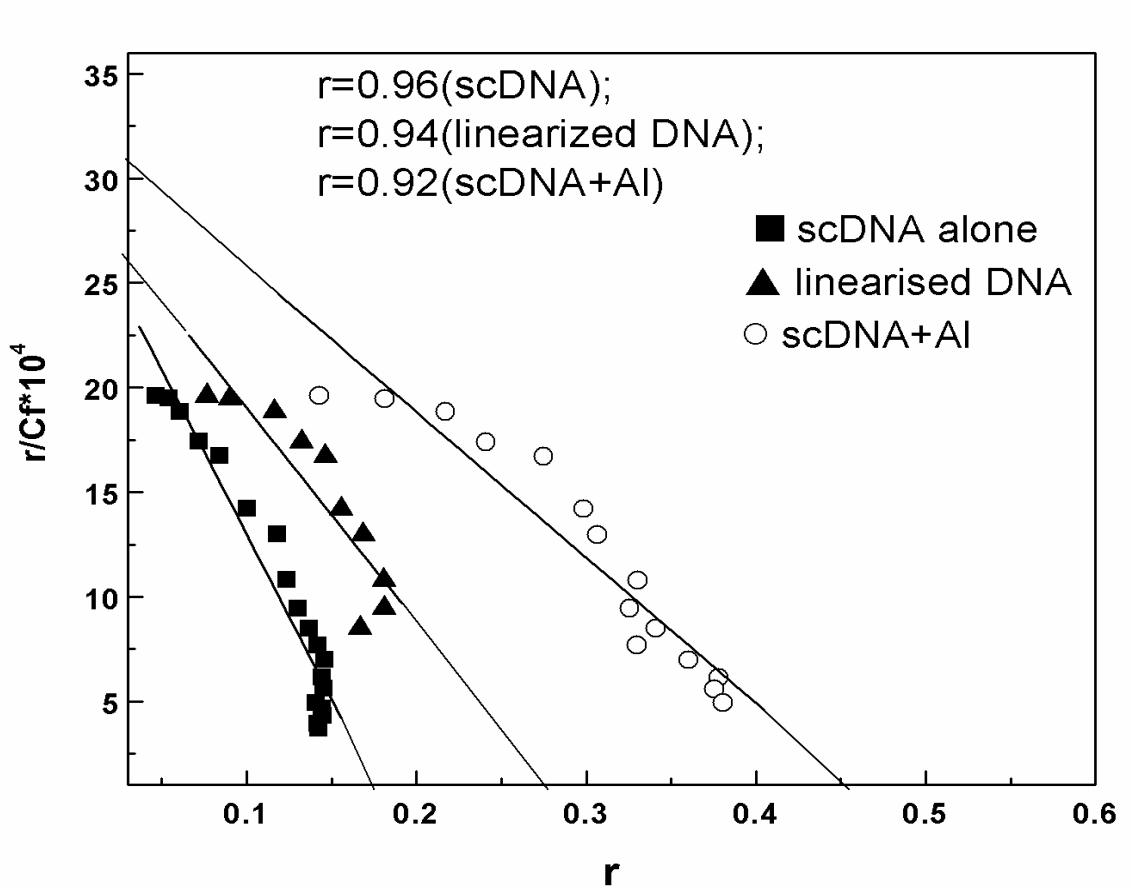


Figure 8. Scatchared plot of EtBr binding to pUC 19 scDNA; Al-maltol treated scDNA; linearized scDNA under 50mM NaCl salt concentration. [1µg of DNA was titrated with increasing amount of EtBr in 2mL containing 10mM Tris-HCl (pH 7.4), 50mM NaCl. Flourescence measurements were done at room temperature setting excitation at 545nm and emission at 600nm respectively. The slit corresponding to the band pass of 15 nm for excitation and 20 nm for emission were used. The light path length was 10. The plot was drawn using least square method].

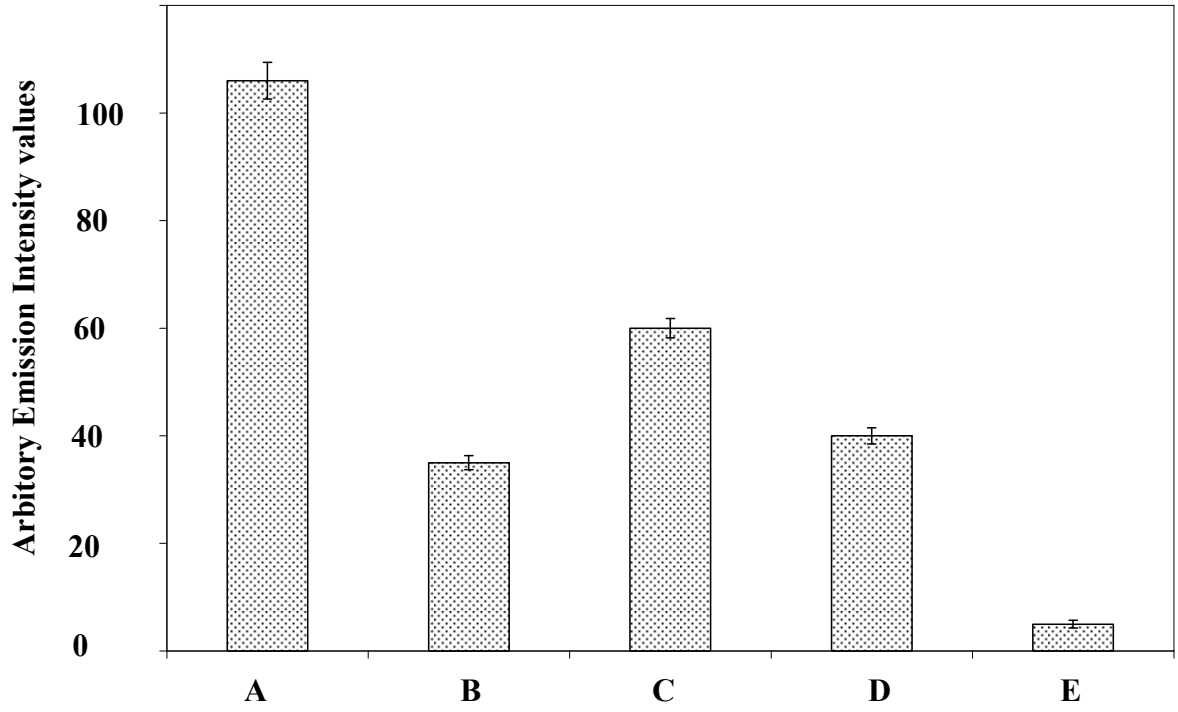


Figure 9. EtBr binding pattern to the ctDNA. The solution were excited at 535 nm and emission monitored at 600nm. The emission intensity values for ctDNA were 106 (A); ctDNA + Al, 35.29 (B); ct-DNA + Ga, 60 (C); ctDNA + In, 40 (D); ctDNA + Tl, 5.5 (E).

3A. 3. 5. Melting Profile

The melting temperature study provides insight on the stability of DNA. Therefore the melting profile of ctDNA in the absence or presence of Al-maltol was examined [Fig 10]. The t_m value for ctDNA was 69.5^oC (±0.5) (Fig 10a) and Al-maltol ($1x10^{-3}$ M) concentration on ct DNA enhanced the t_m to 78^oC (Fig 10d). On other hand metals like In reduced the t_m of ctDNA (Fig.10c). In other experiments the changes in the t_m of scDNA were also examined. It was found that scDNA showed biphasic t_m pattern with the t_m values of 59^oC and 88^oC (Fig 11A) and Al–maltol (monophasic pattern) at concentration

(10^{-5} to 10^{-4} M) did not change the t_m and at higher concentration $1x10^{-3}$ M reduced the t_m of scDNA from 88°C to 74 °C (Fig 11B). Thus the data clearly shows that Al-maltolate destabilizes the DNA.

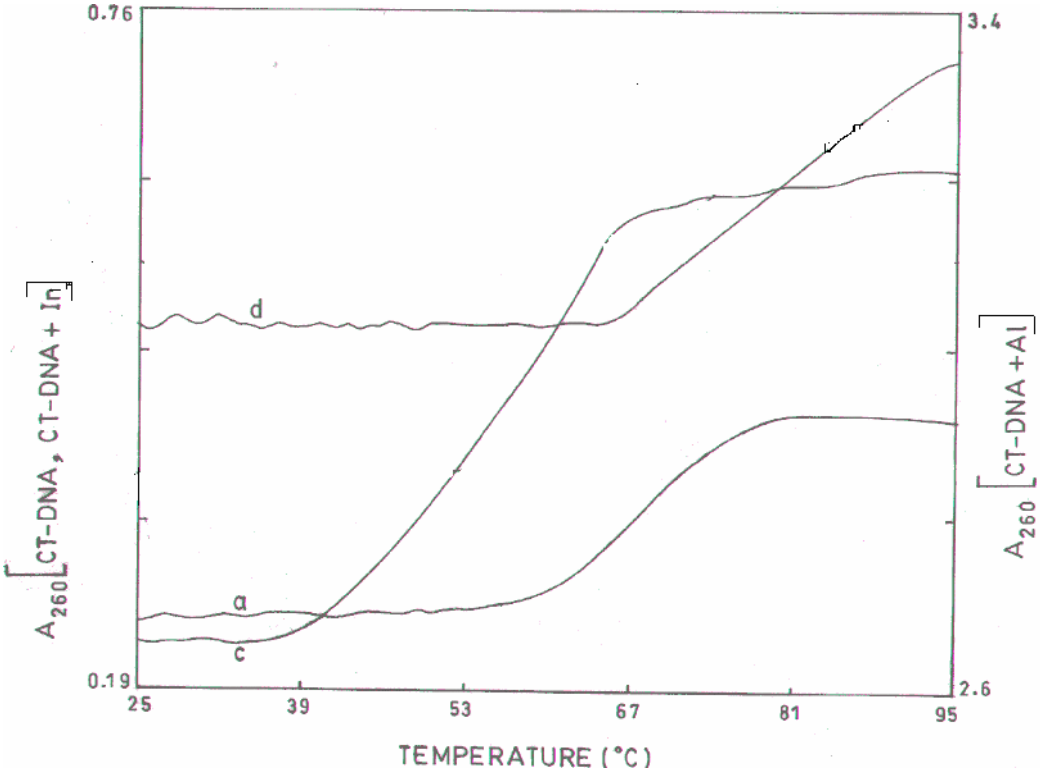


Figure 10. Effect of Al on melting profile of ctDNA. The UV absorbance at 260nm was recorded for ctDNA and ctDNA –Al complexes at different temperatures. The melting curves (10°C/min, 25-95°C) for ctDNA and ctDNA-Alcomplexes in 10^{-4} HEPES, pH (7.4), were recorded in a UV spectrophotometer with thermostat. Tm values were determined from hyperchromicity vs temperature plots. t_m for ctDNA was 69.5°C (A). Al-maltol enhanced the t_m 78°C (D), $InCl_3$ reduced the tm58°C(C). The results are representative of three independent experiments.

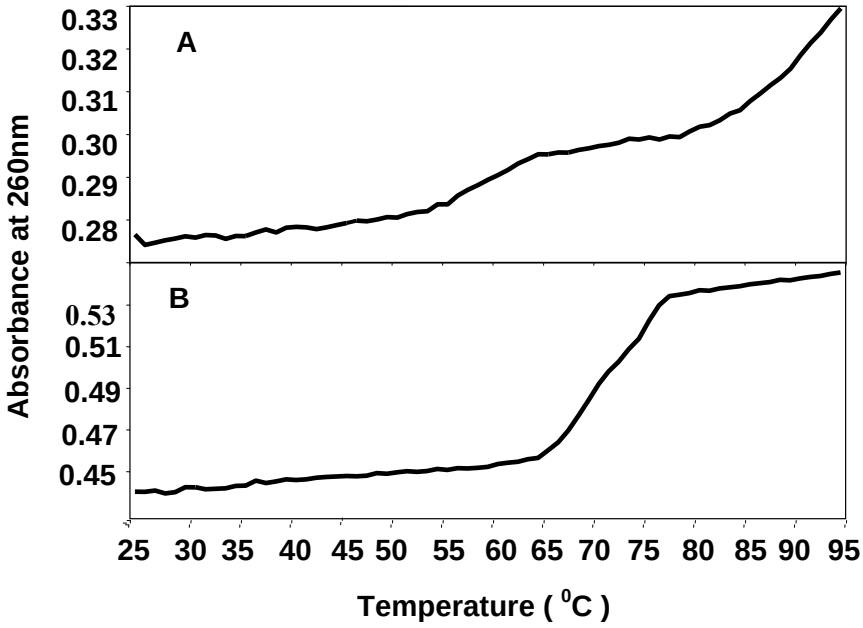


Figure 11. Effect of Al on melting profile of scDNA. The UV absorbance at 260nm was recorded for scDNA and scDNA–Al complexes at different temperatures. The melting curves (10^oC/min, 25-95^oC) for scDNA and scDNA-Al-maltol complex in 10^{-4} HEPES, pH (7.4), were recorded in a UV spectrophotometer with thermostat. Tm values were determined from hyperchromicity vs temperature plots. t_m for scDNA was biphasic with t_m values at 59^0C and 88^0C (A). Al-maltol enhanced the t_m 74^oC (B). The results are representative of three independent experiments.

3A. 3. 6. Estimation of single/double strand breaks

The number of Single Strand Break (SSB) and Double Strand Break (DSB) were estimated in scDNA in the presence and absence of Al-maltol by nick translation methods. In scDNA alone, few single strand breaks and double strand were recognized by polymerase I enzyme (which is in keeping with the agarose gel pattern of scDNA

where a faint band for nicked circular DNA was observed). On incubation with Al-maltol, there was considerable increase in DSBs (Table I).

TABLE I

Assessment of SSBs and DSBs breaks on two different concentrations of Al with scDNA at 37^{o}C for 40 minutes.

Conc.of metals	SSBs/ug of DNA	DSBs/ug of DNA
$1.0x10^{-8}$ M	0	$54.4x10^{6}$
$2.5x10^{-8}$ M	0	81.6×10^{6}

3A. 3. 7. Transmission electron microscopy Purified scDNA in 50mM NaCl, 10mM Tris-Cl pH (7.4) have been investigated by TEM to assess the effect of Al-maltol on the alteration of scDNA. Natural negatively supercoiled DNA of 2686bp deposited onto a pentalamine glow discharged carbon surface in the presence of 50mM NaCl, showed a tight supercoiled configuration (Fig.12A) and $1x10^{-3}$M Al caused linear DNA(Fig 12B).

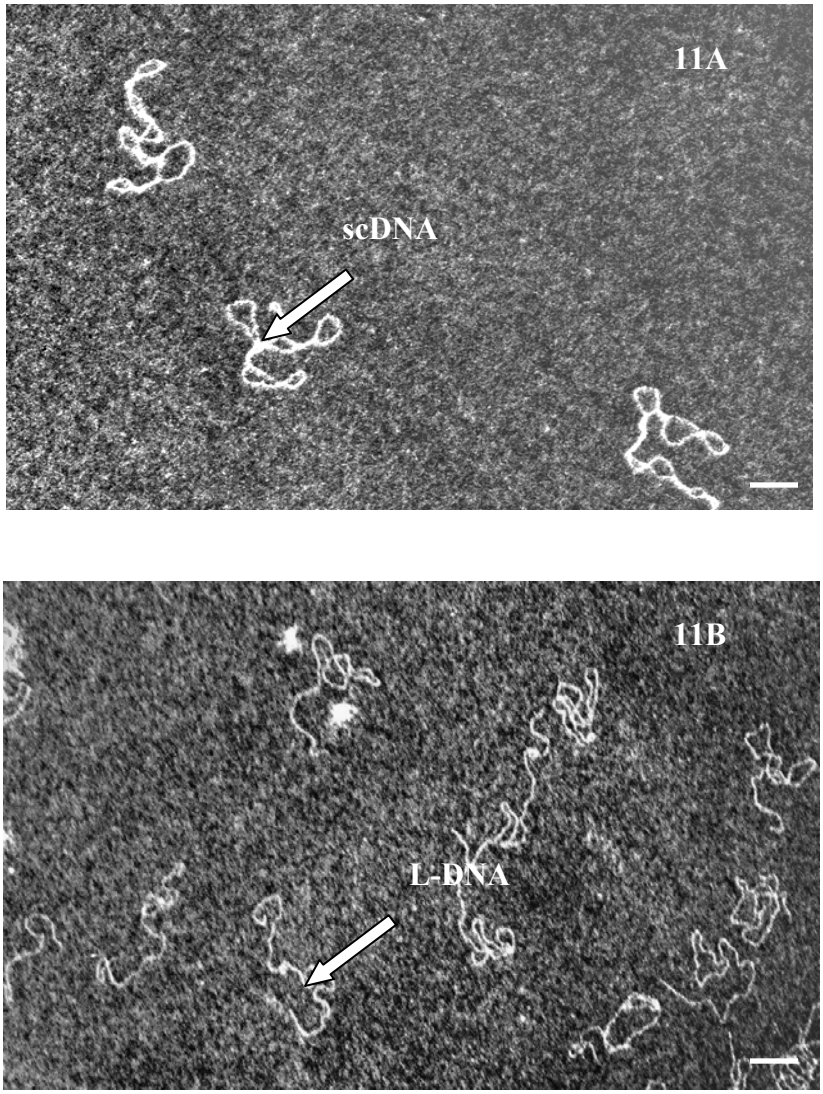


Figure 12. Electron microscopy of scDNA and Al-maltol with scDNA. Supercoiled pUC19 was diluted to ~0.5mg/ml in 10mM Tris-Hcl, pH 7.4 containing sc DNA alone (a), 1mM Al maltol (b) and deposited on a carbon film glow-discharged in the presence of pentalamine. Samples were stained with 2% (W/V) aqoues uranyl acetate. (a) scDNa alone showed the supercoiled configuration and (b) at 1mM Al showed linear DNA. The scale bar represents 200nm.

3A. 4. Discussion

Al is one of the strongly suspected etiological factors in neurodegenerative disorders like AD, PD and Guam Parkinsonism/amylotrophic lateral sclerosis [Perl *et al*.,1982; Garruto *et al*.,1986; Savory *et al*., 1996; Rao *et al*., 1999; Kaneko et al., 2006; Zatta et al., 2003]. Recently our lab for the first time evidenced the presence of left-handed rigid Z-DNA in hippocampus region of severely affected AD brain [Anitha et *al.,* 2002]. It has been hypothesized that one of the etiological factors like Al might be playing a role in right to left handed helical change associated with AD. Based on this DNA conformational change, it was hypothesized an explanation for the unusual phenomenon- like nucleosome misassembly, G* specific DNA oxidation, terminal differentiation and altered gene expression associated with AD brain [Anitha *et al*., 2001, 2002].Further they gave the explanation that presence of Z-DNA in AD brain cells might be reason for the adoption of above unusual phenomenon. Studies from our laboratory [Champion *et al*., 1998; Rajan *et al*., 1996] and others [Karlik *et al*., 1980] showed that Al preferentially binds to DNA. Our lab also showed that Al could strongly bind to AT*-specific oligomers [Rajan *et al*., 1996] and could bring about conformational transition from Z to A conformation in GC*-rich oligomers [Champion *et al*., 1998]. It was reported that Al-(maltol)$_3$ could induce Z-DNA in $(CCG)_{12}$ repeats at neutral pH [Latha *et al*., 2002].

In the present study, we have evidenced the interaction of Al with scDNA and ctDNA. It is known that Al not only binds to scDNA but also able to alter the conformation of the DNA. Al also caused DNA strand breaks and altered the stability of the DNA. Al induced helical transitions from (B to A, B to C or BCA mixed) in scDNA. BCA mixed DNA conformation is energetically weak and is likely to go into Z-DNA conformation

[Anitha *et al*., 2002]. Further, Al induced helical transition from B to Z in case of genomic ctDNA. This may be biological important as Z-DNA was observed in AD brain [Anitha *et al*., 2002]. Previous studies showed that interaction of Al with Poly d(GC) using aluminum chloride results in cross linking of DNA without conformational change [Karlik and Eichhorn 1989]. Alterations in the CD spectra for DNA and polynucleotide had been observed for base – binding metals [Shin, 1973]. The present study also showed that only Al is capable of inducing the DNA conformation, while other thirteen group elements were able to bind the DNA and destabilize the DNA.

A concept of Al-induced genetic malfunction was brought out by Lukiw et al., (1987) and they showed that H1 and $H1^0$ histone binding to chromatin was enhanced by Al. An Al atom has an extremely high ratio of charge (*3) to ionic radius (0.05nm) and is capable of penetrating the nuclear compartment [Rao et al., 1993]. It is conceivable that the addition of small and extremely high density species of Al to the system could provide an electrostatic bridge between the DNA-phosphate groups and the few electro negatively charged amino acids of the linker histones to increase histone-DNA binding resulting in more condensed chromatin. Moreover, a study by Karlik *et al*. (1980) showed that DNA helix may be hyperstabilized in the presence of Al. The binding of Al to chromatin proteins might alter the charge distribution along the surface of these molecules resulting in an increased affinity for DNA. scDNA pockets are known to be involved in genetic function. Rao *et al*. (1993) showed that Al at very low concentration 10^{-6}M (physiological concentration) irreversibly unwinds the scDNA. In the present study it was shown that Al caused the double strand breaks and inturn resulted in the linearization of scDNA. Previous investigators have used Al- nitrate or Al- chloride for Al- DNA studies instead of Al-maltol.

All these above findings indicated that Al caused genetic malfunction through unwinding of scDNA to linearization of supercoiled pockets in genome.

Previous studies have shown that lower Al concentrations enhance the t_m of the sequence specific oligomeric DNA d(GCCCATGGC) but higher concentration does not alter the t_m of the oligomeric DNA [Champion *et al*. 1998]. Rajan *et al*. (1996) reported that Al did not alter the t_m of the DNA sequence d(GCGTACGC) at both lower and higher metal/DNA ratios. Reports also indicate that lower concentration of Mn enhanced t_m and higher concentration decreased t_m while Co enhanced the t_m of genomic DNA irrespective of ionic strength [Eichhorn and Shin, 1968].The present study showed that the t_m values of scDNA+Al complex were decreased compared to scDNA alone. The t_m value for the ctDNA+Al complex were increased compared to ctDNA alone. This significant increase in the Tm value of ctDNA + Al complex might be due to Z–DNA conformation because Z requires more energy to break the bonds. The t_m reduction in case of scDNA+Al complex may be due to the result of opening of super helicity and linearization of scDNA. This study provides insights that Al causes the destabilization of DNA.

Metal ions alleviate electrostatic repulsion between phosphates thereby stabilizing the base pairing and base stacking. This is more clearly evidenced by the increase in melting temperature and hypochromism, respectively. The alkali or alkali earth metals can neutralize repulsive interactions between the negatively charged sugar phosphate backbones and stabilize the interaction between base pairs. This further stabilizes the hydrogen bonding that pairs the two strands, resulting in higher melting temperature and leads to enhanced base stacking with increased hypochromism

[Cowan, 1993]. Like alkali metals, Al can also do the same to stabilize the hydrogen bonding between two strands, resulting in higher melting temperature seen in the Al-ct DNA complex. In contrast, reduction of the t_m in Al-scDNA complex may be due to loss of superhelicity and formation of a weak B-C-A mixed conformation and also may be due to Al binding to bases which destabilize the DNA. The nick translation results also reveal that Al ions can cause double strand breaks to scDNA and produce linear DNA and dimers which were also supported by gel and TEM studies.

The potential reactive sites found on DNA molecules are heterocyclic nitrogen atoms, exocyclic carbonyls on purines and pyrimidine bases and the phosphates oxygens. PO_3^- and base binding have rather different consequences for the structure of the bound DNA. Generally PO_3^- binding stabilizes the double helix while base binding causes the double helix to unwind. Electrostatic forces play a major role in the stability of the double stranded structures, and the double helix can be considered to be equilibrium under two types of opposite forces. The flexibility of double stranded structure will increase with the ionic strength. In low ionic strength the formation of ion pair between the PO_3^- and the counter ion or anion and water shell plays a critical role in the interaction of metal with DNA. However, in the presence of high ionic strength, the negative potential will vanish very close to each Po_3^- group. Accordingly, the anions which are excluded under low ionic strength can penetrate the grooves of the double helix, together with the cations, facilitating van der Waals interactions with the bases [Duguid *et al*., 1995]. Thus changes in the melting profiles of scDNA and genomic ctDNA indicate that binding of metal ions to DNA affects the thermal stability of the DNA based on metal ion charge/ionic radii and coordination potential [Duguid *et al*., 1995].

Another useful spectroscopic technique employed to study metal ion effects is the reduction of EtBr fluorescence. EtBr intercalates between the DNA bases and in doing so produces fluorescence [LePecq and Paoletti, 1967]. In addition to intercalation, EtBr also shows phosphate binding. The present fluorescence studies on metal-DNA interaction indicate that scDNA–Al and ctDNA-Al complexes showed less EtBr binding compared to the scDNA and ctDNA alone. The significant decrease in the fluorescence intensity in the case of the scDNA +Al-maltol complex might be the formation of the linearized form and consequent conformational change. But less EtBr intensity in case of Ga, In and Tl may be due to precipitation of DNA.

However, the reduction in the EtBr intensity in case of Al + ctDNA complex might be a result of the change in conformation from B to Z induced by Al. On the other hand, reduced EtBr intensity pattern in other metals down the group shows that they bind to DNA and at higher concentration they aggregate DNA. Lepecq and paeletti (1967) showed that EtBr at low salt concentrations shows a second mode of binding, which appears to be electrostatic binding of the cationic dye to negatively charged phosphate groups. Since metal ions show a greater affinity for phosphate groups and due to this decrease placement for the EtBr binding to phosphate group might be the reason for the reduction in fluorescence emission. The effect of cations on the stability of the double helix may enhance the overall effect of metal ions in reducing fluorescence.

Previously our lab had reported that Al at low concentration uncoils the super helical state of DNA [Rao *et al*., 1993]. The present agarose gel, TEM and nick translation studies showed that Al at higher concentrations linearizes the scDNA. Al

after binding to Po_3^- groups might have created DSBs to the phosphodiester bond between the nucleotides causing the linearization.

Previous studies on Al- induced conformational transitions in ctDNA indicates that at low concentrations Al did not alter the conformation while at higher concentrations Al caused B to A conformation as evidenced by Ahmed *et al*. (1996). The higher concentrations of Mg and Co hexamine were found to cause B-Z transitions in poly (dG-DC) [Ramesh and Brahmachari, 1983]. Behe and Felsenfeld, (1981) reported that heavy metals cause B-Z transition in poly (dG-dC) and poly (dG-m5dC) in the presence of Na. It was shown that the interaction of Al with DNA produces several types of Al-DNA complexes depending on pH and metal ion concentration [Karlik *et al*., 1980]. However, exact cations binding site and effect of Al interactions on DNA secondary structure have not been determined. Ahmed et al., (1996) showed through FTIR spectroscopy studies that Al at low concentrations binds mainly to the backbone PO_2 group and the guanine N-7 site of the G-C base pairs (chelation). Evidence for cation chelate formation comes from major shifting and an intensity increase of the phosphate antisymmetric stretch at $1222cm^{-1}$ and the mainly guanine band at $1717cm^{-1}$. The perturbations of A-T base pairs occur at high cation concentrations with major double helix destabilization. Evidence for cation binding to A-T bases comes from major spectral changes of the bands at 1663 and $1609cm^{-1}$ related mainly to thymine and adenine in plane vibration. A major reduction of the B-DNA structure occurs in favour of A-DNA upon trivalent cation coordination. There are very limited studies on Al -induced transitions in ctDNA [Rao and Divakar, 1993].

The CD spectra of the scDNA and ctDNA indicated a normal B form of DNA in solution containing 10mM Tris-HCl and 50mM NaCl. The present results showed that Al

could alter the normal B-DNA conformation to B-C-A mixed in scDNA. Moreover CD spectra of Al-ctDNA complex demonstrated the presence of Z-DNA conformation. None of the other IIIA group elements (Ga, In and Tl) were able to change the conformation of DNA but they were found bound to DNA and higher concentration of metals aggregated the DNA. Hence Al is the only trivalent metal ion in the 13 group of periodic table capable of inducing conformational change in DNA. Taken together, these results suggest that the effect of Al on DNA conformation does not represent a general effect of group 13 metals but rather is specific feature of Al. However, the latter three metal ions of group 13 showed strong binding to the DNA as evidenced by lowering of CD spectra at both 275 nm and 245 nm. Martin, (1996) indicated the nitrogen bases of the DNA and RNA do not bind to Al strongly. The weakly basic phosphate group of DNA binds Al (III) weakly, while the basic and chelating phosphate groups of nucleoside di and triphosphate do bind Al strongly. However, studies from Zhang *et al*., (2002) showed that Al ions bind to phosphate group of the DNA strongly.

The reasons attributed for structural changes in DNA are due to metal specific binding sites on DNA (base nitrogen, phosphate oxygen). It is known that Mg binds to PO_3^- and the N-7 atom of the nucleic acid base via a water bridge [Glassman *et al*., 1971], while Mn binds to the PO_3^- and N-7 site of the bases through the participation of a C6-NH_2 group [Martin, 1996]. Co binds the PO_3^-, C_6-NH_2 and C_5-OH groups of the bases [Shulman *et al*., 1965], whereas Al binds only to the PO_3^- moiety [Karlik *et al*., 1989]. Al co-ordination number being 6, its preferred geometries are octahedral and its biological ligands are oxygen, phosphates and carboxylates. Al forms a complex with oxygen donor ligands especially with phosphate group. It is generally believed that Al has a great affinity for

phosphate sites so that the interaction of naturally intaken and circulating Al with phosphate bearing molecules is expected to be a major circumstance in Al biology. Nucleotides contain three different metal binding sites: phosphate groups in the mono-, di- or tri phosphate moieties, alcoholic hydroxyl(s) at the ribose or deoxyribose unit and carbonyl oxygen or ring nitrogen donors in the nucleic base functions. The phosphate-binding site can be weakly basic or non-basic, as in the nucleic acids, DNA, and RNA, or it can be basic, as the terminal phosphate in nucleoside phosphates and in many other pyrophosphates [Kiss *et al*., 1996].

The studies from the interaction of Al with ATP showed that the basic terminal phosphate group is the primary binding site for the Al [Kiss *et al*., 1996; Bock and Ash, 1980]. So far no significant interaction with the other two binding sites for Al has been established by any structural investigation method. Studies from ^{31}P data revealed that Al binding strongly to P_β and P_γ in the phosphate chain resulting in the complexes containing octahedral Al(III) coordinated to six oxygen atoms as suggested by ^{27}Al NMR measurements [Kiss *et al*., 1996]. On the basis of ^{1}H NMR spectroscopic results, it was proposed that Al (III) binds to N-7 of the adenine base [Kiss *et al*., 1996]. Al (III) binds to all nucleoside phosphates predominantly through the phosphate group. ^{27}Al NMR spectral measurements showed that there was a great similarity in the Al (III) binding ability of various adenine and guanine nucleotides [Karlik *et al*., 1982].

In contrast with nucleotides, nucleic acid contain only weak acidic phosphates (pKa<2), whose metal binding ability is weak. The interaction of Al (III) with DNA was found to be so weak that quantitative study was limited to pH<5.5 because of metal ion hydrolysis and precipitation [Dyrssen *et al*., 1987]. In early work [Karlik *et al*., 1980,

1989] several mono- and dinuclear Al (III) complexes were detected with DNA at micromolar Al(III) concentrations using t_m and CD methods. No definite mechanism is available to explain the role of Al causing conformational transitions. It has been suggested that reduction of DNA phosphate repulsion (charge screening) and polymer hydration states were important determinants of conformational and structural transitions in DNA [Soumpass *et al*., 1987]. Rossetto and nieboer (1994) proposed that metals that bind to nitrogen bases facilitate DNA structural transitions. Drew *et al*. (1980) proposed that transition in DNA conformation occurs by lowering the activity coefficient or the effective concentration of the surrounding water molecules. Daune, (1974) reported that metal ions cause the transition due to the formation of an ion pair between the phosphate groups of DNA.

Based on the above findings and reports so far, it emerges that Al's sates as debatable etiological factor for AD as it is likely to play a role in modulating DNA conformation to the Z form. Z-form of DNA has been evidenced in AD brain.

3A. 5. References

Ahmed R, Naoui M, Neault JF, Diamantoglou S, Taimir-Riahi HA. An FTIR spectroscopy study of calf-thymus DNA complexation with Al(III) and Ga(III) cations, J.Biomol. Struct. Dyn. 1996; 13:795-780.

Alfrey AC. The physiology of aluminum in man. In: Gitelman HJ,(Eds), Aluminum and health. Marcel Decker Pub, New York, 1989; pp101-124.

Anitha S, Rao KSJ, Latha KS, Viswamitra MA. First evienced to show the topological change of DNA from B-DNA to Z-DNA conformation in the hippocampus of Alzhiemer's brain. J. Neuromol. Med. 2002; 2: 287 – 295.

Anitha S, Stein R, Rao KSJ, Latha KS, Viswamitra MA. A link between apoptopic DNA damage and DNA topology in Alzheimer's brain: a hypothesis. Alz Rep. 2001; 4: 121-131.

Bauer W.R, Crick F.H.C, White H. Supercoiled DNA, Sci. Am. 1980; 16: 243- 245.

Behe M, Felsenfeld G. Effect of methylation on a synthetic polynucleotide: the B-Z transition in poly(dG-m5dC),poly(dG-m5dC). Proc.Natl.Acad.Sci.(USA). 1981; 78: 1619 – 1624.

Berlyne GM, Yagil R, Ari JB, Weinberger G, Knopf E, Danovitch GM. Aluminium toxicity in rats. Lancet. 1972; 1:564-568.

Bock JL, Ash DE. NMR and infrared spectroscopic investigations of the Al (III), Ga(III), and Be(II) complexes of ATP, J. Inorg. Biochem. 1980; 13:105-110.

Candy JM, Oakley AE, Klinowski J, Carpenter TA, Perry RH, Atack JR, Perry EK, Blessed G, Fairbairn A, Edwardson JA. Aluminosilicates and senile plaque formation in Alzheimer's disease. Lancet. 1986; 1: 354 -357.

Champion CS, Kumar D, Rajan MT, Rao KSJ, Viswamitra MA. Interaction of Co, Mn, Mg and Al with d(GCCATGGC) and d(CCGGGCCCGG): a spectroscopic study, Cell. Mol. Life Sci. 1998; 54: 488-496.

Chatterjee B, Rao GR. Superhelical density of goat mitochondrial DNA: fluorimetric studies. Indian J Biochem Biophys. 1994; 31:77-79.

Corain B, Bombi GG, Tapparo A, Perazzolo M, Zatta P. Aluminum toxicity and metal speciation: established data and open questions, Coord. Chem. Rev. 1996; 149: 11-22.

Cowan JA. Inorganic Biochemistry; VCH, NewYork, 1993, pp1-45.

Crapper DR, Karlik SJ, De Boni, U, Senile dementia and related disorders. In: Katzman R, Terry RD, Bick KL, (Eds), Alzheimer's disease. Raven Press, New York, 1978, pp 471-485.

Crapper DR, Quittkat S, Krisnan SS, Dalton AJ, DeBoni U. Intranuclear aluminum content in Alzheimer's disease and dialysis encephalopathy and experimental aluminum encephalopathy. Acta Neuropathol. 1980; 50: 19-24.

Daune M. Interaction of metal Ions with Nucleic Acid, Marcel Decker, New York, 1974, 3, pp1-43.

De Boni, U, Scott JW, Crapper DR. Intracellular aluminum binding; a histochemical study. Histochemistry. 1974; 40: 31-37.

Drew H, Takano J,Tanaka S, Itakuva K, Dickerson RE. High salt d(CpGpCpG), a left handed Z-DNA double helix, Nature. 1980; 286: 567-568.

Dubochet J, Ducommun M, Zollinger M, Kellenberger E. A new preparation method for dark-field electron microscopy of biomacromolecules, J Ultrastruct Res, 1971; 35: 147-167.

Duguid JG, Bloomfield VA, Benevides JM, Thomas GJ Jr. Aggregation of melted DNA by divalent metal ion-mediated cross-linking, Biophys. J. 1995; 69:2623-2632.

Dyrssen D, Haraldsson C, Nyberg E, Wedborg M. Complexation of aluminum with DNA, J.Inorg.Biochem. 1987; 29: 67 -75.

Edwardson JA, Ferrier IN, McArthur FK, McKeith IJ, McLaughlin I, Morris CM, Mountfort SA, Oakley AE, Taylor GA, Warda MK, Candy JM. In: Nicolini M, Zatta PF, Corain B (Eds), Aluminum in Chemistry Biology and Medicine, Cortina International Raven Press, New York. 1991, pp 85.

Eichhorn GL, Shin YAJ. Interaction of metal ions with polynucleotide and related compounds. XII. The relative effect of various metal ions on DNA helicity. J.Am.Chem. Soc.1968; 90: 7323-7329.

Exley C. The aluminium-amyloid cascade hypothesis and Alzheimer's disease. Subcell Biochem. 2005; 38:225-234.

Finnegan MM, Retigg SJ, Orvig C. A neutral water-soluble aluminum complex of neurological interest. J. Am. Chem. Soc. 1986; 108: 5033-5035.

Garruto RM, Swyt C, Yanagihara R, Fiori CE, Gajdusek DC. Intraneuronal co-localization of silicon with calcium and aluminum in amyotrophic lateral sclerosis and Parkinsonism with dementia of Guam. N Engl J Med. 1986; 315: 711-712.

Ghiribi O, Herman MM, Savory J. The endoplasmic reticulum is the main site for caspase-3 activation following aluminum-induced neurotoxicity in rabbit hippocampus. Neurosci Lett. 2002a; 324:217-221.

Ghiribi O, Herman MM, Spaulding NK, Savory J. Lithium inhibits aluminum-induced apoptosis in rabbit hippocampus, by preventing cytochrome c translocation, Bcl-2 decrease, Bax elevation and caspase-3 activation. J Neurchem. 2002b; 82: 137-145.

Glassman TA, Cooper C, Harrison LW, Swiff TJ. A proton magnetic resonance study of metal ion-adenine ring interactions in metal ion complexes with adenosine triphosphate. Biochemistry. 1971; 10:843-851.

Gray DM, Taylor TN, Lang D.Dehydrated circular DNA: circular dichroism of molecules in ethanol solutions. Biopolymers. 1978; 17:1-45.

Gray M, Dratliff RL, Vaughan MR. Circular Dichroism Spectroscopy of DNA. Methods Enzymol. 1992; 211: 389 – 406.

Griffioen KJ, Ghiribi O, Fox N, Savory J, DeWitt DA. Aluminum maltolate-induced toxicity in NT2 cells occurs through apoptosis and includes cytochrome c release. Neurotoxicology. 2004; 25: 856-867.

Gupta VB, Anitha S, Hegde ML, Zecca L, Garruto RM, Ravid R, Shankar SK, Stein R, Shanmugavelu P, Rao KSJ. Aluminium in Alzheimer's disease: are we still at a crossroad? Cell Mol Life Sci. 2005; 62:143-158.

Hanlon S, Brudno S, Wu TT, Wolf B. Strucrural transitions of deoxyribonucleic acid in aqueous electrolyte solutions. I. Reference spectra of conformational limits. Biochemistry. 1975; 14:1648-1660.

Hanlon S, Chan A, Berman S. Specific cation effects on conformational transitions of DNA in aqueous solutions. Biochim Biophys Acta. 1978; 519:526-36.

Kaneko N, Takada J, Yasui H, Sakurai H. Memory deficit in mice administered aluminum-maltolate complex. Biometals. 2006; 19: 83-89.

Karlik SJ, Chong AA, Eichhorn GL, De Boni U. Reversible toroidal compaction of DNA by aluminum. Neurotoxicology. 1989; 10: 167-176.

Karlik SJ, Eichhorn GL, Lewis PN, Crapper DR. Interaction of aluminum species with deoxyribonucleic acid. Biochemistry. 1980; 19: 5991-5998.

Karlik SJ, Eichhorn GL. Polynucleotide cross-linking by aluminum. J Inorg Biochem. 1989; 37: 259-269.

Karlik SJ, Elgavish GA, Pillai RP, Eichhorn GL. Aluminum-27 NMR studies of Al(III)-phosphate complexes in aqueous solution. J. Magn. Reson. 1982; 49: 164-167.

Kiss T, Zatta P, Corain B. Interaction of aluminum (III) with phosphate binding sites: biological aspects and implications. Coord. Chem. Rev. 1996; 149:329-346.

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

LePecq JB, Paoletti C. A fluorescent complex between ethidium bromide and nucleic acids.Physical-chemical characterization. J.Mol.Biol.1967; 27: 87-92.

Lukiw WJ, Krishnan B, Wong L, Kruck TP, Bergeron C, Crapper McLachlan DR.Nuclear compartmentalization of aluminum in Alzheimer's disease. Neurobiol Aging. 1992; 13: 115-121.

Lukiw WJ, Kruck TP, McLachlan DR. Alterations in human linker histone-DNA binding in the presence of aluminum salts in vitro and in Alzheimer's disease. Neurotoxicology. 1987; 8:291-301.

Maestre MF, Wang JC. Circular dichroism of superhelical DNA. Biopolymers. 1971; 10:1021-1030.

Martell AE, Hancock RD, Smith RM, Motekaitis RJ. Coordination of Al(III) in the environment and in biological systems. Coord. Chem. Rev. 1996; 149:311 -328.

Martin CN. Osmond C, Edwardson JA, Barker DJP, Lacey RE. Geographical relation between Alzheimer's disease and aluminum in drinking water. Lancet. 1989; 1: 59-62.

Martin RB. Chemistry of Aluminum in the central nervous system. In: Yasui M, Strong JM, Ota K, Verity AM (Eds), Mineral and Metal Neurotoxicology. CRC press, New York. 1996, pp75-80.

Martin RB. The chemistry of aluminum as related to biology and medicine. Clin Chem. 1986; 32:1797-1806.

McLachlan DR, Dam TV, Farnell BJ, Lewis PN. Aluminum inhibition of ADP-ribosylation in vivo and in vitro. Neurobehav Toxicol Teratol. 1983; 5: 645-647.

McLachlan DR, Dam TV, Farnell BJ, Lewis PN. Aluminum inhibition of ADP-ribosylation in vivo and in vitro, Neurobehav Toxicol Teratol. 1983; 5: 645-647.

Meiri H, Banin E, Roll M, Rousseau A. Toxic effects of aluminium on nerve cells and synaptic transmission. Prog Neurobiol. 1993; 40:89-121.

Perl DP, Brody AR. Alzheimer's disease: X-ray spectrometric evidence of aluminum accumulation in neuro-fibrillary tangle bearing neurons. Science, 1980; 208: 297-299.

Perl DP, Gajdusek DC, Garruto RM, Yanagihara RT, Gibbs CJ. Intraneuronal aluminum accumulation in amyotrophic lateral sclerosis and Parkinsonism-dementia of Guam. Science. 1982; 217:1053-1055.

Rajan MT, Champion CS, Kumar D, Vishnuvardhan D, Rao KSJ, Viswamitra MA. Interaction of Co, Mn, Mg and Al with d(GCGTACGC): A spectroscopic study, Mol. Biol. Rep. 1996; 22:47-52.

Ramesh N, Brahmachari SK. Critical cation balance in B leads to Z transition: role of Li, FEBS Lett. 1983; 164:33-38.

Rao GV, Rao KSJ. Evidence for a hydroxy-aluminum polymer (Al_{13})in synaptosomes, FEBS Lett. 1992; 311: 49-50.

Rao KSJ, Divaker S. Spectroscopic studies on the effects of Aluminum ion on Calf-Thymus DNA. Bull. Environ. Contam. Toxicol. 1993; 50: 92-99.

Rao KSJ, Katestos CD, Hermann MM, Savory J. Experimental aluminum encephalopathy. Clin Lab Med. 1998; 18: 687 – 698.

Rao KSJ, Rao BS, Vishnuvardhan D, Prasad KVS. Alteration of superhelical state of DNA by aluminum (Al), Biochim Biophys Acta. 1993; 1172:17-20.

Rao KSJ, Rao RV, Shanmugavelu P, Menon RB. Trace elements in Alzhiemer's disease brain: A new hypothesis. Alz Rep. 1999; 2: 241-246.

Rossetto FE, Nieboer EJ. The interaction of metal ions with synthetic DNA: induction of conformational and structural transitions, Inorg. Biochem. 1994; 54: 167-172.

Sanderson C, McLachlan DR, De Boni U. Inhibition of corticosterone binding in vitro, in rabbit hippocampus, by chromatin bound aluminum. Acta Neuropathol. 1982; 57: 249-254.

Sarkander HI, Balb G, Schlosser R, Stoltenburg G, Lux RM. In: Cervos- Navarro J, Sarkander HI, (Eds), Brain aging: Neuropathology and Neuropharmacology, Raven Press, New York, 1983, pp 259-274.

Savory J, Exley C, Forbes WF, Haung Y, Joshi JG, Kruck T, McLachlan DR, Wakayama I. Can the controversy of the role of aluminum in Alzheimer's disease be resolved? What are the suggested approaches to this controversy and methodological issues to be considered? J Toxicol Environ Health. 1996; 48: 615-635.

Savory J, Herman MM, Ghiribi O. Intracellular mechanisms underlying aluminum-induced apoptosis in rabbit brain. J Inorg Biochem. 2003; 97: 151-154.

Shin YA. Interaction of metal ions with polynucleotide and related compounds. XXII. Effect of divalent metal ions on the conformational changes of polyribonucleotides. Biopolymers. 1973; 12:2459-2475.

Shulman RG, Sternlight H, Wyluda BJ. Study of metal - ion binding to nucleic acids by 31-P nucleic magnetic resonance. J. Chem. Phys. 1965; 43:3116-3122.

Soumpass Dm, Wiechen J, Jovin TM. Relative stabilities and transitions of DNA conformations in 1:1 electrolytes: a theoretical study, J.Biomol. Struct. Dyn. 1987; 4:535-552.

Suh D, Sheardy RD, Chaires JB. Unusual binding of ethidium to a deoxyoligonucleotide containing a B-Z junction. Biochemistry. 1991; 30:8722-8726.

VanBerkum MF, Wong YL, Lewis PN, Crapper McLachlan DR. Total and poly (A) RNA yields during an aluminum encephalopathy in rabbit brains. Neurochem Res. 1986; 11:1347-1359.

Walton JR. Aluminum in hippocampal neurons from humans with Alzheimer's disease. Neurotoxicology. 2006; 27:385-394.

Wedrychowski A, Schmidt WN, Hnilica LS. The in vivo cross-linking of proteins and DNA by heavy metals. J Biol Chem. 1986; 261:3370-3376.

Wen GY, Wisniewski HM. Histochemical localization of aluminum in the rabbit CNS. Acta Neuropathol. 1985; 68: 175-184.

Zatta P, Lucchini R, van Rensburg SJ, Taylor A. The role of metals in neurodegenerative processes: aluminum, manganese, and zinc. Brain Res Bull. 2003;62:15-28.

Zhang RY, Liu Y, Pang DW, Cai RX, Qi YP. Spectroscopic and Voltammetric Study on the Binding of Aluminium (III) to DNA. Anal Sci. 2002; 18:761-766.

3B. 1. Introduction

Iron is an essential element for normal cellular functions. Iron is found in the active centers of many enzymes and oxygen carrier proteins. It is a key component of cytochrome a, b, and c, and cytochrome oxidase which use iron as cofactor for adenosine triphophate (ATP) synthesis in the electron transport chain [Gordon, 2003]. Iron also plays a specific role in the central nervous system (CNS). It is involved in myelin formation [Connor *et al.,* 1995] as well as in the synthesis of several neurotransmitters and in generation of GABAergic activity [Li, 1998]. Iron is needed as a cofactor for many enzymes that are involved in the normal function of neuronal tissue such as the non-haem iron enzyme 'tyrosine hydroxylase' which is essential for dopamine synthesis [Ponting, 2001] and as a cofactor for the production of norepinephrine and serotonin [Li, 1998]. However, iron overload has been implicated as one of the causes of neuronal death [Berg *et al.,* 2001] and can lead to vast range of disorders of the central nervous system. Abnormally high levels of iron in specific brain regions related to PD and AD where neurons undergo degeneration have been reported [Zecca et al., 2004]. These studies suggest the involvement of altered iron homeostasis including transferrin and ferritin in these neurodegenerative disorders [Zecca *et al.*, 2004; Jenner, 1994; Dexter *et al.*, 1989; Lovell *et al*., 1998; Griffiths *et al.,* 1999; Rao *et al.,* 1999; Good *et al.,* 1992; Connor *et al.,* 1992a and 1992b; Thompson *et al.,* 1988].

The neurotoxicity of iron has been widely reported [Chiueh et al., 2000; Liu et al., 2006]. It is a potentially toxic molecule because it contributes to the enhanced generation of ROS, and causes oxidative damage to biomolecules like DNA, proteins and lipids [Halliwell, 1978a; 1978b, Wolin *et al.,* 1997; Hampton and Orrenius, 1997; Halliwell and

Gutteridge, 1984; Crichton, 2001] and thus suspected to play a major role in AD [Sayre *et al.,* 1997; Smith *et al.,* 1997; Smith and Perry, 1995] and other CNS disorders [Floyd,1990; Hall *et al.,* 1993; Sayre *et al.,* 1999]. The increase in iron concentration in neurons, astrocytes and microglia have been reported in regions such as the cortex, hippocampus and substantia nigra, which are particularly susceptible to neuropathological changes that would characterize AD and PD [Jellinger *et al.,* 1990; Griffiths and Crossman, 1993; Zecca et al., 2004]. Normally ferritin, considered as a cytoplasmic iron-storage protein, is also found in the nuclei of neuronal cells [Surguladze, 2004]. Neurons and microglia as well as oligodendrocytes express ferritin, indicating that all of these cell types have the capacity to store iron [Zecca, 2004]. Thompson *et al.* (2002) reported that H-ferritin subunits are seen in neuronal nuclei and cross-linked to chromosomal DNA *in vivo*. Moreover, a recent report has shown that iron is localized in the chromatin region of the neurons in AD brain [Smith *et al.,* 1997].

Metals like Al, Mn, Zn etc are known to modulate secondary conformation of synthetic DNA sequence or oligonucleotides [Rajan *et al.,* 1996; Champion *et al.,* 1998; Latha *et al*., 2002]. Although iron induced oxidative DNA damage through Fenton reaction has been extensively studied [Sagripanti *et al.,* 1987; Toyokuni and Sagripanti, 1992, 1993; Muiras *et al.,* 1993; Kobayash *et al.,* 1990; Stohs and Bachi, 1995; Halliwell and Aruama, 1991; Henle *et al.,* 1999; Barbauti *et al.,* 2001; Rai et al., 2001]. Very few reports are available on studies of iron binding to DNA [Bertoncini *et al.,* 1999; Eisinger *et al.,* 1961; Luo *et al.,* 1994; Trotta *et al.,* 2002]. However, there are limited studies on iron - induced DNA conformations [Asin *et al.,* 2000] and to date no comprehensive data exists on role of iron induced DNA topological changes. Further, the discovery of Z-

DNA conformation in AD brain hippocampus and nuclear localization of iron and ferritin in the hippocampal neurons of AD brain offers new prospectives for the present study, to unravel the DNA topology as observed in AD brain [Anitha *et al*., 2002]. It is intriguing to consider that the localization of iron (cationic) in the nuclear region may facilitate its interaction with polyanionic DNA. With this aim interaction of iron (Fe^{2+}, Fe^{3+}) with Supercoiled DNA (pUC 19- scDNA) and Fe^{2+} with linearised scDNA have been analyzed in the present study. Plasmid scDNA used as model system makes an interesting study, in view of the observation that vast arrays of small scDNA packets have been found in animal and human cells and known to be involved in gene expression [Bauer *et al.,* 1980]. The scDNA is plasmid pUC-19 scDNA which resembles mammalian genomic system. Hence the outcome of the present study can be correlated to human brain genomic DNA to provide an insight into the possible role of iron in the progression of AD pathology with reference to DNA topology.

3B. 2. Materials and Methods

3B. 2. 1. Materials

pUC 19 scDNA (cesium chloride – purified, 90% supercoiled conformation) and agarose, were purchased from Genie, India. Chloroquine, ethidium bromide (EtBr), Hepes, Tris(hydroxymethyl) aminomethane (Tris) were purchased from Sigma (USA). Linearized DNA fragments were prepared as outlined below. A Samples of (5-10μg) pUC 19 scDNA were cut with the restriction enzyme of interest (New England Biolabs, USA) until completion as judged by Transmission Electron Microscopy (TEM) observation and scDNA were purified using Qiagen kits (Qiagen, Germany).

E. coli DNA Polymerase I and Terminal deoxynucleotidyl transferase enzymes were purchased from Amersham chemicals, UK. ^{3}[H]- TTP (Sp. Act.40Ci/mmol) was purchased from Amersham Radiochemicals, UK.^{3}[H] Thymidine (17Ci/mmol) was purchased from Bhaba Atomic Research Center, Bombay, India. dATP, dTTP, dCTP and dGTP were purchased from Sigma (USA). $FeSO_4$ and $Fe(NO_3)_3$ were from Merck(Germany), Stock solution of $FeSO_4$ was prepared freshly in 5mM H_2SO_4 (to prevent oxidation). Stock solution of $Fe(NO_3)_3$ was prepared in MQ water. All other chemicals used were of analytical grade and were purchased from local chemical companies. MQ water was used for preparing the stock solutions and washing the vials.

3B. 2. 2. Methods

3B. 2. 2. 1. CD Spectroscopy - CD spectra were recorded on a Jasco J 750 Spectropolarimeter at 25^{o}C with 2mm cell length. scDNA and ctDNA were titrated with different concentrations of $FeSO_4$ (2.5 x 10^{-4} to 1.5 x 10^{-3} M), and Fe $(NO_3)_3$ (2.5 x 10^{-4} to 1.5 x 10^{-3} M). CD spectra (200-300nm) were recorded for each concentration in 10mM Hepes buffer (pH 7.4). All other measures and methods are the same as those described for Al [see material and methods section of chapter IIIA].

3B. 2. 2. 2. Agarose Gel electrophoresis - Integrity of the scDNA was studied by 1% agarose gel electrophoresis. The DNA samples (scDNA, scDNA + $FeSO_4$, scDNA + $Fe(NO_3)_3$ complexes) were loaded in 1% agarose gels in TA buffer [40mM Tris-acetate (pH 8.0)] and electrophoresed at 4V/cm at room temperature for 8-10 hours. DNA concentration loaded in all lanes was 1μg. All samples prior to loading onto the gel were incubated with $FeSO_4$ (1x10^{-6} M to 1x10^{-3}M) and $Fe(NO_3)_3$ (1x10^{-6} M to

$1x10^{-3}$M) in the absence of H_2O_2 for 45min at RT. To rule out the free radical induced DNA damage in one mixture of sample we added 50µg/ml of catalase. The gels were stained with EtBr and photographed using the gel documentation.

3B. 2. 2. 3. Fluorescence Measurements (EtBr binding Studies) - The EtBr binding patterns to scDNA(1µg), scDNA + ($7.5x10^{-4}$ M to $1x10^{-3}$M) $FeSO_4$ and scDNA+ $Fe(NO_3)_3$ ($7.5x10^{-4}$ M to $1x10^{-3}$M) complexes were analyzed by mixing 1:1 (w/w) DNA/EtBr before measuring fluorescence emission. All other measures and methods are the same as those described for Al [see material and methods section of chapter IIIA]. The amount of EtBr bound and the average number of EtBr molecule bound per base pairs in the Fe-DNA complexes was calculated using Scatchard's equation [Suh *et al.,* 1991; Chatterjee and Rao, 1994].

3B. 2. 2. 4. Melting Profile - The melting profile curves (1^oC/min, 28-95^oC) for scDNA (1µg), scDNA+ ($5x10^{-4}$ M to $7.5x10^{-3}$M) $FeSO_4$, scDNA + ($5x10^{-4}$ M to $7.5x10^{-3}$M) $Fe(NO_3)_3$ complexes were performed using a Amersham Ultraspec 4300pro UV/Visible spectrophotometer with thermostat according to the method described for Al [see chapter IIIA].

3B. 2. 2. 5. Estimation of single/double strand breaks.

Single Strand Breaks (SSB) - SSBs in 1µg of scDNA, Single strand circular DNA(sscDNA) and double strand circular DNA (dscDNA) are induced by $FeSO_4$ ($7.5x10^{-4}$M) and $Fe(NO_3)_3$ ($7.5x10^{-4}$M) were determined through a nick translation type of reaction using E.coli DNA Polymerase I (pol I). The assay mixture and protocols followed are the same as those for Al.

Double Strand Breaks (DSB) – DSBs in 1µg of scDNA, and double strand circular DNA (dscDNA) are induced by $FeSO_4$ ($7.5x10^{-4}$M) and $Fe(NO_3)_3$ ($7.5x10^{-4}$M) were determined through a nick translation type of reaction using E.coli DNA Polymerase I (pol I).The reaction mixture for terminal transferase enzyme assay and protocols followed are the same as those for Al and Liquid Scintillation Counter were used to measure the radioactivity of incorporated ^{3}[H]-dTTP.

3B. 2. 2. 6. Transmission electron microscopy - 1µg of scDNA, scDNA+ ($7.5x10^{-4}$M to $1.5x\ 10^{-3}$M) $FeSO_4$ complex, linearized scDNA(1µg), linearised scDNA + ($7.5x10^{-4}$M to $1x10^{-3}$M) $FeSO_4$ complex were aliquoted for 10µl containing 10mM Tris-Cl (pH 7.4) buffer containing 50mM NaCl and incubated for 45min at RT. The rest of the procedure followed for transmission electron microscopy is same as outlined in chapter IIIA.

3B. 3. Results

3B. 3. 1. Circular Dichroism Spectroscopy – The CD spectrum of the scDNA showed B-DNA conformation which was characterized by a strong positive peak at 275nm and a negative peak at 245nm. CD studies were carried out on addition of increasing concentrations ($2.5\ x\ 10^{-4}$ to $1.5\ x\ 10^{-3}$ M) of $FeSO_4$ and $Fe(NO_3)_3$ to scDNA. In both cases the CD spectra were measured after 10min incubation at RT. On addition of increasing concentrations ($2.5\ x\ 10^{-4}$ to $1\ x\ 10^{-3}$ M) of $FeSO_4$ to the scDNA (Fig.1A), sign of the ellipticity of the band at 275 nm region decreased with no concomitant change in the magnitude of the negative ellipticity at 245 nm. This transition, decrease in the positive peak at 275 nm without any change in the negative peak at 245 nm is a characteristic structural feature of C-DNA indicating that a significant portion of the B-

DNA was converted to a C-DNA conformation. In case of $Fe(NO_3)_3$ (Fig. 1B), there was a decrease in the magnitude of both the positive peak at 275 nm and the negative peak at 245 nm. These spectra indicated a strong binding of Fe^{3+} ions to phosphate backbone of scDNA without any change in the normal B-DNA conformation. The magnitudes of the change in the positive peak at 275 nm and negative peak at 245 nm were plotted with molar ellipticity values versus the concentration of the iron complexes, to determine the percentage change at both wavelengths for each concentration (Fig.2A and 2B). The results indicated a consistent decrease in the molar ellipticity of the positive peak at 275 nm, with no change in the negative peak at 245 nm for Fe^{2+} which reveals a C-DNA conformation. To exclude the possibility of the similar spectral change may be due to the lowering of superhelicity of the scDNA, we studied the CD spectra of scDNA in the presence of chloroquine (10ng/μl), which uncoils the scDNA like topoisomerase-I. In the CD spectra there was no change in the positive or negative peak on uncoiling of scDNA with chloroquine and the spectra was similar to that of scDNA CD spectra (Fig.3). Hence it is clear that relaxation of supercoils is probably not associated with the decrease in the positive peak at 275nm.

Further we also studied CD spectra of ctDNA in the presence of Fe^{2+} and Fe^{3+} ions to understand the role of iron modulating the normal B DNA conformation. Interestingly CD spectra showing the similar results were observed comparable to that of scDNA-iron interactions. Fe^{2+}caused C-DNA conformation [Fig. 4A] while Fe^{3+} could not induce DNA conformation except a decrease in both peaks at 275nm and 245nm [Fig 4 B]. This study also clearly indicates that conformational change is not due to simple uncoiling process but conformational change from B to C – DNA caused by Fe^{2+} ions.

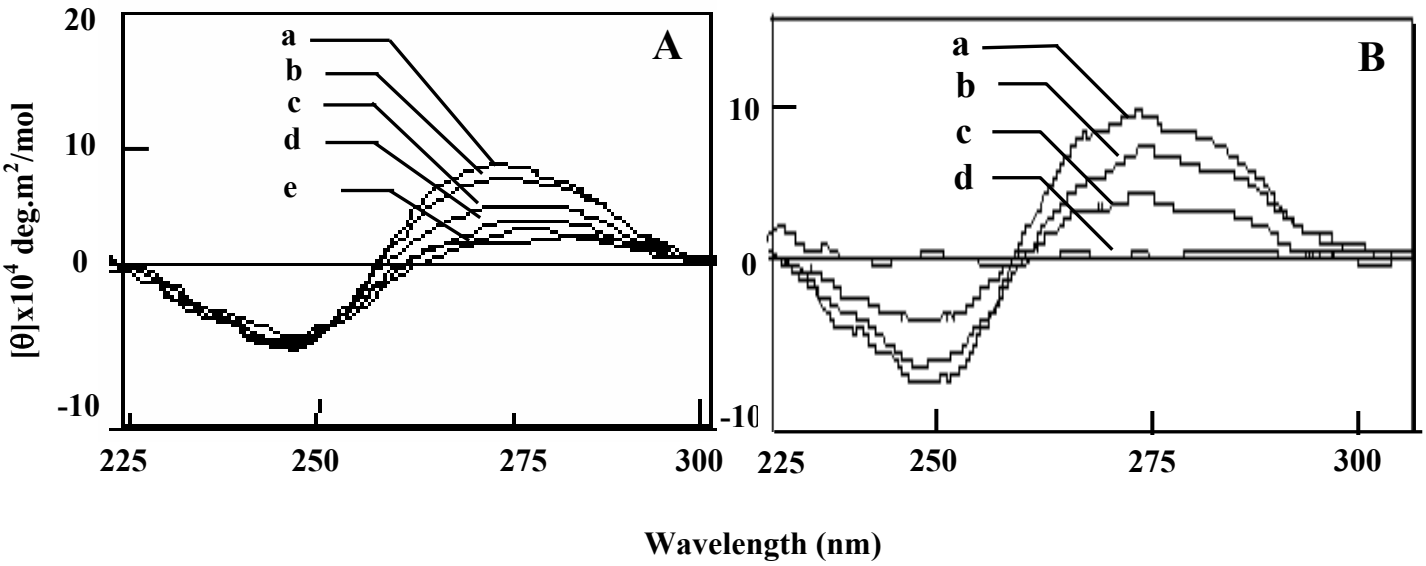


Figure 1A and 1B. CD spectra of scDNA alone(a) and in presence of $Fe(SO)_4$ measured at different concentration 250μM (b), 500μM (c), 750μM(d), and 1.0mM(e) in 10^{-4} M HEPES buffer (pH 7.4). The scDNA alone showed B-DNA conformation. On addition of increasing concentrations of $Fe(SO)_4$, the native B-DNA conformation was transformed to C conformation while in Fig.1B, $Fe(NO)_3$ could not induce the conformational change except binding but, at 750μM it aggregate the DNA.

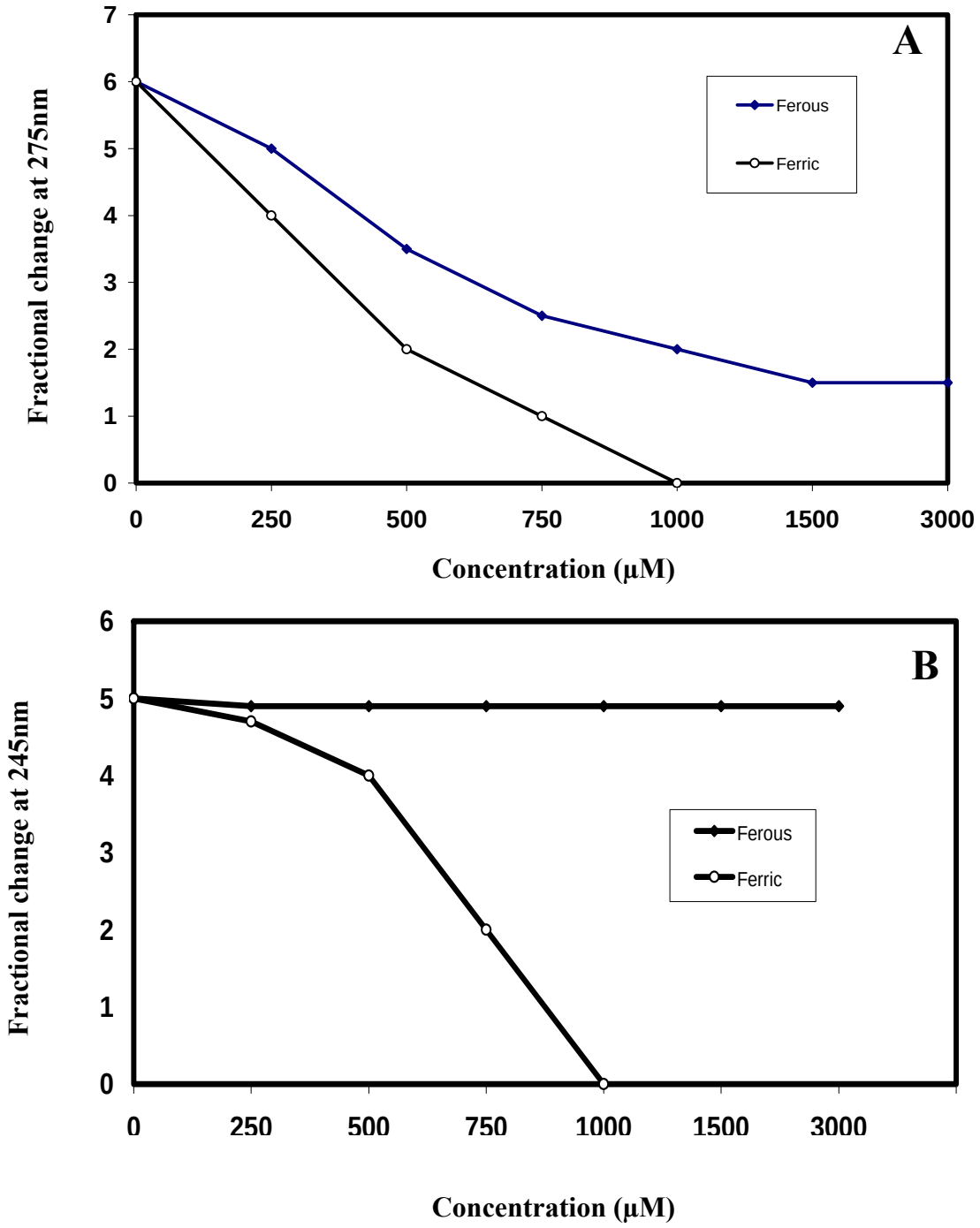


Figure 2. Magnitudinal change in CD spectra of Iron scDNA complexes at 275nm and 245nm. Magnitudinal change in CD spectra of Fe^{2+} and Fe^{3+} with scDNA at 275nm (A), magnitudinal change in CD spectra of $Fe^{2+}$and Fe^{3+} scDNA at 245nm (B).

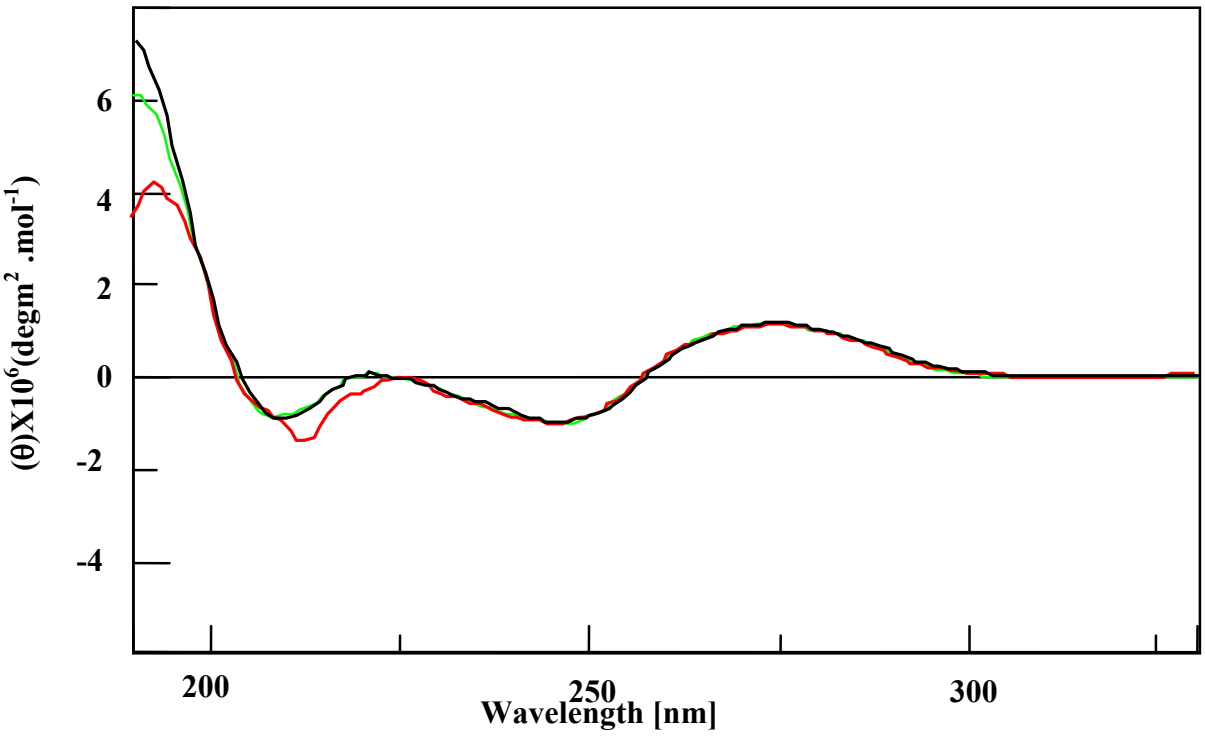


Figure 3. CD spectra of scDNA alone (a) and presence of chloroquine (10ng/µl) (b). There is no change in positive and negative peak of scDNA after treating with chloroquine.

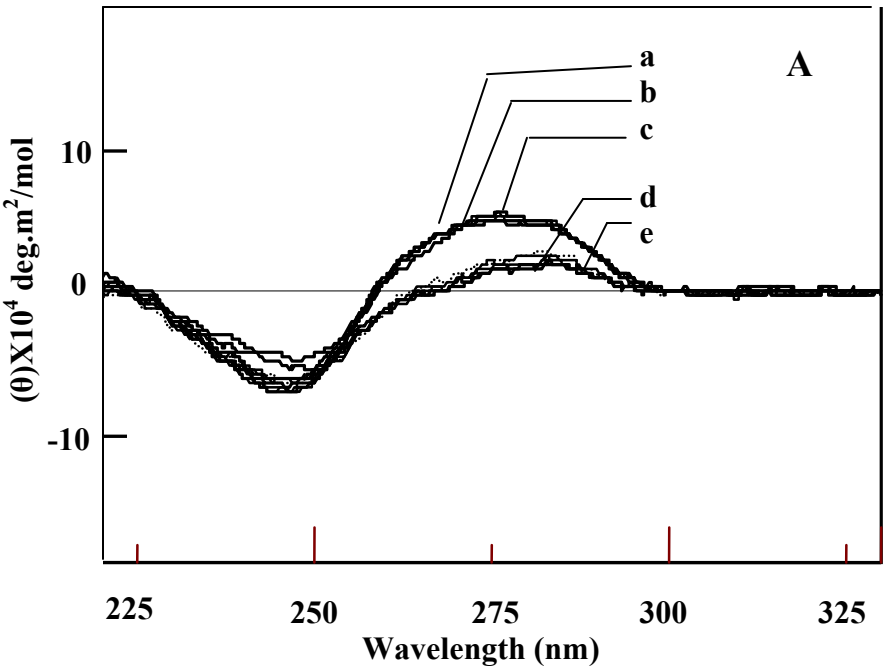


Figure 4 A. CD spectra of Fe^{2+} interaction with ctDNA. Effect of Fe^{2+} on conformation of ctDNA in 10^{-4} M hepes buffer (pH 7.0). The ct- DNA alone showed B-DNA conformation. On addition of increasing concentrations of Fe^{2+} ($2.5x10^{-4}$, $5x10^{-4}$ M, $7.5x10^{-4}$ M, $1x10^{-3}$ M), the native B-DNA conformation transformed to C conformation.

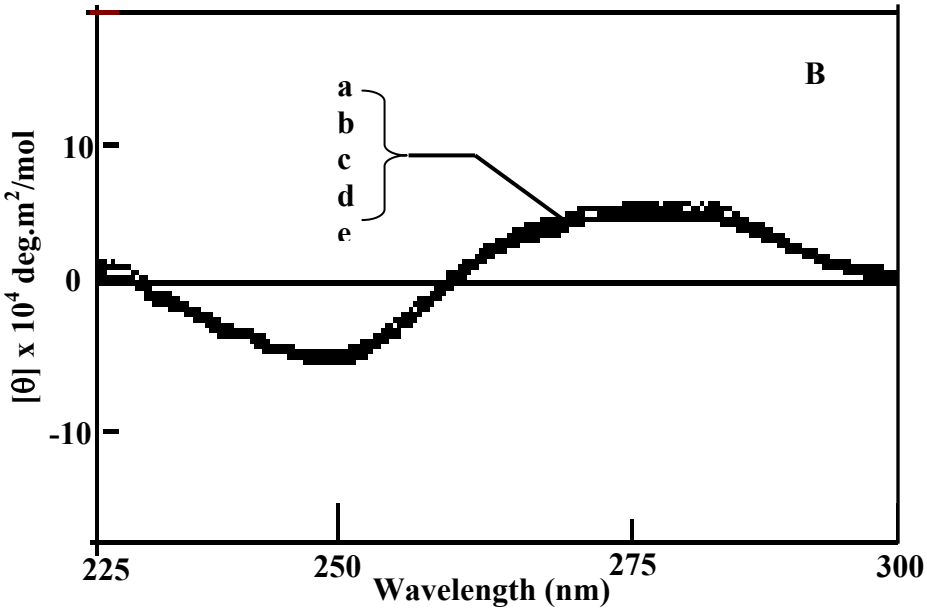


Figure 4B. CD spectra of Fe^{3+} interaction with ctDNA. Effect of Fe^{3+} conformation of ctDNA in 10^{-4} M hepes buffer (pH 7.0). The scDNA alone showed B-DNA conformation. On addition of increasing concentrations of Fe^{3+}($2.5x10^{-4}$ M, $5x10^{-4}$ M,$7.5x10^{-4}$ M, $1x10^{-3}$ M), the native B-DNA conformation was not altered.

3B. 3. 2. Agarose Gel Electrophoresis -scDNA(1µg) was incubated with $FeSO_4$ ($1x10^{-6}$to $2.5\ x10^{-3}$M), $Fe(NO_3)_3$ ($1x10^{-6}$to $1x10^{-3}$M) in buffer containing 10mM Tris-Cl (pH 7.5), 50mM NaCl and subjected to 1% agarose gel electrophoresis (Fig.5A and 5B).Untreated plasmid DNA showed a major band corresponding to the supercoiled form (form I) and a minor band corresponding to the circular DNA (form II). When DNA was incubated with a Fe^{2+}, single strand breaks were increased as shown by the decrease in the amount of form I (remaining supercoiled DNA) and concomitant increase in the bands corresponding to nicked circular DNA (Figure 5A, lane C to J). When different concentrations of Fe^{3+} were incubated with scDNA up to $2x10^{-4}$ M no change was observed in the integrity of scDNA. But as concentrations increased from $2x10^{-4}$M onwards, scDNA started condensing and at higher concentration it began to precipitate. It was also noticed that there was no effect of catalase (50µg/ml) on Fe^{2+} induced open circular form through single strand nick [Fig. 6]

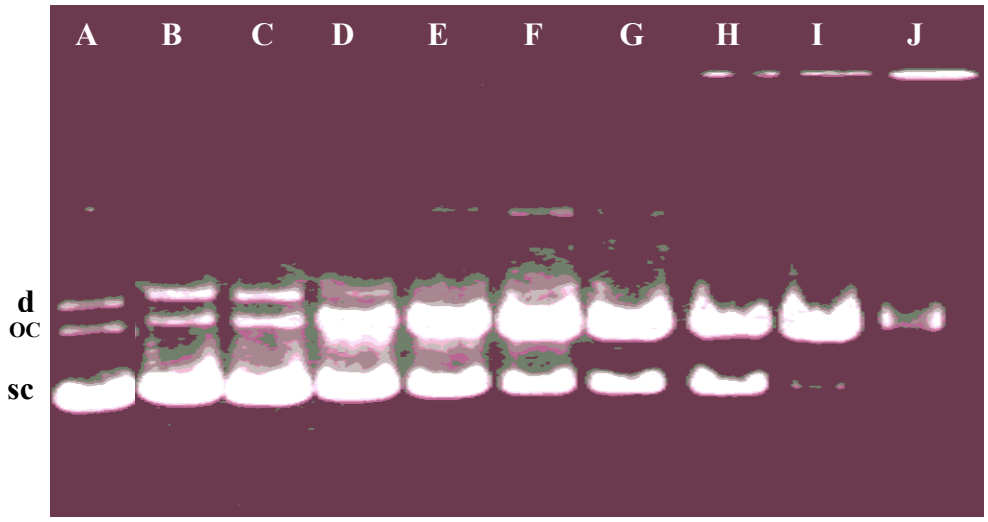


Figure 5A. Effects of Fe^{2+} ions on scDNA in 1% agarose gel. The control scDNA is presented in supercoiled (sc) and open circular (oc) forms and as dimer plasmid (d). Lane A, scDNA alone; Lane B, plus $1x10^{-6}$M Fe^{2+}; Lane C, plus $1x10^{-5}$M Fe^{2+}; Lane D, plus $1x10^{-4}$M Fe^{2+}; Lane E, plus $2x10^{-4}$M Fe^{2+}; Lane F, plus $2.5x10^{-4}$M Fe^{2+} ; Lane G, $5x10^{-4}$M Fe^{2+}; Lane H, $7.5x10^{-4}$ M Fe^{2+}; Lane I, $1x10^{-3}$ M Fe^{2+}; Lane J, $2.5x10^{-3}$M Fe^{2+} all the reaction mixture were incubated at RT in buffer containing 10mM Tris-cl (pH 7.4) and 50mM NaCl for 45minutes.

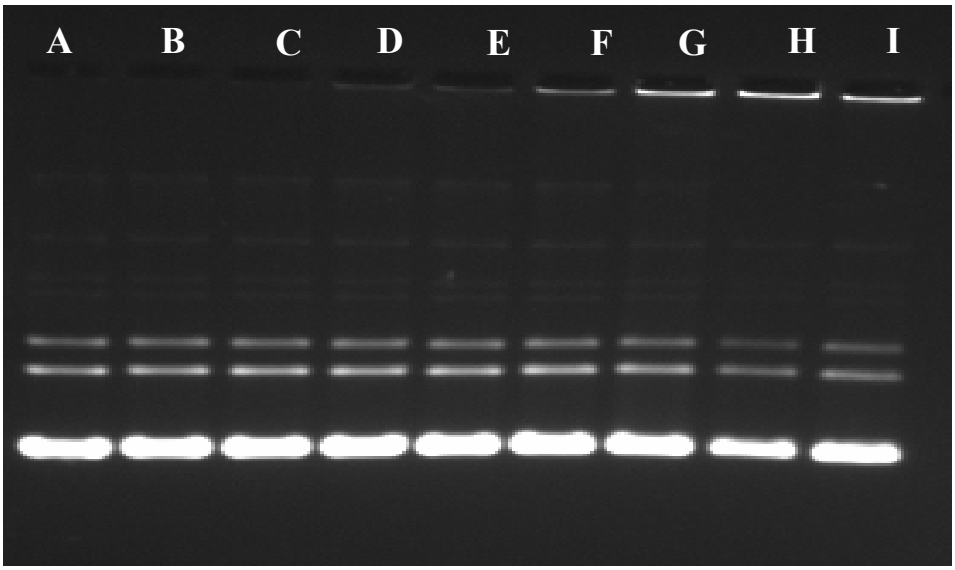


Figure 5B. Effects of Fe^{3+} ions on scDNA in 1% agarose gel. The control scDNA is presented in supercoiled (sc) and open circular (oc) forms and as dimmer plasmid (d). Lane A, scDNA alone; Lane B,plus $1x10^{-6}$M Fe^{2+}; Lane C, plus $1x10^{-5}$M Fe^{2+}; Lane D, plus $1x10^{-4}$M Fe^{2+}; Lane E, plus $2x10^{-4}$M Fe^{2+}; Lane F, plus $2.5x10^{-4}$M Fe^{2+} ; Lane G, $5x10^{-4}$M Fe^{2+}; Lane H, $7.5x10^{-4}$ M Fe^{2+}; Lane I, $1x10^{-3}$ M Fe^{2+} all the reaction mixture were incubated at RT in buffer containing 10mM Tris-cl (pH 7.4) and 50mM NaCl for 45minutes.

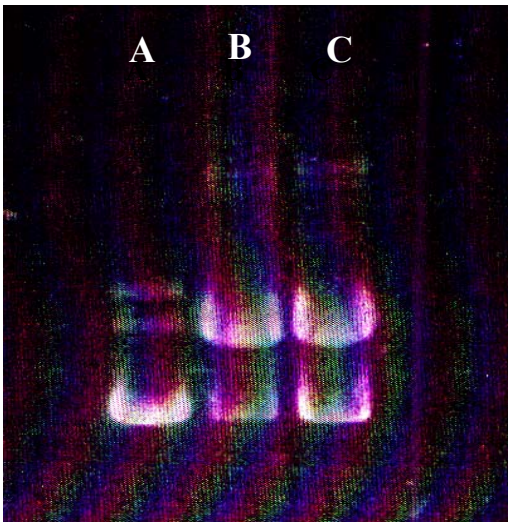


Figure 6. ScDNA was incubated with Fe^{2+} absence and presence of catalase in order to rule out the free radical induced DNA damage. Lane A is 1µg Sc DNA alone., lane B is ScDNA and 7.5X 10^{-4} Fe^{2+} lane C is ScDNA , 7.5X 10^{-4} Fe^{2+} and 50µg/ml catalase.

3B. 3. 3. Fluorescence measurements - The EtBr binding pattern to DNA was monitored in the presence and absence of iron. EtBr fluorescence intensities were recorded for scDNA, with iron salts ($7.5x10^{-4}$ to $1x10^{-3}$M). The data clearly indicate that both form of Fe (2+ and 3+) complexes reduced the EtBr fluorescence intensity (Fig 7). EtBr intensity was observed to be low both the case of iron Fe^{2+} and Fe^{3+} -scDNA complexes. The Scatchard's plot showed that the average number of EtBr molecule bound per base pairs for scDNA alone, Fe^{2+}-scDNA complexes and Fe^{3+}-scDNA complexes were 0.18, 0.062, 0.016 respectively (Fig. 8). From this data, it is clear that Fe^{2+} - scDNA complexes have less EtBr binding capacity confirming C DNA. The less EtBr binding in case of Fe^{3+}-scDNA complex might be due to aggregation of the DNA.

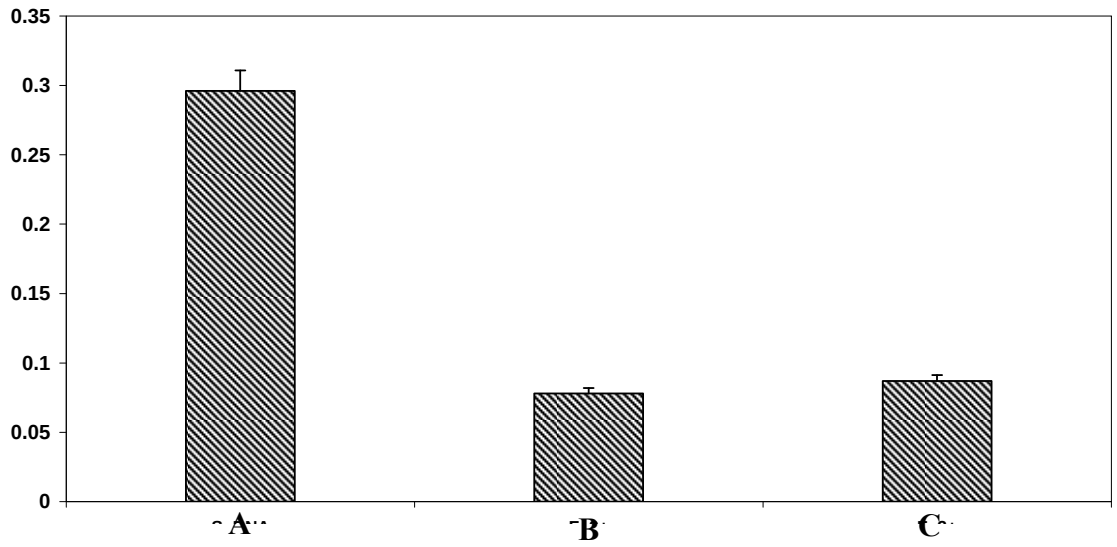


Figure 7. Equimolar concentrations of DNA and EtBr (w/w) were used as a model to study the effect of Fe ions on scDNA. The alteration of scDNA was quantified by measuring the EtBr fluorescence intensity of 1:1 (w/w) DNA/EtBr solutions. The solutions were excited at 535nm and emission monitored at 600nm. The emission intensity values for scDNA (A), sc DNA + Fe^{2+} (B) and Sc DNA +Fe^{3+} (C) were 2.9, 0.07, and 0.09 respectively.

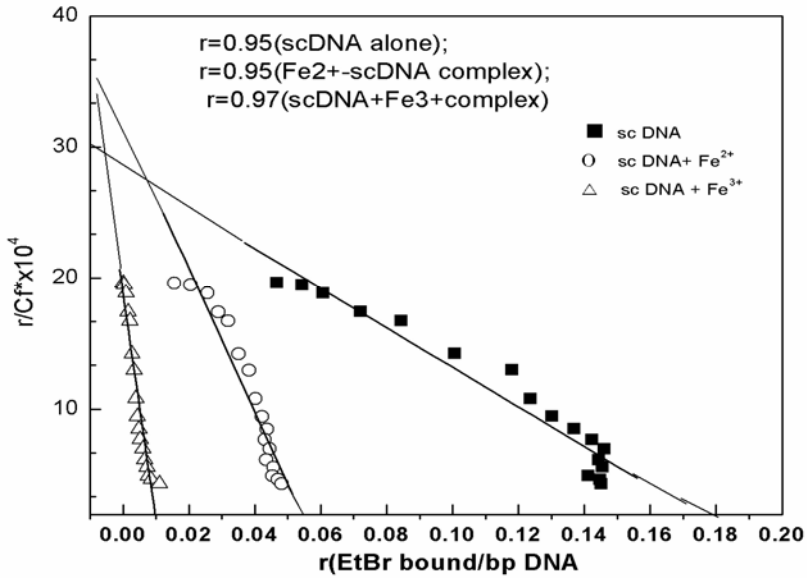


Figure 8. Scatchared plot of EtBr binding to pUC 19 scDNA under 50mM NaCl salt concentration. [1µg of DNA was titrated with increasing amount of EtBr in 1mL containing 10mM Tris-HCl (pH 7.4), 50mM NaCl. Flourescence measurements were done at RT setting excitation at 545nm and emission at 600nm respectively. The light path length was 10. The plot was drawn using least square method]. ■ sc DNA alone, ○5x10^{-4}M Fe^{2+} + sc DNA; △ 5x10^{-4}M Fe^{3+}.

3B. 3. 4. Melting Profile - The melting temperature study which provides insight on stability of the DNA was used to examine the melting profile of the scDNA in the absence and presence of Fe^{2+} ions or Fe^{3+} ions (Table I). The t_m value for scDNA was 88^{o}C (± 0.5). Fe^{3+} mildly altered the t_m by 5^{o}C at lower concentrations (50µM). But t_m is reduced to 65^{o}C by Fe^{2+} ions at 50µM. This shows that Fe^{2+} cause more instability to the DNA than Fe^{3+} ions i.e., less concentration of Fe^{2+} enough to make DNA instable whereas high concentration of Fe^{3+} are required to make same amount of DNA to instable.

TABLE I

The effects of iron ions on the melting profile of scDNA.

Metal- DNA	Tm values ^{o}C
scDNA alone	88 ± 0.5
scDNA alone + 50µM Fe^{2+}	65 ± 0.5
scDNA alone + 50µM Fe^{3+}	83 ± 0.5

3B. 3. 5. Estimation of SSB and DSB - The number of SSBs and DSBs were estimated on the interaction of Fe^{2+} and Fe^{3+} with dscDNA (double strand circular DNA) (Table II) and scDNA (Table III) by nick translation methods. The amount of SSBs caused by Fe^{2+} ions are more than Fe^{3+} ions. For example, $0.5x10^{-8}$ M (Fe^{2+}) is caused 66.8 x 10^{4} SSBs/µg of DNA whereas $1.0x10^{-8}$M (Fe^{3+}) is required to cause same amount of SSBs/µg of DNA. However, no DSBs by the two forms of iron (Fe^{2+}; Fe^{3+}) on any of these DNA could be detected. Our observation is that Fe ions caused only single strand breaks not double strand breaks in both scDNA and dscDNA.

TABLE II

Assessment of SSBs and DSBs formation per 1µg of dsc-DNA on its interaction with Fe^{2+} and Fe^{3+} ions at RT for 40 minutes.

Conc.of metals	**SSBs/ug of DNA**	**DSBs/ug of DNA**
$0.5x10^{-8}$ M (Fe^{2+})	37.7 x 10^{7}	0
$0.5x10^{-8}$ M (Fe^{3+})	23.4x10^{7}	0

TABLE III

Assessment of SSBs and DSBs breaks on increasing concentration of Fe^{2+} and Fe^{3+} ions with scDNA at RT for 40 minutes.

Conc.of metals	SSBs/ug of DNA	DSBs/ug of DNA
$0.5x10^{-8}$ M (Fe^{2+})	66.8 x 10^4	0
$1.0x10^{-8}$ M (Fe^{2+})	$20.0x10^5$	0
$0.5x10^{-8}$M(Fe^{3+})	$25.0x10^4$	0
$1.0x10^{-8}$M(Fe^{3+})	$70.0x10^4$	0

3B. 3. 6. Transmission electron microscopy - Natural negatively supercoiled DNA of 2686bp deposited onto a pentylamine glow discharged carbon surface in the presence of 50mM NaCl, showed a tight supercoiled configuration (Fig. 8A). Purified scDNA and Linearized scDNA in 50mM NaCl, 10mM Tris-Cl pH (7.4) were investigated by TEM to assess the effect of Fe^{2+} ions on the alteration of scDNA, and its effects on the linearized scDNA. Deposition from solution containing 0.75mM Fe^{2+} with Sc DNA in buffer (10mM Tris-Cl pH (7.4), 50mM NaCl) showed the open circular form (Fig 8B) while more than 0.75mM Fe^{2+} produced condensation of the DNA. The TEM image demonstrated the circularization of linearized scDNA having cohesive ends by 0.75mM Fe^{2+} (Fig.8C). At higher concentrations of Fe^{2+} ions produced daisy shaped aggregates as showed in TEM images (Fig 8D).

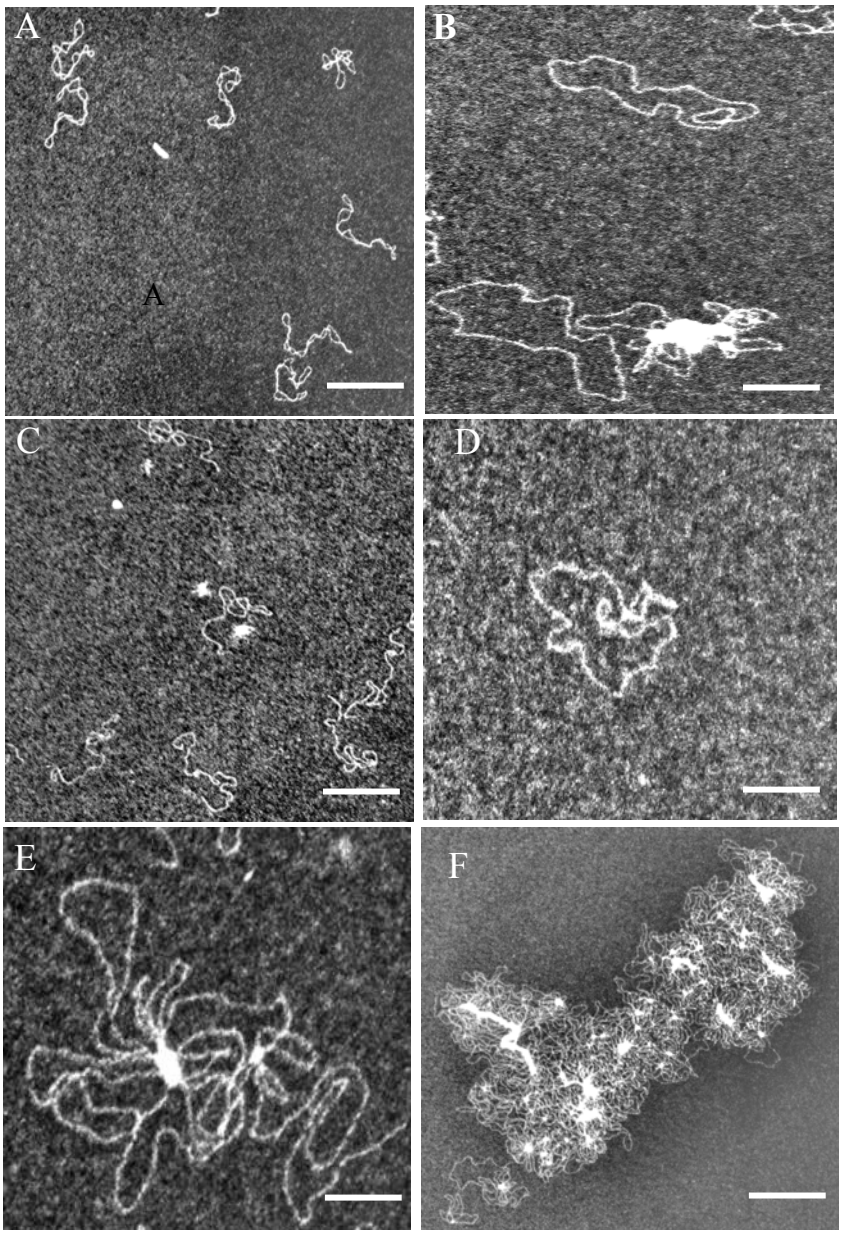


Figure 8. Electron microscopy images showing the effects of Fe^{2+} ions on both scDNA and linearized scDNA fragments. (A) scDNA alone. (B) Open circular form from scDNA by Fe^{2+}at 0.75mM (C) purified linearized DNA fragments with cohesive end, (D) and (E) circularized DNA by 0.75mM Fe^{2+}, (F), at high concentration (>0.75mM) of Fe^{2+} incubation shows the condensation of the DNA. Scale Bar, A 0.25µM; B to F 1µM.

3B. 4. Discussion

DNA conformation is polymorphic in nature. Depending on the precise environmental conditions, DNA can adopt a variety of different non B-DNA conformations [Mirkin and Frank-Kamenetskii, 1994; Rich 1993]. Metals like iron and aluminum accumulate in specific brain regions of neurodegenerative disease like AD and PD [Zecca et al., 2004; Griffiths and Crossman, 1993]. These diseases are associated with several known neuropathological events such as accumulation of iron and aluminum, deposition of Abeta, α-synuclein, abnormal phosphorylation of tau, aggregation of these proteins into NFTs, oxidative stress, and DNA damage [Iqbal *et al.,* 1994; Lyras *et al.,* 1997; Ostrerova-Golts *et al.,* 2000; Uversky *et al.,* 2001; Munch *et al.,* 2000]. Recently studies reveal the presence of left-handed, rigid Z-DNA conformation in severely affected AD brain in contrast to normal young and aged brains which have the usual right – handed Watson-Crick DNA conformation [Anitha *et al.,* 2002]. Metals play a pivotal role in inducing DNA conformational change. Studies from our lab showed that metals like aluminum alters the conformation of $(CCG)_{12}$ triplet repeats, and sequence specific oligonucleotides [Rajan *et al.,* 1996; Champion *et al.,* 1998; Latha *et al.,* 2002]. Most investigators have shown that iron is one of the potent risk factor for neurodegenerative diseases because it induces formation of ROS causing oxidative stress in neuronal cells [Thompson *et al*., 2003; Barnham *et al*., 2004]. Interestingly iron was found to be localized in the chromatin region of the nucleus in AD [Smith et al., 1997].

The present study reports the effects of iron cations (Fe^{2+}, Fe^{3+} ions) on the conformation, stability and strand breaks on scDNA. Furthermore the possible effects

of Fe^{2+} ions on linearized scDNA have been examined. The experiment was not based on free radical generation via Fenton reaction. We hypothesize that metals like Al^{3+} and Fe^{2+} might play important role in right-to-left DNA handedness change associated with AD. Based on DNA conformational change an explanation for unusual phenomenon like G* specific oxidation, terminal differentiation, transcriptional defects and altered gene expression associated with AD brain have been provided [Anitha *et al.,* 2002, 2001].

It is known that in its native undamaged state, the double stranded pUC19 plasmid exists in a tightly compact "supercoiled conformation" and as such it has relatively high electrophoretic mobility. Upon formation of one or more single strand breaks the supercoiled tertiary structure is disrupted resulting in an open – circle conformation and a reduced electrophoretic mobility in agarose. The present findings from the agarose gel electrophoresis, nick translation assay, TEM images studies confirm that iron ions cause only single strand nicks and not double strand break in scDNA which is contradictory to previous report [Netto *et al.,* 1991; Lloyd *et al.,* 1998; Lloyd and Phillips, 1999].

The present finding shows that Fe^{2+} not only binds to scDNA but is also able to alter the conformation of DNA from normal B to C. Importantly the study shows that for the conformation of DNA to occur, iron in the reduced state (II) is essential since iron in the oxidized state (III) lacks such ability to alter the transition. However, according to Maestre and Wang, (1971) the lowering of superhelical density of the scDNA also involved similar spectral change. To exclude this possibility, CD spectrum of uncloiled scDNA by chloroquine was observed but there were no change in the CD spectra with

respect to scDNA. Hence this study also made clear that the relaxation of supercoils is probably not associated with the decrease in positive peak intensity at 275 nm. Therefore, the changes observed in the CD spectra were not because of simple uncoiling phenomenon but a conformational change caused by Fe^{2+} ions. This conformation belongs to C-DNA conformation. EtBr binding studies also showed that scDNA + Fe^{2+} complex had low binding due to C-DNA conformation. Hanlon *et al*. (1978) reported that the grooves were shallower in C-DNA because the bases are off-axis, rather narrower compared to the B- form where the base stacking was significantly high. Hence the EtBr binding towards C-DNA was apparently low, as the EtBr could not intercalate between the base pairs. A previous study from our lab showed that spermine could convert C-DNA to Ψ-DNA [Bharathi *et al.,* 2003]. It is known that Ψ-DNA, an ordered, twisted, tight-packing arrangement of the double helix is structurally and immunologically closely related to the left handed Z-DNA family [Thomas and Thomas, 1989]. This indicates that DNA topological changes induced by Fe^{2+} ions could contribute to the DNA conformational changes as seen in AD brain.

However, the present study also analyzed the effect of Fe^{2+} ions on the linearized scDNA with a cohesive end. Recently Revet and Fourcade, (1998) had demonstrated that Mg^{2+} cations with uranyl acetate help to stabilize end - to - end DNA interactions in cohesive end fragments regardless of their size in the absence of DNA ligase. Interestingly, the present study on Fe^{2+} - linearized DNA interaction using TEM demonstrated that Fe^{2+} can also circularize the sticky ends of the restricted fragments in a similar manner to Mg^{2+}ions. This study therefore showed for the first time the role played by iron in the circularization of linearized scDNA.

The complex mechanism that underlies the Fe-induced DNA conformation is yet to be elucidated. The important question that needs to be answered is why the reduced form of iron (Fe^{2+}) alters DNA conformation and stability than oxidized form of iron (Fe^{3+})? Metal ions and its complexes interact with nucleic acids in a variety of ways, both covalent and non-covalent interactions have been investigated [Bertini *et al.,* 1994]. The binding of transition metals to DNA was thought to involve association with phosphate residues followed by transfer to preferred sequence regions to form a complex whose stability depends upon particular metal ions, the DNA sequence, and the secondary structure of the DNA in the region [Pullman and Pullman, 1981; Mattes *et al.,* 1986; Sagripanti and Kraemer, 1989; Hartley *et al.,* 1990]. Iron (2+, 3+) could react with both oxygen and nitrogen from phosphate or bases respectively [Bertoncini *et al.,* 1999]. Fe^{3+} forms stable complexes with DNA and causes DNA condensation by charge neutralization of the phosphodiester groups, DNA condensation was not observed by Fe^{2+} unless when cooperatively induced by alcohol [Kasyanenko *et al.,* 1998]

Iron ions Fe^{2+} and Fe^{3+} can bind to DNA by two qualitatively different mechanisms. Electrostatic forces play a major role in the stability of the DNA double helix and generally phosphate binding stabilizes the double helix. The first mechanism is non-specific and probably involves electrostatic interactions with backbone phosphate groups. Here Fe^{2+} acts like divalent magnesium cations. There are numerous reports that ferrous ions can form complexes with phosphates [Svoboda and Harms-Ringdahl, 2002; Miller *et al.,* 1990], pyrophosphates, polyphosphates [Biaglow and Kachur, 1997], DNA [Floyd, 1981], or nucleotides in the di –or tri phosphates form [Floyd, 1983; Floyd and Lewis, 1983; Kachur *et al.,* 1997], where by ferrous ions readily oxidized to ferric ions.

Svoboda and Harms-Ringdahl, (2002) reported that DNA-phosphate can oxidize bound Fe^{2+} ions to Fe^{3+} ions up to equimolar concentrations producing H^+ ions near the vicinity of the DNA. Several studies have shown that during oxidation, superoxide ($O_2\bullet^-$) is formed, leading to the subsequent production of H_2O_2 and Fenton type of reactions [Minotti and Aust, 1987; Gutteridge, 1991].

Binding of ferrous ions with DNA phosphates would be expected to increase oxygen radical formation close to DNA and cause a broad spectrum of different types of DNA damage [Floyd, 1981; Blakely *et al.,* 1990; Henle *et al.,* 1996]. This may be one of the reasons for creation of the open circular form. It is also possible that iron may bind to nucleotides in cellular nucleotide pool causing generation of oxygen radicals forming modified nucleotides which, if incorporated into the newly synthesized DNA could lead to mutations of genes responsible for neurodegeneration.

On the other hand, Fe^{3+} ion can bind to oxygen of DNA-phosphate, neutralize repulsive interactions between the negatively charged sugar-phosphate backbones and stabilize the interaction between the base pairs. Our studies using gel electrophoresis and reduction of EtBr fluorescence support the binding of Fe^{3+} to phosphate residues of DNA. EtBr not only intercalates between the DNA bases but also shows phosphate binding whereby fluorescence is produced [LePecq and Paoletti, 1967]. Fluorescence studies on metal/DNA interaction indicate that Fe ions reduce EtBr fluorescence. Since metal ions show a greater affinity for phosphate groups, there are probably fewer sites left out to bind EtBr molecule to phosphate. This may be the reason for this reduction in EtBr fluorescence in case of Fe^{3+}.

The second mechanism suggest that at higher concentrations of Fe^{2+} can bind to both phosphates and bases with presumable involvement of co-ordination with the latter [Saenger, 1984]. It was known that the preferred site for the metal ion co-ordination to the DNA bases is the N^7 group of purine residues, principally of guanine [Saenger, 1984]. Many authors have pointed out that the strongest base component for chelation of transition metals on DNA is the purine N^7 because those regions around a G-rich site are especially electronegative specifically around the N^7 atoms due to strong stacking interactions [Sagripant, 1989; Saenger, 1984; Mattes *et al.,* 1986; Sissoeff *et al.,* 1976]. The complex might also be stabilized by phosphate residues on the same strand which are 5' to the respective dG residues [Sissoeff *et al.,* 1976].

Such an association with guanine N^7 and the 5' proximal phosphate would place the Fe^{2+} in close proximity of the deoxyribose moiety 5' to the dG residues. Rossetto and Nieboer (1994) proposed that a metal that binds to N-bases facilitates DNA conformational transition. DNA phosphate repulsion and polymer hydration state are important determinants of conformational transition in DNA [Soumpasis *et al.,* 1987]. Drew *et al*. (1980) proposed that transition in DNA conformation occur by lowering the activity co-efficient or effective concentration of surrounding H_2O molecules. This model fits nicely to explain the cleavage observed by Pullman and Pullman, (1981) in GGG and with decrease in ionization potential in the 3' to 5' direction Furthermore, they latter reported that GGG sequences might form a sink for oxidizing radicals [Hall *et al.,* 1996] and also studies from Dervan, (1986) showed that metal-DNA sequence specific interactions are more likely to occur in the major groove than in the minor groove. Therefore it is likely that the iron – specific conformation of DNA arises due to the

specific co-ordination of the metal to the bases. Thus we assumed that susceptibility of G* specific oxidation occurring more in AD due to Fe^{2+} binding to guanine base of the DNA. Henle *et al.* (1999) showed that Fe in the presence of 50mM H_2O_2, preferentially cleaves at the nucleoside 5' of each of the dG moieties in the sequence RGGG. This sequence is found in a majority of telomere repeats and cleaves thymidine within the sequence RTGR, a sequence frequently found to be required in promoters for normal responses of many prokaryotic and eukaryotic genes to iron or oxygen stress. Their model suggests that thymine methyl plays a very important role in iron binding.

Thus the present results suggest that iron (2+) induces only the single strand break and not a double strand break to scDNA to form an open circular DNA and this open circular form has a C-DNA conformation which is different from B conformation seen in original scDNA. But Fe^{3+}could not induce any transitional change rather it stabilized the scDNA by neutralizing the charge, by binding to phosphate groups; at higher concentrations it precipitated. Furthermore, interestingly the interaction of Fe^{2+} ion on linearized scDNA revealed that at lower concentrations Fe^{2+} ions could circularize linearized scDNA, but at higher concentrations it leads to DNA condensation.

Based on the present findings, we hypothesize that iron is implicated in the pathogenesis of AD by changing DNA topology. From our study, we observed the differential effect of Fe^{2+} and Fe^{3+}on the DNA conformation and its stability. It is known that DNA conformation change can affect the normal transcription and subsequently lead to gene alteration [Asin *et al.,* 2000; Latha *et al.,* 2002]. To make any definite conclusion, it is essential to further investigate the relation between DNA conformation and function relationship.

3B. 5. References

Andronikashvili E.L, Mosulishvili L.M, Belokobilskiy A.I, Kharabadze N.E, Shonia N.I, Desai L.S, Foley G.E. Human leukaemic cells. Determination of trace elements in nucleic acids and histones by neutron-activation analyses, Biochem. J. 1976; 157: 529-533.

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

Anitha S, Reuvin S, Rao KSJ, Latha KS, Viswamitra MA. A link between apoptotic DNA damage and DNA topology in Alzheimer's disease brain: a hypothesis. Alz Rep. 2001; 4:121-131.

Asin J, Martos AP, Silva PF, Montoya J, Andreu AI. Iron (II) induces changes in the conformation of mammalian mitochondrial DNA resulting in a reduction of its transcriptional rate. FEBS Lett. 2000; 480:161-164.

Barbauti A, Doulias PT, Zhu BZ, Frei B, Galaris D. Intra cellular iron, but not copper, plays a critical role in hydrogen peroxide-induced DNA-damage. Free Radic Biol Med. 2001; 31: 490- 498.

Barnham KJ, Masters CL, Bush AI. Neurodegenerative diseases and oxidative stress. Nat Rev Drug Discov. 2004; 3:205-214.

Bauer WR, Crick FHC, White H. Supercoiled DNA. Sci. Am. 1980; 16: 243- 245.

Berg D, Gerlach M, Youdim MB, Double KL, Zecca L, Riederer P, Becker G. Brain iron pathways and their relevance to Parkinson's disease. J Neurochem. 2001; 79:225-236

Bertini I, Gray HB, Lippard SJ, Barton JSK. In: Valentine (Eds.), Bioinorganic Chemistry, University Science Books, Sausalito, CA, 1994, pp 455-503.

Bertoncini C, Meneghini R, Cruz DZ, Martins Alves MC, Tolentino H. Studies of Fe(II) and Fe(III)-DNA complexes by XANES spectroscopy. J Synchrotron Radiat. 1999; 6:417-418.

Bharathi, Rao KSJ, Stein R. First evidence on induced topological change in supercoiled DNA by an aluminium D-aspartate complex. J. Biol. Inorg Chem. 2003; 8: 823- 830.

Biaglow JE, Kachur AV. The generation of hydroxyl radicals in the reaction of molecular oxygen with polyphosphate complexes of ferrous ion. Radiat Res.1997; 148:181-187.

Blakely WF, Fuciarelli AF, Wegher BJ, Dizdaroglu M. Hydrogen peroxide-induced base damage in deoxyribonucleic acid. Radiat Res. 1990; 121:338- 343.

Champion CS, Kumar D, Rajan MT, Rao KSJ, Viswamitra MA. Interaction of Co, Mn, Mg, and Al with d(GCCCATGGC) and d(CCGGGCCCGG): a spectroscopic study. Cell. Mol. Life Sci. 1998; 54: 488 - 496.

Chatterjee B, Rao GR. Superhelical density of goat mitochondrial DNA: fluorimetric studies. Indian J Biochem Biophys. 1994; 31: 77-79.

Chiueh CC, Andoh T, Lai AR, Lai E, Krishna G. Neuroprotective strategies in Parkinson's disease: protection against progressive nigral damage induced by free radicals. Neurotox Res. 2000; 2:293-310.

Connor JR, Menzies SL, St Martin SM, Mufson EJ. A histochemical study of iron, transferrin, and ferritin in Alzheimer's diseased brains. J Neurosci Res. 1992a; 31:75-83.

Connor JR, Pavlick G, Karli D, Menzies SL, Palmer C. A histochemical study of iron-positive cells in the developing rat brain. J Comp Neurol. 1995; 355:111-123.

Connor JR, Snyder BS, Beard JL, Fine RE, Mufson EJ. Regional distribution of iron and iron-regulatory proteins in the brain in aging and Alzheimer's disease. J Neurosci Res. 1992b; 31:327-335.

Crichton RR. Inorganic Biohemistry of Iron Metabolism: From Molecular Mechanism to Clinical Consequences. Wiley- Chichester, New York, 2001, pp 319.

Dervan PB. Design of sequence-specific DNA-binding molecules. Science. 1986; 232:464-471.

Dexter DT, Wells FR, Lees AJ, Agid F, Agid Y, Jenner P, Marsden CD. Increased nigral iron content and alterations in other metal ions occurring in brain in Parkinson's disease. J Neurochem. 1989; 52:1830-1836.

Drew H, Takano T, Tanaka S, Itakura K, Dickerson RE. High-salt d(CpGpCpG), a left-handed Z' DNA double helix. Nature. 1980; 286:567-573.

Dubochet J, Ducommun M, Zollinger M, Kellenderger E. A new preparation method for dark-field electron microscopy of biomacromolecules. J Ultrastruct Res. 1971; 35:147-167.

Eisinger J, Shulman RG, Blumberg RG. Relaxation enhancement by paramagnetic ion binding in deoxyribonucleic acid solutions. Nature.1961; 192:963-964.

Floyd RA, Lewis CA. Hydroxyl free radical formation from hydrogen peroxide by ferrous iron-nucleotide complexes. Biochemistry. 1983; 22:2645-2649.

Floyd RA. Direct demonstration that ferrous ion complexes of di-and triphosphate nucleotides catalyze hydroxyl free radical formation from hydrogen peroxide. Arch Biochem Biophys. 1983; 225:263-270.

Floyd RA. DNA-ferrous iron catalyzed hydroxyl free radical formation from hydrogen peroxide. Biochem Biophys Res Commun. 1981; 99:1209-1215.

Floyd RA. Role of oxygen free radicals in carcinogenesis and brain ischemia. FASEB J.1990; 4:2587-2597.

Good PF, Perl DP, Bierer LM, Schmeidler J. Selective accumulation of aluminum and iron in the neurofibrillary tangles of Alzheimer's disease: a laser microprobe (LAMMA) study. Ann Neurol. 1992; 31:286-292.

Gordon N. Iron deficiency and the intellect. Brain Dev. 2003; 25:3 -8.

Gray D.M, Ratlief R.L, Vaughan M.R. Circular dichroism spectroscopy of DNA, Methods Enzymol. 1992; 211: 389- 406.

Gray D.M, Taylor T.N, Lang D. Dehydrated circular DNA: circular dichroism of molecules in ethanol solutions, Biopolymers. 1978; 17:1- 45.

Griffiths PD, Crossman AR. Distribution of iron in the basal ganglia and neocortex in postmortem tissue in Parkinson's disease and Alzheimer's disease. Dementia. 1993; 4: 61-65.

Griffiths PD, Dobson BR, Jones GR, Clarke DT. Iron in the basal ganglia in Parkinson's disease, An in vitro study using extended X-ray absorption fine structure and cryo-electron microscopy. Brain. 1999; 122:667-673.

Gutteridge JM. Hydroxyl radical formation from the auto-reduction of a ferric citrate complex. Free Radic Biol Med. 1991; 11:401-406.

Hall DB, Holmlin RE, Barton JK. Oxidative DNA damage through long-range electron transfer. Nature. 1996; 382:731-735.

Hall ED, Braughler JM. Free radicals in CNS injury. Res Publ Assoc Res Nerv Ment Dis. 1993; 71:81-105.

Halliwell B, Aruama OI. DNA damage by oxygen-derived species: its mechanism and measurements in mammalian systems. FEBS Lett.1991; 281: 9-19.

Halliwell B, Gutteridge JM. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J. 1984; 219:1-14.

Halliwell B. Superoxide-dependent formation of hydroxyl radicals in the presence of iron chelates: is it a mechanism for hydroxyl radical production in biochemical systems?. FEBS Lett. 1978; 92:321-326.

Halliwell B. Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts. Its role in degradation of hyaluronic acid by a superoxide-generating system, FEBS Lett. 1978; 96:238-242.

Hampton MB, Orrenius S. Dual regulation of caspase activity by hydrogen peroxide: implications for apoptosis, FEBS Lett. 1997; 414:552-556.

Hanlon S, Chan A, Berman S. Specific cation effects on conformational transitions of DNA in aqueous solutions. Biochem Biophys Acta. 1978; 519:526-536.

Hartley JA, Forrow SM, Souhami RL. Effect of ionic strength and cationic DNA affinity binders on the DNA sequence selective alkylation of guanine N7- positions by nitrogen mustards. Biochemistry. 1990; 29:2985-2991.

Henle ES, Hanz NT, Rai P, Luo Y, Linn S. Sequence specific DNA cleavage by Fe^{2+}-mediated Fenton reaction has possible biological implications. J. Biol Chem. 1999; 274: 962-971.

Henle ES, Luo Y, Gassmann W, Linn S. Oxidative damage to DNA constituents by iron-mediated fenton reactions, the deoxyguanosine family. J Biol Chem. 1996; 271: 21177-21186.

Iqbal K, Alonso AC, Gong CX, Khatoon S, Singh TJ, Grundke-Iqbal I. Mechanism of Neurofibrillary degeneration in Alzheimer's disease. Mol Neurobiol. 1994; 9:119-123.

Jellinger K, Paulus W, Grundke-Iqbal I, Riederer P, Youdim MB. Brain iron and ferritin in Parkinson's and Alzheimer's diseases. J Neural Transm Park Dis Dement Sect. 1990; 2:327-340.

Jenner P. Oxidative damage in neurodegenerative disease. Lancet. 1994; 344:796-798.

Kachur AV, Manevich Y, Biaglow JE. Effect of purine nucleoside phosphates on OH-radical generation by reaction of Fe^{2+} with oxygen. Free Radic Res. 1997; 26:399-408.

Kasyanenko N, Arikainen N, Frisman E. Investigation of DNA complexes with iron ions in solution, Biophys Chem. 1998; 70:93-100.

Kobayash S, Ueda K, Komano T. The effects of metal ions on the DNA damage induced by hydrogen peroxide. Agri Biol Chem. 1990; 54: 69-76.

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

LePecq JB, Paoletti C. A fluorescent complex between ethidium bromide and nucleic acids. Physical-chemical characterization. J Mol Biol.1967; 27:87-106.

Li D. Effects of iron deficiency on iron distribution and gamma-aminobutyric acid (GABA) metabolism in young rat brain tissues. Hokkaido Igaku Zasshi. 1998; 73: 215-225.

Liu G, Men P, Harris PL, Rolston RK, Perry G, Smith MA. Nanoparticle iron chelators: A new therapeutic approach in Alzheimer disease and other neurologic disorders associated with trace metal imbalance. Neurosci Lett. 2006; 406:189-193.

Lloyd DR, Carmichael PL, Phillips DH. Comparison of the formation of 8-hydroxy-2'-deoxyguanosine and single- and double-strand breaks in DNA mediated by Fenton reactions. Chem Res Toxicol. 1998; 11:420-427.

Lloyd DR, Phillips DH. Oxidative DNA damage mediated by copper (II), iron (II) and nickel (II) Fenton reactions: evidence for site-specific mechanisms in the formation of double-strand breaks, 8-hydroxydeoxyguanosine and putative intrastrand cross-links**.** Mutat Res. 1999; 424:23-36.

Lovell MA, Robertson JD, Teesdale WJ, Campbell JL, Markesbery WR. Copper, iron and zinc in Alzheimer's disease senile plaques. J Neurol Sci. 1998; 158:47-52.

Luo Y, Han Z, Chin SM, Linn S. Three chemically distinct types of oxidants formed by iron-mediated Fenton reactions in the presence of DNA. Proc Natl Acad Sci (U S A). 1994; 91:12438-12442.

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

Maestre MF, Wang JC. Circular dichroism of superhelical DNA. Biopolymers. 1971; 10:1021-1030.

Mattes WB, Hartley JA, Kohn KW. DNA sequence selectivity of guanine-N7 alkylation by nitrogen mustards, Nucleic Acids Res. 1986; 14:2971-2987.

Miller DM, Buettner GR, Aust SD. Transition metals as catalysts of "autoxidation" reactions. Free Radic Biol Med. 1990; 8:95-108.

Minotti G, Aust SD. An investigation into the mechanism of citrate-Fe^{2+}-dependent lipid peroxidation. Free Radic Biol Med. 1987; 3:379-387.

Mirkin SM, Frank-Kamenetskii MD. H-DNA and related structures. Annu Rev Biophys Biomol Struct. 1994; 23:541-576.

Muiras ML, Giacommoni P, Tachon P. Modulation of DNA breakage induced via the Fenton reaction. Mutat Res. 1993; 295: 47-54.

Munch G, Luth HJ, Wong A, Arendt T, Hirsch E, Ravid R, Riederer P. Crosslinking of alpha-synuclein by advanced glycation endproducts--an early pathophysiological step in Lewy body formation? J Chem Neuroanat. 2000; 20:253-257.

Netto LES, Costa Ferreira, AMD, Augusto O. Iron (III) binding in DNA solutions: Complex formation and catalytic activity in the oxidation of hydrazine derivatives, Chem. Biol. Interac. 1991; 79:1-14.

Ostrerova-Golts N, Petrucelli L, Hardy J, Lee JM, Farer M, Wolozin B. The A53T alpha-synuclein mutation increases iron-dependent aggregation and toxicity. J Neurosci. 2000; 20:6048-54.

Ponting CP. Domain homologues of dopamine beta-hydroxylase and ferric reductase: roles for iron metabolism in neurodegenerative disorders? Hum Mol Genet. 2001; 10:1853-1858.

Pullman A, Pullman B. Molecular electrostatic potential of the nucleic acids, Q Rev Biophys. 1981; 14:289-380.

Rai P, Cole TD, Wemmer DE, Linn S. Localization of Fe(2+) at an RTGR sequence within a DNA duplex explains preferential cleavage by Fe(2+) and H_2O_2. J Mol Biol. 2001; 312:1089-1101.

Rajan MT, Champion C.S, Kumar D, Vishnuvardhan D, Rao KSJ, Viswamitra MA. Interaction of Co, Mn, Mg, and Al with d(GCGTACGC): A spectroscopic study. Mol. Biol. Rep. 1996; 22: 47-52.

Rao KSJ, Rao RV, Shanmugavelu P, Menon RB. Trace elements in Alzhiemer's disease brain: A new hypothesis. Alz Rep. 1999; 2: 241-246.

Revet B, Fourcade A. Short unligated sticky ends enable the observation of circularised DNA by atomic force and electron microscopies. Nucleic Acids Res. 1998; 26:2092-2097.

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

Rossetto FE, Nieboer E. The interaction of metal ions with synthetic DNA: induction of conformational and structural transitions. J Inorg Biochem. 1994; 54:167-186.

Saenger W. Principles of Nucleic Acid Structure; Springer-Verlag, Berlin. 1984, pp201-219.

Sagripant JL, Kraemer KH. Site-specific oxidative DNA damage at polyguanosines produced by copper plus hydrogen peroxide, J Biol Chem. 1989; 264:1729-1734.

Sagripanti JL, Swicord ML, Davis CC. Microwave effects on plasmid DNA. Radiat. Res.1987; 110: 219-231.

Sayre LM, Perry G, Smith MA. Redox metals and neurodegenerative disease. Curr Opin Chem Biol.1999; 3:220-225.

Sayre LM, Zelasko DA, Harris PL, Perry G, Salomon RG, Smith MA. 4-Hydroxynonenal-derived advanced lipid peroxidation end products are increased in Alzheimer's disease. J Neurochem. 1997; 68:2092-2097.

Sissoeff I, Grisvard J, Guille E. Studies on metal ions-DNA interactions: specific behaviour of reiterative DNA sequences. Prog Biophys Mol Biol. 1976; 31:165-199.

Smith MA, Harris PL, Sayre LM, Perry G. Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. Proc Natl Acad Sci U S A.1997; 94:9866-9868.

Smith MA, Perry G. Free radical damage, iron, and Alzheimer's disease. J Neurol Sci. 1995;134 (Suppl):92-94.

Soumpasis DM, Wiechen J, Jovin TM. Relative stabilities and transitions of DNA conformations in 1:1 electrolytes: a theoretical study. J Biomol Struct Dyn. 1987; 4:535-552.

Stohs SJ, Bachi D. Oxidative mechanisms in the toxicity of metal ions. Free Radic.Biol. Med. 1995; 18: 321-336.

Suh D, Shardy RD, Chaires JB. Unusual Binding of Ethidium to a deoxyoligonucleotide containing a B-Z Junction. Biochemistry. 1991; 30: 8722 - 8726.

Surguladze N, Thompson KM, Beard JL, Connor JR, Fried MG. Interactions and reactions of ferritin with DNA. J Biol Chem. 2004; 279:14694-14702.

Svoboda P, Harms-Ringdahl M. Kinetics of phosphate-mediated oxidation of ferrous iron and formation of 8-oxo-2'-deoxyguanosine in solutions of free 2'-deoxyguanosine and calf thymus DNA. Biochem Biophys Acta. 2002; 1571:45-54

Thomas TJ, Thomas T. Direct evidence for the presence of left-handed conformation in a supramolecular assembly of polynucleotides. Nucleic Acids Res. 1989; 17: 3795 – 3810.

Thompson CM, Markesbery WR, Ehmann WD, Mao YX, Vance DE. Regional brain trace-element studies in Alzheimer's disease. Neurotoxicology. 1988; 9:1-7.

Thompson K, Menzies S, Muckenthaler M, Torti FM, Wood T, Torti SV, Hentze MW, Beard J, Connor J. Mouse brains deficient in H-ferritin have normal iron concentration but a protein profile of iron deficiency and increased evidence of oxidative stress. J Neurosci Res. 2003; 71:46-63.

Thompson KJ, Fried MG, Ye Z, Boyer P, Connor JR. Regulation, mechanisms and proposed function of ferritin translocation to cell nuclei. J Cell Sci. 2002; 115:2165-2177.

Toyokuni S, Sagripanti J.L. Iron-Mediated DNA damage: Sensitie Detection of DNA Strand Breakage Catalyzed by Iron. J Inorg Biochem. 1992; 47: 241-248.

Toyokuni S, Sagripanti JL. DNA Single and double strand breaks produced by ferric nitrilotriacetate in relation to renal tubular carcinogenesis. Carcinogensis.1993; 14: 223-227.

Trotta A, Paulsen AB, Silvestri A, Ruisi G, Girasolo MA, Barberi R. The dynamics of ^{57}Fe nuclei in Fe^{III}–DNA condensates. J Inorg Biochem. 2002; 88:14-18.

Uversky VN, Li J, Fink AL. Metal-triggered structural transformations, aggregation, and fibrillation of human alpha-synuclein. A possible molecular NK between Parkinson's disease and heavy metal exposure. J Biol Chem. 2001; 276:44284-44296.

Wolin MS, Mohazzabk M, Scandalios JG. Oxidative stress and the Molecular biology of antioxidant defenses. Cold Spring Harbor Press: Cold Spring Harbor, New York, 1997, pp 21-48.

Zecca L, Youdim MB, Riederer P, Connor JR, Crichton RR. Iron, brain ageing and neurodegenerative disorders. Nat Rev Neurosci. 2004; 5:863-873.

4. 1. Introduction

Bipolar disorder (BPD) or manic-depressive illness is one of the two major psychiatric disorders characterized by recurrent depressive and manic episodes [Kato, 2001]. BPD affects about 1% of the population and causes sever neuropsychological impairments. The illness is implicated in functional impairment and represents an important risk factor for suicide [Oquendo and Mann, 2001]. Twin, adoption and family studies showed that genetic factors contribute to the etiology of the disorders and is proposed that it is not caused by single major gene but multifactorial inheritance [Kato, 2001]. More recently remarkable progress has been made in identifying changes in the brain related to pathophysiology of BPD. Both structural, chemical, functional brain imaging [Brambilla *et al*., 2005] and postmortem [Rajkowska, 2002] studies have demonstrated volume loss in subjects with BPD brain. Similarly a reduction in the numerical density of neurons has also been demonstrated in several brain regions including the anterior cingulated cortex [Benes *et al*., 2001] and hippocampal formation [Benes et al., 1998] of subjects with BPD. Further, studies have consistently reported that an increase in lipid peroxidation and decrease in major antioxidant enzymes in individuals with BPD [Ozcan *et al*., 2004; Ranjekar *et al*., 2003; Kuloglu *et al*., 2002], suggesting that oxidative stress may play a role in pathophysiology of BPD. Oxidative stress in BPD could result from several mechanism, such as generation of ROS by redox active metal ions, reduced antioxidant enzyme and presence of increased products of lipid peroxidation [kuloglu *et al*., 2002; Ranjekar *et al*., 2003; Frey *et al*., 2006a; 2006b; Benes *et al*., 2005]. One of the consequences of oxidative stress is apoptosis and /or necrosis and recent study showed that apoptosis is associated with etiology of BPD [Benes *et al*., 2005].

Moreover no studies to date have presented quantitative evidence for the presence of neurodegeneration in major psychiatric disorders. A dysregulation of apoptotic mechanism believed to play a role in a variety of neuropsychiatric disorders [Margolis *et al*., 1994; Catts and catts 2000]. Apoptosis and necrosis can be distinguished from histopathologically from one another on the basis of their energy requirement, histological profile, plasma membrane changes, phagocytic mechanisms, presence or absence of an inflammatory reaction, and pattern of DNA breakdown [Hetts, 1998]. The damage to DNA noted in the apoptosis is believed to be involving a DNA fragmentation factor that is turn on by activated caspase enzyme [Evan and Littlewood, 1998]. The resulting DNA nicks that are induced in the nucleotide strands can appear as either single-stranded or double stranded breaks [Ansari *et al*., 1993]. If the cell is unable to repair such damage with a DNA polymerase enzyme, these structural DNA changes may persist indefinitely in surviving neurons. Recently, Benes *et al*. (2003) evidenced decreased DNA fragmentation in BPD anterior cingulated cortex brain region by in situ terminal deoxynucleotidyl transferase (TdT) –mediated dUTP nick-end labeling - TUNEL/ISEL method. Further, DNA fragmentations have been well shown to associate with neurodegenerative disorder like PD [Alam *et al*., 1997; Tatton and Olanov, 1999]. More recently, Hegde *et al*., (2006) reported presence of DNA fragmentation in some region of PD brain. Further, it was shown that presence of an altered DNA conformation in hippocampus of AD brain [Anitha *et al*., 2002]. However such information on topology and stability of DNA is lacking in BPD depression brain. Moreover, there is only one study on DNA fragmentation focused in anterior cingulated cortex of BPD [Benes *et al*., 2003] and no study have been done on DNA fragmentation with respect to

other region of BPD brains compared to control brains. Recently Frey *et al*., (2006b) reported that DNA isolated from blood of BPD patients has more damage than compared to controls.

The regions like frontal cortex and thalamus are great interest for understanding the pathophysiology of BPD, because they participate in modulating decision making, planning, mood regulation and emotional processing [Brambilla *et al*., 2005]. Recently significant decreases in volume and grey matter density in anterior cingulated cortex particularly left side hemisphere have been reported in adult bipolar patients as compared with healthy controls [Sassi *et al*., 2004; Lyoo *et al*., 2004]. Recently, MRI studies showed that regions like thalamus, temporal lobe, and cerebellum size are reduced in BPD brain compared to control [Brambilla et al., 2005]. Recent postmortem studies in BPD also provided direct evidence for reductions in number and density as well as changes in cell body size and shape of neurons and glia, implicating specific cell pathology in the mood disorders [Rajkowska, 2002]. Studies also showed that frontal cortex and hippocampus brain regions are associated with BPD [Soars and Mann 1997; Antonova *et al*., 2004]. Till late no information is available on DNA integrity and damage in BPD brain regions.

In this current study, we have assessed the presence of DNA fragmentation, conformation and DNA stability to ascertain whether these factors play a significant role in the pathophysiology of BPD.

4. 2. Materials and Methods

4. 2. 1. Chemicals

Radiolabeled ^{3}[H]-TTP (Sp.Act.40Ci/nmol) was purchased from Amersham Radiochemicals, UK. Ribonuclease A (RNAse a), Proteinase k, Deoxyribonuclease I (DNAse I), dATP, dTTP, dCTP, dGTP, low melting agarose, DNA polymerase I (from Escherichia coli), terminal deoxynucleotidyl transferase enzymes, 1kb and 100bp DNA ladders, and lamda DNA ladder were purchase from Genei, India. Ethidium bromide (EtBr), Hepes, and Tris buffers were purchased from Sigma Chemicals (USA). All other chemicals were of analytical grade and were purchased from Sisco Research Labs, Mumbai, India.

4. 2. 2. Brain Samples

Eight normal and ten depressions affected BPD postmortem human brain samples were collected from the JSS medical hospital and College, Mysore, India. Autopsies were performed on donors with written informed consent obtained direct next of kin. The control human brains were collected from accident victims, who had no history of long-term illness, psychiatric diseases, dementia, or neurological disease prior to death. The depression affected BPD brains were postmortem specimens and Dr. Sathyanarayana Rao, Professor of psychiatry, diagnosed the cases by using postmortem psychiatric analysis and also from their medical records. The average postmortem interval between the time of death and collection of brain and freezing was ≤ 5h. Within one hour after death the body was kept in refrigerator maintained at 4˚C. Dissected brain tissue was stored frozen at -80˚C till the analysis.

4. 2. 3. Isolation of DNA from brain tissue

Genomic DNA was isolated from various regions (Parietal, temporal, and occipital lobe, hippocampus, thalamus, cerebellum, hypothalamus, medulla, pons, and frontal cortex) of frozen brain tissue by standard 'phenol-chloroform extraction' method after Sambrook *et al*.,(1989) with some modifications to prevent DNA fragmentation during isolation. The method involved the following steps:

1. Brain tissue was cut into small pieces and a weighed amount of tissue was transferred into an autoclaved porcelain mortar and pestle. All glass wares, mortar, pestle etc., were autoclaved to avoid bacterial contamination.
2. Liquid nitrogen was poured into the mortar and the tissue was allowed to freeze.
3. Tissue was ground 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 (a sterile spatula was used to transfer the powdered tissue into a graduated tube).
5. The tissue homogenate was incubated with lysis buffer (50mM Tris-HCI (pH 8.0), 10 mM EDTA (pH 8.0), 100 mM NaC1) was added into the tube along with 15µg/mL of proteinase K and 2% SDS final volume. One milliliter of lysis buffer was used for every 500 mg of tissue. (Note 1: lysis buffer should be pre warmed, 2: add proteinase K after first 2 h, optimum: 3 h.)
6. The homogenate was incubated at 37˚C in a water bath for 12-16 h or over night.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 10 min.

7. The lysate was centrifuged for 10 min at 10,000 rpm at 13˚C.
8. 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. One part phenol: one 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).
9. The supernatant and Tris-saturated phenol-chloroform mixture was centrifuged at 5000 rpm at 4˚C.
10. The upper aqueous layer was collected into a fresh tube and 1/30th volume of sodium acetate (pH 5.5) and equal volume of chilled absolute ethanol were added.
11. 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).
12. DNA was washed twice with 70% alcohol and once with absolute alcohol to remove excess salt and vacuum dried.
13. The vacuum dried DNA was dissolved in 1 mL of TE buffer (10 mM Tris-HC1, 1mM EDTA, pH 8.0). The DNA isolated from cells also contains RNA which was removed by digesting the preparation with RNAse Enzyme. RNAse solution was kept in boiling water for 10 min before use so as to inactivate any DNAse, because the RNAse may also contain DNAse.

The method provides high quality genomic DNA with good yield.

Precautions taken in order to prevent in vitro DNA damage during phenol-chloroform genomic DNA extraction.

1. Employed proper microbiological aseptic technique when working with DNA.
2. Wore latex gloves when isolating and handling DNA to prevent nuclease contamination from the surface of the skin and to minimize the activity of endogenous nucleases.
3. Used sterile, disposable plastic ware. Autoclaved pipette tips and 1.5 ml microcentrifuge tubes were used for additional protection against nucleases. Pipettes were wiped with DNAse-removal solutions (diethylpyrocarbonate) when transitioning between handling crude extracts to handling more purified material.
4. Pipette tips with blunt ends (the tips cut with blade) were used as a precaution to prevent DNA fragmentation.
5. Liquid nitrogen was used to keep the frozen tissue hard before grinding. This step will prevent activation of endonucleases.

4. 2. 4. DNA Concentration and purity

The concentration of DNA was measured by noting absorbance at 260 mm and the purity of DNA preparation in terms of any protein contamination was checked by recording the ratio of absorbance at 260 nm/280 nm which should be ideally between 1.6 and 1.8.

4. 2. 5. Neutral and alkaline agarose gel electrophoresis

The integrity or damage in genomic DNA was assessed by running neutral and alkaline gel electrophoresis. The migration pattern in neutral gels reflects the double strand breaks present in the DNA and the migration pattern in alkaline gels shows both

single and double strand breaks [Sutherland and Shih, 1983]. Neutral gels were electrophoresed on 1.8% agarose gels in Tris-acetate-EDTA buffer (pH 8.0) at 4 V/cm for 4 h and stained with EtBr for 15 min. Three micrograms of DNA was loaded in each well. DNA ladders (1 kb and 100 bp) were used as molecular weight markers.

For alkaline gels, the DNA samples were mixed with alkaline stock mix solution consisting of 1N NaOH and 2xBCG dye. BCG dye consists of 0.25% Bromocresol green and 50% (v/v) glycerol. Two micrograms of DNA sample was mixed with the alkaline stop mix and loaded on to the gel and run with alkaline running buffer consisting of 30 mM NaOH and 2mM EDTA for 4 h at 4V/cm. After the run, the alkaline gel was neutralized in 100 mM Tris buffer, pH 8.0 for 30 min and then stained with EtBr for 15 min. The stained gels were analyzed in UV gel documentation system.

4. 2. 6. Estimation of single strand and double strand breaks in genomic DNA isolated from control and BPD- affected postmortem brain samples.

Single strand breaks: Single strand breaks (SSBs) are calculated through incorporation of ^{3} [H]-TMP into DNA samples when incubated with E. coli DNA polymerase I (Klenow Fragment) in a nick translation assay. DNA polymerase I adds nucleotides at the 3'-OH end of a SSB, generated by various means, using the other strand as template. When one of the deoxynucleotide triphosphates is labeled, then the incorporation of radioactivity into substrate DNA would be proportional to the number of SSBs present in the DNA sample. During the standardization of the essay conditions with the plasmid DNA(Cos T fragment of ג phage) having known number of SSBs, it was found that 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

Double strand breaks: Terminal deoxynucleotidyl transferase catalyzes the addition of deoxynucleotides to the 3' termini of DNA and does not need direction from template strand. Here, 3'- ends of duplex DNA also serve as substrates. Similar conditions to incubate DNA with terminal transferase as in the case of E. coli polymerase I assay were used. The incorporation of the ^{3}[H]-dTTP into DNA would be proportional to the number of double strand breaks (DSBs) in the DNA. From the conditions and incubation [Bhaskar and Rao, 1994; 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 1.2 x 10^{7} 3'-ends or half of that number minus one DSBs. The assay mixture for terminal transferase reaction consisted of a total volume of 50 µ1:100 mM sodium cacodylate buffer, pH 7.0, 1 mM $CoCl_2$, 0.2 mM DTT, 1 µ Ci of ^{3}[H]-dTTP, 1 µg DNA, and 1 U of the enzyme.

4. 2. 7. Melting temperature and hyperchromicity of the genomic DNA

To determine the physical state of the DNA in BPD depression brain, the melting profiles of DNA isolated from different regions of BPD depression brains and corresponding control groups were examined. DNA was dissolved in 0.01M Hepes buffer, pH 7.4. The DNA was used at a concentration of 20µg/mL. The melting profiles (t_m) of DNA from the control and BPD depression were recorded in Spectrophotometer, equipped with a thermo programmer and data processor (Amersham, Hong Kong). The

hyperchromicity changes of the DNA were recorded from 20 to 95˚C with 1˚C increment/minute. The temperature point at which there is a 50% hyperchromic shift is taken as t_m of the DNA sample. t_m values were determined graphically from the hyperchromicity vs. temperature plots. The precision in t_m values estimated in triplicate with ±0.05˚C deviation.

4. 2. 8. Ethidium bromide binding studies

The quantification of ethidium bromide (EtBr) bound in moles per base pair of genomic DNA was measured in 0.01 M Hepes, pH 7.4 [Chatterjee and Rao, 1994] using HITACHI F-2000 Fluorescence Spectrophotometer. The fluorescence was measured using a constant amount of DNA [2µg] with increasing EtBr [1nanogram to 2microgram] against the blank containing no DNA. The measurements were performed keeping excitation at 535 and emission at 600 nm with 10 mm path-length.

The maximum amount of EtBr bound per base pair of DNA was calculated using Scatchard plots of 'r' vs. 'r/Cf' [Chatterjee and Rao, 1994]. The concentration of bound EtBr in 1.0 mL dye-DNA mixture (Cb') was calculated using the equation:

$Cb' = Co' [(F-F_0) / (V \times F_0)]$.

Where,

Co = EtBr (pmoles) present in the dye-DNA mixture

F = observed fluorescence at any point of dye-DNA mixture

F_0 = observed fluorescence of EtBr with no DNA,

V = experimentally derived value, ratio of bound EtBr/ free EtBr at saturation point.

The concentration of free dye (Cf') was then calculated by the relation.

Cf' = Co' – Cb'.

Where, Cf', Co', and Cb' were expressed in pmoles. The amount of bound EtBr/base pair 'r' was calculated by

r= Cb' (pmoles)/DNA concentration (pmoles of base pair).

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

Cf = Cf' x 10^{15} M.

4. 2. 9. Circular dichrosim studies

The Circular Dichrosim (CD) spectra (190-330 nm) were recorded for genomic DNA in 0.01 M Hepes buffer (pH 7.4) on a JASCO-J720 Spectropolarimeter. The cell length and width in 1mm each. Each spectrum is the average of quadruplicate recordings. Twenty five micrograms of DNA from each sample was used. The DNA conformations were characterized from the CD spectra using the reference of Gray *et al*., (1992).

4. 2. 10. Statistical analysis

All the data obtained in this study were statistically treated and the significance of differences between control and BPD depression were calculated according to Student's *t* test. The statistical analysis was carried out using Microsoft Excel 2000 Soft-ware.

4. 3. Results

The clinical characteristics and agonal states of 8 healthy controls and 10 subjects with BPD depression matched for age, postmortem interval, sex and freezer storage time are summarized in (Tables 1). As presented in Table-2, the mean age, postmortem interval

(PMI) and sex ratio were remarkably similar between controls and patients with BPD depression. Representative anatomical areas have been dissected in frozen state, transported on dry ice and subjected to standard DNA extraction and further studies. DNA was isolated from ten different regions namely, Parietal, temporal, occipital lobe, hippocampus, thalamus, cerebellum, hypothalamus, medulla, pons, and frontal cortex from eight control and ten BPD depression brains.

Table I. List of Cases and Demographics for Healthy Control and BD-depression subjects

Number	**Age, Y**	**PMI, h**	**Sex**	**Hemisphere, L/R**	**Cause of Death**
Healthy controls					
N-1	25	4	F	L	RTA
N-2	38	5	F	L	Snake bite
N-3	21	4	F	L	Accidental burns
N-4	18	4	M	L	RTA
N-5	22	5	M	L	RTA
N-6	24	6	M	L	RTA
N-7	30	3	F	L	Accidental burns
N-8	37	8	M	L	Fall from height
Bipolar-depression					
BD-1	25	4	M	L	Suicide by hanging
BD-2	20	5	F	L	Suicide by burning
BD-3	19	4	M	L	Suicide by hanging
BD-4	18	3	M	L	Suicide by hanging
BD-5	20	4	F	L	Suicide by hanging
BD-6	21	4	F	L	Suicide by burning
BD-7	35	4	M	L	Suicide by hanging
BD-8	23	6	M	L	Suicide by hanging
BD-9	21	4	F	L	Suicide by hanging
BD-10	30	5	F	L	Suicide by burning

Abbreviations: PMI, postmortem interval. RTA, Road traffic accident

Table II. Average Demographics of Healthy Control and Bipolar depression subjects

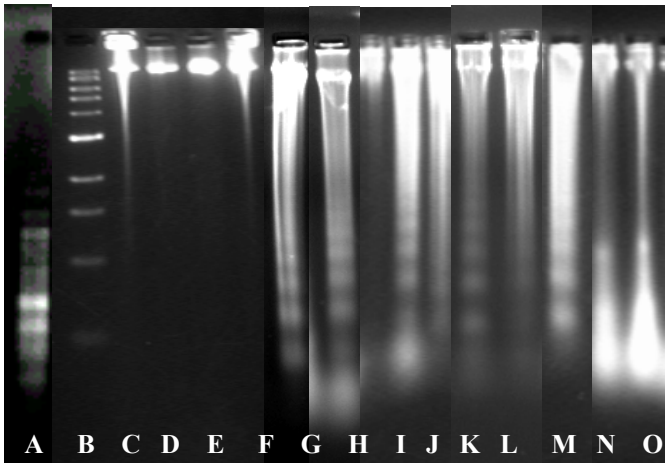
Diagnosis	No. of Subjects	Mean ± SD Age, Y	Mean ± SD PMI, h	Sex, M/F
Healthy control	8	26± 7.4	4.9 ± 1.6	5/3
BPD depression	10	23.2 ± 5.4	4.4 ± 1.1	5/5

4. 3. 1. Agarose gel electrophoresis

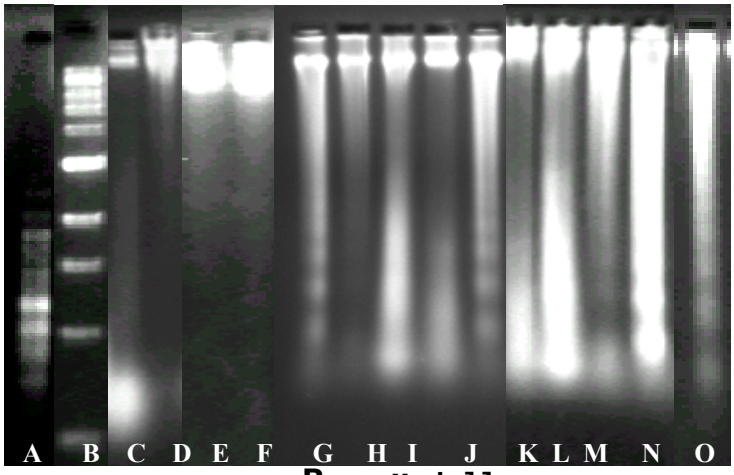
Fig. 1 shows the neutral agarose gels of genomic DNA from various regions of control and BPD – depression affected postmortem brains. In each gel DNA isolated from ten BPD depression and four control brains are given and lane A and B in all the gel represents the 100bp and 1kb marker respectively. In pons (Fig. I A), lane C to F are control subjects DNA showed intact DNA, lane from G to P are depressive pons brain DNA which shows DNA fragmentation (DNA laddering). The DNA isolated from medulla region (Fig. 1B) lane from C to D represents control subjects DNA shows intact, while G to P showed the DNA laddering or fragmentation. In frontal cortex (Fig. 1C) lane C to F are control brain DNA which is intact, lane from G to P observed DNA fragmentation. In thalamus (Fig.1D) the control thalamus DNA observed intact form (lane C to F), while DNA is fragmented in thalamus of BPD depression brain are observed (lane from G to O). In cerebellum (Fig. 1 E) lane C to F is control DNA showed intact DNA; lane G to P are from BPD DNA shows DNA fragmentation but not laddering. In temporal lobe (Fig. 1 F) lane C to F represents the intact control DNA; lane G to O represents fragmented DNA from BPD brain. In parietal lobe (Fig.1 G) lane observed C to F shows the intact DNA, lane H to O showed little fragmented DNA of BPD depression brain. However, interestingly the hippocampus (Fig.1 H) lane C to F shows intact control DNA and lane from G to P showed no DNA fragmentation except lane K and O. In hypothalamus (Fig.1 I) lane C to F are control DNA showed intact form and lane G to L showed fragmentation, but other lanes such as H to K and M to O are intact like control DNA. In occipital lobe, lanes D to F shows control DNA and are intact, lane G to O were found little fragmentation. This study showed that pons, medulla,

thalamus, frontal cortex and cerebellum showed more DNA fragmentation (DNA laddering) in genomic DNA of BPD-depression compared to healthy controls as indicated in agarose gel. The next DNA damage was found more in temporal lobe and parietal lobe compared to controls. While hypothalamus, occipital lobe and hippocampus showed very less DNA fragmentation.

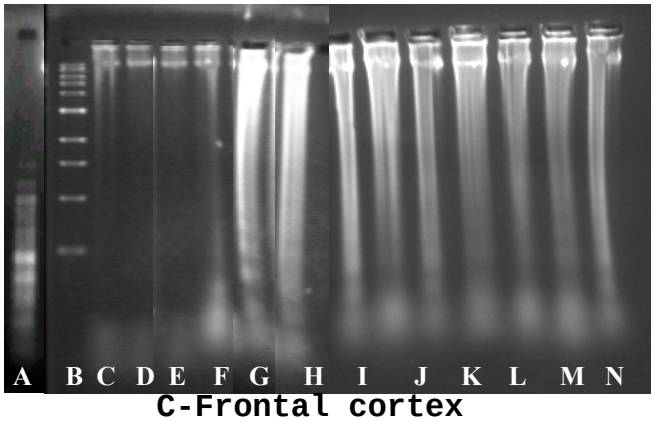
Fig.2 shows the mobility of DNA in alkaline gel of BPD-depression and control brains. One representative DNA sample from pons, medulla, thalamus, frontal cortex, cerebellum from control subjects and BPD depression brain are shown. Lane A, C, E, G and I represent the DNA from control brain and lanes B, D, F, H and J represent DNA from BPD-depression brain. It was observed that thalamus, medulla, frontal cortex and pons showed more strand breaks than the age matched controls.



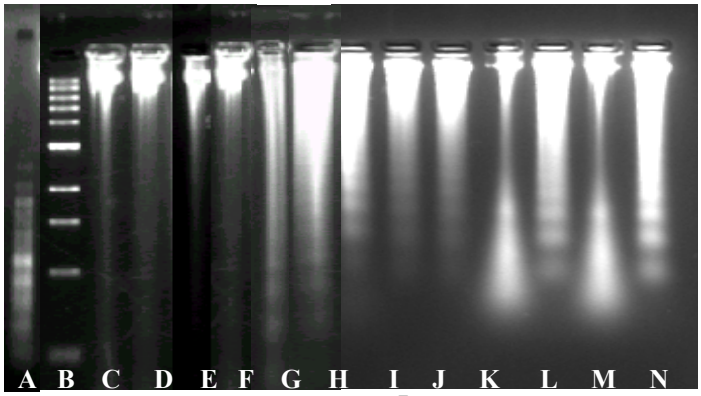
A- Pons



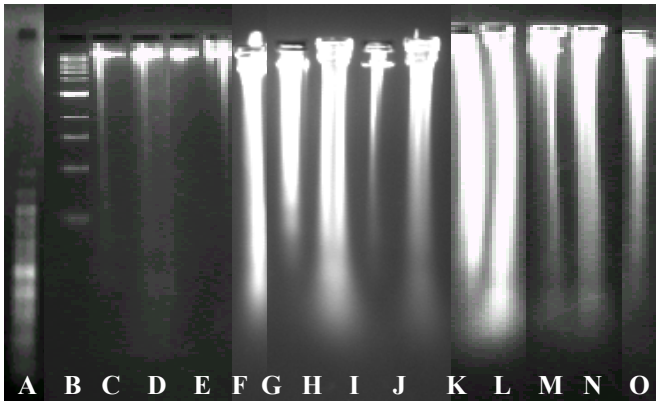
B - Medulla



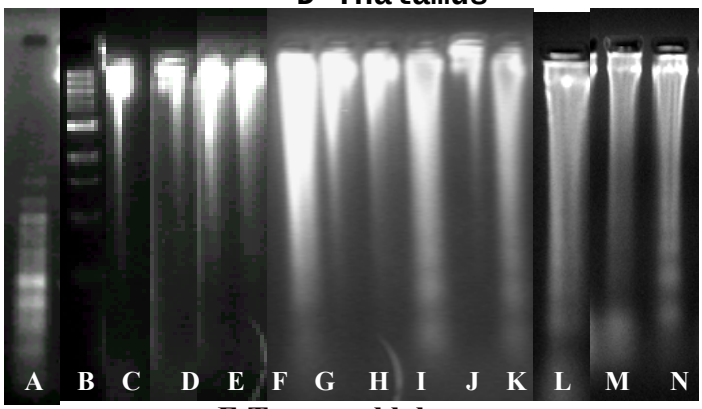
C-Frontal cortex



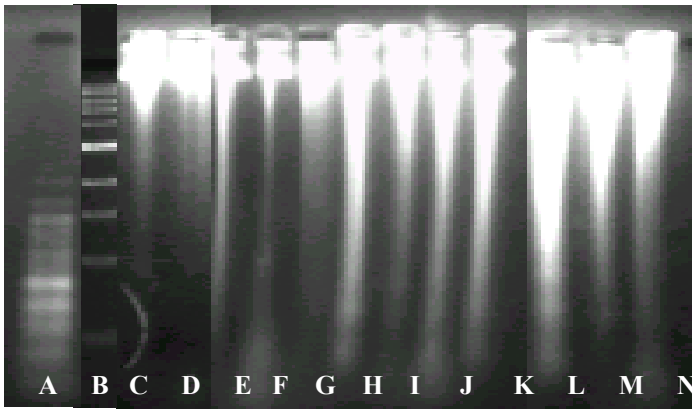
D-Thalamus



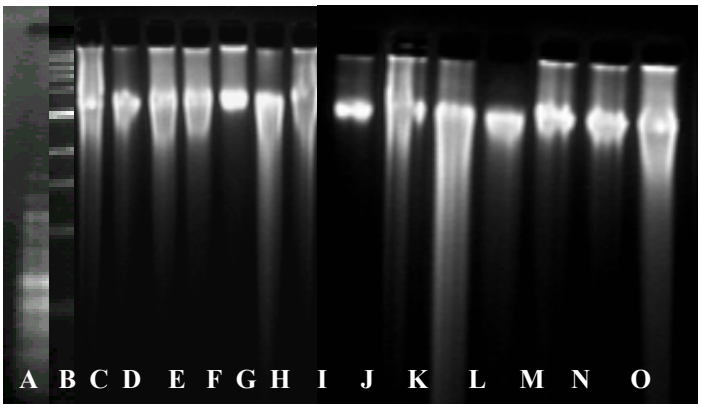
E-Cerebellum



F-Temporal lobe

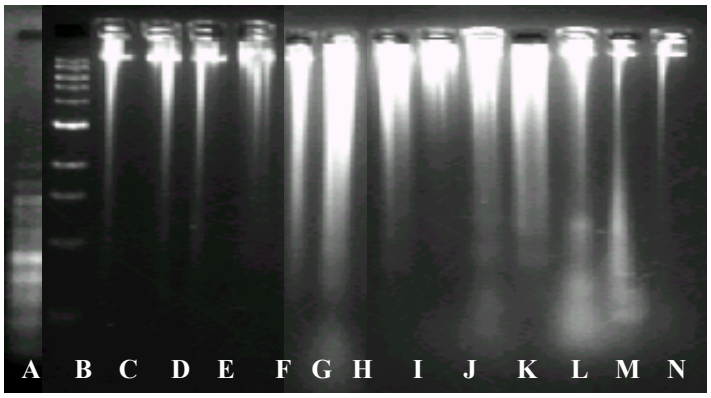


G-Parietal lobe



H- Hippocampus

T- Hypothalamus



J-Occipital lobe

Fig.1. Neutral agarose gel electrophoresis pattern of genomic DNA isolated from BPD depression and age matched control brains. Neutral gels were run on 1.8% agarose. 2µg of each DNA sample was used for gel studies. Lanes A and B in each gel represent 100bp ladder and 1kb DNA marker respectively. Gel studies were done on the ten samples of BPD-depression cases and four controls. The result indicates that DNA from pons, medulla, frontal cortex, thalamus, temporal, parietal lobes and cerebellum showed DNA fragmentation (DNA laddering) in neutral gel compared to control DNA. There was not much DNA fragmentation in hippocampus, occipital lobe and hypothalamus regions.

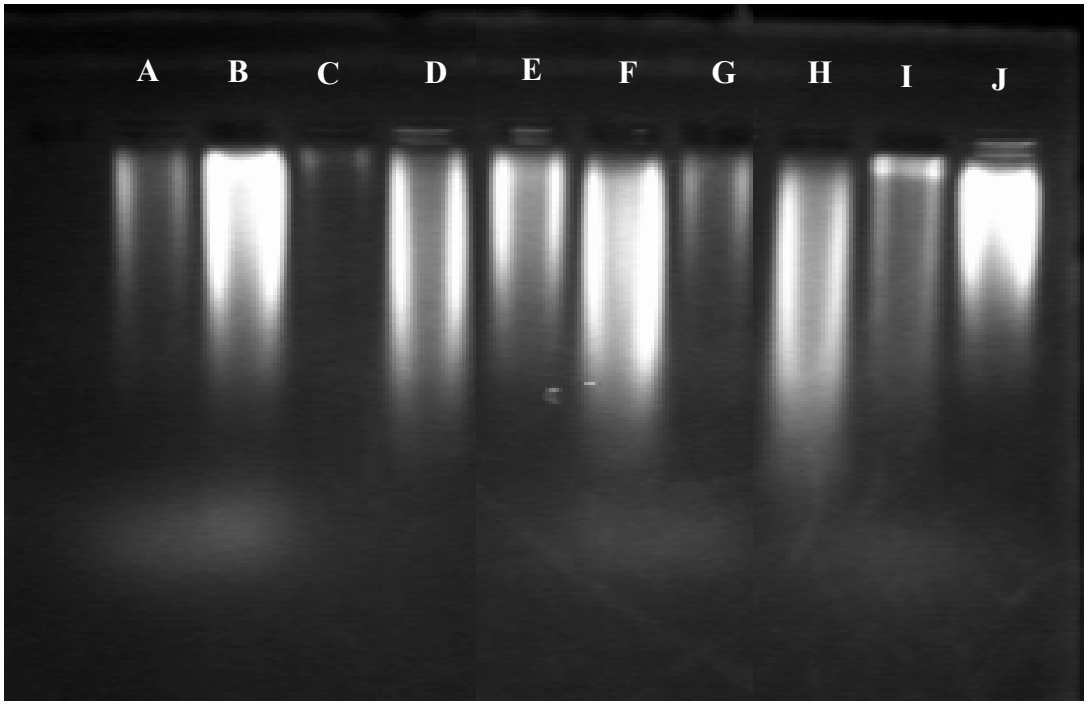


Figure 2. Alkaline gel electrophoresis for observation of SSBs and DSBs DNA. Alkaline gel was run on 0.8% agarose gels and 2µg of each DNA was used. Lane A, C, E, G and I represent the DNA from control brain and lanes B, D, F, H and J represent DNA from BPD-depression brain. A/B, thalamus; C/D, pons; E/F, medulla; G/H, frontal cortex; I/J, cerebellum. Alkaline studies were done on eight control and ten BPD-depression samples in each brain region and one BPD DNA sample (sample No. BPD-1 from Table I, age 25years) and its age matched control (sample No. N-1 from Table I, age 25 years) have been represented in the figure. DNA isolated from medulla, pons, thalamus, cerebellum and frontal cortex showed more DNA fragmentation in BPD-depression than controls. Similar trend was observed in all the BPD-depression and control samples studied.

4. 3. 2. Single Strand Breaks

The most prevalent type of DNA damage in mammalian cell is the SSBs. Single-stranded breakage is the end point of several types of structural insults inflicted on the genome by both endogenous and exogenous agents [Rao, 1993]. Fig.3 showed number of SSBs per microgram of genomic DNA isolated from various brain regions. Accumulations of SSBs were more frequent in BPD-depression than in healthy controls in the following regions. The result showed that thalamus ($p<0.001$), pons ($p<0.01$), cerebellum ($p<0.05$), parietal lobe ($p<0.02$) and frontal cortex ($p<0.05$) accumulated considerably higher number of SSBs compared to healthy controls from their respective regions (Fig.3). There were no single strand breaks in hippocampus.

4. 3. 3. Double Strand breaks

The DNA isolated from thalamus, frontal cortex, cerebellum, pons and temporal lobe showed significantly higher number of DSBs than respective controls ($p<0.01$) (Fig 4). Hippocampus showed no DSBs in both control and BPD-depression samples. The region such as thalamus, pons, medulla, cerebellum and parietal lobe were accumulated both SSBs and DSBs more in BPD than respective controls, whereas frontal cortex, accumulated more DSBs than SSBs. We also observed that little amount of DSBs in hypothalamus DNA but this damage was less compared to other region from the BPD brain. The present result showed that frontal cortex has more DSBs than SSBs whereas thalamus, cerebellum, parietal lobe and pons have presence of both DSBs and SSBs accumulated. Interesting finding is that hippocampus region were not seen any accumulation of SSBs and DSBs in both control subjects and BD-depression human brain samples.

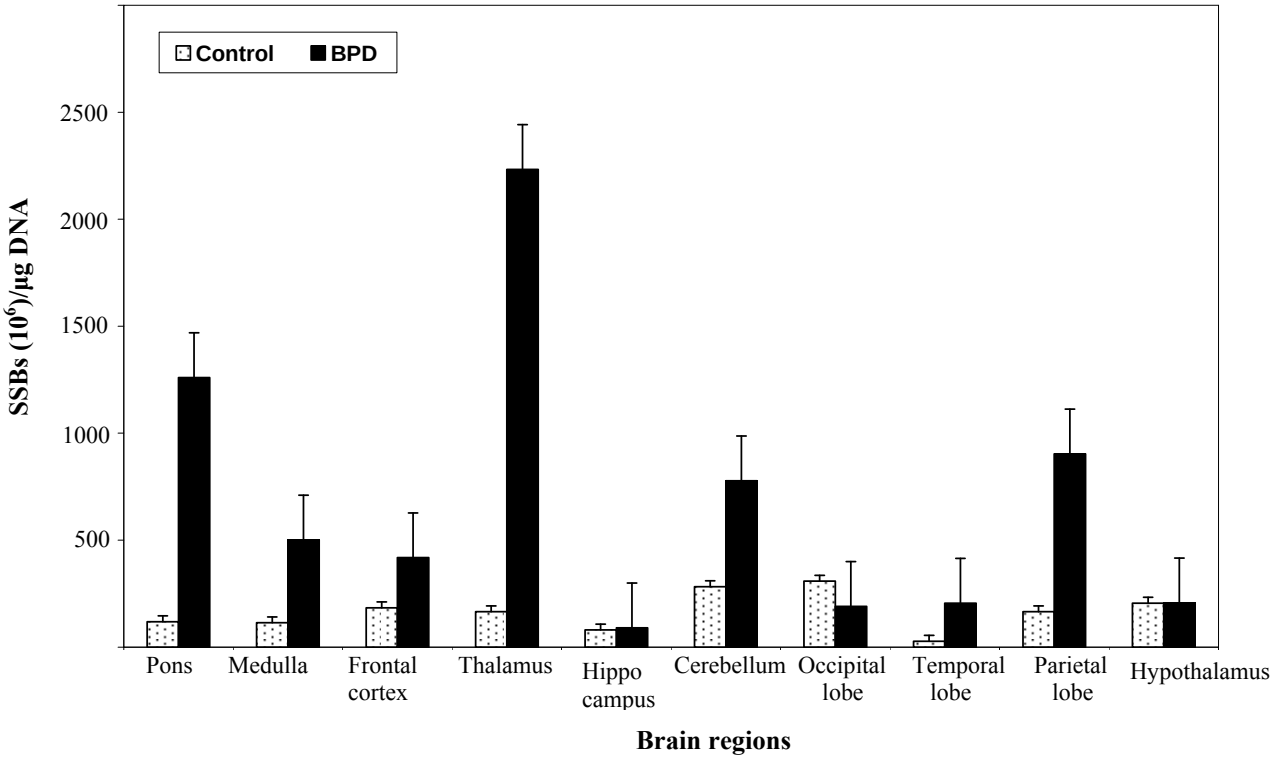


Figure 3. Assessment of single strand breaks (SSBs) in DNA isolated from different regions of BPD-depression and control brains. The SSBs in DNA were determined through nick translation type of incubation with E.coli DNA-polymerase I. The bar chart represents SSBs values ±SD for eight control and ten BPD-depression brain DNA samples.

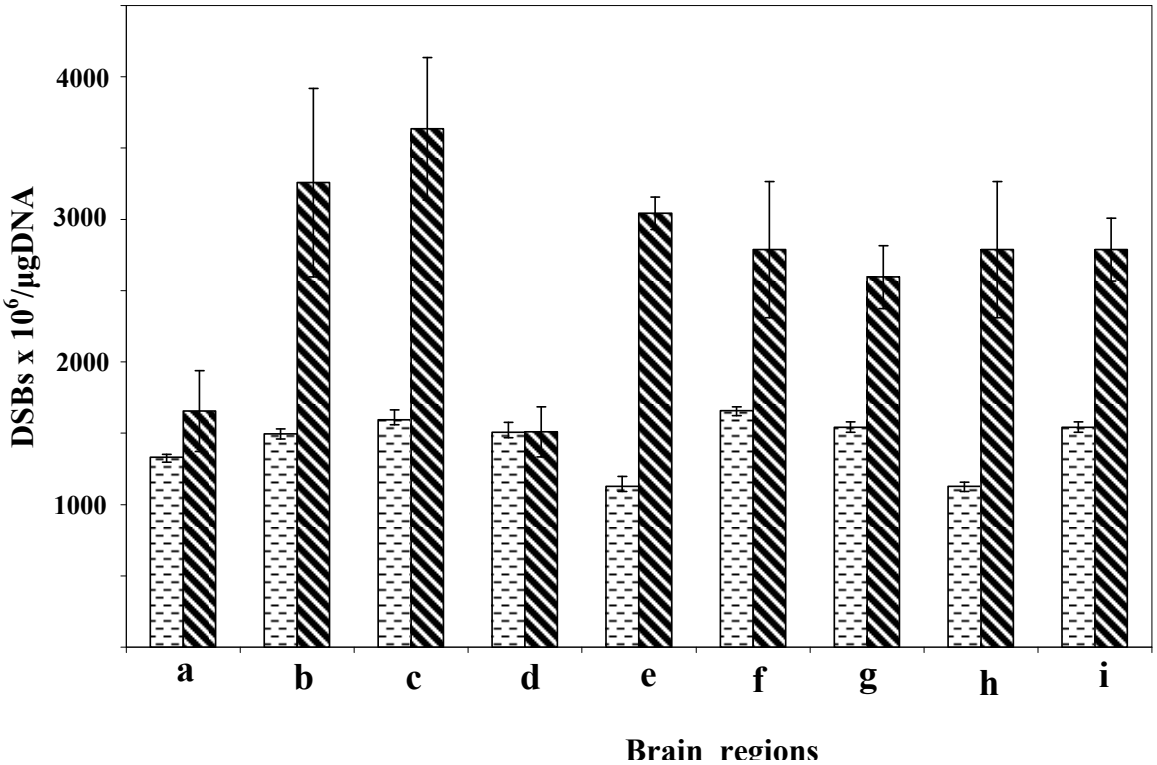


Figure 4. Assessment of double strand breaks (DSBs) in DNA isolated from different regions of BPD depression and control brains. (a) Hypothalamus, (B) Frontal cortex, (C) Thalamus, (D) Hippocampus, (E) Cerebellum (F) Temporal lobe, (G) Pons, (H) Medulla, (I) Parietal lobe. DNA were measured using terminal transferase assay. The bar-chart represents mean of DSBs values ±SD for eight controls and ten BPD brain DNA samples.

4. 3. 4. Melting temperature and hyperchromicity of DNA isolated from different regions of BPD-depression and control brain samples

Heating the DNA leads to destruction of double stranded hydrogen bonded region of DNA. The process of denaturation is accompanied by an increase in absorbance at 260 nm as double stranded DNA converted to single strands. This increase in absorbance is referred as the hypochromic effect. A method to analyze DNA is to denature with heat, monitor the increase in A260 with increasing temperature and determine the thermal melt temperature (t_m). The t_m is defined as the temperature at which 50% of the base pairs of the duplex are separated [Markey and Breslauer, 1987; Wetmur, 1991]**.**

Table 3 and Fig. 5 represent the t_m and hyperchromicity values of genomic DNA dissolved in 0.01M Hepes buffer, pH 7.4. The data showed that the t_m is significantly low for BPD-depression DNA; temporal lobe ($p<0.001$), medulla ($p< 0.001$), frontal cortex ($p<0.001$), thalamus ($p<0.001$), pons ($p<0.05$) and cerebellum ($p<0.05$) compared to their respective controls indicating DNA damage or decrease in stability. No significant difference was observed in t_m for DNA isolated from hippocampus and hypothalamus from both control and BPD depression brains (Table 3). The decrease in t_m of DNA from thalamus, temporal lobe, medulla, frontal cortex, cerebellum and pons indicate more strand breaks and resulting destabilization of DNA.

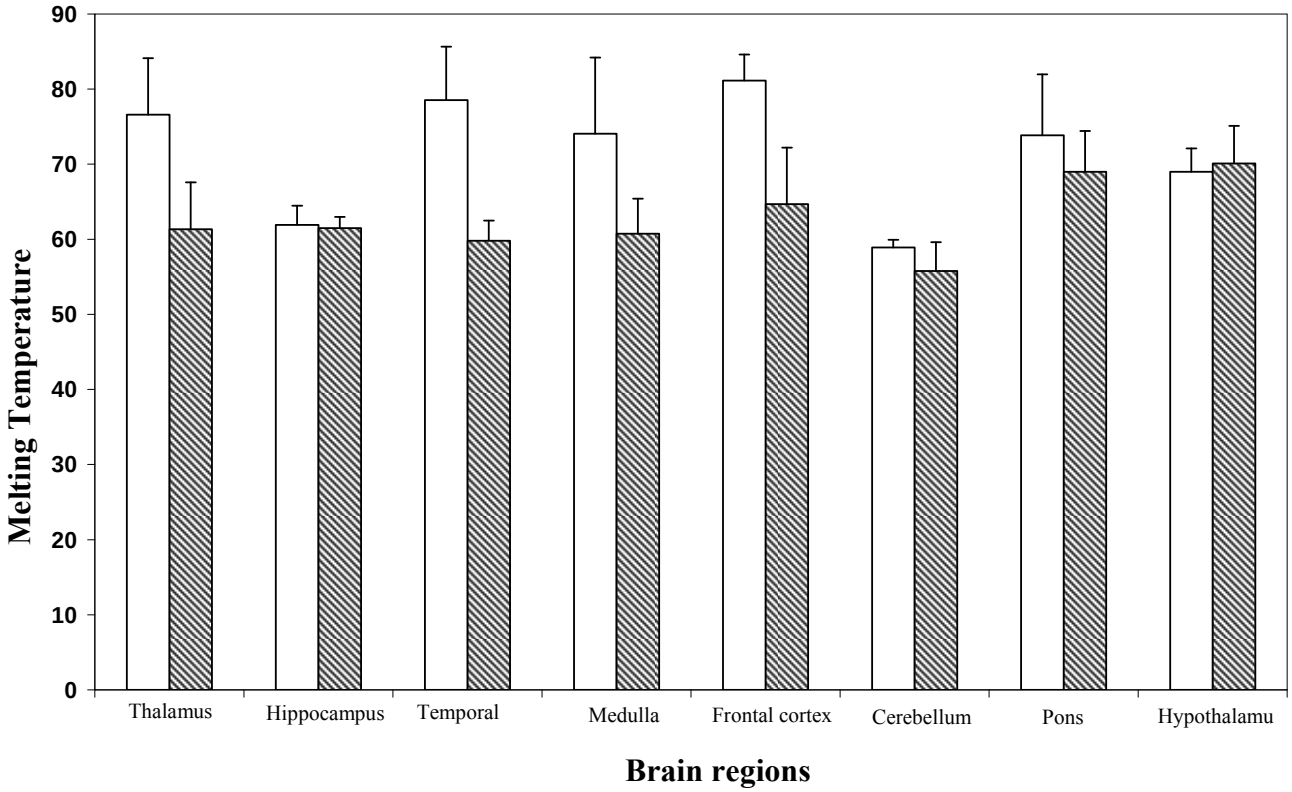


Figure.5. Melting profile of DNA isolated from different regions of BPD-affected postmortem human brain with reference to control. DNA samples were taken at concentration of 20µg/mL in 0.1 Hepes buffer, pH 7.4. t_m was calculated graphically as the point of 50% hyperchronic shift. Mean values ± SD for eight control and ten BPD brain DNA were calculated.

Table 3
Melting temperature (t_m) of DNA isolated from control and BPD- affected postmortem human brains

Brain regions	Control	BPD-depression
Thalamus	76.6 ± 7.5*	61.34 ± 6.25
Frontal cortex	81.6 ± 3.5*	64.7 ± 7.5
Medulla	74.17 ± 10.1**	60.8 ± 4.7
Temporal lobe	78.5 ± 7.1*	59.8 ± 2.7
Cerebellum	58.9 ± 1.01**	55.8 ± 3.8
Pons	73.8 ± 8.2*	69 ± 5.4
Occipital lobe	68 ± 5	61.2 ± 4.4
Hypothalamus	69 ± 3.1	70.1 ± 5
Hippocampus	61.9 ± 2.6	61.5 ± 1.49

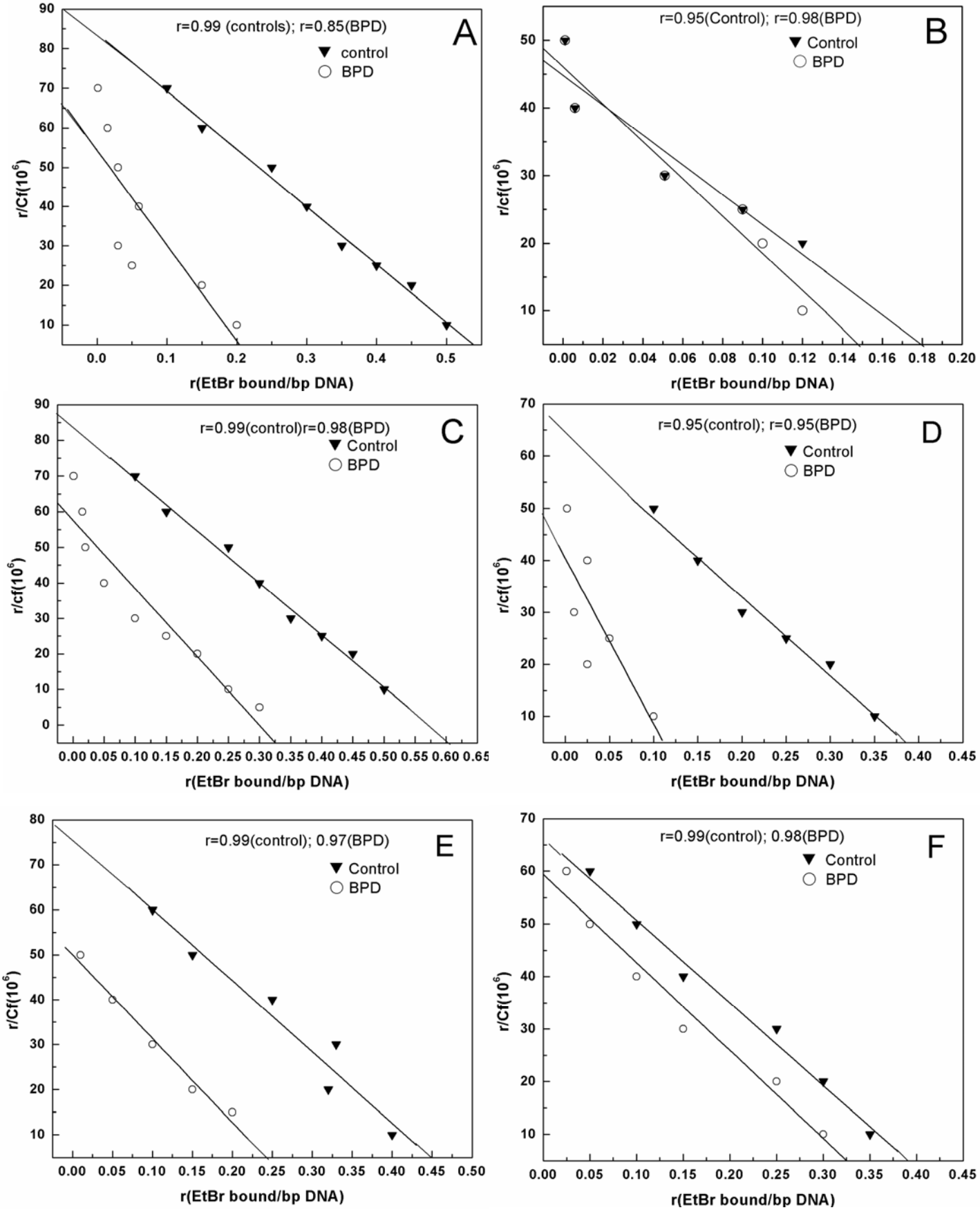
Values are as degrees centigrade. All the values are means ± SD of eight control and ten BPD samples. The melting temperature(t_m) was calculated as the point of 50% hyperchromic shift. The statistical significance was calculated using Student's t test.

*These values are significantly different from the corresponding control values at $p< 0.001$.

** These values are significantly different from the corresponding control values at $p<0.02$.

4. 3. 5. EtBr binding studies

The amount of EtBr molecules bound per base pair (bp) of DNA was represented in Table 4. Scatchard plots of ten regions from the eight controls and ten BPD-depression brain DNA are shown in Fig 6. The EtBr binding data showed (Table 4) that DNA isolated from thalamus, medulla, pons, cerebellum, temporal lobe and frontal cortex have less EtBr/bp in BPD compared to corresponding controls at a statistical significance of $p<0.02$. EtBr bound/bp to DNA from hippocampus, occipital lobe and hypothalamus did not differ significantly between BPD depression and control subjects. This directly reflects DNA topology and stability.



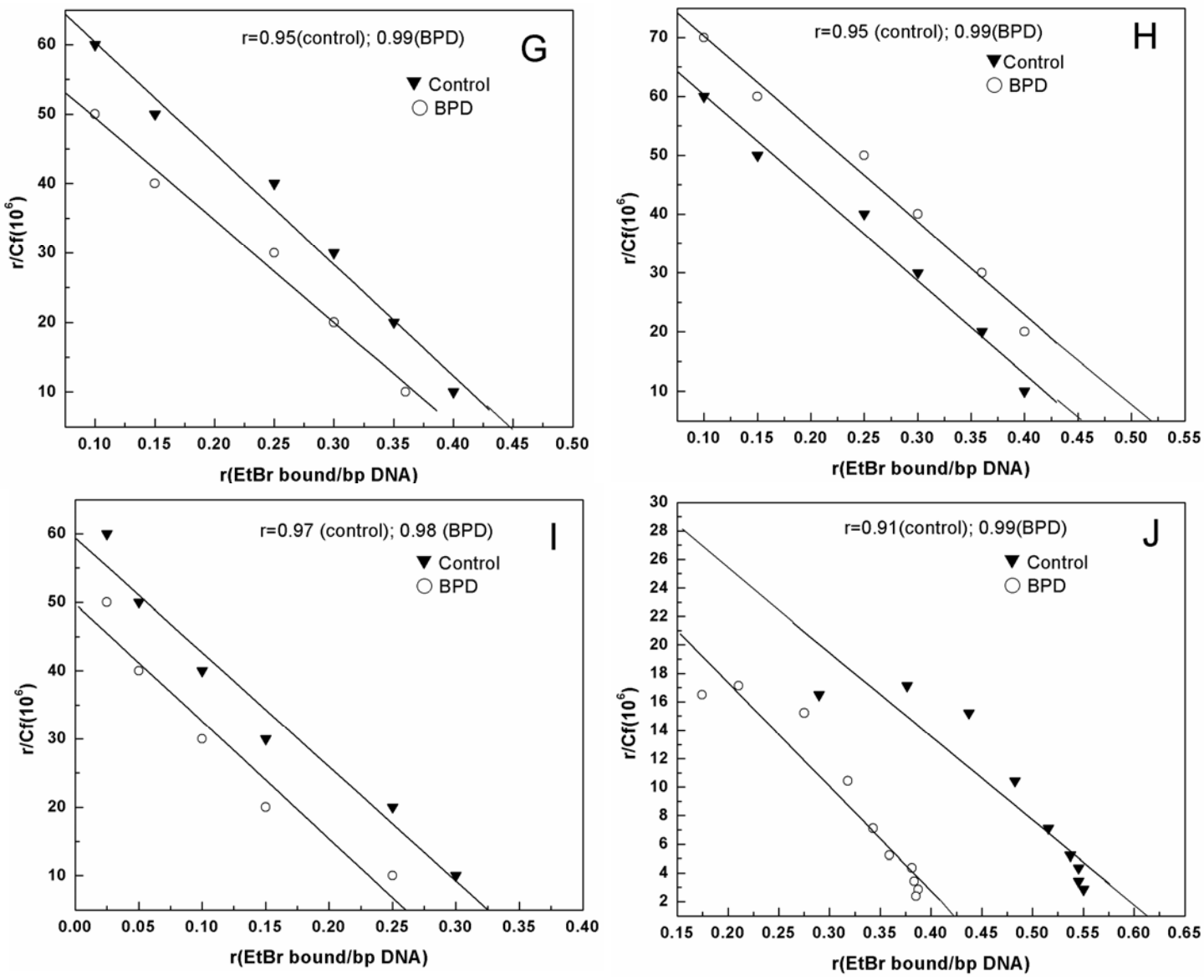


Fig. 6 Scatchard plot of EtBr binding to DNA isolated from human BPD and control subjects. (A) Cerebellum, (B) Hippocampus, (C) Medulla, (D) Pons, (E) Frontal cortex, (F) Temporal lobe, (G) Occipital lobe, (H) Parietal lobe, (I) Hypothalamus, (J) Thalamus. Increasing amount of EtBr was added to a fixed concentration of DNA in a 1mL reaction mixture (0.01M Hepes, pH 7.4). Fluorescent measurements were done at RT setting excitation at 535nm and emission at 600nm. The Scatchard plot was drawn using least square method. The same analysis was carried out for all the DNA samples extracted from ten regions of eight control and ten BPD-depression brain samples. This data as 'means ± SD' and statistical significance (p value) (calculated from student's t- test) have been calculated.

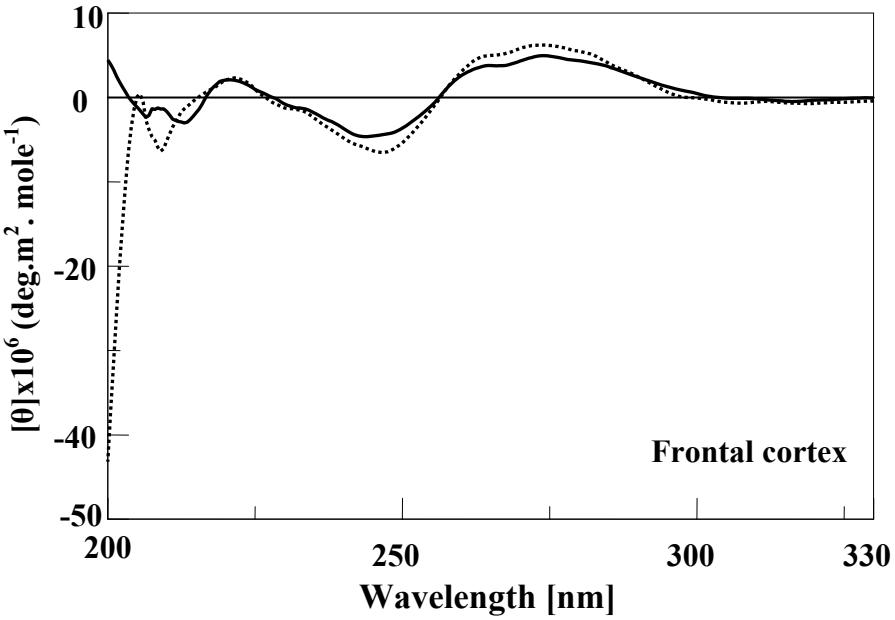
Table 4
EtBr binding assay

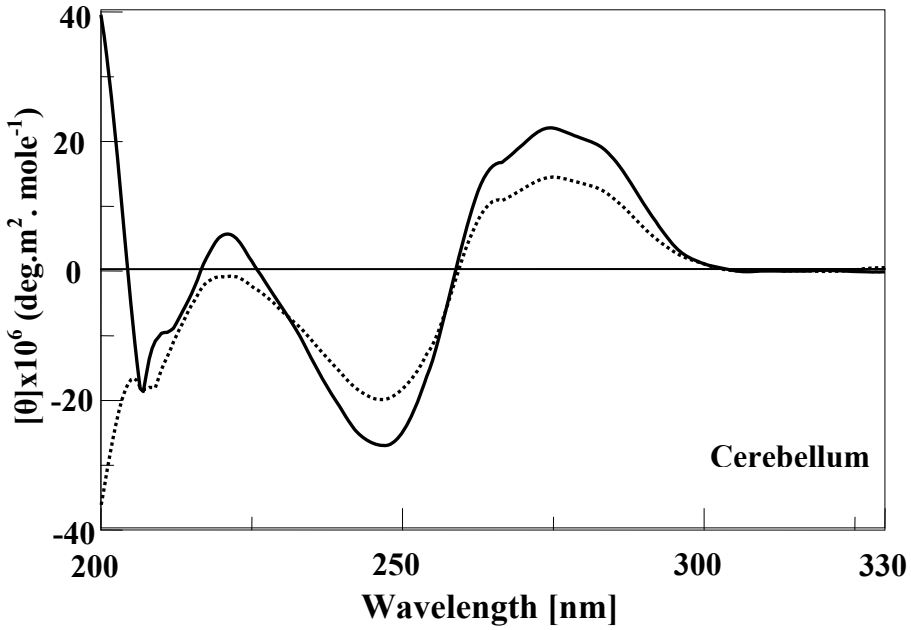
Brain regions	Control	BPD
Thalamus	0.63 ± 0.09	0.44 ± 0.07*
Medulla	0.61 ± 0.11	0.32 ± 0.15*
Pons	0.38 ± 0.1	0.12 ± 0.03*
Cerebellum	0.53 ± 0.16	0.20 ± 0.04*
Hippocampus	0.18 ± 0.06	0.15 ± 0.1
Temporal lobe	0.39 ± 0.08	0.32 ± 0.03*
Occipital lobe	0.45 ± 0.12	0.40 ± 0.08
Parietal lobe	0.45 ± 0.15	0.51 ± 0.11
Hypothalamus	0.32 ± 0.08	0. 26 ± 0.13
Frontal cortex	0.44 ± 0.1	0.25 ± 0.03*

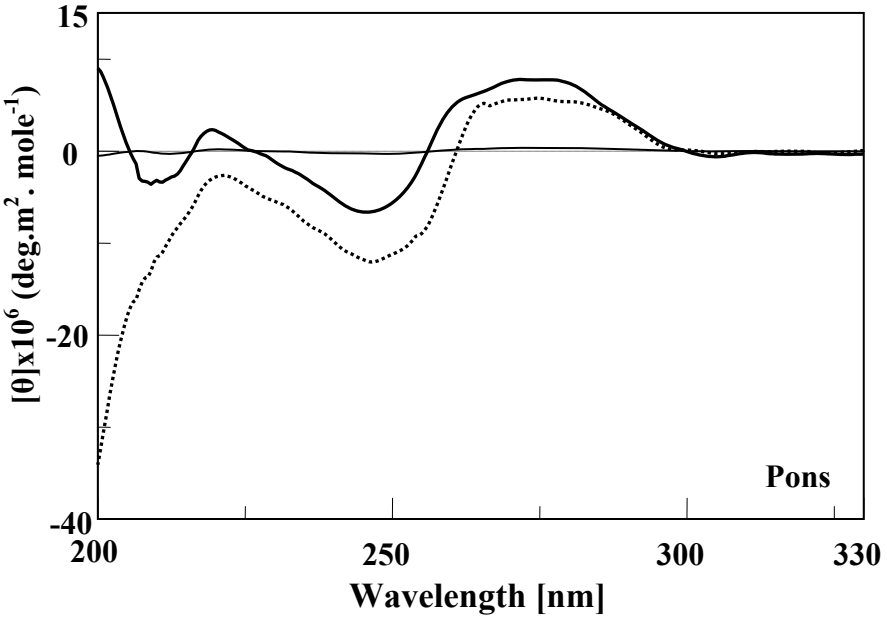
Bound EtBr per base pair (bp) of DNA from BPD depression and control samples were calculated using Scatchard plots [Scatchard, (1949); Chatterjee and Rao (1994)]. Values are as mean of number of EtBr molecules bound per base pair of DNA ± SD. These values with asterisk (*) are significantly different from the corresponding control values at $p< 0.02$ of eight control and ten BPD DNA samples. The statistical significance was calculated using Student's t test.

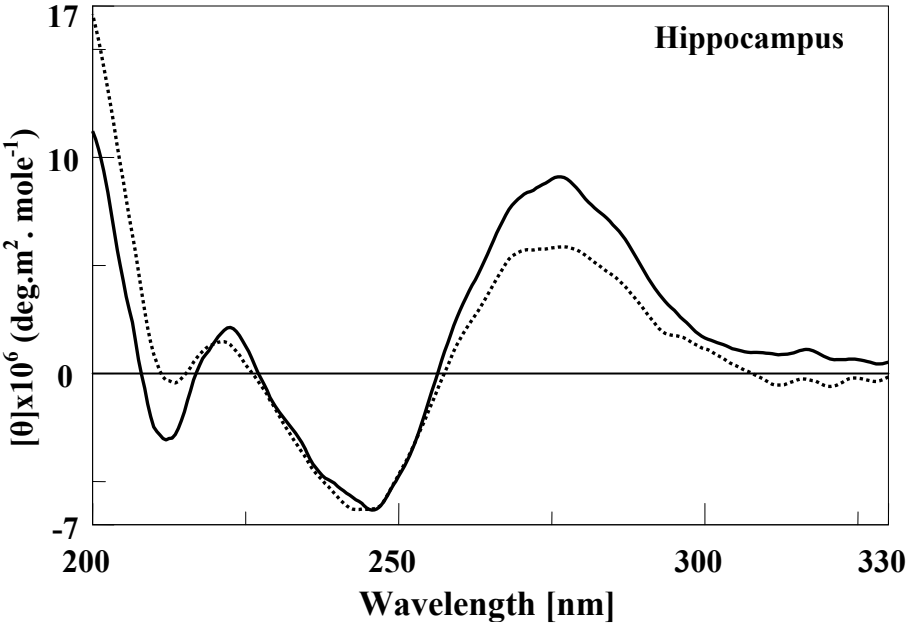
4. 3. 6. Circular Dichroism studies

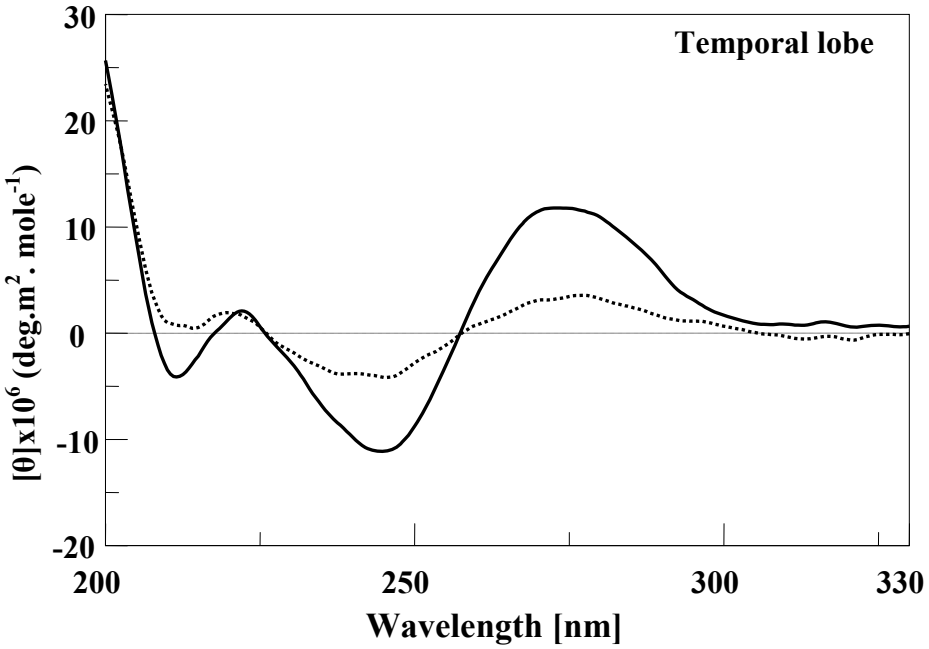
The CD studies showed that the conformation of DNA isolated from hippocampus did not differ from corresponding controls. However, CD signal of DNA for frontal cortex, cerebellum, pons, thalamus, parietal lobe, temporal lobe, hypothalamus and occipital lobe differs from respective controls (Fig.7). The negative peak at 200 nm was observed in frontal cortex, cerebellum, pons and parietal lobe of BPD-depression brain compared to control subjects. Presence of negative peak at 200 nm is the characteristic feature of secondary DNA. The results showed that the positive peak at 275 nm and negative peak at 245 nm were consistently low for DNA isolated from temporal lobe, hypothalamus, occipital lobe and thalamus of BPD brain than control subjects. CD spectra of BPD- thalamus and pons showed the blue shift from left to right compared to controls.

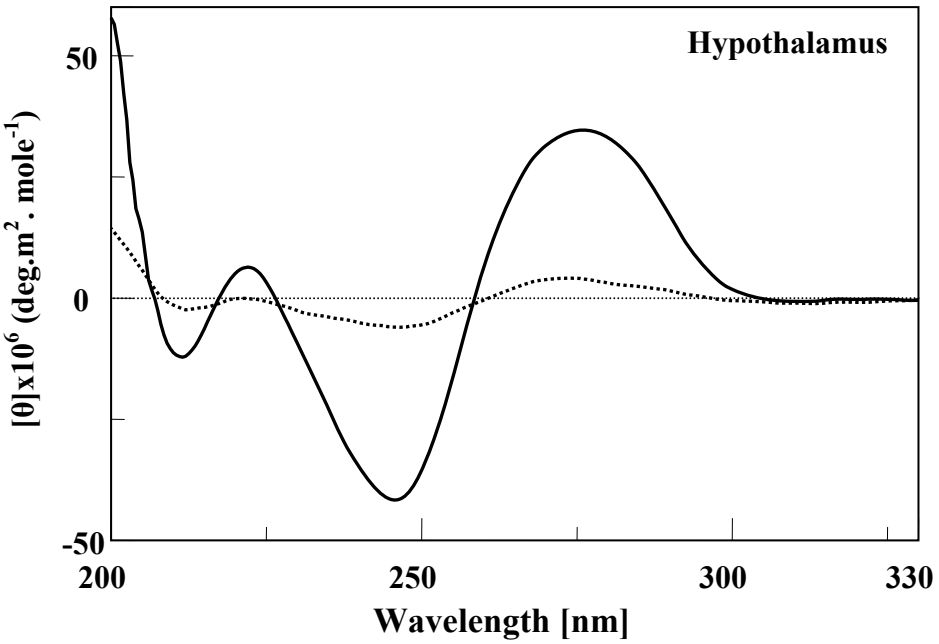


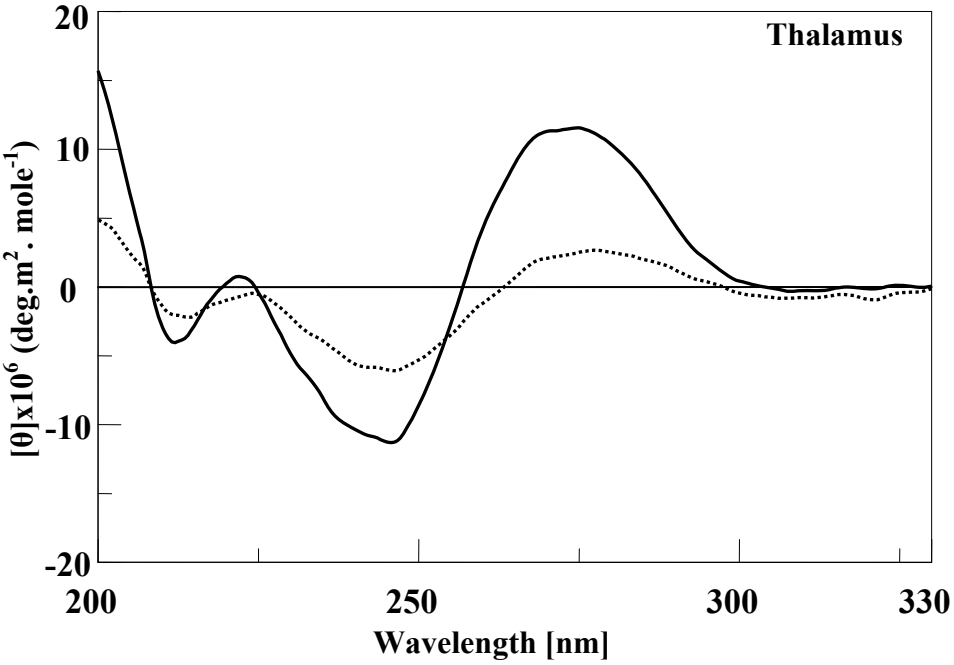


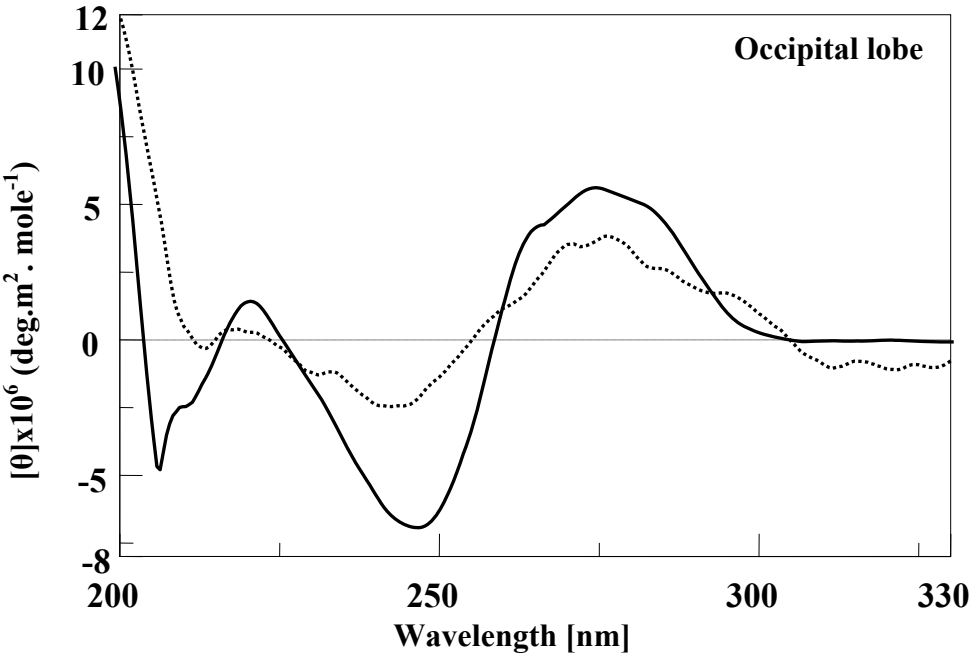












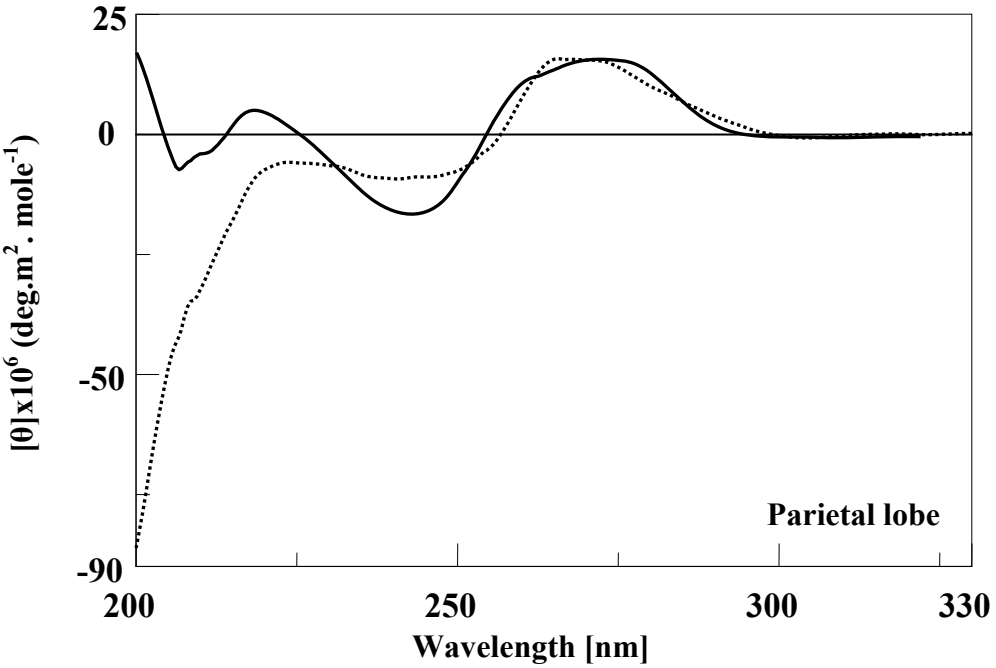


Figure 7. Circular Dichroism (CD) spectra of DNA isolated from frontal cortex, cerebellum, pons, parietal lobe, temporal lobe, hypothalamus, thalamus, occipital lobe and hippocampus. Solid line represents control DNA and dotted line represents BPD DNA. The recordings were performed in 0.01M Hepes buffer, pH 7.4. Each spectrum representative average of three recordings. CD was performed on eight control and ten BPD DNA samples and one spectrum from each group has been represented.

4. 4. Discussion

Bipolar disorder is a common, lifelong illness that typically begins in late adolescence. Genomic integrity is very essential for the survival of any organism and genomic damage will affect the function of the cell. Recent study showed that oxidative stress, mitochondrial dysfunction, mitochondrial DNA-deletion, altered gene expression, apoptosis are associated with pathophysiology of Bipolar disorder [Kato *et al*., 1997; Kato and Kato, 2000; Ranjekar *et al*., 2003; Benes *et al*., 2005; Frey *et al*., 2006a; 2006b]. MRI studies showed that frontal cortex, temporal lobe, hippocampus, thalamus

and cerebellum are important brain regions in the pathology of BPD [Brambilla *et al*., 2005]. Frontal cortex linked to expression of emotional state; temporal lobe linked to speech, language and phonologic/auditory processing; Hippocampus together with the amygdala and anterior cingulated cortex involved in information processing and in creation of emotional and declarative memories. The thalamus is a relay station through cortical and sub cortical brain regions crucial for motor and cognitive coordination; cerebellum involved in motor and ocular regulation, cognitive integration and modulation of mood [Makris *et al*., 2003; Brambilla *et al*., 2005]. The cerebellum has extensive connections through out the brain through two primary pathways: the thalamic pathway to the limbic and cortical associative areas such as dorsolateral, prefrontal cortex, medial frontal cortex, anterior cingulated and posterior hypothalamus and the pons/red nucleus pathway to cortical associative areas [Leiner *et al*., 1993; Snider *et al*., 1976]. The MRI studies reports that smaller cerebellum [DelBello *et al*., 1999], reduction in thalamus volume in adolescent [Dasari *et al*., 1999] and frontal cortex reduction size [Coffman *et al*., 1990; Kieseppa *et al*., 2003] in BPD compared to controls. Studies also showed that frontal cortex dysfunction plays a major role in the pathophysiology of the bipolar illness and is correlated with reduced frontal lobe size, neuropsychologic deficits [Coffman *et al*., 1990; Sax *et al*., 1999] and loss of bundle coherence in prefrontal white matter tracts [Adler *et al*., 2004].

The present study assessed the topology and damage of DNA isolated from BPD and corresponding age matched control subjects brains. The present study showed that DNA damage was seen specific to some brain region of BPD. DNA from thalamus, pons, medulla and frontal cortex are damaged accumulating significantly higher number of

single and double strand breaks compared to controls. While, regions like cerebellum, parietal lobe and temporal lobe are also showed DNA damage in BPD brain but it was observed less DNA damage compared above mentioned region. However the complex brain region hippocampus did not show any DNA fragmentation both in control and BPD brains. The gel, t_m, EtBr, nicktranslation studies also supported the above results of hippocampus.

It is interesting to note that present results in natural agarose gel electrophoresis showed classical apoptotic DNA laddering pattern in some regions of BPD brains like thalamus, pons, medulla and frontal cortex. According to Didier *et al*., (1996) the DNA laddering on gel electrophoresis is a hallmark features of end-stage apoptotic cell death and by this apoptosis can be distinguished from necrosis. However, significant DNA fragmentations with no laddering pattern upto 200bp were observed in cerebellum, temporal lobe. Earlier studies showed that cell death can also be preceded by DNA fragmentation by Ca^{2+}, Mg^{2+} dependent DNAase into 180 and 200bp fragments with endonuclease activation occurring early in the process of cell death [Clarke, 1999; Wyllie *et al*., 1980; Kerr *et al*., 1995]. Further studies from Benes *et al*.(2003) and (2005), showed that apoptosis may be one of the contributory factors for the cell dysfunction in BPD.

The influence of perimortem conditions, antimotem hypoxia on DNA fragmentation in postmortem tissue have been demonstrated in some previous studies [Kingsbury *et al*., 1998]. However, we evaluated our results on DNA stability/damage by several approaches with matching case controls and established that postmortem delay (<7) related DNA damage does not account for the changes in BPD and control brains.

The present study showed that structural integrity and topology of genomic DNA is altered in many regions of BPD brain. The changes include DNA damage in the form of single and double strand breaks and DNA instability as indicated by t_m and EtBr binding experiments. Interestingly, the changes observed appear to be region specific in the brain. The DNA isolated from thalamus, frontal cortex, medulla, cerebellum, temporal lobe and pons are largely affected while parietal lobe and hypothalamus are less affected. Further our study showed that hippocampus region was not affected in both control and BPD depression brain. It is fascinating to understand the implication of above findings in relevance to pathology of BPD.

It was previously shown that the genomic DNA extracted from severe AD hippocampus is predominantly in Z-DNA conformation rather than the usual B-DNA conformation [Anitha *et al*., 2002] and it was proposed that the change in conformation of DNA alters the chromatin integrity and thus have relevance to the cell system. It was earlier shown that DNA fragmentation reduces the high activation energy barrier required to induce the conformational and topological changes in DNA [Anitha *et al*., 2002]. The present CD spectral studies showed that frontal cortex, cerebellum, pons and parietal lobe of BPD brain DNA were in B-A mixed DNA conformation. However, both control and BPD hippocampus DNA showed right handed helical B-DNA conformation.

Our result on nick translation studies showed that DNA from thalamus, cerebellum, pons, frontal cortex and parietal lobe showed significantly high single and double strand breaks in BPD brain than controls. In contrast to this finding, earlier study were done only on one brain region namely anterior cingulated cortex of BPD brain and showed decreased DNA fragmentation by ISEL method [Benes *et al*., 2003]. Our study is

first report (to our knowledge) to show that there is selective increase of single strand and double strand breaks in DNA of thalamus, pons, cerebellum, frontal cortex and medulla in BPD brains. The hippocampus DNA was not affected in terms of SSBs and DSBs in both control and BPD brains. This finding was supported by the preliminary study in which apoptotic pathways did not show any changes in the hippocampus of BPD and controls [Benes *et al*., 2003]. Since both control subjects and BPD patients were well matched for age, postmortem interval, sex ratio, and freezer storage time, it seems unlikely that these variables account for the difference observed in the subjects observed with BPD. Although one report on reduction of right hemisphere hippocampal volume in bipolar patient [Manji *et al*., 2000], but most of the studies showed that there is no change in hippocampal size in BPD compared to control [Sax et al., 1999; Hauser *et al*., 1989; Altshuler *et al*., 1998; Brambilla *et al*., 2003].

There is variety of mechanisms through which an increased DNA fragmentation occurs in a BPD as compared with controls. The etiology of BPD include many endogenous and exogenous factors such as trace metals, oxidative stress, mitochondrial dysfunction, apoptosis, decrease antioxidant enzymes, genetic factors etc., [kuloglu *et al*., 2002; Ranjekar *et al*., 2003; Frey *et al*., 2006a; 2006b; Benes *et al*., 2005; Kato and Kato, 2000]. The first and most obvious possibilities are that neurons may be exposed to oxidative stress. The genes that play a central role in the clearance of free radicals generated by mitochondrial oxidation reactions such as glutathione synthase, catalase and SOD [Kingsbury *et al*., 1998] also showed substantial decreases expression in BPD. The Study showed that upregulation of NOS1 could potentially contribute to the apoptosis through increased excitocity and decrease in SOD enzyme [Benes *et al*., 2005]. This

suggests that the accumulation of ROS associated with the oxidative stress would tend to cause potential damage to DNA, proteins and lipids [Pollack and Leeuwenburgh, 2001]. However, the intact DNA from hippocampus may represent either an adaptive compensation to oxidative stress. The manner in which DNA can be damaged has been extensively reviewed by Friedberg,(1985) and Rao, (1997). Our previous data have shown that metals like Fe, Al can bind and nicks DNA. Many of these insults potentially lead to single strand and double strand breaks in DNA leading to genomic instability in BPD.

Another possible reason for accumulated DNA fragmentation in BPD brain could be due to decreased antioxidant enzymes glutathione synthase, catalase and SOD in BPD. Under the condition of BPD with environmental insults causing oxidative stress and genotoxic stress, the genomic DNA's structural integrity is under constant threat [Davydov *et al*., 2003]. Hence, any insufficiency in the machinery to counteract the damage lead to accumulation of DNA breaks [Rao, 1993]. A decline in DNA stability signifies the shift between DNA damage and repair. Presence of oxidative DNA damage in various brain region of PD was studied [Alam *et al*., 1997] but there are no such studies on BPD brain. Further there is no information available till date on the topology and stability pattern of genomic DNA in BPD depression brain. To our knowledge to date, this is the first study that showed the apoptotic DNA fragmentation and genomic instability and conformation in region specific BPD brains compared to control. Genomics involving topology and integrity in mood disorders is still a challenging and puzzling pathway.

4. 5. References

Adler CM, Holland SK, Schmithorst V, Wilke M, Weiss KL, Pan H, Strakowski SM. Abnormal frontal white matter tracts in bipolar disorder: a diffusion tensor imaging study. Bipolar Disord. 2004; 6: 197-203.

Alam ZI, Jenner A, Daniel SE, Lees AJ, Cairns N, Marsden CD, Jenner P, Halliwell B. Oxidative DNA damage in the parkinsonian brain: an apparent selective increase in 8-hydroxyguanine levels in substantia nigra. J Neurochem. 1997; 69:1196-1203.

Altshuler LL, Bartzokis G, Grieder T, Curran J, Mintz J. Amygdala enlargement in bipolar disorder and hippocampal reduction in schizophrenia: an MRI study demonstrating neuroanatomic specificity. Arch Gen Psychiatry.1998l; 55:663 - 664.

Anitha S, Rao KSJ, Latha KS, Viswamitra MA. First evidence to show the topological change of DNA from B-DNA to Z-DNA conformation from hippocampus of Alzheimer's brain. J Neuromol Med. 2002; 2:289-297.

Ansari B, Coates PJ, Greenstein BD, Hall PA. In situ end-labelling detects DNA strand breaks in apoptosis and other physiological and pathological states. J Pathol.1993; 170:1-8.

Antonova E, Sharma T, Morris R, Kumari V. The relationship between brain structure and neurocognition in schizophrenia: a selective review. Schizophr Res. 2004; 70:117-145.

Benes FM, Kwok EW, Vincent SL, Todtenkopf MS. A reduction of nonpyramidal cells in sector CA2 of schizophrenics and manic depressive. Biol Psychiatry.1998; 44: 88-97.

Benes FM, Matzilevich D, Burke RE, Walsh J. The expression of proapoptosis genes is increased in bipolar disorder, but not in schizophrenia. Mol Psychiat. 2005; 1-11.

Benes FM, Vincent SL, Todtenkopf M. The density of pyramidal and nonpyramidal neurons in anterior cingulate cortex of schizophrenic and bipolar subjects. Biol Psychiatry. 2001; 50:395-406.

Benes FM, Walsh J, Bhattacharyya S, Sheth A, Berretta S. DNA fragmentation decreased in schizophrenia but not bipolar disorder. Arch Gen Psychiatry. 2003; 60:359-364.

Bhaskar MS, Rao KS. Altered conformation and increased strand breaks in neuronal and astroglial DNA of aging rat brain. Biochem Mol Biol Int. 1994; 33:377-384.

Brambilla P, Glahn DC, Balestrieri M, Soares JC. Magnetic resonance findings in bipolar disorder. Psychiatr Clin N Am. 2005; 28: 443-467.

Brambilla P, Nicoletti MA, Sassi RB, Mallinger AG, Frank E, Kupfer DJ, Keshavan MS, Soares JC. Magnetic resonance imaging study of corpus callosum abnormalities in patients with bipolar disorder. Biol Psychiatry. 2003; 54:1294-1297.

Catts VS, Catts SV. Apoptosis and schizophrenia: is the tumour suppressor gene, p53, a candidate susceptibility gene? Schizophr Res. 2000; 41: 405-415.

Chatterjee B, Rao GR. Superhelical density of goat mitochondrial DNA: fluorimetric studies. Indian J Biochem Biophys. 1994; 31:77-79.

Clarke PGH, In: Koliatsosue M, Ratan RR (Eds). Cell death and Disease of Nervous system, Human Press, Totowa, New York, 1999, pp.3-28.

Coffman JA, Bornstein RA, Olson SC, Schwarzkopf SB, Nasrallah HA. Cognitive impairment and cerebral structure by MRI in bipolar disorder. Biol Psychiatry. 1990; 27:1188-1196.

Colye JT, Puttfarcken P. Oxidative stress, glutamate and neurodegenerative disorder. Science. 1993; 262: 689-695.

Dasari M, Friedman L, Jesberger J, Stuve TA, Findling RL, Swales TP, Schulz SC.A magnetic imaging study of thalamic area in adolescent patients with either schizophrenia or bipolar disorder as compared to healthy controls. Psychiatry Res 1999; 91:155-162.

Davydov V, Hansen LA, Shackelford DA. Is DNA repair compromised in Alzheimer's disease? Neurobiol Aging. 2003; 24:953 – 968.

DelBello MP, Strakowski SM, Zimmerman ME, Hawkins JM, Sax KW. MRI analysis of the cerebellum in bipolar disorder: a pilot study. Neuropsychopharmacology. 1999; 21: 63-68.

Deng G, Wu R. Terminal transferase: use of the tailing of DNA and for in vitro mutagenesis. Methods Enzymol. 1983; 100:96 -116.

Didier M, Bursztajn S, Adamec E, Passani L, Nixon RA, Coyle JT, Wei JY, Berman SA. DNA strand breaks induced by sustained glutamate excitotoxicity in primary neuronal cultures. J Neurosci. 1996; 16: 2238- 2250.

Evan G, Littlewood T. A matter of life and cell death. Science.1998; 281: 1317-1322.

Frey BN, Andreazza AC, Kunz M, Gomes KM, Quevedo J, Salvador M, Goncalves CA, Kapczinski F. Increased oxidative stress and DNA damage in bipolar disorder: A twin-case report. Prog Neuropsychopharmacol Biol Psychiatry. 2006b; xx:xxx-xxx

Frey BN, Valvassori SS, Gomes KM, Martins MR, Pizzol DF, Kapczinski F, Quevedo J. Increased oxidative stress in submitochondrial particles after chronic amphetamine exposure. Brain Res. 2006a; 1097:224-229.

Friedberg EC. Nucleotide excision repair of DNA in eukaryotes: comparisons between human cells and yeast. Cancer Surv. 1985; 4: 529-555.

Garruto, RM., Flaten, TP, Wakayama I. Natural and experimental models of environmentally induced neurodegenaration: Implications for Alzheimer's disease. In: Corain B, Winsneiwski H and P. Zatta P.(Eds), Alzheimer's disease: Advances in Clinical and Basic Research, John Wiley and Sons Ltd, New York, 1993, pp.257 – 266.

Gray M, Dratliff RL, Vaughan MR. Circular Dichroism Spectroscopy of DNA. Methods Enzymol.1992; 211: 389 – 406.

Hauser P, Dauphinais ID, Berrettini W, DeLisi LE, Gelernter J, Post RM. Corpus callosum dimensions measured by magnetic resonance imaging in bipolar affective disorder and schizophrenia. Biol. Psychiatry. 1989; 26: 659-668.

Hegde ML, Gupta VB, Anitha M, Harikrishna T, Shankar SK, Muthane U, Rao KS, Rao KSJ. Studies on genomic DNA topology and stability in brain regions of Parkinson's disease. Arch Biochem Biophys. 2006; 449:143-156.

Hetts SW. To die or not to die: an overview of apoptosis and its role in disease.JAMA.1998; 279: 300-307.

Houser P, Dauphinais ID, Berrettini W, DeLisi LE, Gelemter J, Post RM. Corpus callosum dimensions measured by magnetic resonance imaging in bipolar affective disorder and schizophrenia. Biol Psychiatry. 1989; 26:659 - 668.

Kato T, Kato N. Mitochondrial dysfunction in bipolar disorder. Bipolar Disord. 2000; 2:180-190.

Kato T, Stine OC, McMahon FJ, Crowe RR. Increased levels of a mitochondrial DNA deletion in the brain of patients with bipolar disorder. Biol Psychiatry.1997; 42: 871-875.

Kato T. Molecular genetics of Bipolar disorder. Neurosci Res. 2001; 40:105-113.
Kerr JFR, Gobe GC, Winterford CM, Harmon BV. In: Schwartz LM, Osborne BA (Eds), Methods in Cell biology: Cell Death, Academic press, New York, 1995, pp 1-27.

Kieseppa T, van Erp TG, Haukka J, Partonen T, Cannon TD, Poutanen VP, Kaprio J, Lonnqvist J. Reduced left hemispheric white matter volume in twins with bipolar I disorder. Biol Psychiatry. 2003; 54: 896 – 905.

Kingsbury AE, Marsden CD, Foster OJ. DNA fragmentation in human substantia nigra: apoptosis or perimortem effect? Mov. Disord. 1998; 13: 877-884.

Kuloglu M, Ustundag B, Atmaca M, Canatan H, Tezcan AE, Clinkilinc N. Lipid peroxidation and antioxidant enzyme levels in patients with schizophrenia and bipolar disorder. Cell Biochem Funct. 2002; 20:171-175.

Leiner HC, Leiner AI, Dow RS. Cognitive and language functions of the human cerebellum. Trends Neurosci 1993; 16: 444- 447.

Lyoo IK, Kim MJ, Stoll AL, Demopulos CM, Parow AM, Dager SR, Friedman SD, Dunner DL, Renshaw PF. Frontal lobe gray matter density decreases in bipolar I disorder. Biol Psychiatry. 2004; 55:648-651.

Makris N, Hodge SM, Haselgrove C, Kennedy DN, Dale A, Fishl B, Rosen BR, Harris G, Caviness VS Jr, Schmahmann JD. Human cerebellum: surface assisted cortical parcellation and volumetry with magnetic resonance imaging. J Cogn Neurosci. 2003; 15:584-599.

Manji HK, Moore GJ, Chen G. Lithium up regulates the the cytpprotective protein Bcl-2 in the CNS in vivo: a role for neurotrophic and neuroprotective effects in manic depressive illness. J Clin Psychiatry. 2000; 61(Suppl 9):82-96.

Margolis RL, Chuang DM, Post RM. Programmed cell death: implications for neuropsychiatric disorders. Biol Psychiatry. 1994; 35:946-956.

Marky LA, Breslauer KJ. Calculating thermodynamic data for transitions of any molecularity from equilibrium melting curves. Biopolymers. 1987; 26:1601-1620.

Marti MJ, James CJ, Oo TF, Kelly WJ, Burke RE. Early developmental destruction of terminals in the striatal target induces apoptosis in dopamine neurons of substatia nigra. J Neurosci. 1997; 17:2030-2039.

Martin LJ. Neuronal cell death in nervous system development, disease, and injury. Int J Mol Med. 2001; 7: 455-478.

Oquendo MA, Mann JJ. Identifying and managing suicide risk in bipolar patients. J Clin Psychiatry. 2001; 62(Suppl 25): 31-34.

Ozcan ME, Gulec M, Ozerol E, Polat R, Akyol O. Antioxidant enzyme activities and oxidative stress in affective disorders. Int Clinpsychopharmacol. 2004; 19:89-95.

Pollack M, Leeuwenburgh C. Apoptosis and aging: role of the mitochondria. J Gerontol A Biol Sci Med Sci. 2001; 56:B475-482.

Rajkowska G. Cell pathology in bipolar disorder. Semin Clin Neuropsychiatry. 2002; 7:281-292.

Ranjekar PK, Hinge A, Hegde MV, Ghate M, Kale A, Sitasawad S, Wagh UV, Debsikdar VB, Mahadik SP. Decreased antioxidant enzymes and membrane essential polyunsaturated fatty acids in schizophrenic and bipolar mood disorder patients. Psychiatry Res. 2003; 121:109-122.

Rao KS. DNA-damage & DNA-repair in ageing brain. Indian J Med Res. 1997; 106: 423-437.

Rao KS. Genomic damage and its repair in young and aging brain. Mol Neurobiol. 1993; 7:23- 48.

Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning-A Laboratory Manual, 1989; 2, pp 9.14-9.19.

Sassi RB, Brambilla P, Hatch JP, Nicoletti MA, Mallinger AG, Frank E, Kupfer DJ, Keshavan MS, Soares JC. Reduced left anterior cingulated volumes in untreated bipolar patients. Biol psychiatry. 2004; 56:467-475.

Sax KW, Strakowski SM, Zimmerman ME, DelBello MP, Keck PE Jr, Hawkins JM. Frontosubcortical neuroanatomy and the continuous performance test in mania. Am J Psychiatry. 1999; 156: 139 – 141.

Snider RS, Maiti A, Snider SR. Cerebellar connections to catecholamine systems: anatomical and biochemical studies. Trans Am Neurol Assoc. 1976; 101: 295-297.

Soares JC, Mann JJ. The functional neuroanatomy of mood disorders. J Psychiatr Res. 1997; 31:393-432.

Sutherland BM, Shih AG. Quantitation of pyrimidine dimer contents of nonradioactive deoxyribonucleic acid by electrophoresis in alkaline agarose gels. Biochemistry. 1983; 22:745-749.

Tatton WG, Olanov CW. Apoptosis in neurodegenerative diseases: the role of mitochondria. Biochem Biophys Acta. 1999; 1410:195-213.

Wetmur JG. DNA probes: applications of the principles of nucleic acid hybridization. Crit Rev Biochem Mol Biol. 1991; 26:227-259.

Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol. 1980; 68:251-306.

5. 1. Summary and Conclusions

Metal ions play a very important role in biological processes. Some are required in trace quantities and are termed as essential trace elements. However, High or low concentrations of any essential trace elements lead to deleterious effects. For example, essential metals like Fe^{2+} in the free form catalyses the production of OH* in the presence of H_2O_2 and cause DNA strand breaks. Low concentrations also lead to other deleterious effects.

The metal imbalances are reported as one of the etiology of many neurological diseases like Alzhiemer's disease (AD), Parkinson's disease (PD), Bipolar disorders (BPD), Amyotrophic lateral sclerosis (ALS) etc., Metals like Al and Fe are found to be increased in the specialized region of brain which undergoes neurodegeneration in AD and PD. These two metals play a pivotal role in neurotoxicity and neurodegeneration. These cause neurofibrillary tangles formation, abeta, α-synuclein aggregation and Al^{3+} in conjunction with iron, promote oxidative stress. These metals also can alter Bcl-2: Bax ratio, induces apoptosis. Thus these metals play a multiple role in altering the normal physiological function of nervous system in AD and PD.

The interaction of metal ions with nucleic acid is well studied during the last two decades. It is well known that all the reactions in which nucleic acids generally participate in biological systems are mediated by metal ions. While some metals tends to interfere with the activity of the DNA and lead to genetic error. Due to interaction with metal ions, DNA molecule shows significant changes in structure, conformation. Al and Fe are reported to cause cellular toxicity through their interaction with DNA. DNA damage causes genomic instability and cell death and oxidative DNA damage is reported

in AD and PD brain. Moreover nuclear localization of Al and Fe has been observed in AD brain. Recent report from our lab showed the presence of Z-DNA in hippocampus of AD brain. It was also hypothesized that environmental input of metals such as Al, Fe, oxidative stress, and trace metal homeostasis imbalance might be responsible for the DNA topological change in AD brain. There are no such studies are available in BPD brain DNA.

Collectively, there are no stuudies on inter elemental relationship between the control subjects and three types of BPD. Interaction of Al, Fe with scDNA, ctDNA and its effects on conformation, stability and single strand and double strand breaks relevance to AD brain genomic DNA. Further there are no studies on genomic DNA conformation, DNA stability and DNA damage of bipolar depression brain.

The present work is focused on

1. To study the trace elemental levels in serum and brain samples of bipolar disorders and to develop trace metals interrelation pattern.
2. To study A-Fe DNA interactions *in vitro* with reference to supercoiled DNA linearized DNA and genomic ctDNA.
3. To study the DNA conformation and stability in genomic DNA of bipolar depressive brain.

Chapter 1: General Introduction

1. Trace elemental imbalances and oxidative stress are one of the main etiological factors for the neurological diseases such as BPD, AD and PD.
2. Imbalances in iron, zinc, copper and aluminium may adversely affect biological processes and are associated with many neurological diseases.

3. Al and Fe are the two main trace elements which are reported to be high in specific brain region of AD and PD compared to control brain.
4. These two elements have some similar properties, as they are carried by the same serum proteins and enter the brain through BBB mainly by transferrin–transferrin receptor mediated mechanism.
5. Al and Fe toxicity has a role in NFT formation, Aβ and α-synuclein aggregation, oxidizing environment, induction of apoptosis and DNA damage.
6. DNA damage plays very important role in the genomic instability and neuronal cell death.
7. The DNA molecule shows significant changes in structure, conformation and stability upon interaction with metal ions.
8. DNA conformation, damage and stability have been altered in few brain region of AD and PD brain DNA.
9. Cross talk between genetic and environmental factors like metals is responsible for the neurological diseases like AD, BPD and PD.

Chapter 2: Trace elements in Serum & Brain

1. Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) was used for analysis of eleven elements in three type's of BPD serum (I, II hypomanic, II depressives and V) compared to controls.
2. The total concentrations of trace elements are relatively higher in the bipolar groups than the control group, indicating imbalance in the trace elemental homeostasis in bipolar subjects.

3. Major elements which showed significant increases were Al, Cu, Na, K and Mn in BPD-I, Al, Cu, Na and Mn in BPD–II hypomanic, Na, K, Cu and Al in BPD-II depression, and Na, Mg, P, Cu and Al in BPD-V. Interestingly while Fe, S, and Zn were decreased in all three types of BPD compared to controls.
4. Trace metals such as Al, Fe and Cu are increased and Zn concentration were reduced in the frontal cortex of the BPD depressive brain region compared to age matched control subjects. Intrestingly we observed that there were no change in the levels of these metals in hippocampus of BPD depressive and control human brain regions.
5. Our study on element-to-element ratio and correlation patterns shows that there is definite imbalance in the trace elemental homeostasis and element-to-element inter-relationship in three types of bipolar patients in comparison to a control group.
6. Interdependency in the concentration of certain elements to maintain homeostasis of trace elements pool as is apparent from our studies seems to be crucial in a biological system.
7. A hypothesis has been proposed on the basis of present study that increased Al levels in serum of bipolar disorder is likely to alter the trace elemental homeostasis pool that may cause imbalances in trace elemental levels in the brain leading to oxidative stress and damage to biomolecules like DNA, lipids, and proteins.

8. From our and literature studies suggest that elemental homeostatic imbalance results in the imbalance of biochemical events and oxidative stress in bipolar disorder may later manifest into neurodegeneration.

Chapter 3 A: Aluminium – DNA interactions

1. The nuclear localization of Al in AD brain has been reported and found to accumulate in nuclear chromatin of cortical and hippocampal neurons.
2. Based on this evidence for nuclear localization of Al, it was hypothesized that Al might play a role in modulating DNA topology and possibly contribute to the B to Z helical transition associated with AD.
3. Interaction of scDNA and ct DNA were done using various methods like circular dichroism spectroscopy, agarose gel electrophoresis, nick translation, melting profile, transmission electron microscopy and EtBr binding studies are used for this present study.
4. CD analysis revealed that scDNA and ctDNA exhibit normal B-DNA conformation but upon interaction with Al ($1x10^{-3}$M), it induced BCA–mixed conformation to scDNA and left handed Z-DNA conformation to genomic ctDNA. Other IIIB group elements such as Ga, In and Tl did not alter the conformation of scDNA and ctDNA but showed the strong binding to the DNA. This shows that only Al is capable of inducing the DNA conformational change while other thirteen group elements bind the DNA and destabilize the DNA.
5. Nick translation study showed the Al causes only double stranded breaks to the scDNA.

6. TEM and nick translation analysis showed that Al could linearise the circular scDNA by nicking the double strand of the DNA.
7. The present finding show Al-induced conformational change from B to Z in ctDNA and B to BCA mixed conformation in scDNA and linearization of scDNA by double strand breaks.
8. These findings indicate that Al causes genetic malfunction through unwinding and linearizing of supercoiled pockets in genome.
9. As Z-form of DNA has been observed in AD brain, the results of this study and the hypothetical remarks indicate that Al affects AD brain through structural changes in DNA

Chapter 3 B: Iron -DNA interactions

1. Iron is one of the essential elements and high concentration of this is reported in AD brain and also found to be localized in nucleus of AD brain.
2. The present study reports the effects of iron (Fe^{2+}, Fe^{3+} ions) on the conformation, stability and strand breaks on scDNA and ct DNA by methods described in chapter III A.
3. The present finding shows that Fe^{2+} (1x 10^{-3} M) not only binds to scDNA and genomic ct DNA but is also able to alter the conformation of DNA from normal B to C. while Fe^{3+} (1x 10^{-3} M) could not bring any transitional change to both of the DNA instead showed only binding to DNA. Hence this study shows that for the conformation of DNA to occur, iron in the reduced state (II) is essential since iron in the oxidized state (III) lacks such ability to alter the transition.

4. This indicates that DNA topological changes induced by Fe^{2+} ions may contribute to the conformational changes seen in AD brain DNA.
5. Another interesting observation in this study is that TEM observations suggest that Fe^{2+} ($7.5x10^{-4}$M) can circularize the sticky ends of the restricted scDNA fragments in a similar manner to Mg^{2+}ions.
6. Nick translation evidenced that Fe^{2+} induces only single strand breaks and not double strand break to sc DNA and form an open circular DNA and this also was evidenced by TEM studies.
7. Based on the present findings, we hypothesize that Fe and Al are likely to play important role in the pathogenesis of AD by changing DNA topology

Chapter 4: DNA integrity and stability in brain

1. Genetic factors, oxidative stress, metal imbalances, apoptosis are reported to be etiological factors for the pathophysiology of BPD.
2. The present study provides the information on DNA integrity and damage in ten different regions of BPD-depression brain.
3. Genomic DNA was isolated from ten regions in the human BPD and control brain, namely frontal cortex, thalamus, hypothalamus, pons, medulla, parietal lobe, occipital lobe, temporal lobe, cerebellum and hippocampus and was analyzed for single and double strand breaks and their conformation and topology.
4. The result showed that pons, medulla, thalamus, frontal cortex and cerebellum and temporal lobe showed more DNA fragmentation (DNA laddering) in genomic DNA of BD-depression than healthy controls.

5. It was also observed that thalamus; pons, medulla and frontal cortex are most severely damaged accumulating significantly higher number of single and double strand breaks in BPD brains compared to healthy controls.
6. Hippocampus is not affected in terms of SSB and DSB and helicity in both BPD and healthy control brains.
7. The study suggests that apoptosis might be one of the contributory factors for the cell dysfunction in BD.
8. The present CD spectral analysis showed that frontal cortex, cerebellum, pons and parietal lobe of BD brain DNA were in B to A mixed conformation. However, both control and BD hippocampus DNA showed right handed helical B-DNA conformation without any change.

Conclusions

Imbalances in elemental levels are known to play a major role in neurological diseases. Our study revealed that there is an imbalance in elemental levels in bipolar disorders compared to control. The major elements increased were Na, Al and Cu in the serum of all three types of bipolar disorder and Fe and Zn levels were decreased in serum of bipolar disease. Taken together the observation permit us to hypothesize that imbalance in trace elemental level in serum might effect or alter the trace elemental level in brain and which induce oxidative stress, apoptosis and DNA alteration (genetic error) leads to neurodegeneration.

Metal ions play a role in altering in DNA handedness, stability and fragmentation which leads to mutation or genetic error. It has been shown that Z-DNA conformation

exists in AD brain hippocampus. Studies have revealed the presence of oxidative DNA damage and gene mutation in AD brain. The present study was focused on effects of neurotoxic metals like Al and Fe on DNA helicity, stability and strand breaks. High concentrations of these two metals were detected in hippocampus of AD brain and also localization in the nucleus of hippocampus neurons. In this background, metal like Al and Fe ions interaction with DNA were investigated in-vitro to see whether these two ions play a role in modulating the DNA strand breaks and conformation relevant to AD. This is the first report to show that Al induces BCA –mixed conformation in scDNA and Z-DNA conformation in genomic ctDNA while Fe^{2+} induce C-DNA conformation in both DNA. However, Fe^{3+} could not bring about this helicity change. Further Al can cause the double strand breaks to the circular scDNA and linearise the DNA as evidenced by TEM and nick translation study. On the other hand Fe^{2+} caused only single strand breaks to scDNA and formed the open circular form. Our study also showed that Fe^{2+} at ($7.5x10^{-4}$M) can circularize the sticky ends of linearised scDNA fragments by restricted enzyme. This is the first report to demonstrate the role of Al and Fe in modulating the DNA topology and strand breaks, and this information provides a clue on the possible involvement of Al and Fe at molecular level in neurological disorders like AD.

It has been demonstrated that long term depression might lead to neurodegeneration. Apoptosis plays major role in bipolar disorders. We have analyzed ten human bipolar depression brain samples with regard to DNA helicity, stability and strand breaks and compared it with healthy controls. Present study showed that apoptotic DNA fragmentation (laddering) were seen in frontal cortex, thalamus, pons, medulla, cerebellum and parietal lobe compared to healthy control. Single strand breaks and

double strand breaks were accumulated high in these regions compared to control brain regions. CD spectral analysis showed that frontal cortex, cerebellum, pons and parietal lobe of BD brain DNA were in B - A mixed form rather in B-form of conformation. Interestingly this study has shown that no DNA damage in terms of single strand breaks and double strand breaks; no apoptotic DNA fragmentation; no helicity change in hippocampus of bipolar depression brain compared to control.

List of publications:

1. Muralidhar L. Hegde**,** S Anitha, Kallur S. Latha, **Mahammed S. Mushtak,** Reuven Stein, Ravika Raved, K. S. Jagannatha Rao (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.
2. Shanmugavelu, Muralidhar L. Hegde**,** **Mohammed S Mushtak,** T.S.S. Rao, R.B. Menon, B. Vengamma, R.V. Rao and K.S.J. Rao, (2001) "The complexity of trace elemental inter-relationships in understanding neuro-degenerative (Alzheimer's, Parkinson's diseases) and neuro-psychiatric (Bipolar) disorder", Proceedings of 3rd International Symposium on Trace metals and Health, Athens, Greece, (Eds) Ermidou S. and Pollet S., pp. 1040-1055**.**
3. Satya Prasad V, **Mushtak M S**, Bharathi, Muralidhar L. Hegde, Anitha S, Latha KS, Geetha Bali, Sathyanarayana Rao TS and Jagannatha Rao KS (2003) "First Evidence on Aluminium complexes induced topological changes in supercoiled DNA: Relevance to neurological disorders", Proceedings of 4th International Symposium on Trace Elements in Human: New Perspectives, Athens, Greece, (Eds) S. Ermidou-Pollet and S. Pollet, pp. 267-274.
4. Shanmugavelu P, Sathyanarayana Rao TS, Muralidhar L Hegde, **Mushtak MS,** Menon RB, Rao RV and Jagannatha Rao KS (2004). 'Altered trace elemental homeostasis in neurodegenerative (Alzheimer's and Parkinson's) and neuropsychiatric (bipolar) disorder' In. Neurodegenerative diseases: Alzheimer's disease [P21-1}, ***J Neurochem***. (Abstract) 88 (Suppl. 1), 49.

5. **M.S. Mushtak**, T.S.Sathyanarayana Rao, P.Shanmugavelu, Rani B.Menon, R.V.Rao, and K.S. Jagannatha Rao**. "**Studies on trace elemental levels and the complexity of elemental inter-relationships in serum samples of Bipolar mood disorders" (2006) Communicated to Biological Trace elements International Journal for Psychiatry and Neuroscience**. (In press).**

Workshop and paper/poster Presentation in Symposium

a) Participated in workshop on "Neuroscience Perspectives" organized by IBRO (International Brain Research Organization) at Tata Institute of Fundamental Research, Bangalore, India on March 3-5th 2001.

b) **Musthak M.S** and Rao, KSJ (2002) Presented poster on " Does Aluminium alone causes B to Z transition in Calf thymus DNA? A Comparative study with Group (IIIA) Elements", in Indo-Us Neuroscience meeting held at National Brain Research Center (NBRC), New Delhi, India on 10th to 12 January 2002.

c) **Mushtak MS** and Rao KSJ (2003) " Ferrous iron circularizes the linearised DNA: Relevance to Neurodegenerative disorders", Presented a Poster in International symposium held in National Brain Research Centre (NBRC), Manesar, New Delhi on December 15th to 18th 2003.

d) **Mustak MS**, Rao TSS, Shanmugavelu P, Menon RB, Rao RV, and Rao KSJ **(2004)**, oral presentation on the topic **"**Studies on trace elemental levels and the complexity of elemental inter-relationships in serum samples of Bipolar mood disorders" at 56th Annual National Conference of the Indian Psychiatric Society, in Mysore on January 8th –11th 2004.

e) Jagannatha Rao KS, Anitha S, Latha KS, **Mushtak MS**, Muralidhar L. Hegde, and Bharathi (2003) "Interaction of Trace metals with DNA: Implications in Alzheimer's disease", abstract from invited talk at 10th Congress of Federation of Asian & Oceanian Biochemists and Molecular Biologists, Bangalore, India, 7-11 December 2003.

f) **Mohammed Shafiul Mustak** and Rao KSJ. New evidence on DNA topological changes induced by Aluminium and Iron: Relevance to Alzheimer's disease. An abstract accepted for poster presentation at 34th SFN Annual Meeting held in San Diego, USA on October 23-27, 2004.