STUDIES ON THE INTERRELATIONSHIP BETWEEN BODY MASS INDEX, DNA-REPAIR AND TELOMERE LENGTH IN AGING NORMAL AND DOWN SYNDROME SUBJECTS

A THESIS SUBMITTED FOR THE DEGREE OF

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BY

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DECLARATION

I hereby declare that the work presented in this thesis entitled *"Studies on the interrelationship between Body Mass Index, DNA-repair and Telomere length in aging Normal and Down syndrome subjects"* is entirely original work and was carried out by me in the Department of Biochemistry, University of Hyderabad, under the supervision of Prof. K. Subba Rao. I further declare that to the best of my knowledge this work has not formed the basis for the award of any degree or diploma of any University or Institution.

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CERTIFICATE

This is to certify that the thesis entitled *"Some studies on **the** interrelationship between Body Mass Index, DNA-repair and Telomere length in aging Normal and Down syndrome subjects"* submitted by Ms. N.S. Raji to the University of Hyderabad is based on the studies carried out by her under my guidance and supervision. This thesis or any part of this thesis has not been submitted elsewhere for any other Degree.

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CHAPTER - 1

INTRODUCTION

Aging is regarded as an elusive and inevitable phenomenon occurring in all higher beings; it seems natural that as we grow older various organs will start to deteriorate in their function. In fact, after the completion of development and attainment of maturity, a slow but progressive change in structure and a decline in functions appears in every surviving individual, even in the absence of any disease. These changes seem to occur at all levels of organisation - molecular, cellular, tissue and organismal in all living beings during its lifespan. Such changes occurring after reproductive maturity is attained, comprise the phenomenon of aging or senescence. Gerontologists are confronted with the challenge of identifying the more fundamental cause(s) of this process.

The rapid inroads of scientific research in medicine and biology has enabled control of infectious diseases responsible for the majority of deaths. Due to this progress in medicine and health care practices, the average human lifespan has been on the increase during the past 100 years. Gerontology research in the past 3 decades, mostly due to the advent of molecular biology, which provided the tools to look at the various physiological process at the molecular level, has provided enormous data cataloguing age-related changes, but has yet to identify the basic molecular mechanism(s) responsible for these changes.

Several theories have been adduced in an attempt to explain the precise factors involved in the aging process, yet no single theory explains the various biochemical changes occurring during this process *(Rattan and Clark, 1988; Gensler and Bernstein, 1981).* Some of these emphasise the genetic factors as determinants of the process, while the others give importance to random accumulation of damage such as that caused by mutations, errors and free radicals, in addition to the genetic component *(Smith, 1962; Warner et ai, 1987; Rattan and Clark, 1988, Finch, 1990; Bernstein and Bernstein, 1991; Rao, 1988, 1993;*

Kanungo, 1994). However, the plethora of aging theories existing today can be divided into two major categories.

1. Programmed Aging

According to this, the process of aging is predestined and genetically fixed. Therefore all the developmental changes which take place in an organism are a result of predetermined genetic programme. The manifestations of senescence are brought about by playing out of this programme which contains specified information or due to the presence of "gerontogenes" or "aging genes" which code for these changes.

Weismann's theory of germ plasm *(1889)*, aging clocks, Codon restriction theory, Chromatin reorganisation theory, Gerontogenes *(Rattan and Clark, 1988),* DNA damage and repair theory *(Hart and Setlow, 1974; Bernstein and Bernstein, 1991)* and gene regulation theory *(Kanungo, 1980)* support this view of programmed aging.

However, there is no clear experimental evidence in support of these theories, explaining the primary cause of aging. Also, it is difficult to explain the effect of environmental disturbances, like temperature shift, various antioxidant treatments, altering the strictly determined stable programme for aging *(Holliday and Rattan, 1984; Holliday, 1986 a,b).*

2. Stochastic Aging

This concept assumes that aging occurs from contingencies of living rather than programmed development. It assumes that the genetic apparatus does not programme for senescent changes per se but that these changes result from an accumulation of random events which damage the essential information containing molecules leading to a loss of accurate information and an accumulation of mis-specified proteins.

Theories supporting this concept are cross linkage theory of Bjorksten *(1974),* DNA denaturation theory *(von Hahn, 1970),* somatic mutation theory, error catastrophe theory *(Orgel, 1963),* free radical theory *(Harman, 1956)* and post modification theory

SOMATIC MUTATION THEORY

Ross and Scott *(1939),* first reported that rats exposed to whole body irradiation that was too low to produce any acute syndrome died earlier than unirradiated controls. This was followed by a report that symptoms of aging and death of irradiated rodents and humans was similar to that of normal individuals, except that the former had high incidence of neoplasia.

Based on these observations, Szilard *(1959),* proposed the somatic mutation theory, according to which, accumulation of DNA damage leading to mutations in somatic cells was the basic mechanism underlying the aging process. Mutations that occur randomly and spontaneously destroy genes and chromosomes in post-mitotic cells during the lifespan of an organism and gradually increase the mutation load. This results in loss of functional genes and decreased production of functional proteins. When the mutation load increases beyond a critical level, cell death occurs which when continued, results in decreased functional ability of the organism.

Studies concerning the effects of ionising radiation on the lifespan both *in vivo (Clark and Rubin, 1961)* and at the level of the cells *in vitro (Hoehn et al., 1975; Thompson and Holliday, 1978)* have shown that life shortening due to ionising radiation may be a non-specific effect and may occur due to "radiation syndrome" which is unrelated to natural aging.

One difficulty in evaluating the validity of somatic mutation theory is that there is no objective means of measuring the range of somatic mutations in the

post-mitotic cells. The only way it has been measured is evaluating the mortality rate which may be due to several factors.

It cannot explain why germ cells of long-lived species, humans, are more sensitive to ionising radiation than those of mice and drosophila *(Sinex, 1974).* The higher longevity of Drosophila *(Strehler, 1964)* and Mice *(Walburg et al., 1966)* after exposure to ionising radiation may be due to secondary effects, although at higher doses, the effects of mortality are evident.

ERROR CATASTROPHE THEORY

Orgel *(1963),* proposed the error theory of aging, according to which the progressive decrease in the accuracy of protein synthesis might be one of the factors contributing to the age-related deterioration of cells. Errors occurring during information transfer steps like transcription and translation may cause accumulation of defective proteins and cause aging. Particularly errors in enzymes required for transcription and translation would amplify errors in cells. These errors may self-propagate and cause exponential increase in defective enzymes and proteins culminating in a cascade of errors. This may lead to the so-called "error catastrophe" and result in senescence and death of cells. This theory was later modified by Orgel *(1970),* who proposed that even though the accuracy of the protein synthesising machinery is not absolute and allows in production of errors, such errors may not accumulate since the successive generations of protein synthesis apparatus are discrete.

Subsequently, Orgel *(1973),* postulated that the errors do not accumulate in germ cells as if this occurs then the species would be wiped out. He postulated that some "quality control" processes might operate during oogenesis and development and lead to rejection of ova or embryos having high level of errors. However, this does not hold true because the immortal transformed or tumour cells do make errors.

Several experimental findings contradict the occurrence of errors in protein at levels that may cause aging. Studies from Kanungo's laboratory, on different enzymes *(Kanungo and Gandhi, 1972; Patnaik and Kanungo, 1976; Srivastava, 1971),* show that no changes in the primary structure of enzymes occur as a function of age. Certain enzymes like aldolase *(Gershon and Gershon, 1973a,b)*, isocitrate lyase *(Reiss and Rothstein, 1975),* 3-phospho glycerate kinase *(Gupta and Rothstein, 1976)*, and acid DNase *(Singh and Rao, 1984)* show lowered specific activity (decrease is about 30-70%) and higher temperature sensitivity in old age. The activity of proteases is decreased resulting in increased half life of proteins, thus providing a greater chance for undergoing modifications. Therefore, these changes have been attributed to post-translational modifications or to conformational changes.

RNA polymerase, according to Orgel *(1973),* is one of principal culprits in generating errors. It is shown that this enzyme exhibits no differences in thermolability and specific activity from early and late passage cells W1-38 fibroblasts *(Evans, 1976).* Furthermore, the fixed lifespan of species and the gradual decline in function with increasing age cannot be explained on the basis of errors by genes or other factors which contradicts the concept of error theory. Suggestions for aging, like errors and post-translational modifications are only circular and do not explain the basic cause of aging.

CODON RESTRICTION THEORY

This theory *(Strehler et al., 1971)* is based on the hypothesis that the fidelity or accuracy of translation in a cell depends on its ability to decode the triplet codons in messenger RNAs. Two factors are responsible for accurate reading of codons:

1. Transfer RNAs
2. Aminoacyl-tRNA synthetases

The decoding molecules of the genetic code, the tRNAs are degenerative. Qualitative changes in the iso-acceptor tRNAs of amino acids may alter the rate of decoding of the message and this affects translation. Moreover, tRNAs undergo post-translational modifications that may alter their aminoacylation and hence, the fidelity in decoding.

Aminoacyl-tRNA synthetases are also crucial for the fidelity of translation as they identify the appropriate tRNAs and charge them with specific aminoacids. Any deviation in their activity results in the synthesis of wrong proteins. Old rats have rapidly degradable fractions of tRNA, which have a lower ability to be aminoacylated and also the number of iso-acceptor tRNAs are reduced *(Yang, 1971).* The profiles of $tRNA^{ser}$, $tRNA^{arg}$ and $tRNA^{lys}$ are altered in rooster liver after oestrogen treatment *(Bernfield and Maenpac, 1971).* Since the level of hormones are known to change with age, concomitant alterations in tRNA profiles may follow.

This theory, however, does not explain the factors responsible for altering the gene expression for tRNA and synthetases and those responsible for the post-translational modifications of tRNAs. These changes are secondary in nature, and hence the basic cause of aging remains unexplained.

FREE RADICAL THEORY

Free radicals are extremely reactive chemical species produced in metabolic reactions as well as from those reactions which are spontaneous in nature. They play an important role in accelerating the aging process by damaging important biomacromolecules, DNA and proteins and biologically active structures, cellular membranes *(Harman, 1956).*

Free radical action induces peroxidation of unsaturated fatty acids primarily by the superoxide radical which leads to the formation of aging pigment

called "Lipofuchsin". Membrane damage affects transport processes across cell and organelle membranes. In case of lysosomes, they become leaky and the released hydrolytic enzymes might cause damage to other cytoplasmic and nuclear components. Attack on proteins and DNA causes cross-linking within or between these molecules.

The key enzymes involved in the process of deactivation of dangerous superoxide radical is superoxide dismutase (SOD) and catalase. Reiss and Gershon *(1976a,b),* found that the activity of these enzymes decreases in various organs of rat during aging by 60% which suggest the susceptibility of older animals to the disastrous effects of the free radicals and ionising radiation.

Superoxide radical at the molecular level causes oxidation of -SH groups to the -S-S- and also ferrous ion to ferric ion which results in conformational changes of many key enzymes and proteins containing sulfhydral groups. It also facilitates the production of $[O_2]^-$ and $[OH]^-$ radicals. Antioxidants like Vitamin E, ascorbic acid and glutathione scavenge/neutralise free radicals and stabilise cellular membranes and there seems to exist a link between free radical associated damage, aging and carcinogenesis. However, the exact molecular mechanism remains unresolved.

DNA, the genetic material of all living organisms, is the repository of all biological information. Hence, any impairment in the structure and function of the genetic material may result in changes in the structure and function of the organism. Fundamental to all aging theories is the importance of maintenance of genome integrity.

There are two striking and universal characteristics of all multicellular organisms which appear to be inherited and genetically programmed.

1. All organisms undergo a gradual decline in their adaptability to the environment after attaining reproductive maturity

2. All members of the species have more or less a fixed lifespan.

There are two theories of aging which have genetic basis and also explain the influence of environmental factors on the process of aging and therefore have attracted the attention of many gerontologists.

GENE REGULATION THEORY

This theory states that the expression of various genes occurs sequentially till reproductive maturity is attained. Differentiation and growth occur by sequential activation and repression of certain genes unique to these phases. The products and by-products of these genes after reaching critical levels and at specific time stimulate certain unique genes responsible for reproductive phase. Some of the products of these genes may trigger the expression of these genes whose products like hormones and other factors confer reproductive ability to the organism. Continued reproduction causes alteration in the levels of these products crucial for keeping certain genes expressed or repressed. This may lead to the destabilisation and impairment of the homeostatic control of reproductive phase genes. Failure to maintain a balance of factors and modulators results in the loss of reproductive ability and other functions. Thus, decline in physiological functions may begin and lead to senescence *(Kanungo, 1980).*

For example, the expression of various haemoglobin (Hb) chains is under the control of different genes which appear sequentially during the gestation period in humans *(Zuckerkandl, 1965)*. The haemoglobin of foetus during 1-2 months is a2e2. This is followed by a2y2 predominant foetal Hb. Then the adult Hb comprises of $\alpha 2\beta 2$ which appears just before birth. Thus it is clear that the

expression of genes for e, γ, β follow a particular pattern and duration, while the gene for a remains active throughout. Therefore, switching on these genes occurs in a programmed and sequential manner. The duration of the lifespan of a species may vary within certain limits and may be influenced by intrinsic and extrinsic factors like nutrition, stress and temperature which may account for the variability in the lifespan of individuals within a species. Acceleration or deceleration of sequential activation or repression of genes may start at any point of time which results in differences in lifespan of related mammalian species.

Human genetic diseases like Progeria and Werner's syndrome that occur due to mutation of autosomal genes have shortened lifespans due to abbreviated developmental and reproductive phases respectively *(Setlow, 1978).* Thus alteration in genes at a specific time of a specific phase resulting in change in the duration of lifespan renders support to the basic tenet of this model.

DNA DAMAGE AND REPAIR THEORY

According to this theory, the ability of an organism to repair alterations in the genetic material, DNA, may be directly related to the longevity of that organism. DNA damage can occur either spontaneously or induced by various factors. With time, DNA damage occurs, which if not properly repaired results in accumulation of DNA damage beyond a threshold level which results in the death of the cell and ultimately affects the survival of the organism. A major stimulus for the DNA damage and repair theory of aging was provided by the observation of Hart and Setlow *(1974),* that a direct relationship exists between maximum achievable lifespan of a species and its capacity for UV-induced unscheduled DNA synthesis (as a measure of DNA repair capacity) in fibroblasts from seven species. Similar observations were made using fribroblasts from primates *(Hart and Daniel, 1980)* between two mouse species with a difference in lifespan of 2.5 fold *(Hart et* a/, *1979b),* in skin cells of humans *(Sutherland et al.,*

1980) and in lens epithelial cells from rat, rabbit, dog, cow and horse *(Treton and Courtois, 1982).* However, Francis et al. *(1981),* and Collier et al. *(1982),* studying a variety of mammalian species obtained data with a considerable scatter and could not find similar correlations.

DNA Damage

All the organisms, both prokaryotic and eukaryotic are constantly exposed to various endogenous and exogenous genotoxic substances. Any deleterious mutation in the coding region of an important enzyme or protein results in an adverse impact on the cell. Frame shift mutations and translocation which destroy a major portion of an open reading frame of a gene results in the abnormal expression of the gene. DNA is subject to damage by both endogenous and exogenous events resulting in the modification or loss of bases, the production of mismatched base pairs, strand breaks, DNA-DNA cross-links and cross-links between DNA and other cellular constituents *(Friedberg, 1990; Bernstein and Bernstein, 1991).* Many of these DNA modifications have been shown to be mutagenic *in vitro* and *in vivo (Loeb, 1989).*

Endogenous Sources of DNA Damage

DNA is a very dynamic molecule and under constant threat from various sources, endogenous and exogenous. The more relevant causes of DNA damage with respect to aging seem to be those induced by endogenous biochemical and physical reactions *(Hart et al., 1979a; Ames, 1983; Gensler et al., 1987).*

The most ubiquitous natural cause of DNA damage is body heat. Heat induced DNA alterations measured in double stranded DNA, incubated at different temperatures suggests that about 10,000 depurinations are induced per mammalian cell per day at 37°C. Such apurinic sites (AP sites) are

spontaneously converted into single strand breaks (SSB) in about 100 hours *(Lindahl and Nyberg, 1972; Lindahl and Anderson, 1972).* Bailly and Verly *(1988)* observed that in the presence of basic substances like spermidine and histone H1, conversion of AP sites into single strand breaks is promoted. The other forms of heat induced DNA damage include deamination of cytosine to uracil and to lesser extent of adenine and guanine to hypoxanthine and xanthine which can be removed enzymatically by specific **DNA** gycosylases *(Lindahl and Nyberg, 1974).*

The second most abundant source of endogenous DNA damaging agents are free radicals. The free radicals are generated during several metabolic processes in the cell and also by ionising radiation and they react with many cellular components especially DNA. Free radicals produce a variety of oxidative DNA damages including single strand breaks (SSB), double strand breaks (DSB), AP sites and cross-links and several modified bases such as thymine glycol and 5-hydroxymethyl uracil, two forms of oxidised thymine *(Cadet and Berger, 1985).* It was estimated by Richter et al. *(1988),* that in human cells about 10,000 free radical damages are induced per cell per day.

The reactive species other than oxygen can be the cause of spontaneous DNA damage as well. Lee and Cerami *(1987),* reported that interaction of glucose and other sugars with the amino-group of lysine leads to the formation of DNA-protein cross-links, the occurrence of which has been observed in various cells during aging *(Bojanovic et al., 1970).*

Another spontaneous DNA damage that occurs in the cells is by the alkylation of **DNA** by S-adenosyl-L-methionine (SAM) the normal intra-cellular methyl group donor *(Barrows and Magee, 1982; Rydberg and Lindahl, 1982).* This leads to the formation of N^7-methyl-guanine, N^3-methyl-adenine, N^3-methyl-thymine and small amounts of O^6-methyl-guanine. The methylation of adenine and guanine bases causes destabilisation of the N-glycosydic bond, resulting in

an increased spontaneous cleavage and the formation of AP sites *(Lindahl and Nyberg, 1972).*

Exogenous Sources of DNA Damage

The major exogenous source of DNA damage is sunlight. Ultraviolet light forms a cyclobutane pyrimidine dimer *(Setlow, 1982; Niggli and Rothlisherher, 1988).* Formation of pyrimidine dimers is influenced by the nucleotide composition of the DNA *(Setlow and Carrier, 1966).* UV irradiation also induces DNA-protein cross-links and single strand breaks *(Peak et al., 1985; Lai et ai, 1987).* Ionising radiation (X-rays, y-rays and fast neutrons) cause single and double strand breaks and cross-links *(Scholes, 1983; Hutchinson, 1985).*

Human diet contains a great variety of natural mutagens and carcinogens, such as polycyclic aromatic hydrocarbons (PAH), aflotoxin B1 and Nitrosamines *(Ames, 1983).* These agents can react with DNA, inducing several types of damages including single strand breaks and bulky adducts. Cigarette smoke also contains various carcinogenic compounds including benzo-x-pyrene (BAP) *(Everson et ai, 1986; Randerath et ai, 1986).* DNA adducts were detected in bronchial cells of heavy smokers *(Bann et ai, 1988).*

The various types of damage or alterations that are seen in DNA, irrespective of the causative agent, can be basically divided into the following six types:

AP sites	Altered bases	Thymidine dimers
Crosslinks	Single strand breaks	Double strand breaks

- with the good possibility of one type getting converted to the other. From the existing knowledge the frequency of these types of damage per cell per day are summarised in Table 1.

Table1: APPROXIMATE FREQUENCIES OF OCCURRENCE OF DNA DAMAGES IN MAMMALIAN CELLS *

TYPE OF DAMAGE	Events per day / cell	Reference
Depurination	10,000	Lindhal and Nyberg, 1972
Depyrimidination	500	Lindhal and Karlstrom, 1973
Deamination	100-300	Lindhal and Nyberg, 1974
Base damages (including all types of base damage viz. oxidative damage, adduct formation with reducing sugars, methylation, crosslinks, and so forth)	10,000	Richter et al, 1988
Single-strand breaks	20000-40000	Saul and Ames, 1985
Interstrand crosslinks	8	
Double-strand breaks	9	Bernstein & Bernstein, 1991
DNA-protein crosslinks	Unknown	

* It should be noted that the rates are calculated on the basis of spontaneous (endogenous) damaging events and therefore could be actually much higher depending on the dietary composition and style of living.

DNA REPAIR

Organisms have developed a number of mechanisms to counteract various deleterious alterations of the genetic material *(Hanawalt et al., 1979; Linn, 1982; Friedberg, 1985; Sedgwick, 1986; Collins et al.,1987; Sancar and Sancar, 1988; Friedberg, 1990, 1991).*

Enzymes such as superoxide dismutase, catalase, glutathione peroxidase antagonise the activity of free radicals. Mixed function oxidases present in liver, play an important role in detoxification of various carcinogens. Besides these enzymatic defence systems, there are several substances generated in the cell or present in food, such as Selenium, Vitamin E (α-tocopherol), Vitamin C, glutathione and Cysteine, that have ability to scavenge free radicals.

In addition to this "first line" of defence systems, cells possess a multitude of potentials DNA repair pathways. From the knowledge available today, the repair processes can be divided into three classes: those that simply reverse the damage without involving the breakage of phosphodiester bond, viz., monomerisation of pyrimidine dimers by an enzymatic reaction dependent on light of wavelength more than 300 nm, removal of methyl groups, and simple rejoining of the strand breaks.

The other category of DNA-repair processes is the nucleotide and base excision repair. Except for the initial difference, these two processes actually constitute a common pathway. The third type of DNA-repair, the recombinational repair, is least understood.

The first step in nucleotide excision repair pathway is the recognition and incision of the damage site by an incision endonuclease. From the plethora of endonucleases found in different organisms, it appears that these enzymes have rather broad and varied specificities *(Linn, 1982; Lambert et al., 1988).* Some recent information suggests that the recognition of the site by the endonuclease is facilitated by the binding of damage specific proteins. A protein that has

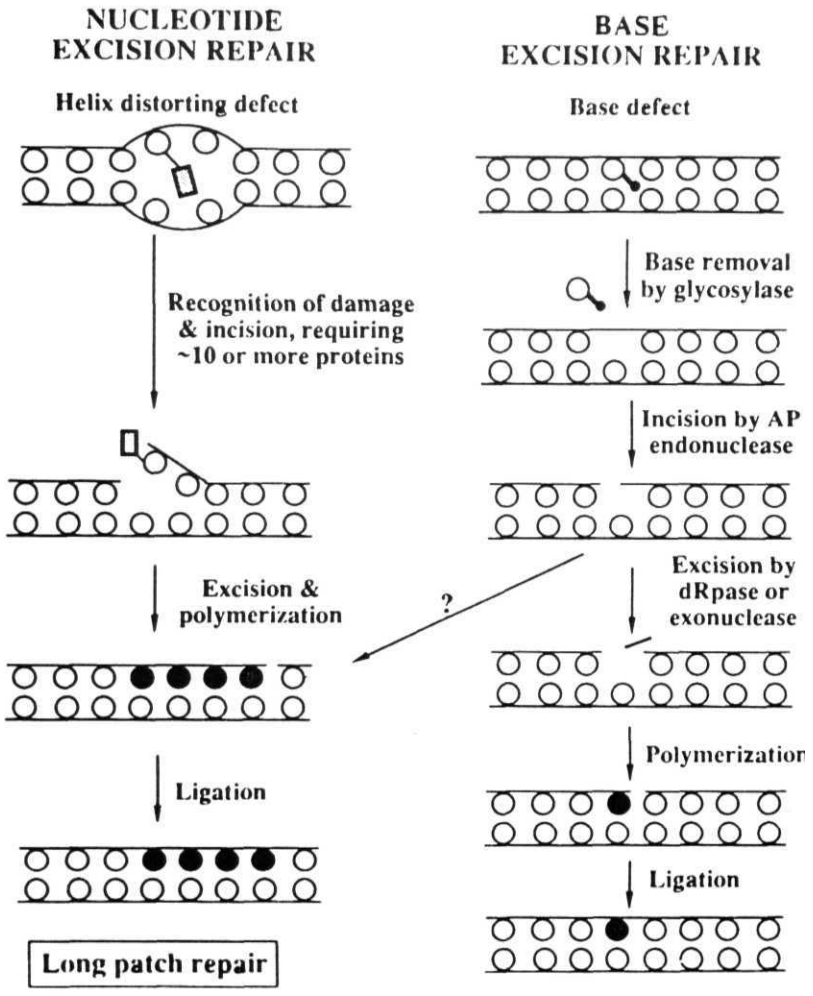
specificity for UV damaged DNA has been isolated from HeLa cells *(Chao et al., 1991).*

The second step is the excision of the damaged region by an exonuclease, This excised region may include some adjacent nucleotides as well. For example, in the case of pyrimidine dimer excision in eukaryotes, a fragment of 27-29 nucleotides in length, with dual incisions flanking the damage, about 22 nucleotides 5' and six nucleotides 3' to the damaged site is released *(Tanaka and Wood, 1994).* However, in prokaryotes the excised region is of 12-13 nucleotides long *(Sancar, 1996).* The third step involves the filling up of the gap by a DNA-polymerase using the other strand as a template and finally sealing off the gap by the DNA-ligase. In the case of base excision repair, first the baseless sites formed either spontaneously or by the action of DNA-glycosylase would be recognised by an apurinic/apyrimidinic endonuclease and the site incised. Thereafter, the subsequent steps are similar to that of the nucleotide excision repair. It is generally believed that the nucleotide excision repair characterised by a long patch repair based on the number of nucleotides incorporated (about 100) per each repair segment, whereas in the base excision repair the patch is a short one comprising only three or four nucleotides. Information regarding enzymes involved in the eukaryotic DNA-repair system is scanty as compared to the extensive information obtained from the prokaryotes like E.coli. DNA-repair systems in eukaryotes appears to be more complex than in prokaryotes *(Sancar, 1996).* The general outline of the nucleotide and base excision repair process are shown in Figure 1.

Post replication repair is one of the mechanisms whereby the cells resume DNA synthesis on template containing replicative blocks, some distance downstream (essentially circumventing them) therefore creating gaps or discontinuities in the daughter DNA strands, which become filled in by recombination or *de novo* synthesis. This mode of cellular response to DNA damage was first suggested by Rupp and Howard-Flanders *(1968).*

Figure 1

An outline of excision repair pathway: On the left side is nuclei excision repair, and on the right is the base excision repair. diagram is kindly provided by Larry H. Thompson of Lawn Livermore National Laboratory, Livermore, CA.


NUCLEOTIDE
EXCISION REPAIR
Helix distorting defect
Recognition of damage
& incision, requiring
~10 or more proteins
Excision &
polymerization
Ligation
Long patch repair
BASE
EXCISION REPAIR
Base defect
Base removal
by glycosylase
Incision by AP
endonuclease
Excision by
dRpase or
exonuclease
?
Polymerization
Ligation
Short patch repair

Cells that are incapable of repairing damage by excision may tolerate template damage by discontinuous semi-conservative **DNA** synthesis associated with recombinational gap filling and translesion DNA synthesis (error-prone post replication repair).

Structures suggestive of post replicative gaps in DNA have been visualised with electron microscopy of **DNA** using direct aqueous spreading or staining with T_4 gene 32 protein (a protein that specifically binds to single-strand regions of **DNA).** Measurements from such electron micrographs have yielded gap sizes ranging from 1,500 to 40,000 nucleotides *(Johnson and McNeil, 1978).*

DNA-repair systems vary greatly among different species and individuals. Hart and Setlow *(1974)* have shown large species-specific differences in the amount of UV-induced excision repair among 7 species which correlated with the maximum lifespan and is therefore indicative of the role of DNA-repair in aging.

TELOMERES AND AGING

During the past few years a new concept has emerged which again adds credibility to the theory of DNA damage and repair in explaining aging as well as cell replication and transformation of somatic cells into malignant cells. This concept/hypothesis is based on the results suggesting that the non-coding genomic DNA (Telomere) located at the tips of the eukaryotic chromosomes may have a telling role in DNA replication (cell replication) and therefore in the phenomena of cancer and aging.

Telomeres are the DNA and protein structures of the termini of eukaryotic chromosomes and are considered to provide stability to chromosomal structure *(Blackburn and Szostak, 1984; Blackburn, 1991; Day et al., 1993).* The telomeric DNA from a variety of organisms, consists of a repeat sequence of 5 to 10 base pairs and variation in the exact repeat sequence is seen from one species to the other. For example, in humans and other vertebrates, the repeating unit is a

hexanucleotide 5'-TTAGGG-3' while in ciliates *Tetrahymena* and *Euplotes* it is GGGGTT and GGGGTTTT respectively *(Price, 1993).* The length of this telomere sequence is found to vary considerably among different species. Some ciliates have as little as 36 bp of length whereas humans have a telomere length of 8 to 14 kbp and mice upto 150 kbp. Although Telomeres are generally considered to be the structures at the tips of the chromosomes, such sequences are also being discovered at internal and interstitial positions in chromosomes *(Katinka and Bourgain, 1992; Day et ai, 1993; Price, 1993).*

Apart from providing stability to the chromosomes, Telomeres carry out another crucial function in replicating cells - the ability to allow the end of the linear DNA to be replicated completely without the loss of terminal bases at 5'-end. Such loss is predicted as a natural consequence derived from the properties of the replicative machinery of conventional semiconservative replication *(Olovnikov, 1973).* The lost sequences of the telomere at each round of replication are synthesised again by an enzyme, telomerase.

Telomerase is a ribonucleoprotein enzyme with RNA-dependent DNA polymerase activity *(Blackburn, 1991).* The RNA component of the enzyme actually serves as a template achieving the addition of species specific telomere sequence onto the 3' end of a telomere primer *(Price, 1993; Bhattacharyya and Blackburn, 1994).* This enzyme activity has been found in many species including ciliates *(Shippen-Lentz and Blackburn, 1990; Romero and Blackburn, 1992)* and humans *(Morin, 1989).* Both RNA and protein components of the enzyme are required for activity. Telomerase is a novel RNA dependent DNA polymerase/terminal transferase where the template RNA is an integral part of the enzyme. In humans the tandem repeat added by the enzyme is TTAGGG *(Morin, 1989).*

The DNA sequence to which telomeric repeats could be added seems quite diverse *(Muller et al., 1991; Beissmann et al., 1992).* Although it was originally thought that a primer consisting of telomeric sequence is needed, it is now

evident that sequence requirements are not very stringent at least under a set of conditions *(Harrington and Greider, 1991; Morin, 1991).* This fact indeed raises an interesting possibility that the functions of telomere repeats could be much wider than presently thought.

The telomere theory of aging, originally hypothesised by *Olovnikov (1973)* and later substantiated by others *(Hariey et al., 1992; Hariey, 1995),* has emerged as an attractive proposition to explain `replicative senescence'. This word refers not only to the finite proliferative capacity of human cells *(Hayflick, 1965; Goldstein, 1990),* but also to the deleterious pattern of gene expression observed in metabolically active, senescent cells at the end of their replicative lifespan *(West et al., 1989; West, 1994).* In short, this theory, correlates cell's capacity to undergo division to a certain critical length of telomere.

For each given species, the length of telomere sequence at the end of the chromosomes seems to be maintained within certain reasonable limits. Human cells exhibit telomeric length in the range of 8-14 kbp *(De Lange et al., 1990),* while rodents seem to have an average length upto several hundred kbp *(Kipling and Cook, 1990),* with one species of mouse *(Mus spretus)* having lengths similar to humans *(Starling et al., 1990).*

The telomeric hypothesis predicts that a small portion of the telomeric DNA is lost with each round of DNA replication/cell division. During division of somatic cells (human fibroblasts) *in vitro,* telomere shortening has been noticed *(Hariey et al., 1990).* In addition, telomeric length correlates well with the lifespan of fibroblasts in culture, cells with longer telomeres undergoing more divisions than those with shorter telomeres *(Hariey et al., 1990; Hariey, 1991; Allsopp et al., 1992).* Telomere shortening is also observed *in vivo (Allsopp et al., 1992; Hastie et al., 1990; Lindsey et al., 1991; Vaziri et al., 1993).* It is now generally accepted that the rate of telomere loss per population doubling *in vitro* is 50-200 bp. The loss noted *in vivo* is equal to 15-50 bp per year *(Hariey, 1995).* When the telomeric length is reduced to a critical point, a signal is given to the cell's machinery and

the cell stops dividing and senescence sets in. This critical point appears to be analogous to the Hayflick's limit *(Hayflick, 1965)* which denotes the finite replicative capacity of a somatic cell.

It is interesting that in certain human syndromes, characterised by features of premature aging viz., Progeria and Werner's syndrome, the average telomeric lengths are significantly shorter than in the normal individuals, thus pointing out a relationship between average telomeric length and aging.

CALORIE RESTRICTION, LONGEVITY AND DNA-REPAIR

Several attempts have been made by many workers to develop an experimental system in which the rate of aging could be altered and manipulated in a reproducible and predictable manner. This would facilitate the distinction between cause and effect, a problem which has made the interpretation of data on aging so difficult. The search for the factors that may play a role in the determination of maximum life time of an individual species has led to the correlation of a number of biological parameters associated with species longevity.

In 1935, McCay and co-workers demonstrated that food restriction increased the lifespan of laboratory rats and therefore, by implication, delayed the process of aging. Subsequently, research concerning the effects of nutritional restriction on aging gained a powerful impetus. Later, several workers *(Berg and Simms, 1961; Everitt, 1973; Sacher, 1977; Harman, 1981; Yu et al., 1984)* have confirmed the reproducibility of the effect of dietary restriction on extending lifespan in spite of widely differing experimental designs, species, strain of rodents, composition and caloric value of diet. They claim that the diversity of the effects on the physiological system strongly indicates that dietary restriction acts on the basic aging process by delaying or preventing the age-related physiological deterioration and the onset of age-related disease processes.

Calorie restriction is the only known experimental paradigm which is the simplest and most effective method in modulating the aging process at the whole animal level. It is now well established that decreased calorie/dietary consumption without sacrificing the quality of diet leads to uniform and proportionate increase in the maximum achievable lifespan in rodents and other species.

The life-prolonging action of calorie restriction has been reported in various animal systems ranging from invertebrates to higher vertebrates: Daphnia *(Ingle et ai, 1937),* rotifers *(Fanestil and Barrows, 1965),* fish *(Comfort, 1963),* Tokophyra lemnarum *(Mackeen and Mitchell, 1975)* rats, hamsters and mice *(Stuchlikova et ai, 1975; Leto et ai, 1976; Yu et ai, 1982; Holehan, 1984).* Payne *(1979)* remarked that an experimental regime which involves restriction of food supply may inevitably introduce an element of both energy and protein deficiency. However, calorie intake appeared to have a greater effect on survival than protein intake, evident in a study where rats fed calorically restricted diets survive longer than those fed ad-libitum even when both groups consume same amount of protein. Therefore, during dietary restriction, it is the calorie restriction and not the source of calories or richness of diet which is the most critical dietary variable in extending lifespan *(Davis et al.,1983; Kubo et al.,1984).*

Dietary restriction and age-related disease processes

Aging is characteristically associated with increased incidence of different types of diseases such as arthritis, atherosclerosis, osteoporosis, diabetes, malignant tumours and finally result in loss of vigour and vitality. Therefore, it is reasonable to speculate if diseases are the primary cause or the consequence of aging process. As it is clear that dietary restriction enhances the survival of animals, early attempts were directed to explain this effect and therefore concentrated on the sensitivity of age-related pathology to nutrition.

The age-related pathologies of rodents are exquisitely sensitive to the dietary history of the animal. There is unequivocal evidence that long-term caloric restriction significantly increased resistance and postponed the rate of appearance of most of the age-associated degenerative diseases *(Weindruch and Watford, 1988; Keenan et al.,1994).*

The effect of caloric restriction is most noticeable on the incidence of chronic glomerulonephritis, myocardial fibrosis, peri-branchial lymphocytosis, periarteritis, prostatitis, bile duct hyperplasia and endocrine hyperplasia in rats *(Berg, 1960; Ross, 1964).* It also retards the development of cardiomyopathy *(Masoro, 1984),* chronic nephropathy *(Maeda et al., 1985)* suppressing cardiac and renal hypertrophy.

Calorie restriction has also been shown to be extremely effective in reducing the incidence and progression of spontaneous tumours *(Sarkar et al., 1982)* and chemically induced tumours in rats and mice *(Kritchevsky et al., 1984; Pollard and Luckert, 1985; Ruggeri et al., 1987).* Appropriate dietary restriction started in middle aged mice can inhibit tumour formation and will extend mean and maximum lifespan. The delay in the onset of neoplastic lesions in chronically underfed rats is attributed to the prolonged sustenance of immunological competence. Thus caloric restriction confers a degree of protection both against exogenously administered carcinogens and genetically predisposed pathology.

Merry and Holehan *(1985)* examined the *in vivo* DNA synthesis in number of organs from fully fed and diet-restricted rats (maintained at 50% body weight of ad-lib fed controls). There was severe inhibition of DNA synthesis *in vivo* in liver, kidney, heart and abdominal skin during the first 6 months of life. The developmental peak of DNA synthesis seen before 100 days of age in control rats was completely inhibited while DNA synthesis remained unaltered in rapidly proliferating cells of small intestine.

Dietary restriction retards polyploidisation in rodent hepatocytes *(Enesco and Samborsky, 1986)* which becomes less pronounced by 15 months of age. Returning 4 month old undernourished rats to ad-lib feeding for 3 weeks negate the effects resulting in recovery and overshoot of hepatocyte ploidy level.

MECHANISM OF ACTION OF DIETARY RESTRICTION

Several theories were proposed to explain the mechanism of action of caloric/dietary restriction on aging. Few of these are discussed below.

1. GROWTH RETARDATION THEORY

McCay et at., hypothesised that slowing of growth and development was causal for the increased longevity of food-restricted rodents. Later several studies showed that food restriction even when initiated in adult life increases lifepsan. Moreover Yu et al. *(1984),* found that food restriction limited to the developmental period was much less effective than when imposed in adult life. Therefore it seems unlikely that life prolonging action is due to delaying maturation or slowing down growth.

2. FAT CONTENT THEORY

In 1961, Berg and Simms were of the opinion that there exists a direct relationship between adiposity and mortality and hypothesised that lack of excess body fat as the major cause of longevity. Subsequent observations by Stuchlikova et al. *(1975),* Harrison et al. *(1984)* that obese, diet-restricted rats lived longer than ad-libitum fed lean mice of the same strain, although the former had greater fat content do not support this theory.

3. METABOLIC RATE THEORY

The essential tenet of this hypothesis *(Sacher, 1977)* was that dietary restriction reduced the metabolic rate thereby allowing the animals longer chronological period before total energy expenditure per gram tissue approached to that observed for ad libitum fed rats. Sacher supported his thesis by analysing the data of Ross in which 5 groups of animals fed on different diets had a similar life time caloric consumption per gram body weight.

This theory cannot be substantiated, for life time caloric consumption per gram lean mass was significantly higher in dietary restricted rats *(Masoro et al., 1982; Holehan, 1984)* and metabolic rate is not depressed when compared to ad lib fed rats *(McCarter, 1985).*

4. IMMUNOLOGICAL THEORY

Walford *(1969)* and Burnet *(1974)* believed that senescence is primarily due to deficiencies in the immune system with age. Comfort *(1979)* suggests that aging could be chiefly or wholly an immunological process whether the immunological changes themselves are genetically programmed as to the involution of thymus or homeostatic control or clonal exhaustion or T-cell maturation decay through a decline in trophic factors or increase in suppresser cell activity.

Data on immune system of rats maintained on low calorie diet does not contradict these suggestions for a delay in thymic involution, enhanced T-cell competence, better response of mitogen induced lymphocyte proliferation and interleukin-2 induction was observed.

Dietary restriction retards the age-related decline in immune system while suppressing the auto-immune pathologies. The retention of a functional immune system to a greater age is important in delaying appearance of diseases.

The immune theory of aging does not however, explain at the molecular level the mechanism which contributes to its functional retention.

5. NEUROENDOCRINE THEORY

In 1973, Everitt postulated that the retardation of the aging process by food restriction was due to a reduced secretion by the pituitary gland of an aging factor. He developed the hypothesis that dietary restriction produces changes in neurotransmitter metabolism within the hypothalamus which decreases the secretion of hypothalamic releasing hormone *(Campbell et al., 1977)* and results in decreased secretion of pituitary hormone. Everitt provided experimental support for this view with their findings in hypophysectomised rats (with cortisone replacement therapy) common with effects of food restriction.

Harrison et al., *(1982)* reported shortened times for hypophysectomised mice despite an apparent increase in motor activity and overall more youthful appearance. Fernstrom and Wurtman *(1971)* demonstrated that brain serotonin levels decrease in rats maintained on tryptophan-deficient diet thus modifying the activity of monoaminergic innervation of hypothalamus directly, since brain serotonin is subject not only to the concentrations of unbound tryptophan but also to the concentrations of other large neutral aminoacids that share the same transport system at the blood-brain barrier. Tryptophan is important in neurotransmission but does not alter the metabolism in dietary restricted rats.

Segall *(1979)* argues that the neuroendocrine axis provides an intricate control of specific gene expression central to other ontogenetic processes which may play a basic role in production of senescent changes. Food restriction could be seen to operate through a complex neuroendocrine response mediated by monoamine transmitters in hypothalamus.

Neuroendocrine theories do account for many physiological and endocrine changes but give no insight to the actual molecular changes basic to enhanced survival.

6. PROTEIN TURNOVER THEORY

Barrows *(1972)* proposed that the various changes seen in an organism throughout its life are the result of new genetic information transcribed and translated at different ages. The synthesis of specific RNAs and proteins influence the rate of occurrence of these programmed events.

Restriction of dietary protein results in increased transcription of rRNA coupled with an increased turnover of rRNA and ribosomes. There was a rapid breakdown of RNA in tissues as shown by an increase in the free nucleotide content in the plasma of undernourished rats *(Srivastava et a!., 1972).*

There is much evidence that the rate of protein synthesis declines with age in many kinds of cells from a wide range of organisms *(Makrides, 1983).* Therefore, it is logical to predict that the rate of protein degradation would also decline and result in an increase in the half-life of proteins and decrease in protein turnover. It appears likely that such a decline in protein turnover is an important contributory factor to the appearance of altered proteins resulting from all sorts of damages and post-translational modifications in older organisms *(Adelman and Dekker, 1985).* Support to this prediction is provided by the work of Richardson and Cheung *(1982),* Rothstein *(1981).*

Data presented by Wulf and Cutler *(1975)* and by Richardson and co workers *(1982,1983)* strongly suggest that at advanced chronological age, in rats dietary restriction retains a higher rate of protein turnover through enhanced rates of polypeptide elongation at the ribosomes. This provides a fine degree of metabolic control, particularly for proteins with a regulatory role.

Buttery *(1983)* reported that there are at least 6 hormones known to affect protein synthesis or sometimes their breakdown. They are insulin, Growth hormone, somatomedins, glucagon, thyroid hormones, corticosteroids **and** prostaglandins. The action of these hormones on protein synthesis may be the molecular expression of the neuroendocrine response to dietary restriction.

However, Lindell *(1982)* proposed that food restriction acts as a `stress' and enhances gene expression at the level of *de novo* mRNA transcription as **a** compensation for the greater fluctuations in diurnal levels of plasma aminoacids or essential nutrients required for protein synthesis. Moreover, there is increased amino acid reutilisation and renewal of mRNA, thus maintaining the cellular homeostasis as the organism ages. It is conceivable that enhanced protein turnover may be one of the operative mechanisms of dietary restriction, providing better metabolic regulation and thus enhancing survival. The molecular mechanisms by which enhanced protein turnover is achieved is yet to be determined.

It has been repeatedly shown in a number of species, except humans, that restricted dietary consumption without sacrificing the quality improves longevity *(Barrow Jr. and Kokkonen, 1978; Hart et al., 1979; Cheney et al., 1983).* Despite some diversity in observations, there appears a consensus about positive correlation between maximum lifespan and cellular DNA-repair capacity. DNA-damage and DNA-repair are important to aging and senescence. Reports on UDS in lymphocytes, hepatocytes and kidney cells of rats by Licastro et al. *(1985, 1988),* Weraarchakul et al. *(1989)* and Lipman et al., *(1989)* also demonstrated that dietary restriction increases the DNA-repair levels. A study on male Wistar rats was done in this laboratory, to examine the effects of calorie restriction imposed during discrete periods of lifespan. The results positively indicated that calorie restriction improves DNA-repair, as evidenced by total DNA-polymerase activity. The extent of modification was observed to be both

age and tissue dependent and the effects of calorie restriction depend on when the paradigm was initiated and not solely a linear function *(Prapurna and Rao, 1996).* Clearly even with the limited number of studies presently available regarding the changes in DNA-repair in rodents, there is an emergence of the positive relationship between dietary restriction and improved DNA-repair potential.

DNA-REPAIR AND HUMAN DISORDERS OF ACCELERATED AGING

Besides the inter-species and inter-individual differences in DNA-repair activities, there are some hereditary disorders associated with a defect in DNA-repair activities. The best known and most extensively studied defect in humans is that in Xeroderma pigmentosum (XP) patients where the defect lies in the lack of initial DNA damage incision enzyme. Other human disorders associated with DNA-repair defect are

1. Ataxia telangiectasia (AT) also referred to as Louis-Bar syndrome is a heredofamilial syndrome of progressive cerebellar ataxia beginning in infancy, progressive oculocutaneous telangiectasia and proneness to sinopulmonary infection, including bronchiectasis. Its multiple facets include characteristic fades; apraxia of eye movements, simulating ophthalmoplegia; choreoathetosis; progeric hair and skin changes; growth retardation; endocrine abnormality, including ovarian agenesis; hypoplasia or absence of the thymus gland, with an impaired cellular immunity; and a tendency to neoplasia. Mental retardation may occur. The basic defect appears to be in the recognition and repair of γ-ray induced damage *(Patterson et al., 1976, McKinnon, 1987).* Also, despite the proven hypersensitivity of AT lymphocytes and cultured fibroblasts to effects of x-irradiation, the cells are found to have a radioresistant replication, initiation and chain elongation *(Painter and Young, 1980).*

2. Fanconi's anaemia is an autosomal recessive disease characterised by refractory anaemia progressing to pancytopenia, congenital and developmental abnormalities, and an increased incidence of malignancy. Patients display panmyelopathy and tend to die early of bone marrow failure or leukemia. Fanconi cells are deficient in repair of dihydroxydihydro thymine residues, hypersensitive to cis-platinum *(Fujiwara et al., 1987),* DNA cross linking agents like mitomycin C *(Fujiwara et al., 1977).*

3. Bloom's syndrome is associated with numerous chromosome abnormalities and increased cancer sensitivity. Clinical features include photosensitivity, butterfly rash across the malar region of the face, normally proportioned dwarfed body and moderate to severe immunodeficiency. Bloom's patients have been found to have 50-75% reduction in DNA ligase I levels suggesting a structural gene mutation *(Chan et at., 1987).*

More than 150 human genetic disease syndromes have been characterised as having some potential relationship to the normal biology of aging. Approximately 20% of the population suffer from genetic conditions that shorten life, including diabetes, the familial hyperlipidemias, the HLA-associated life shortening diseases (multiple sclerosis, juvenile diabetes etc.), common genetic diseases such as alpha-1 antitrypsin deficiency, and cystic fibrosis. Approximately, 40% of infant mortality results from genetically determined conditions *(Childs, 1975).* The great abundance of human genetic variations raises the possibility that certain mutations will effect genes concerned with longevity. Although we know of no single mutation that lengthens maximum human lifespan, it is apparent that a number of mutations shorten life. Whether or not any of these life shortening mutations reflect alterations in some of the genes that might relate to longevity is unclear.

Martin *(1977)* in his analysis selected 21 phenotypes possibly associated with senescence. These included premature grey hair, diabetes mellitus, presenile dementia, increased neoplasms, and defective DNA-repair. Those syndromes that fall into this category include Down syndrome, Werner's syndrome, Cockayne's syndrome, Progeria, ataxia telangiectasia, Seip's syndrome, cervical lipodysplasia, Kleinfelter's syndrome; Turner's syndrome, myotonic dystrophy, with 3 of the 10 syndromes are chromosomal abnormalities implying quantitative and regulatory defects rather than qualitative enzymatic defects.

1. Classical Cockayne's (CS) syndrome consists of severe photosensitivity, dwarfism, cachexia, microcephaly, prognathism and loss of facial adipose tissue *(Arlett, 1986).* The disease appears after a normal first year of life and characterised by initial mental and physical retardation followed by progressive deterioration. Central nervous system manifestations of the syndrome are the most disabling. These include mental retardation, spasticity, sensorineural deafness, cerebellar ataxia, dementia, incontinence, and normal pressure hydrocephalus. There appears no increased incidence of malignant disease. CS fibroblasts and lymphocytes demonstrate hypersensitivity to ultraviolet irradiation and certain carcinogens, although to a lesser degree than XP cells *(Wade and Chu, 1979).* Assays of DNA repair like UDS and repair replication have been normal. There is lack of recovery of replicative DNA synthesis after UV damage in CS cells similar to that of XP cells *(Lehmann, 1987).* It has been reported from different laboratories that CS cells appear to be deficient in the preferential DNA-repair of active genes (Evans *and Bohr, 1994).*

2. Werner's syndrome is a rare, autosomal recessive condition with multiple progeroid features, but it is an imitation of aging rather than accelerated or premature senescence. It is characterised by short stature, canities

(premature greying of hair), premature baldness, scleropoikiloderma, trophic ulcers of the legs, juvenile cataracts, , hypogonadism, tendency to diabetes, calicification of the blood vessels and tendency to occur in siblings. Schellenberg and coworkers *(Yu et al ,1996)* have identified the gene located on the short arm of chromosome 8 by genetic linkage studies, as the cause of accelerated aging seen in these cases. The protein encoded by the gene indicates a helicase, an enzyme that unwinds the paired DNA strands - a necessary prelude to key activities of repair and replication. This could also be an indicator for the normal aging process despite differences in the process between those with Werner's syndrome and normal individuals.

3. Progeria (Hutchinson-Gilford syndrome) is characterised by retarded growth rarely exceeding that of a normal 4- or 5- year old, appearance is likened to a lucked bird or very old man due to baldness and lack of subcutaneous fat; skin atrophy and brown pigmentation, atrophic or absent nails, prominent eyes and beaked nose, recessive chin and hypoplastic maxilla, narrow chest and protruding abdomen. Clinical manifestations are osteoarthritis and arterioscelorosis and death occurs due to myocardial infarction. Survival beyond second decade is rare. The Hayflick's limit is only around ten in cells of these patients as against fifty in normals.

Patients with Down syndrome (trisomy 21) have the maximum number of features common with premature aging. These include: premature greying of hair and hair loss, increased tissue lipofuchsin, increased neoplasms and leukaemia, variations in the distribution of adipose tissue, amyloidosis, increased autoimmunity, hypogonadism, degenerative vascular disease and cataracts. Senile dementia with pathological findings indistinguishable from

Alzheimer's disease is found in all patients over forty years of age *(Smith and Berg, 1976).*

The life expectancy of patients with DS who reach 10 years of age is only 26, and only 8% survive to age 40 *(Smith and Berg, 1976).* Patients with DS have an extra chromosome 21 and this could lead to a disturbance in the normal gene dosage. The basis of the disease is presumably due to quantitative differences in expression of many genes located on the 21st chromosome and perhaps affecting the expression of genes on other chromosomes, rather than a specific qualitative gene defect, such as may underlay the autosomal diseases like Progeria, Cockayne's and Werner's syndromes. That trisomy 21 is compatible with life at all may be because the chromosome 21 is one of the smallest chromosomes. It appears to have the least amount of active genetic material of any of the autosomes *(Martin and Hoehn, 1974).*

History of Down Syndrome

The story of Down syndrome *(Ref. Zellweger, 1977)* begins about 150 years ago with the first complete description of Down syndrome by Seguin in 1846. However, it was the brief report by Langdon Down *(1866),* "Observations on the ethnic classification of idiots", that established the eponym, and misguided the following generations of scientists and physicians by incorrectly comparing the inner epicanthal fold of Down syndrome to the extension of the tarsal epicanthal fold seen in oriental populations. The first extensive description of Down syndrome was provided in the report of 62 cases of "Kalmuc Idiocy" by Fraser and Mitchell *(1876).* This provided a complete physical description, noted the increased risk of Down syndrome with advanced maternal age and described Down syndrome neuropathology. The observation of Down syndrome congenital heart anomalies by Garrod and Thompson *(1908)* then rounded out the basic clinical description of Down syndrome, largely complete by the turn of the century.

The next major advance came 60 years later, requiring a deeper understanding of the biological basis of human heredity. Although abnormality of chromosome number had been suspected earlier from a knowledge of plant chromosomal variation, it was only in 1959 that LeJeunne and Jacobs independently determined that Down syndrome was caused by trisomy 21. This understanding of Down syndrome as resulting from the duplication of genes on chromosome 21 was focused by the suggestion of Niebuhr *(1974),* that the "typical Down syndrome phenotype" might be caused by the duplication of only a part of chromosome 21 band q22 which itself represents about one half of the long arm. The observation of Down syndrome in a chimpanzee *(McClure et al., 1969)* provided compelling evidence that supported the growing understanding that the specific phenotypic features of Down syndrome are caused by the duplication of specific genes located on chromosome 21.

Progress in the genetic and physical mapping of chromosome 21 has now reached a point at which it is possible to begin the correlation of the phenotypic components of Down syndrome with imbalance of specific regions of the chromosome. Several preliminary efforts in this direction have already been made and suggest that the phenotypic and molecular analysis of the relatively rare individuals with chromosome 21 duplications ("partial trisomy") can be used to specify which regions of chromosome 21 are involved in the generation of specific components of the phenotype. The ultimate goal of correlating genotype with phenotype (phenotypic mapping) is to make it possible to discover which particular genes are responsible for which aspects of the phenotype, thereby permitting the pathogenesis of the syndrome to be elucidated and, hopefully, its most serious consequences to be prevented or ameliorated. Down syndrome occurs in all parts of the world. It is not restricted to any race, culture, social class, or historical period.

Along with this growth in knowledge about the nature of the syndrome, there have been noteworthy changes in the life prospects of people with Down

syndrome. Advances in medical treatment have overcome many health problems and reports around the world have indicated longer and healthier lives *(Masaki et al., 1981; Thase, 1982a; Stratford and Steele, 1985; Fryers, 1986; Dupont et al., 1986; Malone, 1988; Eyman et al.,1991).*

Indeed it is important to recognise that the main attribute which distinguishes people with Down syndrome from everyone else is the additional complement of chromosome 21 genes. It may also be added that all people with Down syndrome are not identical, even with respect to this extra genetic material. The majority have a total of 47 chromosomes because of an additional chromosome 21 (trisomy 21). A few people have an extra 21q11 segment attached to another chromosome (translocation), Mosaicism is third, but even less common form of Down syndrome, in which some cells have the normal complement of 46 chromosomes but because of errors in later cell division, other cells show trisomy 21. The path of development for each individual depends not only on the effects of the extra genetic material but also on mediating influence of other genetic material and environmental factors.

Alzheimer morphology has been commonly reported in older persons with Down syndrome but it is not possible to conclude that all develop Alzheimer's Disease. Schweber *(1987)* reported that up to three-fourths of those with Down syndrome who showed signs of Alzheimer's at autopsy had not developed significant mental problems with age. It seems that a distinction has been made between neuropathological and clinical signs of Alzheimer's and, it should never be assumed that Alzheimer's disease is the invariant consequence of longevity in Down syndrome.' *(Thase 1988).* Sensory impairment e.g. cataracts or hearing loss may produce cognitive or behavioural changes in the older adult *(Hewitt et al., 1985)* and thyroid imbalance, other illnesses, depression, and medication should be investigated regarding their possible influence on behaviour *(Thase, 1982b).*

The elderly with down syndrome

Aging in the population with Down syndrome is not well documented. Although the age level of above 65-70 years is regarded as old in the general community, Down syndrome is characterised by an increased speed of aging, including early menopause *(Miniszek, 1983)* and a better definition of 'old[1] for adults with Down syndrome may be over 50 years of age *(Seltzer, 1985)*.

There is a dearth of knowledge about many aspects of aging in Down syndrome, in particular, the timetable of physiological events and the range of individual variation. This is perhaps because only recently have persons with Down syndrome begun to reach middle age as community residents *(Schleien et al., 1981).* This information is important for determining the functional impact of age changes. We need to know when the decline begins, which functions are involved and how these decrements affect the performance of daily living activities. It is evident that the process and consequences of aging is an area that warrants an increased research effort.

SCOPE OF THE PRESENT STUDY

Accumulation of the DNA damage and decrease in the DNA repair capacity is one of the causes for aging and the age associated disorders *(Hart and Setlow, 1974; Gensler and Bernstein, 1981).*

1. One of the factors affecting the process of aging is calorie restriction. As already discussed earlier, it has been shown in experimental animals that dietary/calorie restriction, without sacrificing the quality of the diet, has an unequivocal effect in extending the lifespan in a variety of species. However, this effect is yet to be demonstrated in humans and it has been a nagging question whether the experimental results with laboratory animals could be extrapolated to human situation. We have therefore

taken up this challenging task and measured DNA repair parameters in the peripheral lymphocytes of subjects with normal and low body mass index (an indicator of calorie restriction) to clinch the issue on humans. The results show that indeed the observations with experimental animals could be extended to humans. The results of these studies are presented in Chapter 3.

2. Yet another way of understanding the aging process is to study those syndromes where premature aging is seen as one of the primary features. Most important among such syndromes is Down syndrome. We have therefore, measured the DNA-repair parameters in the peripheral lymphocytes of the Down syndrome subjects alongwith age- and sex-matched normal subjects as a function of age. It is shown that DNA-repair potential is at a low level and also that it declines more rapidly in Down syndrome patients as compared to normals. These results are presented in Chapter 4.

3. Since a relationship has been established between the telomeric length and the replicative capacity of a cell and the onset of senescence, we have taken up a study to measure the average telomeric length in peripheral lymphocytes of human subjects - Down syndrome cases and controls as well as subjects with differences in body mass indices (indicating their nutritional status). These results are presented in Chapter 5

In Chapter 6 all the results presented in earlier chapters have been discussed in the light of existing information.

CHAPTER - 2

MATERIALS AND METHODS

SUBJECTS

All human subjects were from the Indian population and belonging to both sexes.

CHEMICALS

Highly polymerised calf thymus DNA, Bovine serum albumin, Adenosine 5'-Triphosphate (ATP), Histopaque, N-methyl-N'-nitro-N-nitroso guanidine (MNNG), Ethidium Bromide, Ethylene Diamine Tetraacetic Acid (EDTA), Sodium Lauryl sulfate (SDS), Phytohemeagglutinin (PHA), Hydroxyurea (HU), Ribonuclease A, Leupeptin, Pepstatin, Phenylmethylsulfonyl Fluoride (PMSF), Tizma base, Dithiothreitol (DTT), penicillin, streptomycin, and Agarose were purchased from Sigma Chemical Co., St. Louis, MO, USA. Unlabelled nucleotides, 2'-deoxyadenosine 5'-triphosphate (dATP), 2'-deoxycytidine 5'-triphosphate (dCTP), 2'-deoxyguanosine 5'-triphosphate (dGTP) and thymidine 5'-triphosphate (TTP) were purchased from Pharmacia Fine chemicals, Uppsala, Sweden. Poly (dA). Oligo (dT)12-18 was supplied by Midland Certified Reagent Company, Midland, TX, USA. Tritiated TTP (specific activity 30 Ci/mmol) was purchased from (Radiochemicals) Life Science, Amersham, England and Tritiated Thymidine (specific activity 17 Ci/mmol) and radio-phosphorous y-labelled ATP (specific activity 3000 Ci/mmol) was purchased from BRIT, Bombay, India. Restriction enzyme Hinf I and T4 Polynucleotide Kinase were purchased from Bangalore Genei, Bangalore, India. RPMI-1640 was purchased from Hi-media, India. Proteinase K and nylon membrane was purchased from Bohringer Mannheim GmbH., Mannheim, Germany 2,5-Diphenyl-1,3-Oxazole (PPO) and 2,2'-p-Phenylene-bis[5-Phenyloxazole] (POPOP) were purchased from Beckman instruments inc., Fullerton, CA, USA. Foetal Calf serum was purchased from Biological industries, Kibbutz Beit Haemek, Israel. β-Mercaptoethanol was purchased from Biorad laboratories, Hercules, CA, USA. 1 Kb ladder was purchased from Gibco BRL Life Technologies Inc., Green Island, NY, USA. GF/C filters were purchased from Schleicher and Schuell, Dassel,

Germany. Probe $(TTAGGG)_4$ was obtained from Rama Biotechnologies India Pvt. Ltd., Hyderabad, India. All other chemicals used were of analytical grade.

LYMPHOCYTE ISOLATION

Lymphocytes from anticoagulated blood were isolated by Ficoll-paque density gradient centrifugation *(Boyum, 1976 and Smith et al., 1987).*

Anticoagulated peripheral blood collected aseptically is mixed 1:1 with sterile physiological saline and overlaid carefully onto 0.5 volume of Ficoll-paque. The gradient centrifugation was done at 400 xg for 30 minutes at room temperature. The lymphocytes appear a clear white ring above the ficoll-paque layer while the other leukocytes form the interface with the ficoll-paque and the erythrocytes sediment down. The lymphocytes were harvested into ice-cold RPMI-1640 medium with 2 mM L-Glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% foetal calf serum and washed thrice with medium and each time harvested by centrifugation at 400 xg for 8 minutes. The viability of the cells was determined by trypan blue exclusion and found to be >95%.

PHA-STIMULATION

This was done at a concentration of 2 µg PHA (M-form)/10^6 cells and incubating for 72 hours at 37°C in a CO_2 incubator. The cells were harvested and washed as described above.

ULTRAVIOLET (UV) IRRADIATION

Aliquots of the cells at a concentration of 10^6 cells/ml medium without FCS were taken in petriplates (3.4cm diameter), placed on ice and exposed, in the presence of 5mM HU, to UV light (254 nm) at a dose of 20 J/m^2 and 40 J/m^2 using Phillips TUV 8, 15W germicidal lamp adjusted to deliver at the rate of 1 J/m^2 per second. The cells were collected by centrifugation at 400 xg for 10 minutes. These cells were used for the assessment of various DNA-repair parameters alongwith unexposed controls processed identically.

N-METHYL-N'-NITRO-N-NITROSO GUANIDINE (MNNG) TREATMENT

Aliquots of the cells at a concentration of 10^6 cells/ml medium without FCS were taken and treated with 50 μM MNNG in 1% DMSO, in the presence of 5mM HU, and incubated at 37°C for 30 minutes. The cells were collected by centrifugation at 400 xg for 10 minutes and washed once with fresh medium containing 5mM HU, to remove the MNNG. These cells were used for the assessment of various DNA-repair parameters alongwith with unexposed controls.

UNSCHEDULED DNA SYNTHESIS

Unscheduled DNA synthesis [UDS] was assessed as described earlier *(Rao et al., 1996).*

The cells were incubated at a concentration of 10^6 cells/ml in fresh RPMI-1640 medium containing 10% FCS and 5 mM hydroxyurea and 5μCi ^{3}H-thymidine for 2 hours at 37°C and the reaction was stopped with 1 ml ice cold 10% TCA. The cells were washed thrice with cold 5% TCA containing 10 mM sodium pyrophosphate and twice with 95% Ethanol on GF/C filters and the dried filters processed for liquid scintillation counting using a toluene based fluid containing 5g PPO and 0.5g POPOP per litre.

The UDS was expressed as femtomoles of ^{3}H-thymidine incorporated per μg of DNA.

ASSAY PROCEDURE FOR DNases

UV and AP DNases were assayed essentially according to the procedure of Rao and Rao, 1984, with slight modification.

The cells were homogenised in ice-cold double distilled water at a concentration of 10^6 cells/ml. The homogenate was used for the assay of both the DNases.

UV DNase ASSAY

The reaction mixture contained in a final volume of 0.6 ml, 200 μg of UV irradiated DNA, 0.1 M Sodium acetate buffer, pH 5.0 and the homogenate. At the end of 2 hr incubation at 37°C in a water bath shaker, the reaction was terminated by the addition of 0.4 ml of 1 4N perchloric acid (PCA). The tubes were kept in ice for 10 minutes, after which they were centrifuged at 4,000 rpm for 5 minutes. The absorbence of the supernatant was measured spectrophotometrically at 260 nm against an appropriate blank.

The enzyme activity was expressed as μg of acid soluble DNA-phosphorous (DNA-P) liberated per mg protein or DNA in 2 hours at 37°C.

AP DNase ASSAY

The reaction mixture volume, method of assay and the expression of activity and other details are the same as in the case of UV DNase, except that the reaction mixture consisted of depurinated DNA as the substrate while 0.05 M Tris-HCl, pH 8.25 was used as buffer.

PREPARATION OF SUBSTRATES

UV irradiated DNA was prepared by irradiation of highly polymerised calf thymus DNA (2mg/ml water). The DNA solution was taken into petriplates as a thin layer, kept on ice and UV (254 nm) irradiated at a dose of $2x10^4$ J/m^2 using Phillips TUV 8, 15 W germicidal lamp.

Apurinic (AP) or Depurinated DNA was essentially prepared as described by Sharper and Grossman, 1980.

Native calf thymus DNA (2mg/ml water) was mixed with equal volume of depurination buffer containing 40 mM Sodium Citrate, 40 mM NaCl, 40 mM Potassium Phosphate, pH 5.0 and incubated at 70°C for 15 minutes.

PREPARATION OF DNA POLYMERASE ENZYME EXTRACT

The homegenates of the cells were prepared by homogenising them at 10^6 cells/ml homogenisation medium containing 20 mM Tris (pH 7.5), 0.1 mM DTT, 0.1 mM EDTA, 1.0 mM $MgCl_2$, 5% Glycerol, 1% triton X-100, 0.5 mM PMSF, 1μg/ml Leupeptin, 1μg/ml Pepstatin A and 0.5 M KCl. The homogenate was kept on ice for 1 hour, to aid the complete extraction of the DNA polymerase enzymes. Then it was centrifuged at 1,00,000 xg for 1 hour. The clear, particle-free supernatant thus obtained was used as the enzyme source of DNA polymerases.

DNA POLYMERASES ASSAY

The activity of total DNA polymerase was assayed according to the procedure of Prapurna and Rao, 1996 and the activity of DNA polymerase e was according to the procedure of Prapurna and Rao, 1997.

DNA Total POLYMERASES ASSAY

The reaction mixture contained in a final volume of 50μl, 40 mM Tris-HCl, pH 7.5, 8mM $MgCl_2$, 1mM β-mercaptoethanol, 4mM ATP, 100 μM each of dATP, dCTP, dGTP, 25μM TTP, 1μCi of 3H-TTP, 5μg of `activated DNA' and the enzyme. The incubation is carried out at 37°C for 20 minutes. At the end of incubation, 200μg each of DNA and BSA were added as carrier and the reaction was stopped by the addition of 1ml of ice-cold 10% TCA containing 10mM Sodium pyrophosphate. The precipitate was washed thrice with cold 5% TCA and twice with 95% Ethanol on GF/C filters and the dried filters processed for liquid scintillation counting using a toluene based fluid containing 5 g PPO and 0.5 g POPOP per litre.

The specific activity is expressed as picomoles of ^{3}H-TMP incorporated into the acid insoluble portion/mg protein or DNA/hour.

DNA POLYMERASE β ASSAY

The reaction mixture volume, method of assay and the expression of activity and other details are the same as in the case of Total DNA polymerases, except that the reaction was run with activated DNA as template primer at pH 8 25 (pH optimum for DNA polymerase βactivity).

DNA POLYMERASE e ASSAY

The reaction mixture volume, method of assay and the expression of activity and other details are the same as in the case of DNA polymerase (total), except that the reaction mixture contained 40 mM Tris-HCl, pH 7.5, 0.8mM $MgCl_2$, 5μg bovine serum albumin, 2% Glycerol, 2mM DTT, 4μM TTP, 1μCi of ^{3}H-TTP, 0.025 (A 260) units of poly (dA). oligo $(dT)_{12\text{-}18}$ as template primer and the enzyme.

DENATURED DNA

Calf thymus DNA dissolved in distilled water at a concentration of 2 mg/ml was kept in boiling water bath for 10 minutes and then rapidly cooled in ice.

PROTEIN ESTIMATION

Untreated cells were homogenised in ice-cold double distilled water at a concentration of 10^6 cells/ml. The homogenate was used for protein estimation, according to the method of Lowry et al., 1951.

DNA ESTIMATION

Untreated cells were homogenised in ice-cold double distilled water at a concentration of 10^6 cells/ml. The homogenate was incubated in buffer (pH 8.0) containing 10mM Tris.CI (pH 8.0), 1 mM EDTA (pH 8.0), 20μg/ml RNase A at 37°C for 30 minutes and 100μg/ml Proteinase K at 50°C for 1 hour. The cell lysate was precipitated with ethanol and dissolved in TE buffer and the absorbence was read at 260 nm.

ISOLATION OF HIGH MOLECULAR MAMMALIAN DNA

DNA extraction was according to the method of Blinn and Stafford as given by Sambrook et al., 1989.

The cells were suspended at a concentration of 10^6 cells/ml extraction buffer containing (10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0), 0 5% SDS, 20 µg/ml pancreatic RNase) and incubated at 37°C for 1 hour. Then Proteinase K was added to a final concentration of 100 µg/ml and incubated at 50°C for overnight (~16 hours). The solution was allowed to come to room temperature and equal volume of buffer saturated phenol was added and gently mixed. The phases were separated by centrifugation in a swing out rotor at 5000 xg for 15 minutes. The aqueous layer was separated and re-extracted once with phenol, twice each with Phenol:Chloroform:Isoamyl alcohol in ratio of 25:24:1 and with Chloroform:Isoamyl alcohol in the ratio of 24:1. After all the extractions, NaCl was added to the aqueous phase, to a final concentration of 0.2 M. Then two volumes of ice-cold isopropanol was added and left at -20°C overnight (~16 hours) for precipitation. The DNA was pelleted down at 5000 xg for 30 minutes. To the pellet was added 70% cold isopropanol and allowed to stand at room temperature for 30 minutes and the DNA was harvested by pelleting at 5000 xg for 30 minutes, air dried and then dissolved in Tris-EDTA (TE) buffer (pH 8.0). The concentration and purity of the DNA was estimated by measuring the absorbence at 260 nm and 280 nm against TE buffer. The quality of DNA was tested on 0.5% agarose gels.

RESTRICTION DIGESTION OF DNA

Restriction digestion of an aliquot of DNA was carried out at 37°C for 12-16 hours with 2 units Hinf I/µg DNA. To check the digestion, about 5 µl of sample was electrophoresed on a 0.5% agarose minigel (10 cms - 50 ml) at 30V using 1x TAE buffer (0.04 M Tris acetate, 0.001 M EDTA) for a period of 3-5 hours, stained with ethidium bromide and visualised under UV (300 nm). If the digestion was complete, the reaction was stopped with 1µl 0.5M EDTA (pH 8.0) and the

restricted **DNA** was precipitated with 0.2M NaCl and two volumes of ice-cold isopropanol, **left at -20°C** overnight. and pelleted the **DNA** at 5000 xg for 20 minutes at 4°C and then washed with cold 70% isopropanol. The DNA pellet **was** dried briefly. and dissolved in TE buffer, pH 8.0. The **DNA** was quantified by absorbence of the samples at 260 nm and 280 nm against TE buffer.

DETERMINATION OF AVERAGE TELOMERIC LENGTH

Electrophoresis

On an 0.5% agarose gel (volume 200 ml, length 25 cm)in 1x TAE buffer (0.04 M Tris acetate, 0.001 M EDTA), equal amounts (3 to 5 μg) of restriction enzyme digested DNA in 6x Neutral loading dye (0.25% bromophenol blue, 40% (w/v) sucrose in water) was loaded with the standard 1 Kb DNA ladder on either sides; electrophoresed at 25 V at 4°C until the dye front travels to two-third of the gels length (usually this may take about 24 hours). The gel was stained with ethidium bromide solution 0.5μg/ml of water, observed under UV light (310 nm) and the standards were marked. After complete destaining of the gel with several changes of water, it was denatured with 0.5N NaOH, 0.15M NaCl for 15 minutes and then with a change allowed to denature for another 20 minutes. Then the gel was set up for Southern transfer.

Southern Transfer

Alkaline Transfer of DNA onto Nylon membrane using the VacuGene XL unit [Pharmacia Biotech] was done at 30 mbars for 1 hour. The nylon membrane was pretreated by floating the membrane on the surface of a dish of deionised water until it wets completely from beneath, and then immersing the membrane in transfer solution (1 M NaOH) for at least 5 minutes. After the transfer is over the nylon membrane was washed with 2x SSC for 10 minutes at room temperature to remove any adhering gel particles, air dried, UV cross linked for 2 minutes at 254 nm and then baked at 70°C for 2 hours. The membrane is then processed for hybridisation.

Hybridisation :

Prehybridisation of the nylon membrane was in Church buffer (1 % Bovine Serum Albumin, crystalline grade, 7 % SDS, 1 mM EDTA, 0.5 M Phosphate buffer, pH 7.2) at 42°C for 24 hours. After prehybridisation the labelled probe (16 picomoles end-labelled with 50 μCi γ-^{32}P-ATP using 10 units of T_4 Poly nucleotide kinase, incubated at 37°C for 1 hour and purified through Sephadex G-50 column), was added and the hybridisation carried out at 42°C for 24 hours. After hybridisation the membrane was washed with 0.5x SSC + 1% SDS four times of 10 minutes each at 42°C, air dried and subjected to autoradiography to visualise telomeric DNA. The autoradiograms were scanned with UVP gel documentation system (Mitsubishi) and the average length of the telomeric DNA (ATL) is calculated from the formula

$$ATL = \sum(MWi \times Odi) / \sum(Odi)$$

where ODi is the densitometer output and MWi is the length of the DNA at position /.

CHAPTER - 3

BODY MASS INDEX, DNA-REPAIR AND AGING IN HUMANS

BODY MASS INDEX, DNA REPAIR AND AGING IN HUMANS

Age-related deterioration has high variability, dependent on both genetic and environmental factors. Some of these environmental factors may potentially be manipulated, with the possibility that some age-related diseases and disorders may be delayed or even prevented. Calorie restriction is the only known experimental parameter that has been demonstrated to increase the maximum achievable lifespan *(McCay et al.,1935; Fernandes et al., 1976; Masoro, 1984),* while concurrently reducing the rate of occurrence of most age-associated diseases and postponing or preventing the age-related physiological deterioration. It was also shown to be effective in reducing the incidence and progression of spontaneous tumours and chemically induced tumours in rats and mice *(Sarkar et ai, 1982; Pollard and tucker, 1985; Rogers et al., 1987; Ruggeri et al., 1987; Weindruch and Walford, 1988; Wei et al., 1993).* This aspect has been discussed in detail in Chapter 1.

It is also apparent that the integrity of DNA, maintained by a number of DNA-repair systems, is essential for the survival. Numerous physical and chemical factors damage DNA in vivo with their origin being both endogenous and exogenous. The resultant DNA-damage can lead to biological end points such as cancer, mutations, birth defects, aging and, age-associated diseases and disorders. It is therefore, crucial for the living organism to possess repair mechanisms to counteract the damage for continuous survival and resistance to mutational changes.

There are many ways through which dietary restriction could possibly modulate the aging process. Although the basic mechanisms by which calorie restriction increases maximum achievable lifespan and reduces the susceptibility to disease are not clearly understood, several hypotheses have been postulated to explain these phenomena. It is possible that the mechanism behind the beneficial effects of dietary restriction is positive affectation on DNA-repair

potential by that regime. Reports, from this laboratory as well as from elsewhere, have indicated that dietary calorie restriction does lead to improved DNA-repair capacity in experimental animals *(Licastro et al., 1988; Weraarchakul and Richardson, 1988).* However, such effects are yet to be demonstrated in humans. We have therefore, taken up a study to assess the DNA-repair efficiency in relation to age as a function of calorie restriction as indicated by their body mass index *(Naidu and Rao, 1994)* in human subjects of Indian population living in their natural conditions, as an extension of the work with the experimental animals, to clinch the issue on humans.

MATERIALS AND METHODS

All human subjects selected, belonged to both sexes, and with no familial history of organic defects and premature deaths, were divided into three age groups, young (8-14 years), adult (20-35 years), and old (>55 years).

At each age, the subjects were divided into two groups based on their Body Mass Index (BMI), which is calculated by using the formula as suggested by Naidu and Rao *(1994):*

$$BMI = \text{Body Weight (Kg)} / [\ \text{Height (m)}\ ]^2$$

Normal BMI (NBMI) Group:

Consisted of individuals having a BMI of around 20 or more.

Low BMI (LBMI) Group:

Consisted of individuals having a BMI of less than 18.

The range was between 16 and less than 18 with only a few subjects (5 out of 60) showing a BMI marginally less than 16.

LYMPHOCYTE ISOLATION AND STIMULATION

Lymphocytes from anticoagulated blood were isolated by Ficoll-paque density gradient centrifugation and PHA-stimulated as described in Chapter 2.

ULTRAVIOLET (UV) IRRADIATION

Aliquots of the cells were exposed to a dose of 20 J/m^2 as described in Chapter 2.

Unscheduled DNA synthesis [UDS], preparation of extracts and assay of DNA-repair enzymes, DNases (UV and AP DNases) and DNA polymerase β, Protein and DNA estimations were done according to the procedure described under materials and methods section in Chapter 2.

RESULTS AND DISCUSSION

The human subjects selected were divided at each of 3 age groups - designated Young, Adult and Old, into two groups each based on their BMI and designated as NBMI (Normal Body Mass Index) and LBMI (Low Body Mass Index). The average BMI of the subjects in the various age groups are shown in Table 2. Each category consisted of 20 members at each age group. Since the subjects were selected after careful examination of their familial and dietary history (most of these subjects belong to lower to upper middle class society and largely belong to either this university community or old age homes with a steady income behind them) and extensive clinical examination (data shown in Table 3), the low BMI value is taken to indicate a chronic but natural intake of low calories. Thus this LBMI group is assumed to be similar to an experimentally diet restricted animal and therefore considered as `undernourished' without any apparent malnutrition. The peripheral blood lymphocytes from these subjects were examined for various DNA-repair parameters under different conditions.

Table2: AVERAGE BMI [Body Mass Index] OF EXPERIMENTAL SUBJECTS

	AVERAGE BMI	
Group	**NBMI**	**LBMI**
Young	22.5+1.3	16.5±0.5
Adult	21.5± 1.8	17.1±0.7
Old	23.0±2.2	17.0±0.7

Subjects, based on their BMI, were categorized as `Normal BMI' (BMI >20) and `Low BMI' (BMI <18). The average BMI of the subjects ± S.D. (n = 20) in the various groups are shown, where NBMI = Normal BMI and LBMI = Low BMI. Young denotes subjects 8-14 years; Adult denotes 20-35 years; Old denotes > 55 years.

Table3: **CLINICAL PARAMETERS STUDIED IN EXPERIMENTAL SUBJECTS**

	S.Choi (mg/dl)	Hb (Gms%)	RBS (mg/dl)	T.Prot (gms/dl)	S.Alb (gms/dl)	Glob (gms/dl)
YOUNG						
NBMI	155.2±25.2	12 0±1.1	83.0+10.9	6.9+0.53	4.3+0.62	2 6±0.43
LBMI	145.8±22.5	11.8±0.4	83.0±12.7	6.9±0.48	4.1±0.51	2.7±0.50
ADULT						
NBMI	180.0+27.2	14.5±1.3	76.0+11.5	6.7±0.79	4.6+0.91	2.5±0.45
LBMI	150.2±26.2	13.6+0.8	81.0+27.0	6.5±0.77	4.3±0.93	2.3+0.64
OLD						
NBMI	171.5+26.8	13.8+0.4	79.0+14.0	6.5+0.57	4.0±0.43	2.4+0.50
LBMI	163.0±33.8	13.0+1.4	82.0+31.4	6.7±0.98	4.2±1.10	2.4±0.60

Clinical parameters studied in experimental subjects were Hematology: Total leukocyte count, differential leukocyte count, total erythrocyte count, packed cell volume, blood hemoglobin [Hb]; Biochemistry: Random Blood sugar [RBS], serum cholesterol [S.Choi], serum total proteins [T.Prot], serum albumin [S.Alb], serum globulin [S.Glob]; Others: Hepatitis B surface antigen detection, chest X-ray, electro-cardiograph. The mean values of clinical examinations of 20 individuals in each category are given. The differences, if any between any two groups were not found to be statistically and clinically significant. Other details are the same as in Table 2.

Unscheduled DNA-synthesis (UDS), which is a measure of DNA-repair, without and with UV irradiation, was examined in the peripheral lymphocytes. For the statistical analysis the Students *t* Test, (paired) was used and the results are shown in Table 4 and Figure 2. The basal DNA-repair in NBMI subjects is decreased by 33% in old age as compared to 'young'. The difference between the young and adult groups was found to be not significant. In the LBMI group, however, no significant difference was noticed in the basal repair capacity among the three age groups and also the values at adult and old ages were higher than the corresponding age matched normals ($p > 0.05$). When the lymphocytes were challenged with UV irradiation there was a response in the groups by way of increased UDS. This data is also shown in Table 4 and Figure 3. In normal individuals there is a decrease in this response with age. However in the case of LBMI individuals, the response was always higher as compared to the normal group and this trend was similar in all the three age groups studied. This is to be expected because the LBMI groups already had a higher level of basal repair and when this is coupled with the better response to mutagenic challenge, the result is a distinctly improved DNA-repair capacity in these subjects.

The DNA-repair capacity as revealed by UDS was also examined as a function of BMI in each of the age groups by generating computerized regression curves (Sigma plot programme, version 2.01), plotting DNA-repair (UDS) against BMI. Figure 4A, B and C show the regression analyses for `young', `adult' and `old' age groups, respectively, alongwith 95% confidence limits. As can be seen, BMI, therefore the nutritional status, has practically no correlation with DNA-repair level in young, weak correlation in adult, but significant correlation in old. In line with this, the differences between the average values of low BMI group and normal BMI group at different ages were statistically significant at $p > 0.05$ level (Table 4). Similar regression analysis curves are shown for the UV induced fold increase in DNA-repair as a function

Table4: BASAL AND INDUCED UDS IN PERIPHERAL LYMPHOCYTES OF HUMAN SUBJECTS WITH NORMAL BMI AND LOW BMI AT DIFFERENT AGES

STATUS	BASAL REPAIR	UV INDUCED REPAIR
YOUNG		
NBMI	7.9 ± 3.6	83.4 + 36.2 (10.6 ± 4.6;
LBMI	9.2 ± 4.4	123.2 ±49.9* (13.4 ± 5.4)
ADULT		
NBMI	6.5 ± 4.5	43.0 ±29.9 + (6.6± 4.6)
LBMI	11.2 ± 5.8*	117.4 ±50.1* (10.5 ± 4.5)
OLD		
NBMI	5.3 ± 3.5 +	27.6 ±27.2 + (5.2 ±5.1)
LBMI	11.4 ±10.7*	87.3 ± 78.8* (7.7+ 7.0)

Values are expressed as femtomoles of [^{3}H]-Thymidine incorporated per μg DNA and represent average from 20 individual experiments. * These LBMI values are significantly different from those of the corresponding age matched NBMI group at a p value < 0.05. + This value is significantly different from that of the corresponding NBMI group at 'young' age at a p value < 0.05. Other details are the same as in Table 2.

Figure 2

Basal DNA-repair in **PHA** unstimulated and Stimulated periphera lymphocytes of human subjects with Normal Body Mass Index and Low Body Mass Index at young, adult and old ages.

Basal DNA-repair in human lymphocytes

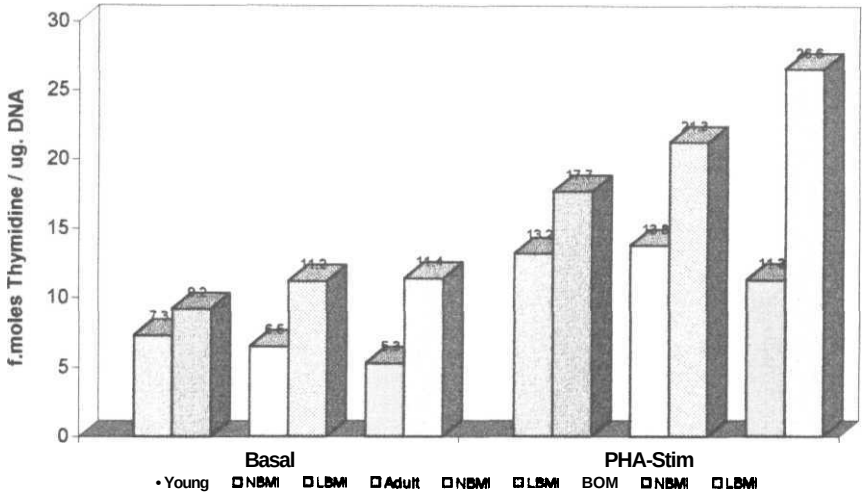


Figure 3

Fold increase of UV (254 nm) induced DNA-repair over the basal repair in PHA unstimulated and Stimulated peripheral lymphocytes of human subjects with Normal Body Mass Index and Low Body Mass Index at young, adult and old ages.

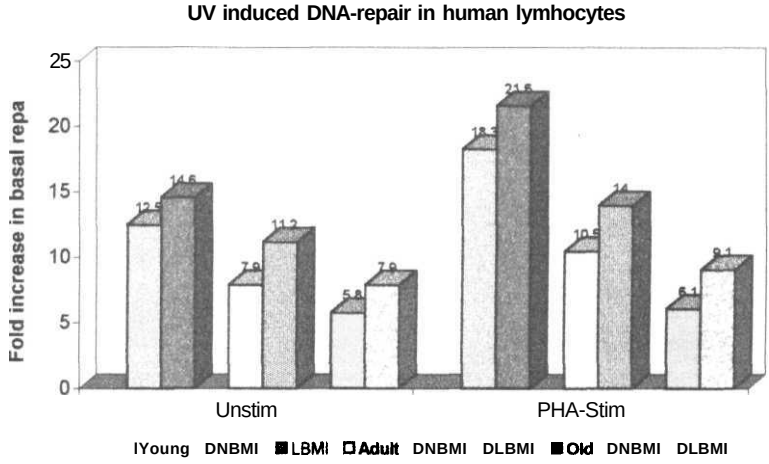
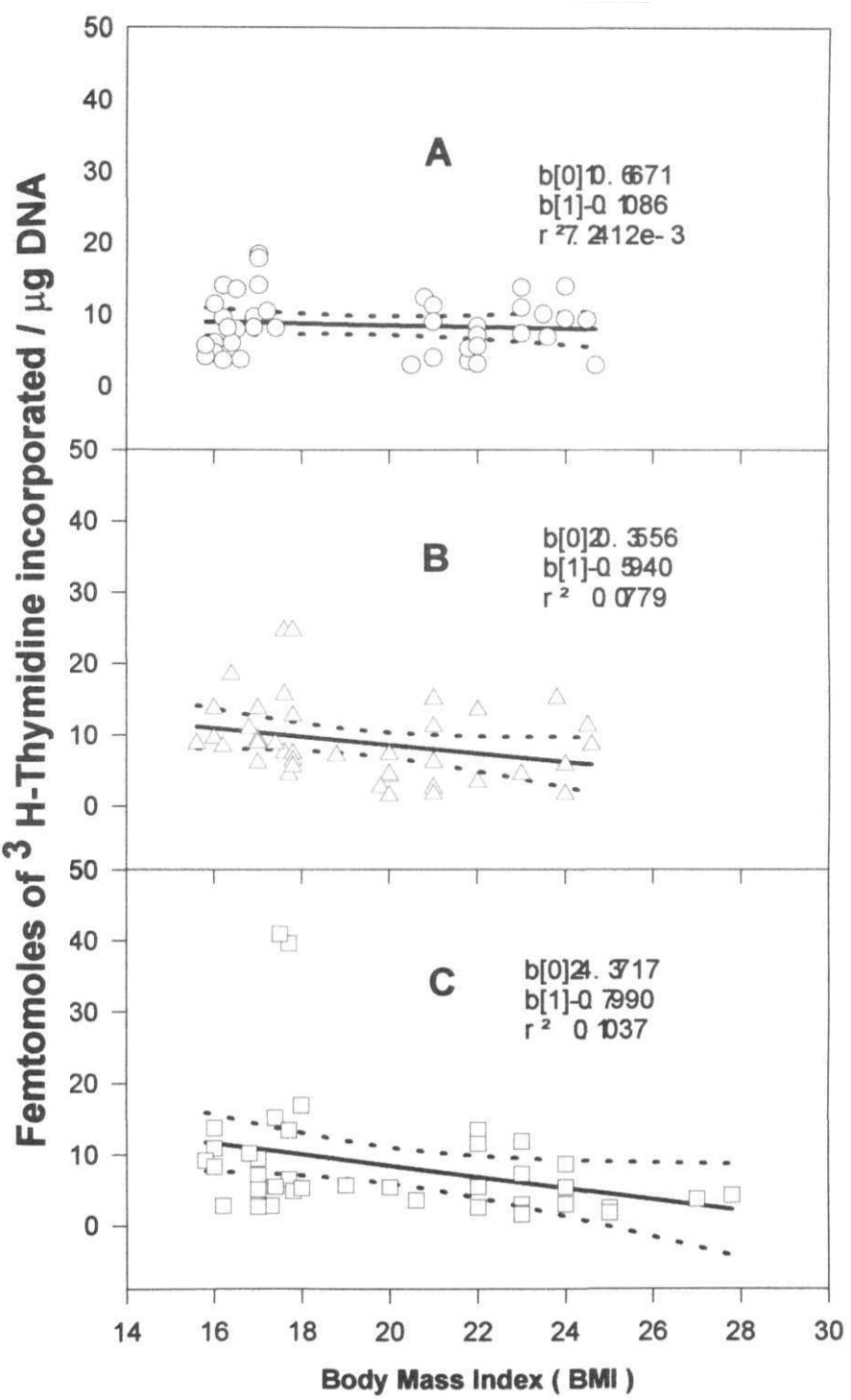

UV induced DNA-repair in human lymhocytes
Fold increase in basal repa
25
20
15
10
5
0
Unstim
PHA-Stim
Young DNBMI LBMI Adult DNBMI DLBMI Old DNBMI DLBMI

Figure 4

Basal DNA-repair in peripheral lymphocytes of human subjects as a function of Body Mass Index (BMI) at young (A), adult (B) and old (C) ages.

Linear regression analyses are shown inside plot-box. b(o) = intercept on y-axis; b(i) = slope of the regression curve; r^2 = regression coefficient. At each age 40 independent values are plotted. The dotted lines represent the 95% confidence limits.


A
b[0]10. 6671
b[1]-0. 1086
r ²7. 2412e- 3
B
b[0]20. 3556
b[1]-0. 5940
r ² 0 0779
C
b[0]24. 3717
b[1]-0. 7990
r ² 0 1037
Femtomoles of 3 H-Thymidine incorporated / µg DNA
Body Mass Index (BMI)
50
40
30
20
10
0
14
16
18
20
22
24
26
28
30

of BMI at all the three ages (Figure 5). It can be seen that there is always an inverse correlation, albeit to varying degrees depending upon the age, between the BMI and the fold increase in DNA-repair. In all these analyses, only the basal DNA-repair in `young' has failed to show any relation with BMI. Also, when the UDS is plotted as a funcion of age in LBMI and NBMI individuals (Figure 6), it is seen once again that the UDS decreases with age in NBMI subjects while there was actualy an increase in the LBMI subjects.

In Table 5 and, Figures 2 and 3, the results of the experiments when the lymphocytes were stimulated with Phytohemagglutinin (PHA) and then the studies carried out are shown. It can be seen that the pattern of the results is similar to that noticed in unstimulated lymphocytes (Table 4).

These results with human subjects are in good agreement with the findings of earlier workers on experimental animals when dietary restriction was shown to improve the unscheduled DNA synthesis in lymphocytes, hepatocytes and kidney cells in rats *(Licastro and Walford, 1985; Weerarchakul et al., 1989; Lipman et al., 1989)*.

DNA-repair in mammalian cells is a complex process involving many gene products *(Thompson, 1989).* The overall process consists of recognition of the damage, excision of the damaged portion, resynthesis of the excised portion and finally ligation of the last nucleotide gap (Figure 1, Chapter 1). Several endonucleases/protein factors were identified for the recognition of the damage and incision at the damaged site *(Thompson, 1989).* The properties of two major endodeoxyribonucleases, one with an acidic pH optimum and the other with alkaline pH optimum, identified in rat brain, suggested a role in DNA-repair at the initial incision step. The acidic pH optimum enzyme had a specificity to UV irradiated DNA *(Suvarchala and Rao, 1994)* while the alkaline pH optimum enzyme could utilize a variety of damaged DNAs including apurinic/apyrimidinic DNA (AP-DNA) *(Venugopal and Rao, 1993).* An endonuclease acting on UV irradiated

Figure 5

Ultraviolet light (UV, 254 nm) induced fold increase in DNA-repair in the peripheral lymphocytes of human subjects as a function of Body Mass Index (BMI) at young (A), adult (B) and old (C) ages.
Linear regression analyses are shown. The dotted lines represent the 95% confidence limits. Other details are the same as in Figure 3.

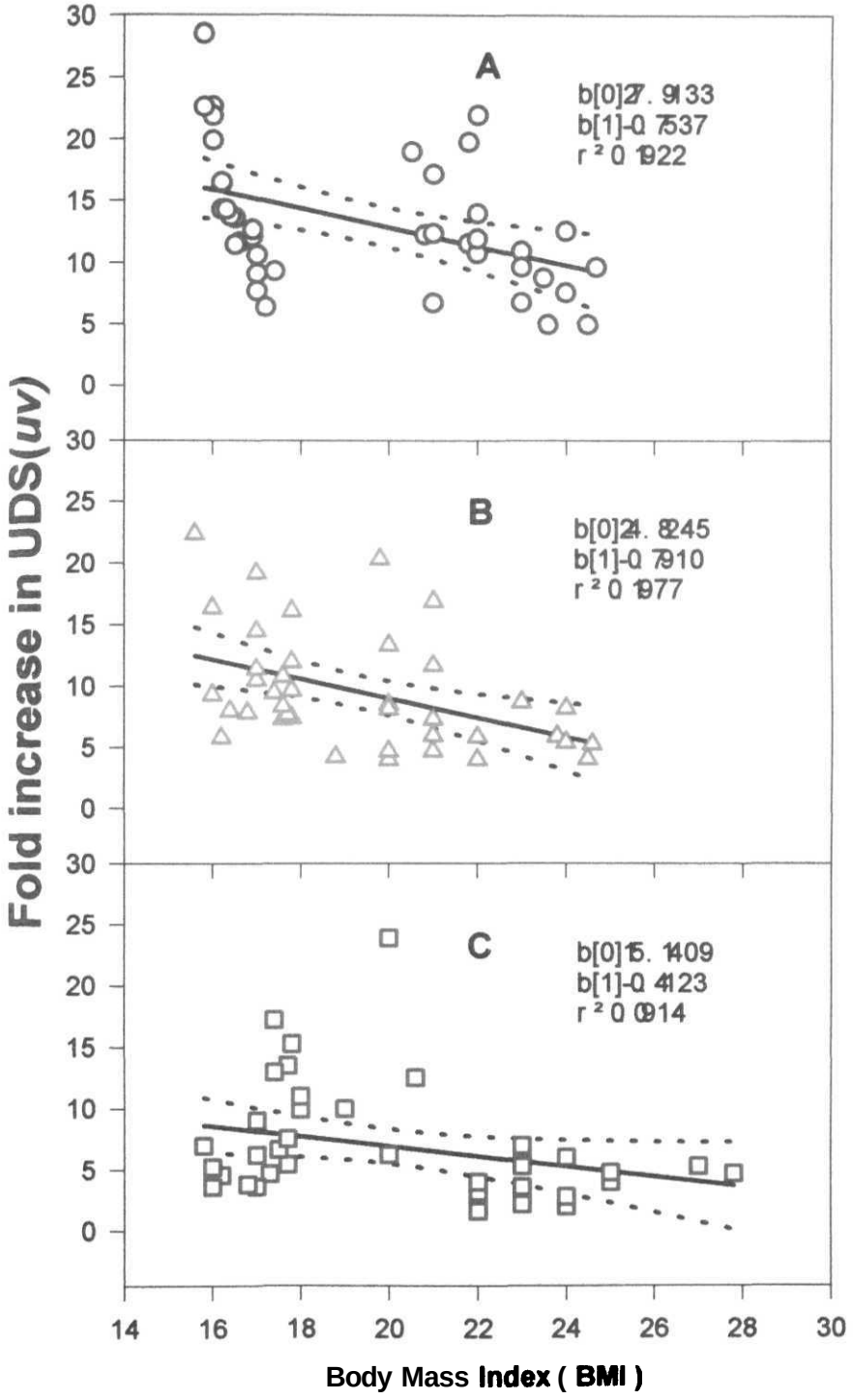

A
b[0]27. 9133
b[1]-0. 7537
r² 0 1922
B
b[0]24. 8245
b[1]-0. 7910
r² 0 1977
C
b[0]15. 1409
b[1]-0. 4123
r² 0 0914
Fold increase in UDS(uv)
Body Mass Index (BMI)

Figure 6

Basal DNA-repair in peripheral lymphocytes of human subjects with Low Body Mass Index and Normal Body Mass Index, as a function of Age.

Linear regression analyses are shown inside plot-box. b(o) = intercept on y-axis; b(i) = slope of the regression curve; r^2 = regression coefficient. At each energy status 60 independent values are plotted. The dotted lines represent the 95% confidence limits.

Unscheduled DNA Synthesis

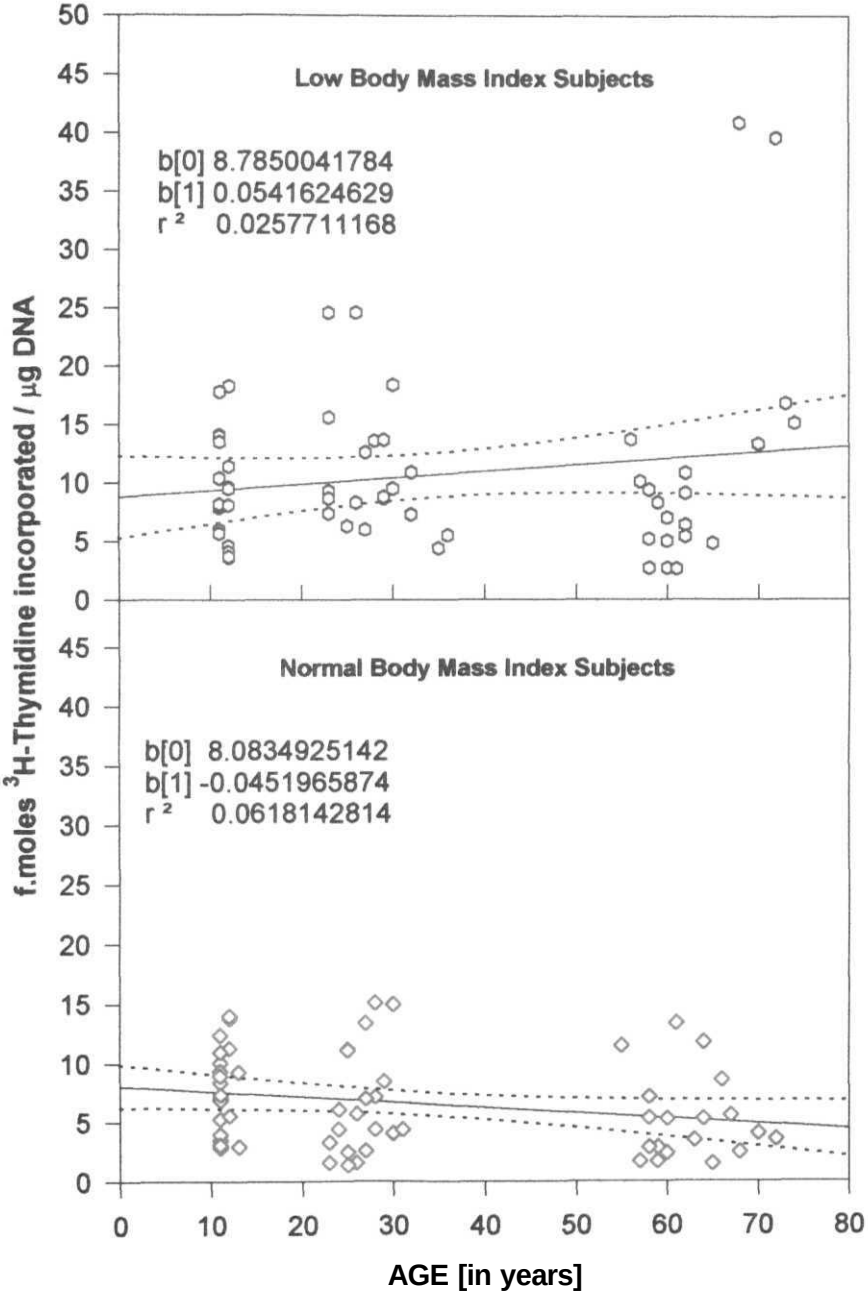


Table5: BASAL AND INDUCED UDS IN PERIPHERAL LYMPHOCYTES OF HUMAN SUBJECTS WITH NORMAL BMI AND LOW BMI AT DIFFERENT AGES AFTER PHA-STIMULATION

STATUS	BASAL REPAIR	UV INDUCED REPAIR
YOUNG		
NBMI	13.2 + 6.0	219.7 ± 89.6 (18.3 ± 6.7)
LBMI	17.7 ±10.4	250.0 ±115.4* (21.6 ± 8.0)
ADULT		
NBMI	13.8 ± 6.1	132.2 ± 76.9 + (10.5 ± 6.0)
LBMI	21.3 ±16.2*	241.6 ±120.2* (14.0 ± 5.9)
OLD		
NBMI	11.3 ± 8.4	57.7 ± 32.3 +# (6.1 ± 3.0)
LBMI	26.6 ±17.8*	198.2 ±112.4* (9.1 ± 4.7)

* These LBMI values are significantly different from those of corresponding age matched NBMI group at a p value < 0.05. + This value is significantly different from that of NBMI group of young' age at a p value < 0.05. # This value is significantly different from that of NBMI group of adult' age at a p value < 0.05. The details are the same as in Table 4.

DNA and an AP endonuclease have been shown in mammalian tissues/cells including brain *(Bacchetti and Beene, 1975; Ivanov, 1987).* Similarly DNA-polymerase p, generally considered to be a repair enzyme especially in base excision repair and also shown to be induced against damage to cellular DNA *(Singhal et ai, 1995; Jinno et al.,1995)* is found to be the major polymerase in adult brain *(Waser et al., 1979; Subrahmanyam and Rao, 1988).* It has also been shown that the activity of this enzyme decreases in the aging rat brain possibly due to post translational modification of the enzyme molecules *(Rao et ai, 1994).* In view of this information, we have measured the activities of DNA-polymerase p, UV DNase and AP DNase as markers of DNA-repair potential in the lymphocytes of the subjects under study.

The activities of DNA polymerase β in lymphocytes with and without UV challenge are shown in Table 6. There is a decrease in the basal activities in both the normal and LBMI subjects, as age progresses, but the enzyme levels are significantly higher in the LBMI when compared to normals in adult and old age groups. Even under induced conditions the pattern remained the same in that while the age dependent decrease is there in both groups, the values were always higher in LBMI group. However, statistical analysis showed that only in the old age the differences between NBMI and LBMI groups was significant (p value < 0.05). It can be seen that at old age the values in LBMI group are 50% higher than those in NBMI group. The activity profile observed in cells stimulated with PHA and with and without UV challenge was also seen to follow a similar pattern The data is shown in Table 7. The observed increase in basal DNA repair before and after PHA-stimulation (Tables 4 and 5) in the LBMI group at adult and old ages may be attributed to the enhanced activities of β polymerase. It is interesting that Srivastava and Busbee *(1992)* have shown that both a and p polymerases of calorie restricted aged mice exhibit a higher level of fidelity than polymerases of *ad libitum* fed aged mice.

Table6: BASAL (BA) AND UV INDUCED (IA) DNA-POLYM ERASE β ACTIVITY IN PERIPHERAL LYMPHOCYTES OF SUBJECTS WITH NORMAL BMI AND LOW BMI AT DIFFERENT AGES

STATUS	DNA POLYMERASE β ACTIVITY	
	BA	IA
YOUNG		
NBMI	395 ±167	732 ± 350 (1.85+ 0.85)
LBMI	424 ±158	911 ± 386 (2.15 ± 0.91)
ADULT		
NBMI	204 ± 51+	361 ±109 + (1.77 ± 0.53)
LBMI	249 ± 63*+	421 ± 113 + (1.69 + 0.45)
OLD		
NBMI	136 ± 69 +#	240 ± 160 +# (1.77 ± 1.18)
LBMI	204 ± 69*+#	362 ±171*+ (1.78 ± 0.84)

Values are expressed as picomoles [^{3}H]-TMP incorporated per mg of Protein per hour. * These values are significantly higher than those of corresponding NBMI group at a p value < 0.05. + This value is significantly different from that of the corresponding group of `young' age at a p value of < 0.05. # This value is significantly different from that of corresponding group of adult' age at a p value < 0.05. Other details are as in Table 2.

It is to be noted that although the conditions of assay are optimal for fi polymerase activity, it is still possible that other polymerase activities are also expressed under the conditions

Table7: BASAL (BA) AND UV INDUCED (IA) DNA-POLYMERASE β ACTIVITY IN PERIPHERAL LYMPHOCYTES OF SUBJECTS WITH NORMAL BMI AND LOW BMI AT DIFFERENT AGES AFTER PHA-STIMULATION

STATUS	DNA POLYMERASE β ACTIVITY BA	IA
YOUNG		
NBMI	546 ± 227	1050 ± 417 (1.92 ± 0.76)
LBMI	659 ± 260	1131 ± 389 (1.72+ 0.59)
ADULT		
NBMI	244 ± 91 +	454 ± 144 + (1.86± 0.59)
LBMI	294 ± 57*+	562 ± 147 + (1.91 ± 0.50)
OLD		
NBMI	202 ± 9 6 +	324 ± 157 +# (1.60 ± 0.78)
LBMI	227 ± 63*+#	377 ± 145*+# (1.66 ± 0.64)

* These LBMI values are significantly different from those of corresponding age matched NBMI group at a p value < 0.05. + This value is significantly different from that of the corresponding group of young' age at a p value of < 0.05. *n* This value is significantly different from that of corresponding group of adult' age at a p value < 0.05. The details are the same as in Table 6.

The activities of two DNases, UV DNase and AP DNase, in the lymphocytes of the subjects before and after PHA-stimulation are shown in Tables 8 and 9, respectively. An age associated decline in basal levels of UV DNase is seen among the normal subjects. The LBMI group at young age showed marginally higher values which were not statistically significant. However, at both adult and old ages, the values were significantly higher in LBMI group as compared to normal. Also the age dependent decline seen in normal individuals is not evident in the LBMI individuals. A similar pattern of changes is seen even after the lymphocytes are exposed to UV except that the values are higher.

In the case of AP DNase, the pattern of changes in basal activities are similar to UV DNase. UV exposure of the lymphocytes resulted in comparable induction of this activity in both normal and LBMI groups at all ages, but in old age both the basal as well as induced activities in LBMI groups were significantly higher than those in NBMI group. Thus the overall picture seems to be that either the activities are unaffected or higher in LBMI individuals. A similar profile was found in PHA-stimulated lymphocytes as well (Table 9).

In substantiation of the above results it has been observed repeatedly by others *(Marini et al., 1987; Nagel et al., 1988; De Greet et al., 1992)* and also by us that PHA-stimulation has become less effective with age. However, such a decline in the PHA-stimulated proliferation was not observed in LBMI subjects.

Age-related decline in UV-induced DNA-repair has been reported in human peripheral lymphocytes *(Roth et al., 1989; Grossman and Wei, 1994)* and also in tissue from the central nervous system *(Gibson and D'Ambrosio, 1982).* The present results while confirming the age dependent decline in the activities of DNA-repair enzymes also show that human subjects with low BMI presumably facing chronic low calorie consumption exhibit either unaffected or even improved activities, with the age dependent decline being distinctly slower.

Table8: BASAL (BA) AND UV INDUCED (IA) ACTIVITIES OF UV DNase AND AP DNase IN PERIPHERAL LYMPHOCYTES OF SUBJECTS WITH NORMAL BMI AND LOW BMI AT DIFFERENT AGES

	UV DNase ACTIVITY		AP DNase ACTIVITY	
STATUS	**BA**	**IA**	**BA**	**IA**
YOUNG				
NBMI	199 ± 37	341 ± 64 (1.71 ± 0.32)	246+ 70	363 ± 70 (1.48± 0.29)
LBMI	210 ±45	368 + 56 (1.75 ± 0.27)	287 ± 66	375+54 (1.31 ± 0.19)
ADULT				
NBMI	140 ± 35+	236 ± 66+ (1.69 ± 0.47)	208 ± 39 +	355 ±115 (1.71 ± 0.55)
LBMI	258 ± 123*	363 ± 159* (1.41 ± 0.62)	253 ±117	365 ± 167 (1.44± 0.66)
OLD				
NBMI	126+57+	160 ± 71 +# (1.27± 0.56)	133 ± 65 +#	176 ± 102 +# (1.32 ± 0.77)
LBMI	222 ± 68*	260 ± 64*+# (1.17 ± 0.29)	237 ± 55*+	276 ± 72*+# (1.17 ± 0.31)

Values are expressed as μg of DNA-P liberated per mg DNA and are the averages of 20 individual experiments. * These values are significantly higher than those of corresponding NBMI group at a p value < 0.05. + This value is significantly different from that of the corresponding group of young' age at a p value of < 0.05. # This value is significantly different from that of corresponding group of `adult' age at a p value < 0.05. Other details are as in Table 2.

Table9: BASAL (BA) AND UV INDUCED (IA) ACTIVITIES OF UV DNase AND AP DNase IN PERIPHERAL LYMPHOCYTES OF SUBJECTS WITH NORMAL BMI AND LOW BMI AT DIFFERENT AGES AFTER PHA-STIMULATION

	UV DNase ACTIVITY		AP DNase ACTIVITY	
STATUS	**BA**	**IA**	**BA**	**IA**
YOUNG				
NBMI	249 ± 53	479 ± 81 (1.96 ±0.30)	390 ± 97	490 ± 86 (1.30 ± 0.23)
LBMI	312+61*	521+63 (1.72 ± 0.34)	406 ± 81	516 + 62 (1.32± 0.33)
ADULT				
NBMI	190 ± 67+	314 ± 95+ (1.76 ± 0.34)	268 ± 97 +	431+126 (1.67 ± 0.32)
LBMI	292+136*	438 + 180* (1.56± 0.29)	304 ±129+	449 ±196 (1.49± 0.35)
OLD				
NBMI	148 ±67+	202 ± 96 +# (1.38 ± 0.47)	156 ± 70 +#	215 ± 122 +# (1.37 ± 0.45)
LBMI	222 ± 68*+	260 ± 68*+# (1.27 ± 0.39)	269 ± 73*+	328 ± 92*+# (1.31 ± 0.67)

* These LBMI values are significantly different from those of corresponding age matched NBMI group at a p value < 0.05. + This value is significantly different from that of the corresponding group of `young' age at a p value of < 0.05. # This value is significantly different from that of corresponding group of adult' age at a p value < 0.05. All details are the same as in Table 8.

This is the first study of its kind on human subjects to demonstrate the beneficial effects of chronic undernutrition, a phenomenon reported earlier in experimental animals *(Licastro and Walford, 1885; Weerarchakul et al., 1989; Lipman et al., 1989).* Walford and his group are conducting an elaborate experiment to examine the effects of diet on general physiology of humans including aging *(1994).* These experiments are still continuing. However the present study was planned and executed with a population that is naturally available in India. The results indicate the possibility of a very intriguing and interesting phenomenon that LBMI (moderate undernutrition with no apparent malnutrition) is actually beneficial in human subjects in maintaining good DNA repair capacity. How undernutrition is able to achieve this is a matter of speculation. It is logical to expect that in LBMI individuals the cellular metabolism would be at a low ebb. However, even against this reduced calorie availability the cell might choose to maintain certain essential pathways at normal rates and DNA-repair pathway could be one such pathway in view of its cardinal role in protecting the genomic integrity. This in itself may be a major contributing factor for improved longevity in such human subjects.

CHAPTER - 4

DNA-REPAIR PARAMETERS IN ACCELERATED AGING: TRISOMY 21 AS A MODEL

DNA-REPAIR PARAMETERS IN ACCELERATED AGING: TRISOMY 21 AS A MODEL

Aging is a complex phenomenon and is the result of a continuing interplay of both genetic and environmental factors, so that although there are common traits with respect to the age of onset of manifestations of the aging processes, the rate of aging varies considerably between individuals.

One of the central theories to explain the mechanisms of aging process is DNA-repair theory which postulates an age dependent accumulation of DNA-damage leading to general degeneracy in vital functions of the cell and finally death. This theory, although it went through a turbulent period, has been revived because of the enormous knowledge that has accumulated about DNA-repair mechanisms and the relationship between this process and several genetic disorders and cancer *(Wei et al., 1993; Rao, 1993; Rao et al., 1994; Bhaskar and Rao, 1994).* It therefore appears that any defect in DNA-repair activity would lead to accumulation of DNA-damage in genetic material which in turn could lead to among other things, cellular senescence and death.

There are a number of hereditary disorders associated with a defect in DNA-repair activities. Some of these are variants of Xeroderma pigmentosum (XP) - with a defect in the lack of initial DNA-damage recognising and incision enzyme(s); Ataxia telangiectasia (or Louis-Bar syndrome) - defect appears to be in recognition and repair of γ-ray induced damage; Fanconi's anaemia - deficiency in repair of dihydroxydihydro thymine residues, hypersensitivity to cis-platinum and DNA cross linking agents; Bloom's syndrome - reduction in DNA ligase I levels. A number of genetic disorders are also known to shorten life. Only three of them, namely, Werner's syndrome, Progeria and Cockayne's

syndrome, have chromosomal abnormalities implying quantitative and regulatory defects rather than qualitative enzymatic defects.

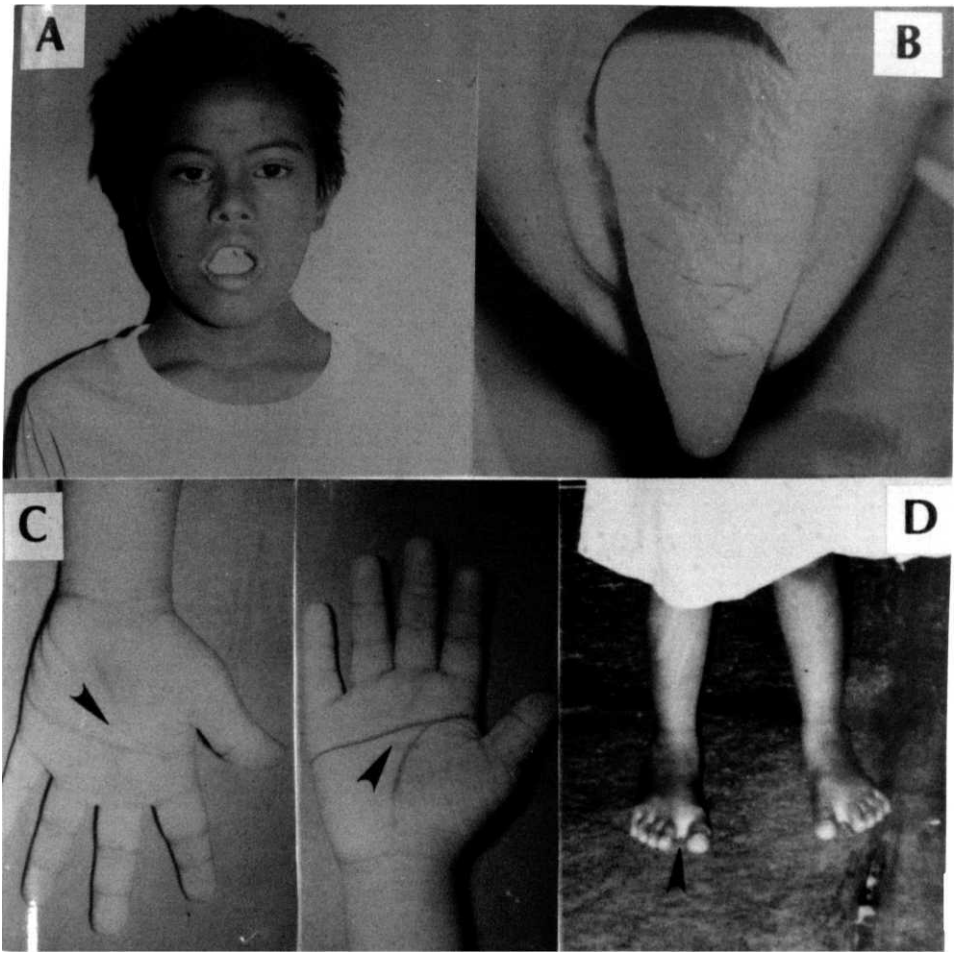
Patients with Down's syndrome have several features in common with premature aging *(Martin, 1977).* These include premature greying of hair and hair loss, increased tissue lipofuchsin, increased neoplasms and leukaemia, variations in the distribution of adipose tissue, amyloidosis, increased autoimmunity, hypogonadism, degenerative vascular disease and cataracts. Senile dementia with pathological findings indistinguishable from Alzheimer's disease is found in all patients over forty years of age *(Smith and Berg, 1976).*

The clinical features distinguishing the Down syndrome patients distinctly are high arched palate, fissured tongue, single palmar fissure, space between the first and second toes, prominent epicanthal folds, high cheek bones, absence of nasal bridge, characteristic slant of eyes and short stature. Some of these features are shown in Figure 7. These patients can be classified into three categories based on their karyotype as classical Down syndrome - patients having 47 chromosomes caused by trisomy 21; Translocation - where part of the chromosome 21, 21q11 segment, is attached to another chromosome mimicking the trisomy condition, and mosaics - where the karyotype in some cells are normal diploid and in some trisomy due to errors in later cell division.

In the last few decades, advances in medicine and changing attitudes towards the care of mentally retarded people have prolonged the lifespan of people with Down syndrome as well as increased their level of cognitive functioning *(Wisniewski et al., 1988).* The average current life expectancy is over 30 years for 80% of the people with Down syndrome who do not have congenital heart defects *(Baird and Sadovnick, 1987),* 25% of this population being expected to survive past the age of 50 years *(Thase, 1982).* They are considered as a good model of accelerated aging owing to the earlier onset of age-associated disorders and diseases attributed in general with aging. We have therefore

Figure 7

Features characteristic of Down syndrome patients - Typical facial feature with characteristic slant of eye (A), Thick fissured tongue (B), Single palmar fissure (C), Space between the first and second toes (D).


A
B
C
D

taken up a study to assess the DNA repair efficiency in relation to aging on Down syndrome subjects (both sexes) of Indian population.

MATERIALS AND METHODS

Human subjects: Down syndrome patients with trisomy 21 are selected after extensive clinical screening. These patients had no associated anomalies. Subjects were of both sexes in the ages of 1-40 years and divided into 3 age groups - Group 1 (upto 12 years), Group 2 (13-25 years) and Group 3 (26 years and above). The age and sex matched control group consisted of subjects with normal diploid complement of chromosomes and clinically healthy.

Lymphocytes isolation from anticoagulated blood, subsequent treatment with UV doses of 20 J/m^2 and 40 J/m^2, and 50μM MNNG and unscheduled DNA synthesis are described in chapter 2.

The cells after mutagenic treatment and prior to preparation of enzyme extract were incubated in fresh RPMI-1640 medium containing 10% Foetal Calf Serum (FCS) and 5mM Hydroxyurea (HU) for 2 hours at 37°C to allow for repair. The untreated cells were also incubated similarly.

The preparation of enzyme extracts and assay for DNA-repair enzymes DNases (UV and AP) and Polymerases (Total, β and e) were as described in chapter 2.

RESULTS

The clinical parameters studied in DS subjects were Haematology - blood haemoglobin, differential and total leukocyte counts; clinical pathology - urine albumin and sugar; biochemistry - Thyroid function tests, random blood sugar and serum cholesterol; and Hepatitis B surface antigen detection. Some of the

results are shown in Table 10. No clinically significant differences between the normal and DS subjects were found in any of the parameters studied.

Unscheduled DNA synthesis (UDS), which is a measure of constitutive DNA excision repair, was measured in the lymphocytes of normal and DS subjects. For the statistical analysis the Student's *t* test (unpaired) was used and the results are shown in Table 11. The basal DNA-repair activity in DS subjects is seen to be lowered significantly with age particularly between the ages of Group 2 and Group 3 *($p < 0.05$)* while the same activity is unchanged with age in the control subjects (Figure 8). When the lymphocytes were challenged with ultraviolet irradiation (UV 254 nm) at a dosage of 20J/m^2 or 40J/m^2, there was a response both in test and control groups by way of increased UDS (Table 11 and Figure 9). It was however, observed that the response to 20J/m^2 UV irradiation in DS group was more than that with 40J/m^2 dose. On the other hand, in normal subjects the DNA-repair response was more with increased UV dose and was significantly higher as compared to DS subjects particularly in age groups 2 and 3. The results are taken to indicate that while the DS subjects' potential to respond to DNA damage reaches saturation level at 20 J/m^2 irradiation level itself and any challenge beyond this level cannot elicit any more response.

When the lymphocytes were treated for 30 minutes with the DNA-methylating mutagenic substance N-methyl-N'-nitro-N-nitroso-guanidine (MNNG) at 50 μM level, UDS was observed to increase in all the groups (Table 12 and Figure 10). In both normal and DS individuals there was a decline in the response with age. However, it could be seen that the decline was more pronounced in DS subjects.

Table10: SOME CLINICAL PARAMETERS IN DOWN SYNDROME SUBJECTS

Subjects	Serum CHOLESTEROL mg/dl	Blood GLUCOSE mg/dl	Blood HAEMOGLOBIN g%
Group 1 : < 12 years			
DS n = 18	123±30	88 ±14*	11.9 ±1.4
NHV n = 12	134±12	101 ± 16	12.6 ±0.7
Group 2 : 13 - 25 years			
DS n = 36	141 ±35	90 ±16*	12.0 ±1.0*
NHV n = 24	136 + 17	102 ±15	13.0 ±1.1
Group 3 : > 26 years			
DS n = 18	135 ±37	90 ±18*	12.7 ±1.2
NHV n = 12	129 ±19	108 + 16	12.8 ±0.5

The values represent the average of clinical parameters of n individual observations of each group. * These values are noticed to be statistically lower than the normal values but observed to be clinically not significant since the DS values fall within the normal range.

Normal values : Serum Cholesterol: 90-220 mg/dl

Blood Sugar Random: 80-160 mg/dl Blood Haemoglobin: 11.0-14.0 g%

DS = Down syndrome cases **NHV** = Normal healthy volunteers.

Table11: BASAL AND INDUCED (UV) DNA REPAIR IN PERIPHERAL LYMPHOCYTES

		FOLD INCREASE	
SUBJECTS	**CONTROL**	**UV20**	**UV40**
Group 1 : < **12 years**			
DS n = 18	5.65 ±1.64*	9.5 ±1.9	6.6 ±1.6*
NHV n = 12	7.48 ±2.49	9.7 ±1.5	12.0 ±1.7
GROUP 2: **13 - 25** years			
DS n = 36	6.28 ± 1.87*	6.2 ± 1.4*♦	4.3 ± 0.9*♦
NHV n = 24	7.97 ± 2 68	8.2 ± 1.9 ♦	10.4 ±2.5
Group 3 : 26 years			
DS n = 18	3.89 ± 1.53*♦♥	3.9 ± 1.3*♦♥	2.6 ± 0.7*♦♥
NHV n = **12**	9.17 + 3.49	6.4±0.9 ♦	8.2 ± 0.9 ♦♥

Values are expressed as femtomoles of [^{3}H]-Thymidine incorporated per μg of DNA and the induced activities as fold increase over the basal value. Cells were exposed to UV as described in Materials and Methods section. The values represent the average of n individual observations in each group. * These values are significantly lower than those of corresponding age matched normal group at a p value < 0.05. • These values are significantly lower than those of the corresponding Group 1, and ♥ these of the corresponding Group 2 , at a p value < 0.05. The actual values after UV exposure are shown in Figure 9.

DS = Down syndrome cases NHV = Normal healthy volunteers.

Figure 8

Basal unscheduled DNA-synthesis in peripheral lymphocytes of Down syndrome patients (DS) and their age- and sex-matched normal healthy volunteers (NHV) as a function of age. The DS group consisted of 72 individuals and the NHV group 48. Linear regression analyses are shown inside plot-box. b(o) = intercept on y-axis; b(i) = slope of the regression curve; r^2 = regression coefficient. The dotted lines represent the 95% confidence limits.

UNSCHEDULED DNA SYNTHESIS

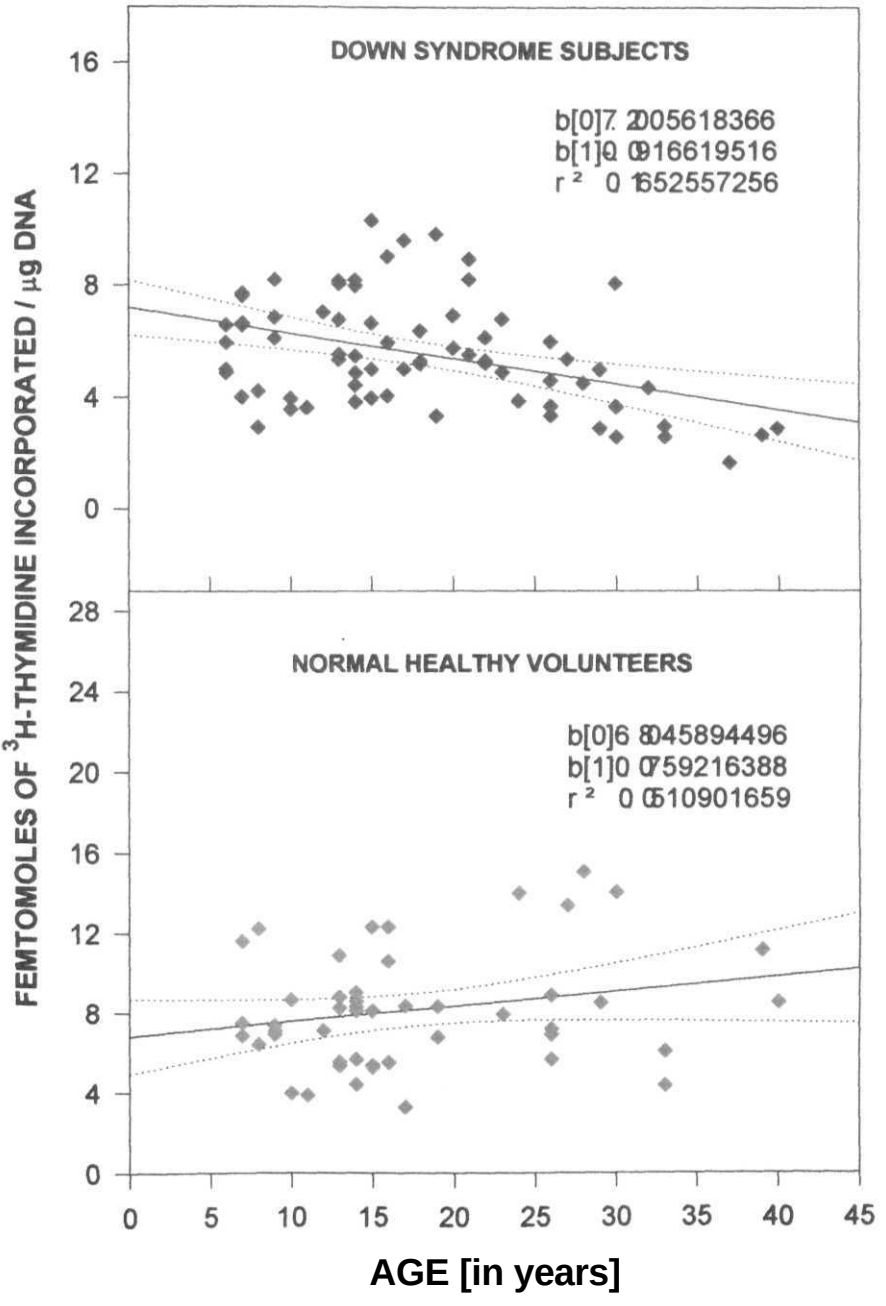


Figure 9

Basal and UV (254 nm) induced unscheduled DNA-repair in peripheral lymphocytes of Down syndrome patients (DS) and their age- and sex-matched normal healthy volunteers (NHV). Group 1: < 12 years, DS n=18 and NHV n=12; Group 2: 13-25 years, DS n=36 and NHV n=24; Group 3: > 26, DS n=18 and NHV n=12.

UNSCHEDULED DNA SYNTHESIS

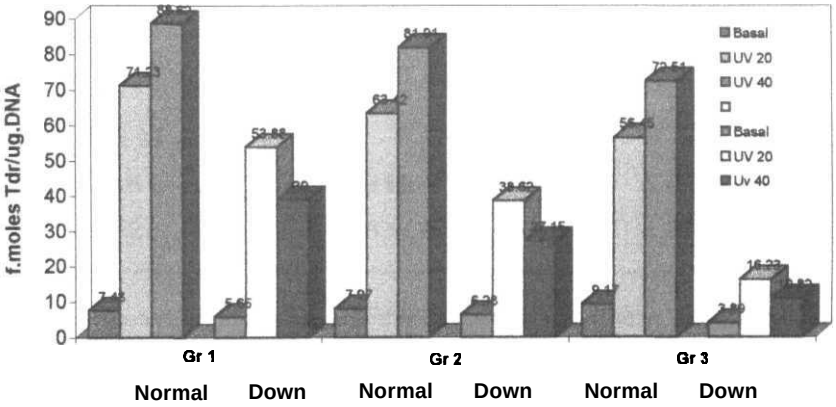


Table12: BASAL AND INDUCED (MNNG) DNA REPAIR IN PERIPHERAL LYMPHOCYTES

	FOLD INCREASE	
SUBJECTS	CONTROL	MNNG
Group 1 : < 12 years		
DS n = 18	5.65 ± 1.64*	7.2 + 1.5*
NHV n = 12	7.48 ±2.49	8 5 + 1.3
Group 2 : 13 - 25 years		
DS n = 36	6.28 ±1.87*	4.7 ± 1.1*♦
NHV n = 24	7.97 ±2.68	6.9 ± 1.4 ♦
Group 3 : > 26 years		
DS n = 18	3.89 ± 1.53*♦♥	2.7 + 0.9*♦♥
NHV n = 12	9.17 ±3.49	5.7 ± 0.8 ♦ V

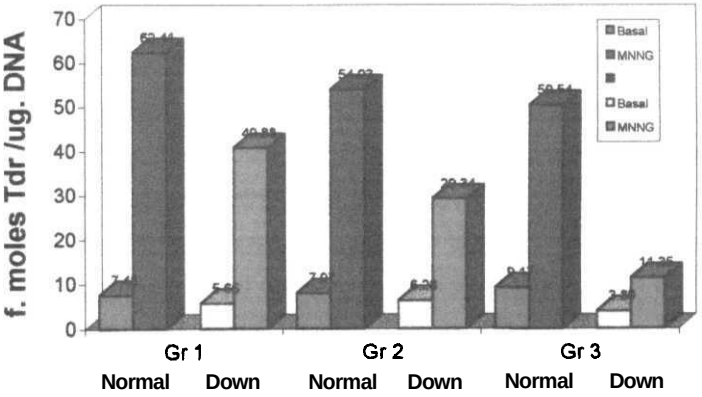
* These values are significantly lower than those of corresponding age matched normal group at a p value < 0.05. Treatment of cells with MNNG were as described in Materials and Methods section. Other details are the same as in Table 11. • These values are significantly lower than those of the corresponding Group 1, and ♥ these of the corresponding Group 2 , at a p value < 0.05. The actual values after MNNG treatment are shown in Figure 10.

DS = Down syndrome cases NHV = Normal healthy volunteers.

Figure 10

Basal and MNNG induced unscheduled DNA-repair in peripheral lymphocytes of Down syndrome patients (DS) and their age- and sex-matched normal healthy volunteers (NHV). The other details are the same as in Figure 9.

Unscheduled DNA Synthesis



During the past several years, two major rat brain endodeoxyribonucleases have been identified in this laboratory. One of them has acidic pH optimum and the other with alkaline pH optimum. Detailed studies on the properties of these enzymes suggested a role in DNA repair possibly at the initial incision step. The former enzyme had a specificity to UV irradiated DNA while the second could act on a variety of damaged DNAs including apurinic/apyrimidinic (AP) DNA *(Suvarchala and Rao, 1994; Venugopal and Rao, 1993).* Since the two activities could form part of the DNA-repair machinery of the cell, these activities were assayed in the lymphocytes of DS subjects before and after challenging them either with UV or with MNNG. The results are shown in tables 13 to 16.

Results concerning UV DNase are shown in Table 13 and Figure 11. While the basal activity in DS subjects has significantly decreased from Group 2 to Group 3, no age dependent variation was seen in normal subjects. However, after treatment with UV, it is interesting to note that the DS subjects have shown a better response as compared to the control individuals. It, thus appears that while the basal levels of the UV DNase is lower in DS subjects at all the ages studied, the fold increase in their activities due to DNA damage inflicted by UV (20 J/m^2) is in the normal range. However, no further stimulation of activity was observed when the dose was increased to 40 J/m^2 unlike in the normal subjects. Actually there was reduction in the fold increase. It is concluded, therefore, that response to DNA damage induced by higher doses of UV (40 J/m^2) is inadequate (Figure 12). The pattern of results seen when the lymphocytes were treated with MNNG is similar to that noticed with 20 J/m^2 UV stimulation (Table 14 and Figure 13).

The activities of the AP DNase in lymphocytes of DS and normal subjects with and without challenge of UV or MNNG are shown in Tables 15 and 16, and

Table13: BASAL AND INDUCED (UV) ACTIVITY OF UV DNase IN PERIPHERAL LYMPHOCYTES

		FOLD INCREASE	
SUBJECTS	CONTROL	UV20	UV40
Group 1 : < 12 years			
DS n = 18	56.33 ±7.73*	2.06 + 0.26*	1.56 + 0.27
NHV n = 12	168.66 ±50.68	1.49 ±0.38	1.76 ±0.32
Group 2 : 13-25 years			
DS n = 36	51.64 ± 9.10*♦	1.88 ±0.35*	1.28 ± 0.21*♦
NHV n = 24	139.50 ±37.71	1.43 ±0.27	1.67 ±0.34
Group 3 : > 26 years			
DS n = 18	38.31 ± 1.53*♦♥	1.70 ± 0.30*♦	1 21 ± 0.17*♦
NHV n = 12	134.55 ±41.75	1.42±0.13	1.73 + 0.19 4

Activities are expressed as μg DNA-Phosphorous liberated/mg DNA and the induced activities as fold increase over the basal value. Values represent the average of n individual observations in each group. Substrate used is UV irradiated calf thymus DNA [$2x10^4 J/m^2$]. Assay conditions are as described earlier by Rao et al. *(1996).* * These values are significantly different from those of corresponding age matched normal group at p value < 0.05. • These values are significantly lower than those of the corresponding Group 1, and ♥ these of the corresponding Group 2 , at a p value < 0.05. The actual values after UV exposure are shown in Figure 12.

DS = Down syndrome cases NHV = Normal healthy volunteers.

Figure 11

Basal activity of DNA-repair enzyme, UV DNase, in peripheral lymphocytes of Down syndrome patients (DS) and their age- and sex-matched normal healthy volunteers (NHV) as a function of age. Linear regression analyses are shown. The dotted lines represent the 95% confidence limits. Other details are the same as in Figure 8.

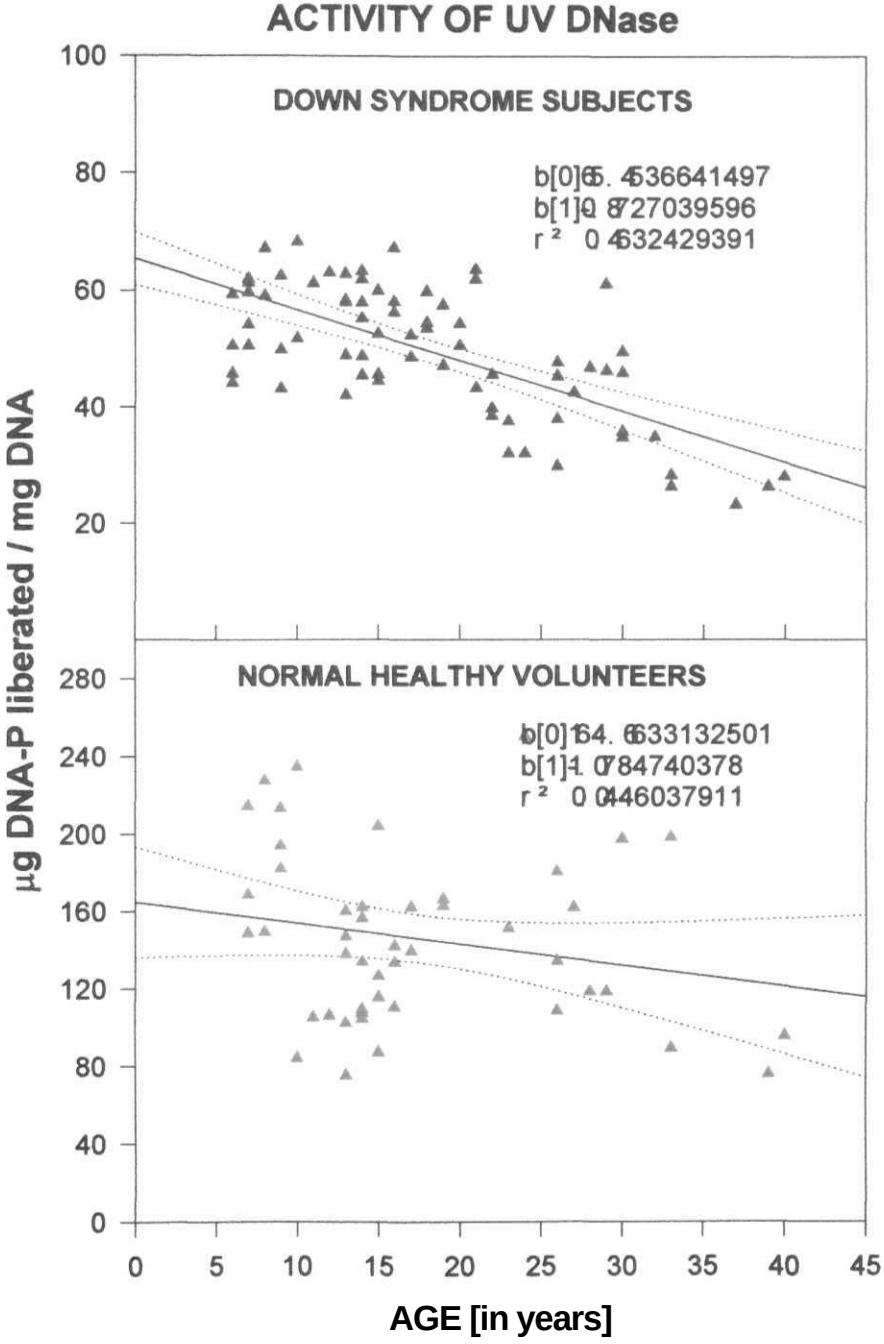

ACTIVITY OF UV DNase
DOWN SYNDROME SUBJECTS
b[0] 65. 4536641497
b[1] -0. 8727039596
r² 0.4632429391
NORMAL HEALTHY VOLUNTEERS
b[0] 64. 6633132501
b[1] -1. 0784740378
r² 0.0446037911
µg DNA-P liberated / mg DNA
AGE [in years]
100
80
60
40
20
280
240
200
160
120
80
40
0
0
5
10
15
20
25
30
35
40
45

Figure 12

Basal and UV (254 nm) induced activity of DNA-repair enzyme, UV Dnase, in peripheral lymphocytes of Down syndrome patients (DS) and their age- and sex-matched normal healthy volunteers (NHV). The other details are the same as in Figure 9.

Activity of UV DNase

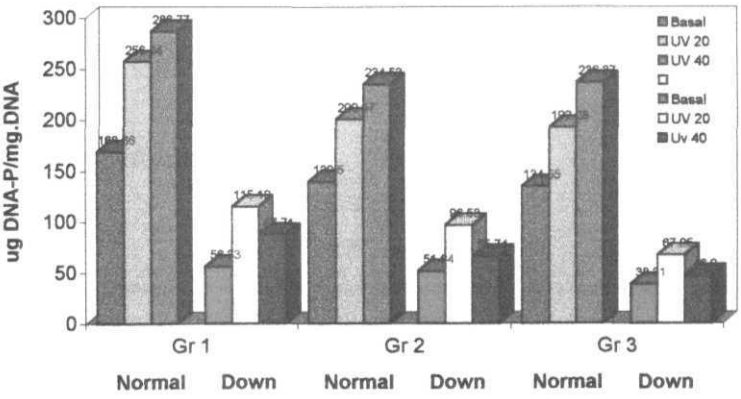


Table14: BASAL AND INDUCED (MNNG) ACTIVITY OF UV DNase IN PERIPHERAL LYMPHOCYTES

SUBJECTS	CONTROL	FOLD INCREASE MNNG
Group 1 : < 12 years		
DS n = 18	56.33+7.73*	1.69 ±0.22*
NHV n = 12	168.66 ±50.68	1.39 ±0.13
Group 2 : 13 - 25 years		
DS n = 36	51.64 ± 9.10*	1.49 ±0.29*
NHV n = 24	139.50 ±37.71	1.28 ±0.23
Group 3 : > 26 years		
DS n = 18	38.31 ±10.30*♦♥	1.32 ± 0.14 ♦♥
NHV n = 12	134.55 ±41.75	1.31 ±0.09

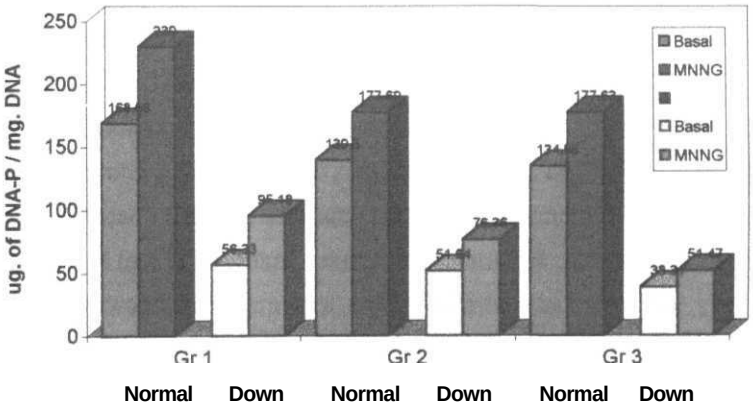
* These values are significantly different from those of corresponding age matched normal group at a p value < 0.05. Other details are the same as in Table 13. • These values are significantly lower than those of the corresponding Group 1, and ♥ these of the corresponding Group 2 , at a p value < 0.05. The actual values after MNNG treatment are shown in Figure 13.

DS = Down syndrome cases NHV = Normal healthy volunteers.

Figure 13

Basal and MNNG induced activity of DNA-repair enzyme, UV Dnase, in peripheral lymphocytes of Down syndrome patients (DS) and their age- and sex-matched normal healthy volunteers (NHV). The other details are the same as in Figure 9.

Activity of UV DNase



Figures 14 to 16. In line with the results obtained with respect to UV DNase, the AP DNase activities were also found to be similarly affected by this chromosomal disorder.

DNA repair in mammalian cells is a complex process involving many gene products *(Thompson, 1989).* The overall process consists of recognition of the damage, excision of the damaged portion, resynthesis of the excised portion and finally ligation of the last nucleotide gap. The resynthesis of the excised portion is carried out by the repair polymerases. Mammalian cells are known to possess 5 different DNA-polymerases a, (alpha), p (beta), *y* (gamma), 5 (delta), and e (epsilon) *(Fry and Loeb, 1986; Burgers, 1989; Burgers et ai, 1990).* Although every known polymerase has been shown to be involved at least to some extent both in DNA replicative and repair mechanisms, it is generally accepted polymerase p and e are more closely associated with atleast those forms of DNA-repair involving a short patch resynthesis *(Wang and Korn, 1980; Singhal et ai, 1995; Sancar, 1995).* Therefore the DNA polymerase activities in the lymphocytes of test and control subjects were studied. Attempts were made to distinguish between polymerase β and e activities as also total polymerase activity by employing reported optimal conditions for assaying these enzymes.

The total DNA polymerases activities are shown in Tables 17 and 18 and Figures 17 to 19. The basal activities are always lower in DS subjects and when exposed to UV at two different doses (Table 17) the fold increase in activity is once again lower in DS subjects at all the three age groups (40 J/m^2). However, at a lesser dose of UV (20 J/m^2) only the Group 3 DS subjects showed significantly lesser fold increase. Results with regard to MNNG treatment are similar to the 20J/m^2 UV dose in that only in Group 3 age (beyond 25 years) the DS subjects are unable to achieve the stimulation comparable to normal.

Table15: BASAL AND INDUCED (UV) ACTIVITY OF AP DNase IN PERIPHERAL LYMPHOCYTES

SUBJECTS	CONTROL	FOLD INCREASE UV20	FOLD INCREASE UV40
Group 1 : < 12 years			
DS n = 18	59.41+6.25*	1.90 ± 0.32*	1.42 ±0.24*
NHV n = 12	149.16 ±44.95	1.49 ±0.23	1.69 ±0.26
Group 2 : 13 - 25 years			
DS n = 36	51.07 ±10.84*♦	1.79 ±0.30*	1.27 ± 0.16*♦
NHV n = 24	137.96+39.54	1.54 ±0.28	1.80 + 0.32
Group 3 : > 26 years			
DS n = 18	35.30 ± 8.15*♦♥	1.68 ± 0.17♦	1.27 + 0.19*♦
NHV n = 12	135.55+33.96	1.51+0.37	1.81 ±0.35

Activities are expressed as μg DNA-Phosphorous liberated/mg DNA and the induced activities as fold increase over the basal value. Values represent the average of n individual observations in each group. Substrate used is calf thymus DNA subjected to depurination [70°C for 15 mins, pH 5.0]. Assay conditions are as described earlier by Rao et al. *(1996).* * These values are significantly different from those of corresponding age matched normal group at a p value < 0.05. • These values are significantly lower than those of the corresponding Group 1, and ♥ these of the corresponding Group 2 , at a p value < 0.05. The actual values after UV exposure are shown in Figure 15.

DS = Down syndrome cases NHV = Normal healthy volunteers.

Figure 14

Basal activity of DNA-repair enzyme, AP DNase, in peripheral lymphocytes of Down syndrome patients (DS) and their age- and sex-matched normal healthy volunteers (NHV) as a function of age. Linear regression analyses are shown. The dotted lines represent the 95% confidence limits. Other details are the same as in Figure 8.

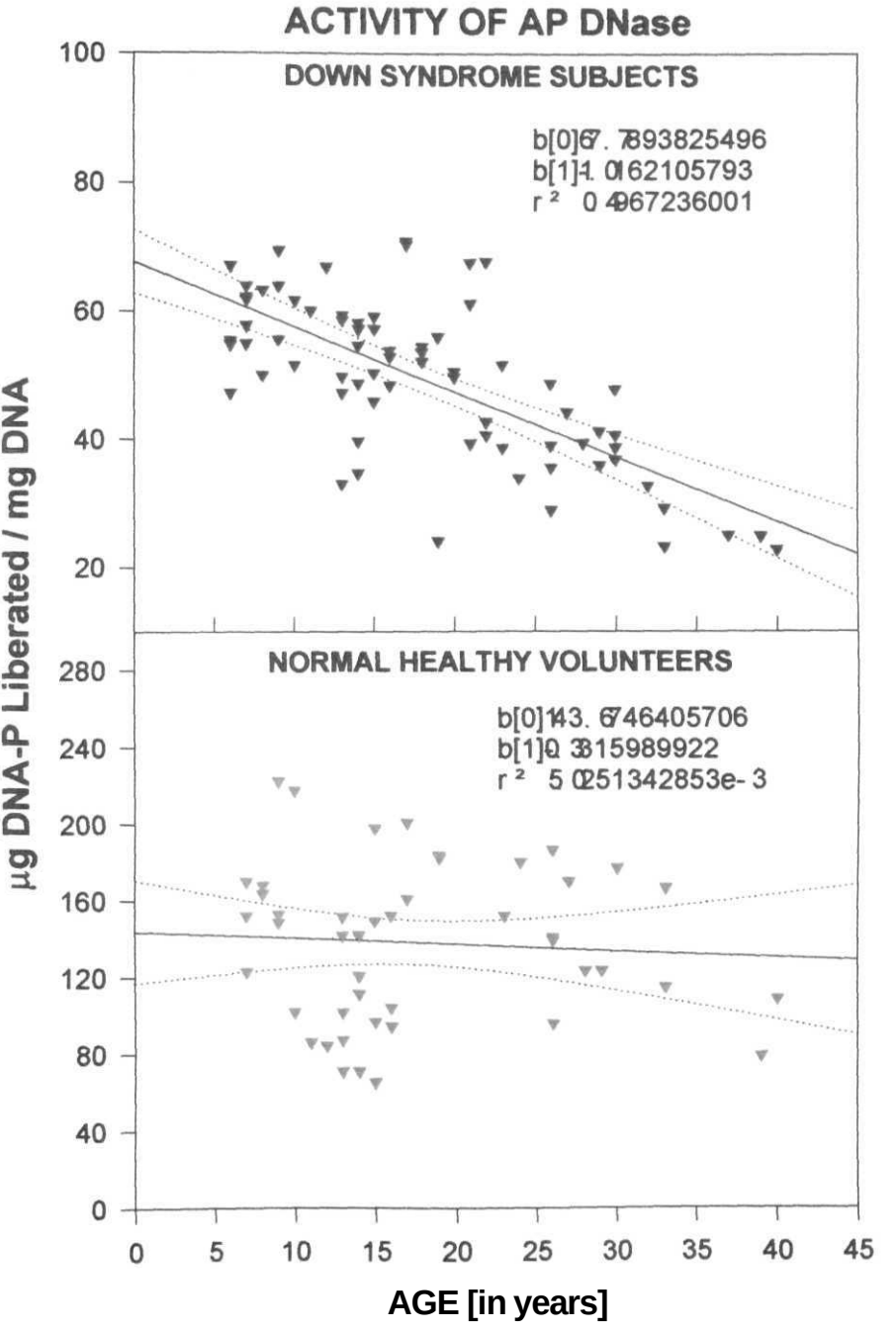

ACTIVITY OF AP DNase
DOWN SYNDROME SUBJECTS
b[0]67. 7893825496
b[1]-1. 0162105793
r² 0 4967236001
NORMAL HEALTHY VOLUNTEERS
b[0]143. 6746405706
b[1]0. 3315989922
r² 5 0251342853e- 3
µg DNA-P Liberated / mg DNA
AGE [in years]
100
80
60
40
20
280
240
200
160
120
80
40
0
0
5
10
15
20
25
30
35
40
45

Figure 15

Basal and UV (254 nm) induced activity of DNA-repair enzyme, AP Dnase, in peripheral lymphocytes of Down syndrome patients (DS) and their age- and sex-matched normal healthy volunteers (NHV) The other details are the same as in Figure 9.

Activity of AP DNase

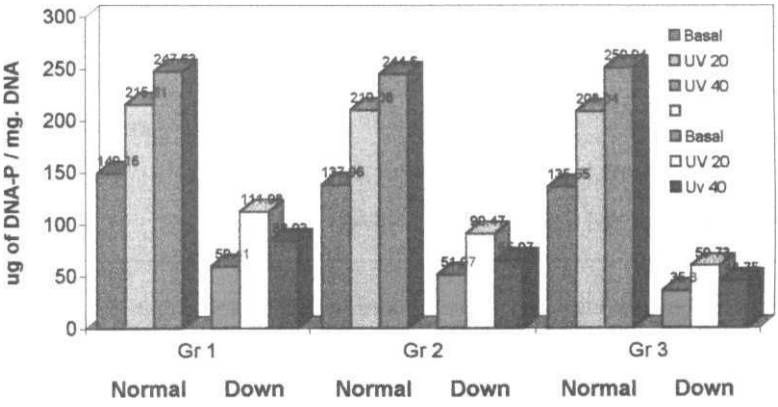


Table16: BASAL AND INDUCED (MNNG) ACTIVITY OF AP DNase IN PERIPHERAL LYMPHOCYTES

SUBJECTS	CONTROL	FOLD INCREASE MNNG
Group 1 : < 12 years		
DS n = 18	59.41 ± 6.25*	1.56 ± 0.18*
NHV n = 12	149.16+44.95	1.36 + 0.14
Group 2 : 13 - 25 years		
DS n = 36	51.07 ±10.84*♦	1.42 ± 0.20♦
NHV n = 24	137.96 ±39.54	1.40 + 0.25
Group 3 : > 26 years		
DS n = 18	35.30 ± 8.15*•♥	1.39 + 0.14♦
NHV n = 12	135.55+33.96	1.35 ±0.19

* These values are significantly different from those of corresponding age matched normal group at a p value < 0.05. Other details are the same as in Table 15. • These values are significantly lower than those of the corresponding Group 1, and ♥ these of the corresponding Group 2, at a p value < 0.05. The actual values after MNNG treatment are shown in Figure 16.

DS = Down syndrome cases NHV = Normal healthy volunteers.

Figure 16

Basal and MNNG induced activity of DNA-repair enzyme, AP Dnase, in peripheral lymphocytes of Down syndrome patients (DS) and their age- and sex-matched normal healthy volunteers **(NHV).** The other details are the same as in Figure 9.

Activity of AP DNase

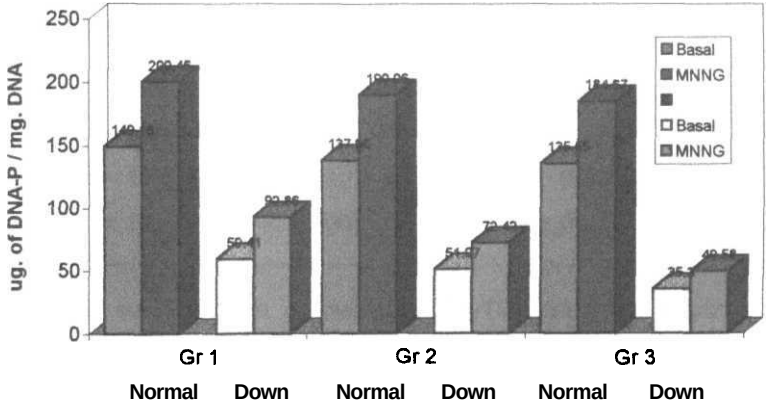


Table17: BASAL AND INDUCED (UV) ACTIVITY OF TOTAL DNA POLYMERASE IN PERIPHERAL LYMPHOCYTES

SUBJECTS	CONTROL	FOLD INCREASE UV20	FOLD INCREASE UV40
Group 1 : < 12 years			
DS n = 18	2125 ±225*	2.05 ±0.20	1.43 ±0.14*
NHV n = 12	3974 +2201	2.21 ± 0.45	2.56 ± 0.44
Group 2 : 13 - 25 years			
DS n = 36	1548 ± 404*♦	1.87 ± 0.23♦	1.32 + 0.16*♦
NHV n = 24	3167 ±575	1.96 ±0.37	2.27 ±0.44
Group 3 : > 26 years			
DS n = 18	985 ± 112*♦♥	1.54 ± 0.09*♦♥	1.17 ± 0.08*♦♥
NHV n = 12	2962 ±706	1.83 ± 0.25♦	2.29 ±0.23

Activities are expressed as picomoles [^{3}H]-TMP incorporated into acid insoluble fraction/mg DNA/hour and the induced activities as fold increase over the basal value. Values represent the average of n individual observations in each group. Substrate used is activated calf thymus DNA. Assay conditions are as described earlier by Prapurna and Rao *(1996).* * These values are significantly lower than those of corresponding age matched normal group at a p value < 0.05. • These values are significantly lower than those of the corresponding Group 1, and ♥ these of the corresponding Group 2 , at a p value < 0.05. The actual values after UV exposure are shown in Figure 18.

DS = Down syndrome cases NHV = Normal healthy volunteers.

Figure 17

Basal activity of DNA-repair enzyme, Total **DNA polymerses**, in peripheral lymphocytes of Down syndrome patients (DS) and their age- and sex-matched normal healthy volunteers (NHV) as a function of age. Linear regression analyses are shown. The dotted lines represent the 95% confidence limits. Other details are the same as in Figure 8.

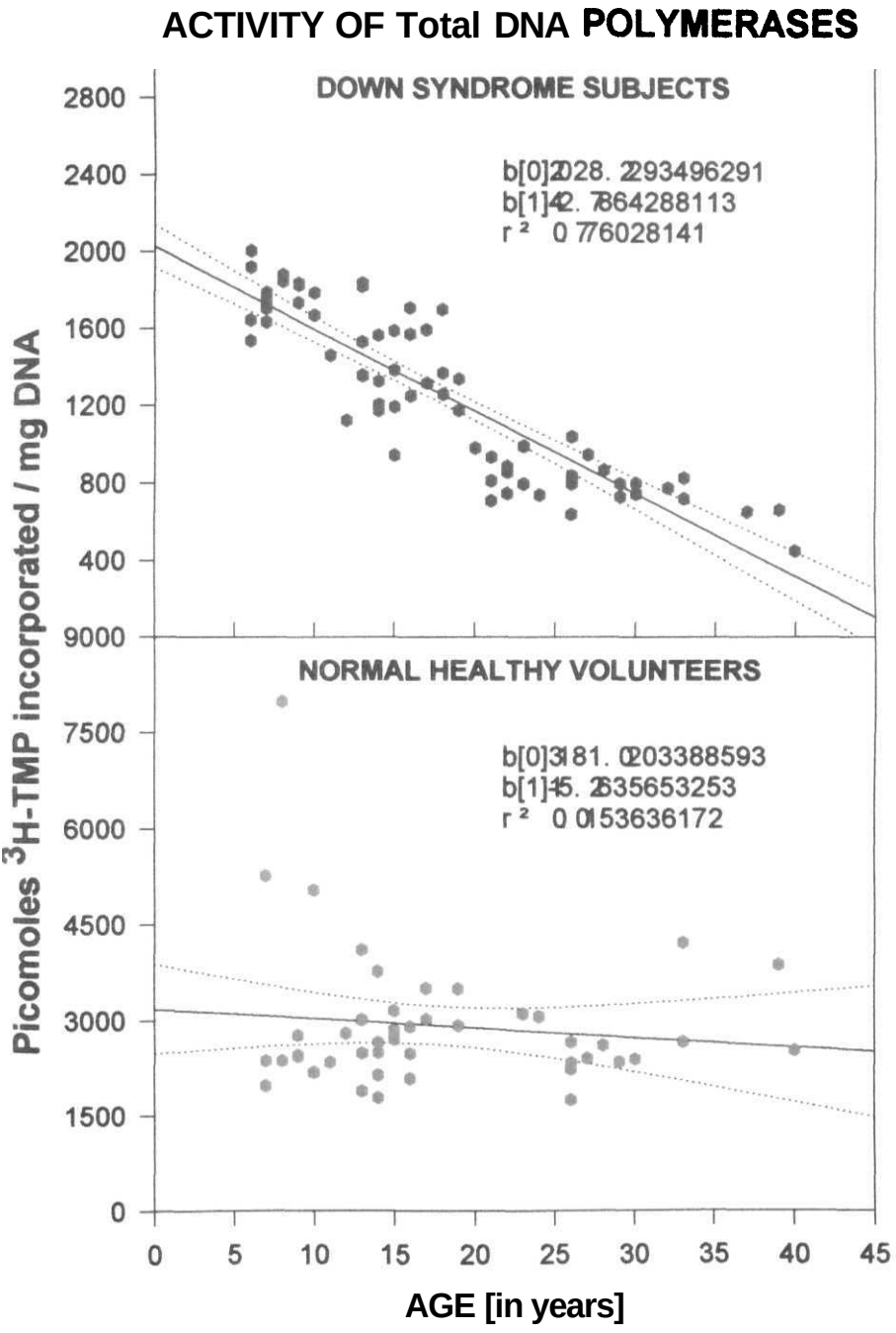

ACTIVITY OF Total DNA POLYMERASES
DOWN SYNDROME SUBJECTS
b[0]2028. 2293496291
b[1]42. 7864288113
r² 0 776028141
NORMAL HEALTHY VOLUNTEERS
b[0]3181. 0203388593
b[1]45. 2635653253
r² 0 0153636172
Picomoles ³H-TMP incorporated / mg DNA
2800
2400
2000
1600
1200
800
400
9000
7500
6000
4500
3000
1500
0
0
5
10
15
20
25
30
35
40
45
AGE [in years]

Figure 18

Basal and UV (254 nm) induced activity of DNA-repair enzyme, Tota DNA Polymerases, in peripheral lymphocytes of Down syndrome patients (DS) and their age- and sex-matched normal healthy volunteers (NHV). The other details are the same as in Figure 9.

Activity of Total DNA-Polymerase

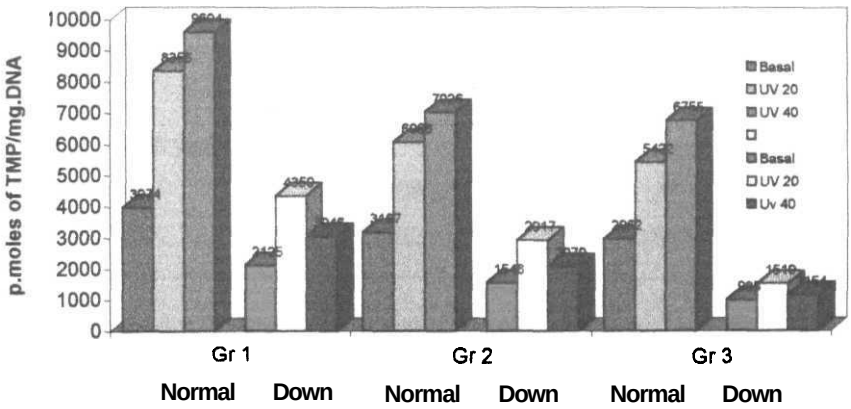


Table18: BASAL AND INDUCED (MNNG) ACTIVITY OF TOTAL DNA POLYMERASE IN PERIPHERAL LYMPHOCYTES

SUBJECTS	CONTROL	FOLD INCREASE MNNG
Group 1 : < 12 years		
DS n = 18	2125 + 225*	1.68 + 0.14
NHV n = 12	3974 ±2201	1.82 ±0.36
Group 2 : 13 - 25 years		
DS n = 36	1548+ 404*♦	1.53 + 0.18♦
NHV n = 24	3167±575	1.56 ± 0.31 •
Group 3 : > 26 years		
DS n = 18	985+ 112*♦♥	1.29 + 0.08*♦♥
NHV n = 12	2962+706	1.50 ±0.20 ♦

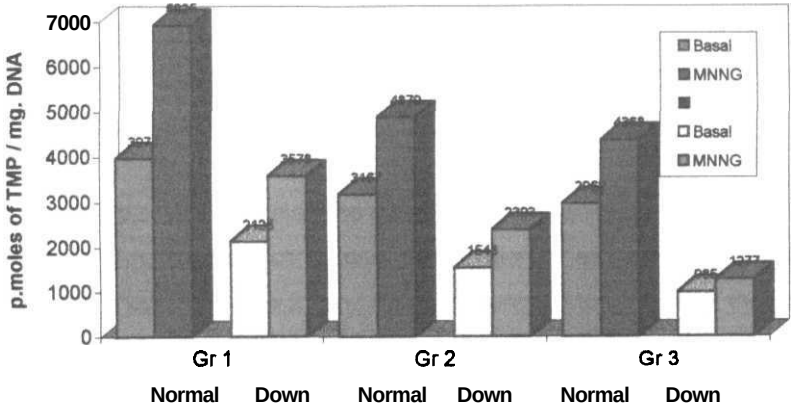
* These values are significantly lower than those of corresponding age matched normal group at a p value < 0.05. Other details are the same as in Table 17. • These values are significantly lower than those of the corresponding Group 1, and ♥ these of the corresponding Group 2 , at a p value < 0.05. The actual values after MNNG treatment are shown in Figure 19.

DS = Down syndrome cases NHV = Normal healthy volunteers.

Figure 19

Basal and **MNNG** induced activity of DNA-repair enzyme, Total DNA Polymerases, in peripheral lymphocytes of Down syndrome patients (DS) and their age- and sex-matched normal healthy volunteers (NHV). The other details are the same as in Figure 9.

Activity of Total DNA Polymerase



When the **DNA** polymerase assay was performed under conditions optimal **for** polymerase β, the activities in the untreated cells of DS subjects **were** distinctly lower than the corresponding controls at all age groups studied. **This** pronounced decreasing trend in the activity with age in Down patients is depicted in Figure 20. The fold increase in this activity due to UV challenge at a dose **of** 20J/m^2 was similar in both test and control except at the 3rd group age of beyond 25 years when the DS subjects' response was limited. Furthermore, UV dose of 40J/m^2 has actually decreased the DS subjects' response, thus making these values distinctly lower than the controls throughout the ages examined (Table 19 and Figure 21). On the other hand, **MNNG** treatment resulted in similar response in both the DS and normal subjects (Table 20 and Figure 22). It is possible that polymerase β may be more involved in repairing **MNNG** induced damage and there seems to be no serious deficiency in this form of repair process in DS subjects.

Attempts were also made to distinguish the DNA-polymerase e activity by adopting optimal reported conditions of incubation for assaying this activity. The results are shown in Tables 21 and 22 and Figures 23 to 25. Firstly, the e activity is higher in DS subjects upto the second group (25 years) and beyond this age (Group 3), the levels are similar to controls. There is an age-dependent decrease in this activity in DS subjects but not in control subjects. At lower dose of UV the induction in the activity in test subjects is either the same or slightly better, but this induction could not be sustained at higher dose of UV (40 J/m^2) and the fold increase therefore, slided down as compared to that in controls. **MNNG** treatment, like in the case of polymerase (3, resulted in similar induction of this activity in both groups of individuals.

It is rather intriguing to note that the percentage of e polymerase out of the total polymerases activity (see Table 17) in DS subjects is 3 times more than that in controls. In DS subjects the percentage ranged from 16 to 18 while in

Figure 20

Basal activity of DNA-repair enzyme, DNA Polymerase β, in peripheral lymphocytes of Down syndrome patients (DS) and their age- and sex-matched normal healthy volunteers (NHV) as a function of age. Linear regression analyses are shown. The dotted lines represent the 95% confidence limits. Other details are the same as in Figure 8.

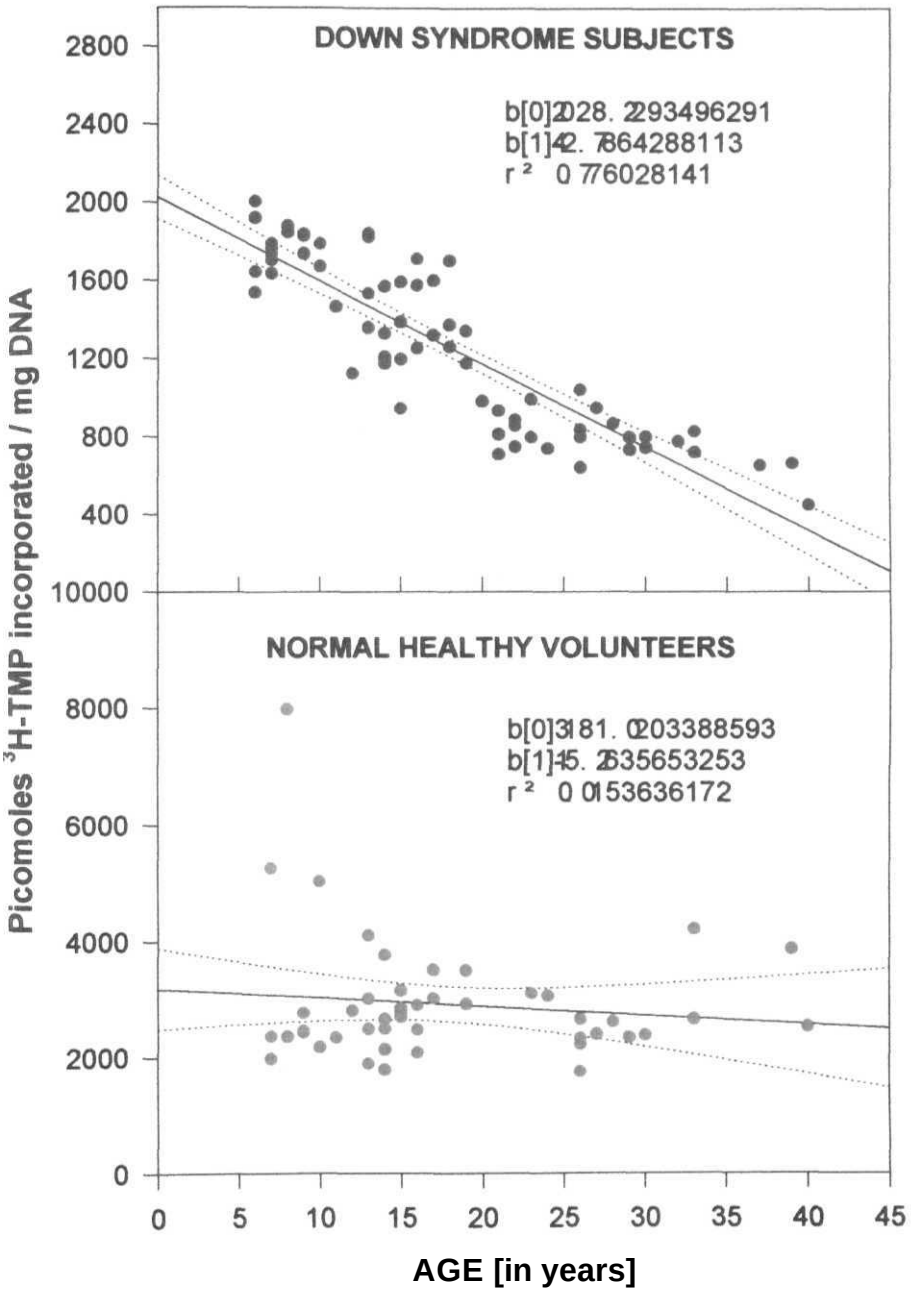

ACTIVITY OF POLYMERASE p
DOWN SYNDROME SUBJECTS
b[0]2028. 2293496291
b[1]42. 7864288113
r ² 0 776028141
NORMAL HEALTHY VOLUNTEERS
b[0]3181. 0203388593
b[1]45. 2635653253
r ² 0 0153636172
Picomoles ³H-TMP incorporated / mg DNA
AGE [in years]
2800
2400
2000
1600
1200
800
400
10000
8000
6000
4000
2000
0
0
5
10
15
20
25
30
35
40
45

Table19: BASAL AND INDUCED (UV) ACTIVITY OF DNA POLYMERASE β IN PERIPHERAL LYMPHOCYTES

SUBJECTS	CONTROL	FOLD INCREASE UV20	FOLD INCREASE UV40
Group 1 : < 12 years			
DS n = **18**	1720 ± 199*	2.03 + 0.26	1.35 ± 0.17*
NHV n = 12	3325 ±1822	2.15 + 0.46	2.48 + **0.49**
Group 2 : 13 - 25 years			
DS n = 36	1247 ± 327*♦	1.90 + 0.17♦	1.35 ± 0.16*
NHV n = 24	2830+559	1.88 ± 0.31 •	2.12 + 0.29 •
Group 3 : > 26 years			
DS n = **18**	766+ 127*♦♥	1.49 + 0.12*♦♥	1.15 + 0.10*♦♥
NHV n = 12	2663 ± 690	1.73 + 0.22 4	2.11 ±0.21 4

Activities are expressed as picomoles [^{3}H]-TMP incorporated into the acid insoluble fraction/mg DNA/hour and the induced activities as fold increase over the basal value. Values represent the average of n individual observations in each group. Substrate used is activated calf thymus DNA. Assay conditions are as described earlier by Prapurna and Rao *(1996).* * These values are significantly lower than those of corresponding age matched normal group at a p value < 0.05. • These values are significantly lower than those of the corresponding Group 1, and ♥ these of the corresponding Group 2 , at a p value < 0.05. The actual values after UV exposure are shown in Figure 21.

DS = Down syndrome cases NHV = Normal healthy volunteers.

Figure 21

Basal and **UV** (254 nm) induced activity of DNA-repair enzyme, DNA Polymerase β, in peripheral lymphocytes of Down syndrome patients (DS) and their age- and sex-matched normal healthy volunteers (NHV). The other details are the same as in Figure 9.

Activity of DNA Polymerase beta

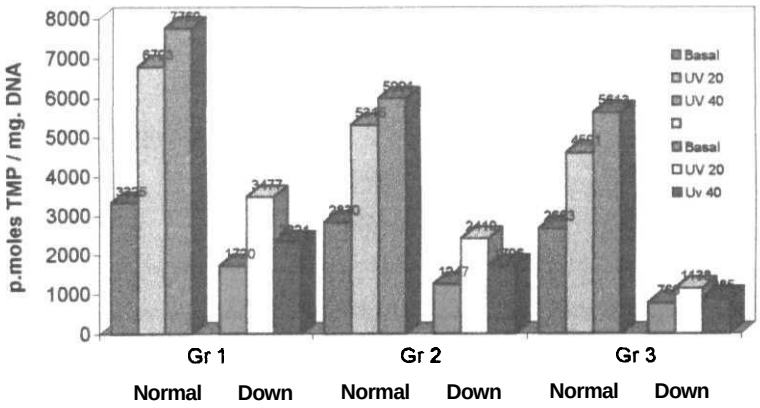


Table20: BASAL AND INDUCED (MNNG) ACTIVITY OF DNA POLYMERASE β IN PERIPHERAL LYMPHOCYTES

SUBJECTS	CONTROL	FOLD INCREASE MNNG
Group 1 : < 12 years		
DS n = 18	1720+ 199*	1.59 ±0.18
NHV n = 12	3325+1822	1.73 ±0.36
Group 2 : 13 - 25 years		
DS n = 36	1247 ± 327*♦	1.55±0.15
NHV n = 24	2830 ± 559	1.49 ± 0.25 •
Group 3 : > 26 years		
DS n = 18	766 ± 127*♦♥	1.28 ± 0.12 ♦♥
NHV n = 12	2663 ±690	1.35 ± 0.22 ♦

* These values are significantly lower than those of corresponding age matched normal group at a p value < 0.05. Other details are the same as in Table 19. • These values are significantly lower than those of the corresponding Group 1, and ♥ these of the corresponding Group 2 , at a p value < 0.05. The actual values after MNNG treatment are shown in Figure 22.

DS = Down syndrome cases NHV = Normal healthy volunteers.

Figure 22

Basal and **MNNG** induced activity of DNA-repair enzyme, DNA Polymerase β, in peripheral lymphocytes of Down syndrome patients (DS) and their age- and sex-matched normal healthy volunteers **(NHV).** The other details are the same as in Figure 9.

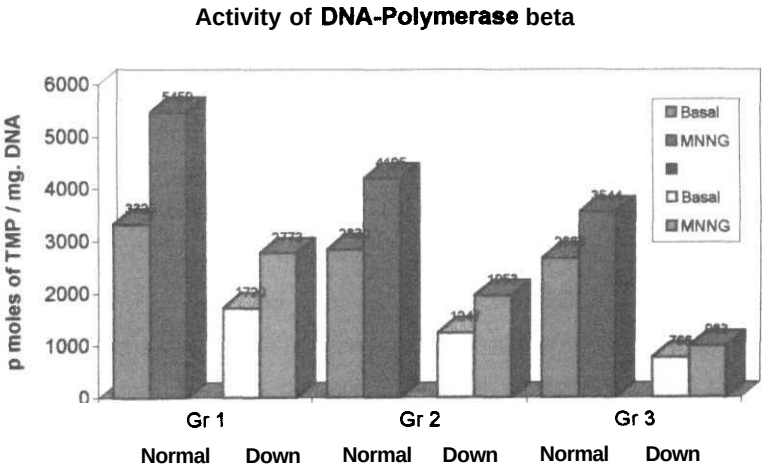

Activity of DNA-Polymerase beta
p moles of TMP / mg. DNA
6000
5000
4000
3000
2000
1000
0
Basal
MNNG
Basal
MNNG
Gr 1
Gr 2
Gr 3
Normal
Down
Normal
Down
Normal
Down

Table21: BASAL AND INDUCED (UV) ACTIVITY OF DNA POLYMERASE e IN PERIPHERAL LYMPHOCYTES

SUBJECTS	CONTROL	FOLD INCREASE UV20	UV40
Group 1 : < 12 years			
DS n = 18	375 ±53*	1.98 ±0.27*	1.37 ± 0.16*
NHV n = 12	124 ±33	1.60 ±0.33	2 09 ±0.36
Group 2 : 13 - 25 **years**			
DS n = 36	248 ± 47*♦	1.78 ± 0.27 ♦	1.29 + 0.17*
NHV n = 24	174 ± 61 ♦	1.87 ±0.85	2.68 ±2.32
Group 3 : > 26 **years**			
DS n = 18	179±21 ♦♥	1.76 + 0.20* •	1.28 ± 0.10*♦
NHV n = **12**	167 ±39	1.42 ±0.20	1.82 ± 0.26 ♦

Activities are expressed as picomoles [^{3}H]-TMP incorporated into acid insoluble fraction/mg DNA/hour and the induced activities as fold increase over the basal value. Values represent the average of n individual observations in each group. Substrate used is Poly dA. Oligo $dT_{[12\text{-}18]}$. Assay conditions are as described earlier by Prapurna and Rao *(1996).* * These values are significantly different from those of corresponding age matched normal group at a p value < 0 05. • These values are significantly lower than those of the corresponding Group 1, and ♥ these of the corresponding Group 2 , at a p value < 0.05. The actual values after UV exposure are shown in Figure 24.

DS = Down syndrome cases NHV = Normal healthy volunteers.

Figure 23

Basal activity of DNA-repair enzyme, DNA Polymerase e, in peripheral lymphocytes of Down syndrome patients (DS) and their age- and sex-matched normal healthy volunteers (NHV) as a function of age. Linear regression analyses are shown. The dotted lines represent the 95% confidence limits. Other details are the same as in Figure 8.

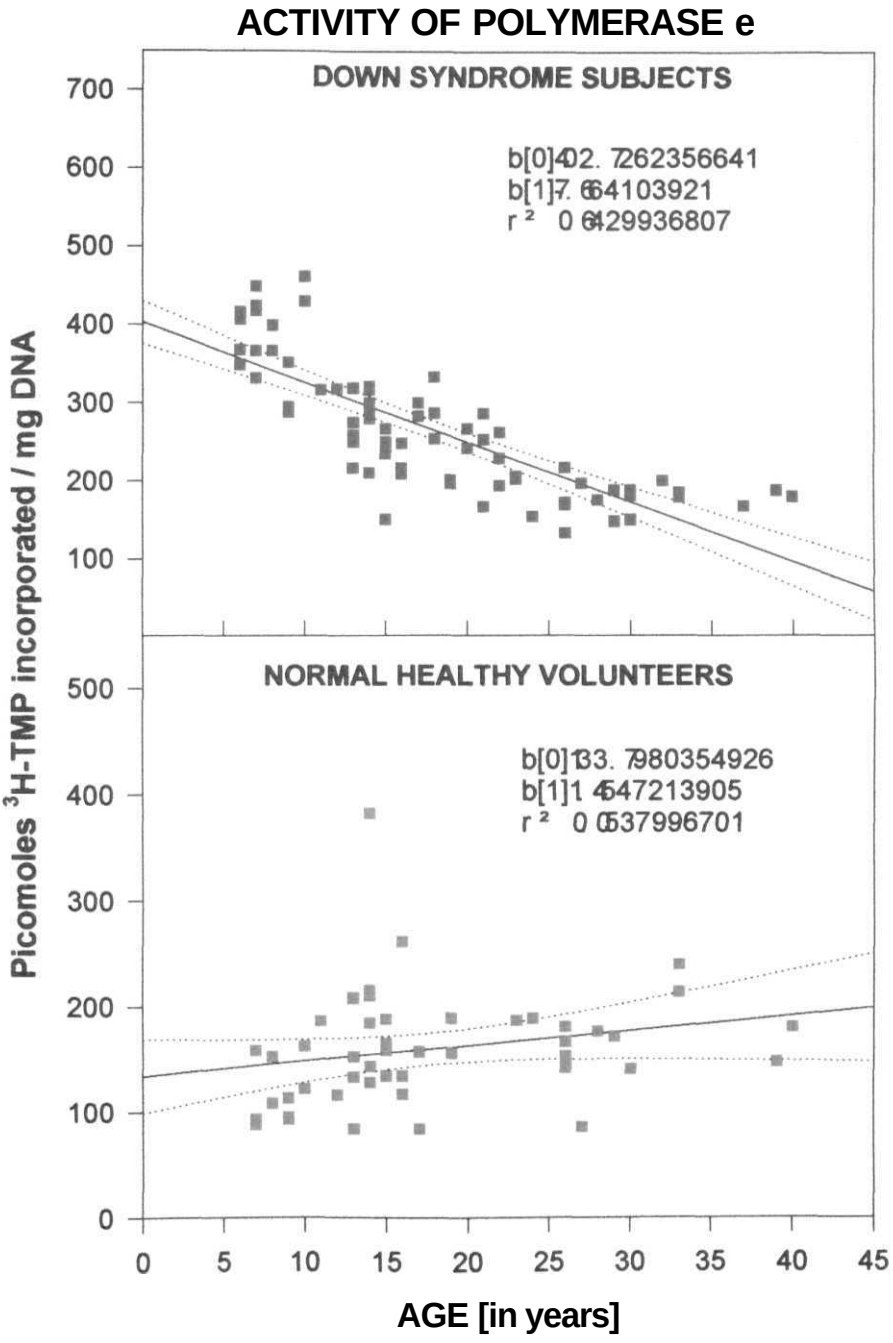

ACTIVITY OF POLYMERASE e
DOWN SYNDROME SUBJECTS
b[0]402. 7262356641
b[1]7. 664103921
r ² 0 6429936807
NORMAL HEALTHY VOLUNTEERS
b[0]133. 7980354926
b[1]1 4547213905
r ² 0 0537996701
Picomoles ³H-TMP incorporated / mg DNA
AGE [in years]
0
100
200
300
400
500
600
700
0
5
10
15
20
25
30
35
40
45

Figure 24

Basal and UV (254 nm) induced activity of DNA-repair enzyme, DNA Polymerase e, in peripheral lymphocytes of Down syndrome patients (DS) and their age- and sex-matched normal healthy volunteers (NHV). The other details are the same as in Figure 9.

Activity of DNA-Polymerase epsilon

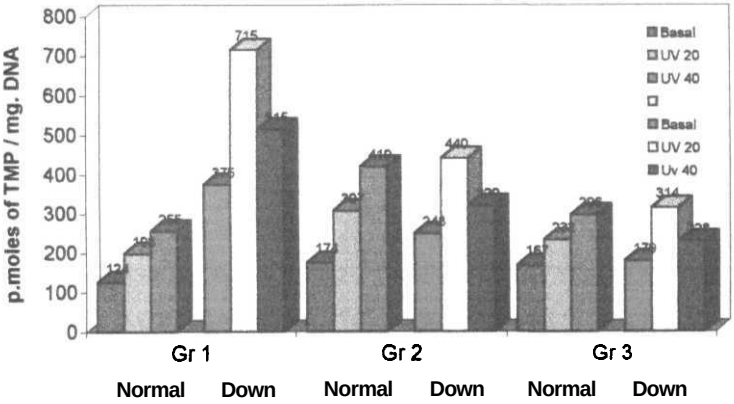


Table22: BASAL AND INDUCED (MNNG) ACTIVITY OF DNA POLYMERASE e IN PERIPHERAL LYMPHOCYTES

		FOLD INCREASE
SUBJECTS	**CONTROL**	**MNNG**
Group 1 : < 12 years		
DS n = 18	375 + 53*	1.63 + 0.24
NHV n = 12	124 ±33	1.64 + 0.34
Group 2 : **13** - **25 years**		
DS n = 36	248 + 47*♦	1.51 ±0.24
NHV n = 24	174 + 61 •	1.58 + 0.64
Group 3 : > 26 **years**		
DS n = 18	179 + 21*♦♥	1.43 ± 0.21♦
NHV n = 12	167 ±39	1.39 ±0.27

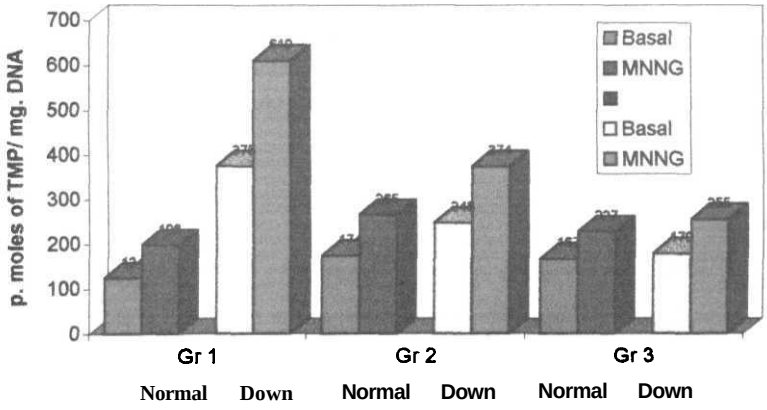
* These values are significantly lower than those of corresponding age matched normal group at a p value < 0.05. Other details are the same as in Table 21. • These values are significantly lower than those of the corresponding Group 1, and ♥ these of the corresponding Group 2 , at a p value < 0.05. The actual values after MNNG treatment are shown in Figure 25.

DS = Down syndrome cases NHV = Normal healthy volunteers.

Figure 25

Basal and MNNG induced activity of DNA-repair enzyme, DNA Polymerase ε, in peripheral lymphocytes of Down syndrome patients (DS) and their age- and sex-matched normal healthy volunteers (NHV). The other details are the same as in Figure 9.

Activity of DNA-Polymerase epsilon



normals it is only 3 to 6 at different ages. It is true that the assays were done with crude extracts and the conditions adopted to differentiate the different polymerases activities are not absolutely specific and in each of the incubation conditions all the polymerases must have been expressing their activity to varying degrees. Nevertheless, the data in Table 21 do seem to indicate that in DS subjects, e polymerase activity is retained at adequate levels.

In all the above studies the DNA-repair markers were also examined after the lymphocytes were given a mitogenic stimulus with phytohemeagglutinin. It was however found that lymphocytes from Down syndrome patients have shown very little response to such stimulus and therefore the attempts were abandoned. It may be mentioned here that Agarwal et al *(1970)* had shown several years ago that lymphocytes of Down patients respond very weakly to phytohemeagglutinin stimulus as judged by the DNA-polymerase activity as a marker of replication.

DISCUSSION

Patients with Down Syndrome have an extra chromosome 21 and this could lead to a disturbance in the normal gene dosage. The basis of the disease is presumably due to quantitative differences in expression of many genes located on the 21st chromosome and perhaps affecting the expression of genes on other chromosomes, rather than a specific qualitative gene defect. That trisomy 21 is compatible with life at all may be because the chromosome 21 is one of the smallest chromosomes. It appears to have the least amount of active genetic material of any of the autosomes *(Martin and Hoehn, 1974).* However, DS seems to exhibit maximum number of premature aging characteristics and only a small percentage of these patients are known to survive beyond 40 years of age *(Smith and Berg, 1976).*

Progress in understanding the genetic mechanisms underlying aging is likely to come from elucidation of the molecular defect that results in premature aging syndromes. While categorising the aging characteristics of various genetic and chromosomal disorders, Martin *(1977)* placed Down syndrome at the top of the list since this syndrome shows maximum number of accelerated aging symptoms. Other genetic disorders like Cockayne's syndrome and Ataxia Telangiectasia, Werner's syndrome, Progeria and Turner's syndrome *(Friedberg, 1985; Warner and Price, 1989; Bohr et al., 1989; Bernstein and Bernstein, 1991)* also exhibit signs of premature aging. However, Cockayne's syndrome and Ataxia Telangiectasia are more akin to Down syndrome in that these three show the common feature of neurodegeneration as well. It thus appears that Down syndrome, Cockayne's syndrome and Ataxia Telangiectasia constitute good models to establish correlation between senescence and DNA-repair. Indeed, it has been shown by Weirich-Schwaiger and Co-workers *(1994)* that there is a striking parallelism between reduced maximal lifespan, elevated levels of spontaneous chromosomal breaks and reduced overall DNA-repair in these syndromes.

Attempts have been going on to identify the locus of DNA-repair defect in the syndromes conspicuous by accelerated aging. Cells from Ataxia Telangiectasia patients are hypersensitive to X-irradiation *(Mckinnon, 1987)* but not to UV light *(Lehmann et al., 1979).* The precise biochemical defect behind this hypersensitivity towards x-ray is still not known. On the other hand Cockayne's syndrome is characterised by extreme sensitivity to UV irradiation *(Schmickel et al., 1977).* There was some suggestion that the defect is associated with repair of actively transcribing DNA strand *(Mayne et al., 1988).* However, recent report of van Oosterwijk et al *(1996)* does not support this view. It is however, becoming increasingly clear that one of the repair steps that is impaired in these subjects is the rejoining of strand breaks *(Schwaiger et al. 1986; Squires et al., 1993).*

Efforts are also being made to delineate the defect in Down syndrome at molecular level. Towards this end, one biochemical defect that has been associated with Down syndrome is overexpression of Cu-Zn superoxide dismutase, which is likely to produce, as a product of the reaction, excessive amounts of H_2O_2 which induces oxidative damage *(Delabar et al., 1987; De La Torre et al., 1996).* Recently a new gene from Down syndrome critical region has been reported to be expressing at higher levels in the brain and heart. The gene codes for a proline-rich protein whose function is yet to be understood *(Fuentes et al., 1995).* Reduction in the activity of ribosomal genes with age is also shown in Down patients although the precise consequences of this are to be studied yet *(Borsatto and Smith, 1996).*

Since cellular aging appears to be related to and perhaps caused by diminished DNA-repair, some studies have been conducted to examine whether there is any direct relationship of DNA-repair capacity in Down syndrome subjects. For example, it has been found that ionising radiation, while inhibiting the DNA-synthesis in normal individuals, does not affect it in the fibroblasts of Down syndrome subjects. This radioresistant DNA-synthesis is considered to cause chromosomal instability *(Barenfeld et al., 1986; 1989).* However, in a different direction, normal repair of single and double strand breaks was seen in both fibroblasts *(Steiner and Wood, 1982)* and lymphocytes *(Leonard and Merz, 1982)* of DS patients. Furthermore, results concerning the repair of UV induced damage and unscheduled DNA synthesis in Down syndrome individuals are conflicting. Thus, while decreased UV induced DNA-repair in fibroblast cultures *(Rehbom and Pfeiffenberger, 1982)* and leukocytes *(Lambert et al., 1976)* of DS subjects was reported, no such impairment was noticed by Yotti et al *(1980).* Thus information regarding the precise biochemical defect in Down syndrome is scanty.

The overall results of the present investigation indicate that in DS one of the distinct biochemical effects seems to be inadequate machinery to repair the DNA-damage induced by UV at a dose of 40 J/m^2 in peripheral lymphocytes (Tables 11,13,15,17,19,21). Also, basal levels of many DNA-repair enzymes are significantly low and deteriorate with age, while in normal individuals there was hardly any decline in their activities during the same age period. These changes are also reflected in a similar fashion in the over all DNA-repair potential, the unscheduled DNA synthesis (Tables 11 and 12).

When the lymphocytes were stimulated either with UV or MNNG, all the DNA-repair parameters responded by way of increased activity but to varying degrees. In particular, polymerase β and polymerase e activities responded to a normal extent against MNNG treatment (Tables 20 and 22). However, against UV treatment the fold increase was pointedly lower in DS subjects against a dose of 40 J/m^2. Both polymerase β and polymerase e are generally considered repair enzymes more so in such repair processes when a short patch synthesis is required *(Singhal and Wilson, 1993; Singhal et al., 1995; Mozzherin and Fisher, 1996).* Since MNNG treatment results in methylated bases and repair of such damages is shown to occur via a short patch synthesis involving polymerase β *(Srivastava et al., 1995)* it may be speculated that in Down syndrome, short patch repair that involves polymerase β and e is not seriously affected. On the other hand repair of UV damage is known to require resynthesis of a long patch and for this other polymerases like a and 8 along with other factors may be required *(Waga and Stillman, 1966; Zeng et al., 1994)* and it appears that DS subjects are unable to gear up this machinery particularly when exposed to higher doses of UV (40 J/m^2). Also, the age at which the DS subjects seem to suffer a distinct decline in DNA-repair potential appears to be around 25 years. Indeed it is known that DS patients usually do not live beyond 40 years and the accelerated decline in DNA-repair capacity could be atleast one of the reasons for such premature death.

Be as it may the present results pointedly indicate a lowered overall DNA-repair potential in DS subjects and also a more rapid decline of this capacity with age. This may well be one of the major causes for accelerated aging symptoms seen in trisomy 21 syndrome. Further detailed studies on the structure and expression of different DNA polymerases, particularly of α and 6, should provide some more information at molecular level.

CHAPTER - 5

AVERAGE TELOMERIC LENGTH IN THE PERIPHERAL LYMPHOCYTES IN AGING HUMAN SUBJECTS OF DIFFERENT NUTRITIONAL STATUS AND OF DOWN SYNDROME PATIENTS

AVERAGE TELOMERIC LENGTH [ATL] IN THE PERIPHERAL LYMPHOCYTES IN AGING HUMAN SUBJECTS OF DIFFERENT NUTRITIONAL STATUS AND OF DOWN SYNDROME PATIENTS.

One of the biological phenomena that has captured the serious attention of not only scientists but also of sociologists and Governments during the past 20 years, is Aging. As already outlined in Chapter 1 of this thesis, many theories have been proposed to explain the molecular basis of the aging phenomenon *(Kanungo, 1994).*

During the past five years a new concept has emerged which adds credibility to the aging theories based on the genetic involvement. This new concept not only explains the transformation of somatic cells to malignant but also explains the onset of replicative senescence. This hypothesis which can be termed as `telomeric hypothesis of aging and immortality' is essentially based on the results suggesting that the non-coding repetitive genomic DNA (telomere) located at the tips of the eukaryotic chromosomes may have a telling role in DNA-replication (cell replication) and therefore in the phenomenon of cancer as well as aging.

Telomeres, consisting of a repetitive DNA of a discrete sequence at the ends of the chromosomes are thought to provide stability to the structure of the chromosome *(Blackburn and Szostak, 1984; Day et ai, 1993).* Telomeres also carry out another function in cells. They ensure that during the replication, the end of the linear DNA is replicated completely without any loss of terminal bases at 5'-end. Such a loss is predicted as a natural consequence of the properties of replicative machinery of conventional semiconservative replication *(Olovnikov, 1973).* The lost sequences of the telomere at each round of replication are synthesised again by an enzyme *telomerase (Harley, 1995).* If this enzyme is not present in the replicating cells, as is the case with most somatic cells *(Rao,*

1996), then each round of replication will result in shortening of the telomeric length. When this length reaches a critical level then the cells stop dividing and senescence sets in. However, tissues and cells that have unlimited potential to replicate, for example, germ cells, cancer cells, immortalised cells etc., are found to have either longer or stabilised telomeric lengths and in such cases the activity of telomerase is always found to be present *(Delange et al., 1990).*

Indeed, reduction in telomeric length has been correlated with the cellular aging. Cells with longer telomeres are found to be capable of undergoing more divisions than those with shorter telomeres *(Harley et al., 1990; Allsopp et al., 1992).* Furthermore, telomeres are shorter in older individuals than the younger individuals *(Hastie et al., 1990; Lindsey et al., 1991; Allsopp et al., 1992; Vaziri et al., 1993).*

From all this it is clear now that in eukaryotes, the telomeric length has an intimate role to play in signalling the cell either to enter senescence period or that of immortality.

In chapter 3 it has been presented that chronic restriction of dietary calorie consumption could sustain better DNA-repair capacity, which might lead to improved longevity. If this were to be the case then it would be interesting to see what would be the connection between improved longevity and the telomeric length in such individuals (persons with low and high Body Mass Index).

Similarly, in chapter 4, the results indicated that DNA-repair capacity is at a low level and deteriorates more rapidly in Down syndrome subjects in comparison with normal subjects. Since Down syndrome is typical example of accelerated aging, it would be of interest to examine the telomeric lengths in these subjects.

Therefore a preliminary study has been undertaken to examine the average telomeric length in the peripheral lymphocytes of normal, low BMI and Down syndrome subjects.

MATERIAL AND METHODS

Selection of the human subjects of various categories including Down syndrome patients has already been described in chapters 3 and 4. However, the present study concerning the telomeric length has been carried out with fewer subjects as the second approach to the subjects has largely been unsuccessful and only a few have obliged. Also this aspect has been taken up as a preliminary extension of the major theme of the thesis presented in chapters 3 and 4.

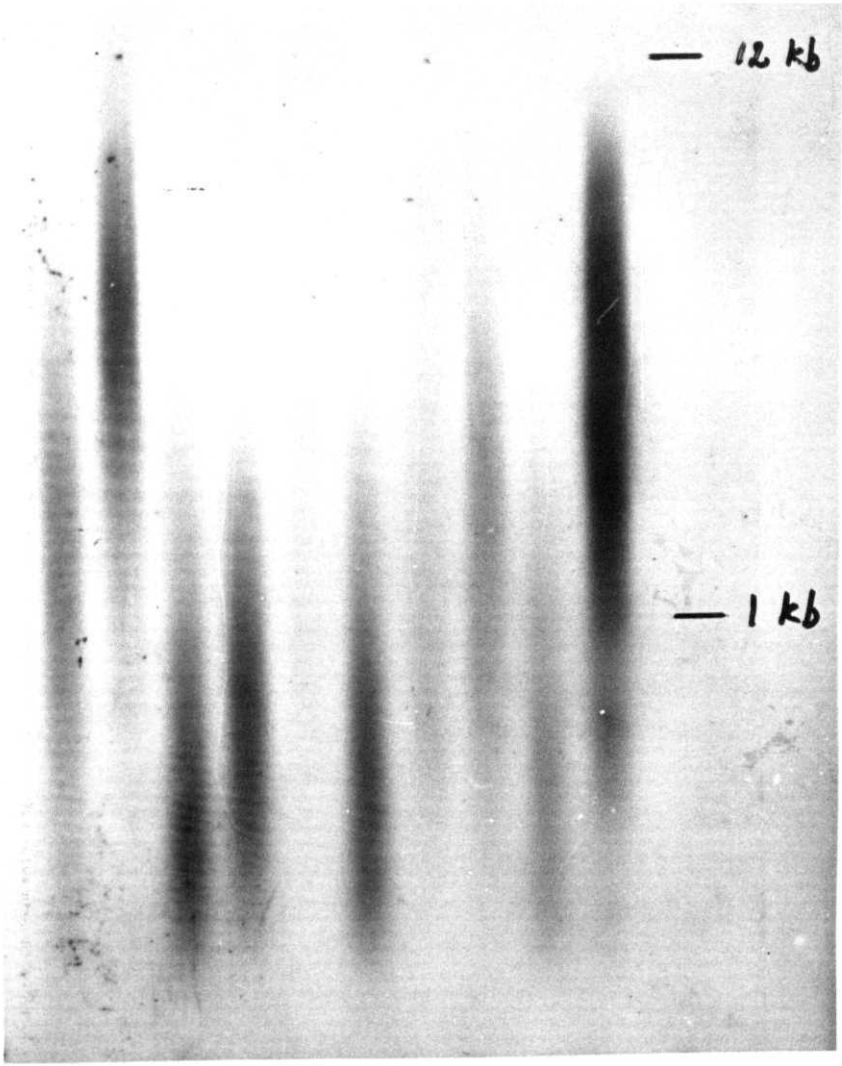
Average telomeric length determination :

The telomeric length was determined essentially as described by Kruk et al. (1995).

Briefly the lymphocytes prepared as outlined in chapter 2, were washed with Phosphate buffered saline, lysed in 10mM Tris HCl, 1mM EDTA, 0.5% SDS, 20μg/ml RNase, 0.1 mg Proteinase K per ml. DNA was isolated by standard organic extraction as described in chapter 2 and the DNA concentration was determined by absorbence at 260 nm. If the ratio of 260/280 absorbence is less than 1.7 then proteinase K digestion was repeated. The DNA was digested to completion with Hinf I as described in chapter 2. About 3 to 5 μg of restricted DNA was loaded onto 0.5% agarose gel, resolved by electrophoresis at 1V/cm for 26-30 hours in TAE buffer pH 7.5-7.8, transferred to a Hybond N+ nylon membrane and hybridised in Church hybridisation buffer (Church and Gilbert, 1984) with (γ-^{32}P)ATP 5'-end labelled TTAGGG probe. Membranes were washed in 0.5x SSC, 0.1% SDS at 42°C and telomeres were visualised by autoradiography. A typical autoradiogram is shown in Figure 26. Each lane

Figure 26

A typical autoradiogram after Southern transfer and hybridisation using the ^{32}P-$(TTAGGG)_4$ probe for determination of average telomeric length from lymphocytes of human subjects.


12 kb
1 kb

alongwith standard molecular weight markers was scanned in a computer aided UVP Gel documentation system and the average telomeric length (ATL) is calculated using the formula

$$\mathbf{ATL} = \Sigma (MW_i \times OD_i \text{ or Area}_i) / \Sigma (OD_i \text{ or Area}_i)$$

where OD, or $Area_i$ is the desitometric output at position / and MW, is the length of the DNA at position i.

RESULTS AND DISCUSSION

The average telomeric length (ATL) in peripheral lymphocytes of 7 individuals whose age range varied from 46 to 80 years (both sexes) are shown in Table 23 and Figure 27. As can be seen the ATL in these subjects varied from 2.84 Kb to 5.78 Kb. It may be noted that there is a perceptible decrease in the ATL with advancing age, which is in line with the earlier observations of shorter telomeres in older individuals *(Hastie et al., 1990; Lindsey at al., 1991; Allsopp et ai, 1992; Vazin et al.,1993).*

When six of the seven subjects studied were divided into two groups based on their Body Mass Index (as an indicator of their nutritional status), the low BMI group (BMI less than 19) showed an ATL of 4.96, whereas the Normal BMI group has exhibited an ATL of 3.41 (Table 24). Although this is a pilot study with only a few subjects, there seems to be a general tendency for the LBMI group in the age range studied to possess longer ATL. While more number of cases must be studied before any meaningful conclusion can be drawn, it is nevertheless tempting to speculate that in LBMI group the aging process may have been delayed and therefore the slightly longer ATL may reflect in these cells the potential to undergo more number of divisions as compared to the NBMI group *(Harley et al., 1990; Hariey, 1991).* However, further studies with an increased sample size are needed to make any affirmative conclusion.

Table23: AVERAGE TELOMERIC LENGTH IN PERIPHERAL LYMPHOCYTES OF AGING HUMANS SUBJECTS

S.NO.	AGE	SEX	BMI	ATL*
1.	46	M	15.63	4.04
2	50	F	18.73	5.05
3.	55	M	24.06	3.41
4.	55	F	18.43	5.78
5.	59	M	20.94	3.40
6.	65	M	19.72	3.41
7.	80	M	16.96	2.84

* The values are expressed in kilo base-pairs (Kb).

Figure 27

Average telomeric lengths in the peripheral lymphocytes of aging subjects of Indian population. For other details, please see text.

ATL in lymphocytes of aging humans

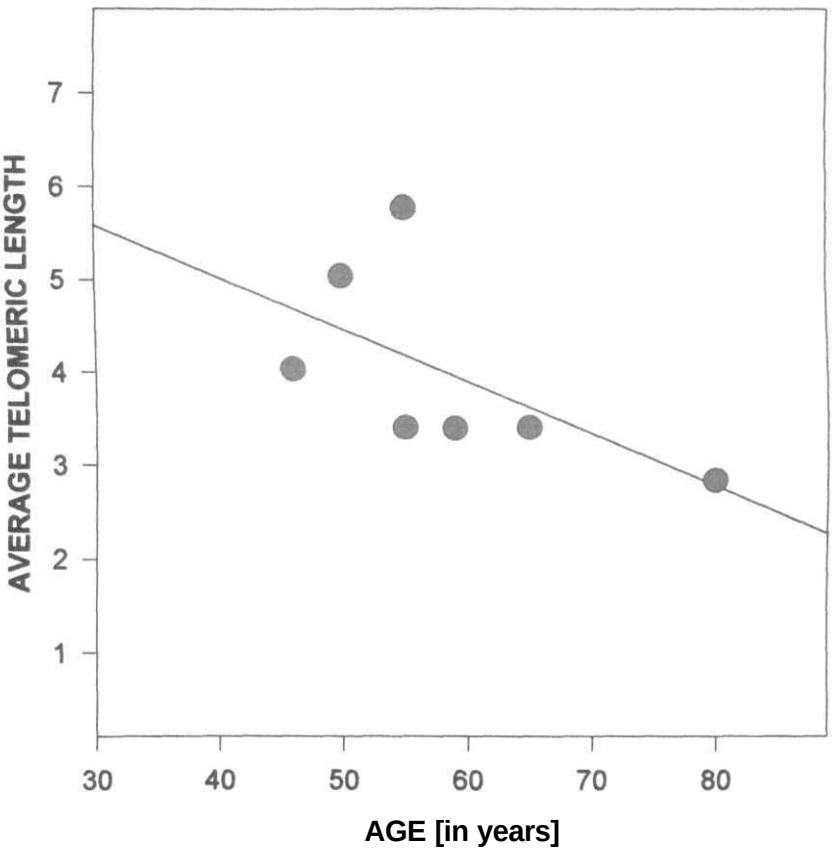


Table24: AVERAGE TELOMERIC LENGTH IN PERIPHERAL LYMPHOCYTES OF LBMI AND NBMI HUMAN SUBJECTS

STATUS	AGE	AVG AGE	BMI	AVG BMI	ATL*	AVG ATL*
LBMI						
1.	46		15.63		4.04	
2	50	50 ± 5	18 73	17.60+1.71	5 05	4.96 ± 0.87 #
3.	55		18.43		5.78	
NBMI						
1.	55		24.06		3.41	
2	59	60 + 5	20 94	21.57+2.24	3 40	3.41 ± 0.01
3.	65		19.72		3.41	

* The values are expressed in kilo base-pairs (Kb).

This value is significantly higher than the average NBMI value at a p value < 0.05.

LBMI = Low Body Mass Index NBMI = Normal Body Mass Index

The ATL in two Down syndrome subjects alongwith age and sex matched controls has been determined and these preliminary observations are recorded in Table 25. The average ATL of the two Down syndrome subjects stands at 2.27 Kb while the same value in control subjects is 3.33 Kb. Thus there is a 1.07 Kb difference in the ATL and this difference could be attributable to the accelerated aging characteristics of Down syndrome subjects. It is already shown that the telomere loss in vivo is equal to 15-50 bp per year *(Harley, 1995).* Taking the value of 50 bp loss per year, it appears that the DS patients are ahead in aging process by 20 years. It is of course, a pure speculation since only two subjects with the disorder have been studied and much more data are needed to reach conclusions with certainty.

It is however, pertinent to mention that shortened telomeres, as compared to the normals, were noticed in lymphocytes of Down syndrome patients *(Vazin et al., 1993),* fibroblasts of Werner's syndrome *(Kruk et al., 1995),* fibroblasts of Ataxia-telangiectasia subjects *(Xia et ai, 1996; Metcalfe et ai, 1996)* and the present preliminary study is in line with the earlier observations.

In the present experiments the ATL found in lymphocytes of normal subjects is somewhat lesser than the values reported by workers from other parts of the world *(Kruk et al., 1995; Xia et ai, 1996; Slagboom et ai, 1996).* They reported 5 to 8 Kb whereas in the present study the values are around 4 Kb. The reason for this is not known. One possibility could be the ethnic differences of the population studied in the present investigation.

Table25: AVERAGE TELOMERIC LENGTH IN PERIPHERAL LYMPHOCYTES OF NORMAL AND DOWN SYNDROME SUBJECTS

S.NO.	AGE	SEX	STATUS	ATL*	AVG ATL*
1.	16	M	Control	4.24	3.33 + 1.29
2	11	M	Control	2.41	
3	16	M	Down Syndrome	2.59	2.27 ± 0.46
4.	11	M	Down Syndrome	1.94	

* The values are expressed in kilo base-pairs (Kb).

CHAPTER - 6

GENERAL DISCUSSION

General Discussion

The aspiration of humanity to achieve a life of immortality, bliss and truth has been a burning flame that could not be put out by circumstances that seemed to deny it altogether. Many things have happened, many wild winds have blown which could easily extinguish the flame of this aspiration of the humanity. There were times when scepticism ruled the roost.

In one of the sacred scriptures of Hindu religion, the *`Bhagvad Gita'* a discourse given by *Lord Krishna* to his disciple, *Arjuna,* Lord Krishna says

`Jatasya hi dhruvo mrityur dhruvam Janma mritasya cha
Tasmad aparihariyartha na tvam sochitam arhasi'

'For certain is death for the born and certain is the birth for the dead:
Therefore, over the inevitable you should not grieve'

It is the observation of very one that every living being that is born has to face death sooner or later. But what is important is that Lord Krishna seems to advise his disciple *not to worry* about the inevitable.

However, man's quest for immortality has not stopped. Scientists with the hallmark of conquering nature could not stop wondering about the mystery of this inevitable phenomenon of aging and death.

Gerontological research all over the world appears to have two main objectives:

1) To understand the molecular basis of aging process

2) To circumvent or eliminate the debilitating physiological functions that are associated with old age.

In other words, one would like to see that the so called old age passes away in a reasonabiy healthy way until the time of death. To conquer death,

which remains a remote possibility, does not seem to be the primary objective since it has its own physiological, social, cultural and philosophical ramifications.

As far as the molecular basis of senescence and aging is concerned, tremendous strides have been made. Several genes responsible for the onset of senescence have been identified and more on the way *(Sugawara et ai, 1990).* In spite of the existence of several thoughts and hypotheses to explain the phenomenon of aging, there appears to be a consensus reached in that unattended or ill attended genomic damage appears to be the root cause of the many deteriorating physiological functions seen in old age. One of the key factors to maintain the genomic integrity is the DNA-repair process , which in itself perhaps operates in a genetically programmed manner. Indeed whether or not DNA-repair potential declines with age and its correlation with the aging process and therefore to the longevity has been a subject of considerable controversy and debate.

Some of the recent findings from other laboratories *(Moriwaki et al., 1996)* as well as those of the present investigation indeed demonstrate that basal DNA-repair capacity does decrease with age. In this unique study, perhaps first of its kind, a gradual decrease with age of the unscheduled DNA synthesis (UDS) in peripheral lymphocytes of normally aging individuals of Indian population, was shown (Please see the results in Chapter 3).

There has been a constant search among various laboratories not only for the biochemical and molecular biological parameters that are associated with aging, but also for those factors that could modulate the process of aging - particulary to delay the process.

In one of the significant observations in the field, Sugawara et al. *(1990)* have found that a gene or genes from human chromosome 1 can induce senescence in cultured immortalised cells. Similarly a couple of genes from

Caenorhabditis elegans were found to induce neurodegeneration *(Driscoll and Chalfie, 1991).*

It is important to note that while a number of genes / factors are identified as contributors to accelerated aging, so far no factor is discovered that can stop or delay aging process.

However, one factor that has been unequivocally shown to retard age-related changes is the restricted dietary calorie consumption. Ever since the original observation of McCay et al. in 1935, much work has been done to show the beneficial effects of limited dietary consumption in many species although the same phenomenon is yet to be demonstrated in humans. These aspects have already been discussed in Chapter 3,

The present investigation provides some evidence to show that indeed reduced dietary calorie consumption for prolonged periods does show beneficial effects in terms of DNA-repair potential as seen in low BMI individuals. Secondly when the lymphocytes were stimulated by phytohemeagglutinin or challenged with UV light (254 nm), the response by way of enhanced DNA-repair parameters is always higher in LBMI group - particularly at adult and old ages - indicating the long term beneficial effects of low calorie consumption.

A weak link in the present studies is whether the low BMI individuals can be compared to those experimental models where dietary restriction was manipulated. In this connection, there are reports to correlate the BMI with nutritional status of the individuals *(James et al., 1988; Luzzi et al., 1991; Naidu and Rao. 1994).* The Indian Council of Medical Research (ICMR) through its established research institutes has conducted extensive surveys / studies and came out with data that correlate the BMI values with the nutritional status of the individuals. Based on these reports a BMI value between 16 - 18 is taken as mild undernutrition essentially due to calorie deficiency without any apparent clinical deficiencies and malnutrition. Extreme care was exercised in selecting the

Extreme care was exercised in selecting the subjects and this is the best one could perhaps do while using a naturally living human population as a model.

It has been a subject of great debate as to how dietary calorie restriction is able to achieve what has been reported in decelerating the aging process. McCay et al. *(1935)* hypothesised that slowing of growth and development was causal for the increased longevity of food restricted rodents. Another thinking is that there is a direct relationship between adiposity and mortality, and lack of excess body fat may be a cause for longevity. Another effect of calorie restriction appears to be reduced metabolic rate, thereby allowing the animals longer chronological period before total energy expenditure per gram tissue approached to that observed for ad-libitum fed rats *(Sacher, 1977).*

There is some evidence to point out that in rats dietary restriction results in higher rate of protein turnover through enhanced rates of polypeptide elongation *(Wulf and Cutler, 1975).* Since accumulation of altered proteins is one of the biochemical markers of aging, the increased turnover of proteins seen in diet restricted animals may not allow such accumulation of altered proteins, thus delaying the process of aging.

It may be logical to assume that when an animal is faced with limited amount of otherwise wholesome food, the whole metabolic machinery may be adapted to utilise the available energy for proper maintenance of soma. In such a situation there may be a trade off between the investment of calories for growth and reproduction and for the maintenance of the soma or adult body *(Holliday, 1989).* For the maintenance of the soma a variety of physiological / biochemical mechanisms may be essential while some other processes may not be so. Thus, the biochemical machinery pertaining to cell replacement, maintenance of genomic integrity of the cell are of paramount importance for the maintenance of soma. For the maintenance of genomic integrity, efficient and accurate DNA-repair is a must. Indeed this could be the reason for the observed

unaltered / improved DNA-repair parameters under calorie restricted conditions observed in earlier studies on experimental animals. The results of the present investigation support this concept and extend it to humans also.

Analysis of human mutations affecting aging may increase our understanding of the basis of genetic diseases. Concurrently we may also learn something about normal mechanism of the action of genes relevant to longevity and whether modifications affecting lifespan will be possible.

Martin *(1977)* has listed a number of genetic and chromosomal disorders that show signs of premature aging, apart from other symptoms. It is noteworthy that of the many syndromes that were listed Down syndrome appears to exhibit prematurely maximum number of features that are associated with aging. In addition, Werner's syndrome, Progeria, Ataxia telengiectasia and Cockayne's syndrome are also characterised by accelerated aging symptoms. Efforts to identify the locus of biochemical defect in these syndromes have revealed that the genetic abnormality in Werner's syndrome is mutation in a gene encoding a trans-acting factor that normally represses the production of an inhibitor of DNA-synthesis until an appropriate time at the end of replicative lifespan. Because of the mutation, the repression is ineffective and DNA-synthesis inhibited prematurely *(Goldstein et al., 1990).* In a more recent report *(Yu et al., 1996)* a gene that encodes a DNA-unwinding enzyme has been implicated with the accelerated aging seen in Werner's syndrome patients. In Ataxia telegiectasia the patients are found to have a defect in recognition and repair of γ-ray induced damage suggesting a reduced activity of endonucleases specific for this damage *(McKinnon, 1987).* A protein, named as ATM (Ataxia telangiectasia gene product) was identified most recently *(Brown et al., 1997)* and it is suspected that this protein may have something to do with fidelity of DNA-synthesis and cell cycle regulation following damage. Altered product of this gene may be responsible for aging characteristics of this syndrome.

It is indeed surprising that inspite of the vast information available about the chromosomal aberration responsible for Down syndrome, information about the precise biochemical defect in these patients is scanty.

Despite the uniqueness of the aetiology of Down syndrome, there is really nothing unique either in the therapies and management strategies that are being employed or the means for preventing Down syndrome that are concurrently available to us. Stated another way, although we know that Down syndrome results from the presence of all or a part of a third chromosome 21 in the genome, we have not been able to capitalise on this knowledge either to prevent Down syndrome from occurring or to prevent or treat many of the components of the syndrome. Among the latter are, in particular, the developmental (mental) retardation, the increased frequency of leukaemia, the greater susceptibility to infection, and - perhaps of greatest concern in the long run - the development of Alzheimer disease.

The recent major advances in molecular biology, cell biology and genetics now make it possible for us to approach the problems of Down syndrome in entirely new ways, and the same holds true for advances in the neural and behavioural sciences as well. Things have never been more promising for developing an understanding of the causation and pathogenesis of Down syndrome and for using that understanding to enhance our ability to improve the physical and intellectual status of persons with this condition.

Chromosome 21 is small in size and one of the genes located on this chromosome and known to be affected is Cu-Zn superoxide dismutase. The levels of this enzyme are shown to be increased in these patients which may result in excessive production of hydrogen peroxide causing macromolecular damage. The few reports that are available on the DNA-repair capacity in Down syndrome subjects are contradictory. While decreased UV induced DNA-repair in fibroblast cultures *(Rehborn and Pfeiffenberger, 1982)* and leukocytes *(Lambert et*

al., 1976) of DS subjects was reported, no such impairment was noticed by Yotti et al. *(1980).* Chiricolo et al. *(1993)* have shown that DNA-repair after γ-radiation is actually enhanced and Zinc supplementation brings this to normal level.

The results of the present investigation show that all the uninduced DNA-repair parameters studied (Unscheduled DNA synthesis, activities of DNases, DNA polymerases, β and e) exhibit lowered levels in Down syndrome subjects, particulary in the age range beyond 25 years as compared to the age and sex matched controls. Also, a conspicuous age-dependent decline, particularly from 15 years of age onwards (compare the values of Group 2 to Group 3 in Tables 11, 13, 15, 17, 19, 21) in DNA-repair markers could be seen only in Down patients but not in normals. To our knowledge this is the first study of its kind dealing with DNA-repair capacity of Down subjects in some detail.

More important is the fact that while exposure to UV at a dose of 20 J/m^2 did show some response in Down subjects, the response did not increase with higher dose of UV (40 J/m^2) but actually decreased. However, if the fold increase due to 20 J/m^2 dose of UV in certain parameters like UV DNase, AP DNase and DNA-polymerase e is considered, the values were either equal or even more than those of the corresponding normals (Tables 13, 15). These results are taken to indicate in Down syndrome that at the damage level induced by 20 J/m^2 of UV, there is some enhancement over the basal repair . However at higher dose of UV (40 J/m^2) the cells were not able to elicit any further response. In fact the decreased fold increase with increased UV dose (Tables 14, 16, 18, 20) may reflect further damage to genomic structure in the absence of adequate repair arsenal.

Another significant finding of this investigation, in this author's opinion, is the manner in which the Down syndrome lymphocytes responded to MNNG treatment (See Tables 14, 16, 18, 20, 22 - Chapter 4). The response by way of fold increase, following treatment with this methylating agent, in the activity of

DNases and DNA-polymerases is similar to that found in normals except in the age group beyond 25 years (Group 3). Moreover, the basal levels of Polymerase e are significantly higher in Down syndrome at all the ages studied. It thus appears that polymerase e activity is spared very preferentially in Down patients and it would be interesting to probe into this aspect further.

MNNG is known to methylate the bases in DNA and thus induce mutations. It is now known that such alterations in DNA structures are repaired by base excision repair involving a short patch resynthesis with polymerase β and e being implicated in such resynthesis *(Singhal et al., 1995; Sobol et al, 1996; Mozzherin and Fisher, 1996).* It will be therefore in line to speculate that in Down syndrome such repair processes requiring resynthesis of a short patch are spared atleast to a reasonable extent while such DNA-damages like UV induced ones which require elaborate nucleotide excision repair involving resynthesis of a long patch are not repaired efficiently. This may well be one of the root causes for the accelerated aging seen in this disorder.

The average telomeric length at the ends of the chromosomes has a direct bearing on the ages of the somatic cell and its donor (a detailed account of this has already been presented in Chapter 5). Thus a longer telomere would indicate a chronologically younger age and the shorter one, the opposite. If low BMI individuals are indeed aging at a slower pace, as reflected by the DNA-repair parameters (Chapter 3), such individuals should also carry longer telomeres as compared to a normally aging person with adequate calorie consumption (the normal BMI subjects). The results reported in chapter 5, Table 24 do indicate that indeed. is the case. Although this was a preliminary study and the sample size was small, still the data are quite pointing a 1 Kb longer telomere in LBMI subjects as compared to age matched NBMI subjects. This indeed would lend support and credibility to some of the conclusions drawn in Chapter 3 about the LBMI individuals being the undernourished and slowly aging population.

The converse must be true in the case of Down subjects. Once again, even with just two cases (Table 25) the trend is quite clear in that Down individuals show about 1 Kb shorter telomeres in their lymphocytes as compared to age and sex matched controls. This preliminary observation is in line with the observations of Vaziri et al. *(1993)* who found higher rate of telomeric loss (133 bp/year) with donor age in lymphocytes of Down patients whereas in age matched normals the rate of loss was 41 bp/year.

It is a matter of surprise that Down syndrome has not been used to adequate extent as a model to unravel the molecular biology of aging. The present investigation shows that one of the major and a fundamental process like DNA-repair is adversely affected in this chromosomal disorder. The rapid deterioration of DNA-repair potential with age and the possibility of the defect being inability to repair such damage which requires long patch resynthesis (e.g. UV induced damage) are the points to be picked up for further studies. It would be important and should be possible to identify the precise type of DNA-polymerase(s) and other enzymes / factors that are involved in long patch repair and to examine how they are affected in this syndrome.

Similarly the present studies also point out the beneficial effects of chronic but mild undernutrition even in human population. Such a model can once again be used to gain answers in the opposite direction i.e., which of the DNA-repair pathway that are preferentially retained or spared under the conditions. These further studies may well provide crucial answers as to the molecular events that may trigger or delay the aging phenomenon.

SUMMARY

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1. Various DNA-repair parameters like unscheduled DNA synthesis (UDS), two putative DNA-repair deoxyriboendonucleases - UV DNase and AP DNase, and DNA-polymerase β were studied in the peripheral lymphocytes of humans under various conditions

2. Subjects of Indian population, living under their natural conditions were divided into three age groups - young (8-14 years), adult (20-35 years) and old (> 55 years).

3. Each of the above three age groups were further subdivided into 2 sub groups - Normal, with a body mass index of 20 or more (NBMI) and undernourished with a body mass index of 18 or less (LBMI).

4. All the subjects were examined for various biochemical and clinical parameters to make sure that they did not suffer from any apparent deficiencies Thus the LBMI group is considered as a population which has been chronically restricted in its dietary-calorie consumption and therefore regarded as mildly undernourished without any apparent malnutrition.

5. The LBMI group (undernourished) showed higher DNA-repair potential, in terms of above parameters, particularly at `adult' and 'old' ages. Also, the LBMI group exhibited reduced decline in DNA-repair potential

6. A similar pattern of results was seen when the lymphocytes were challenged with UV light or stimulated to proliferate with phytoheamagglutinin.

7. These results were taken to indicated a beneficial effect on DNA-repair capacity by reduced calorie consumption in humans also.

8. Down syndrome, with chromosomal aberration of trisomy of chromosome 21, displays maximum number of accelerated aging symptoms. Therefore a study was taken to examine the DNA-repair parameters, mentioned above, in the peripheral lymphocytes of the patients alongwith age and sex matched normals

9. Here also, the patients were divided into three age groups - Group A upto 12 years; Group B - 13-25 years and Group C beyond 25 years to represent the young, adult and old ages in the accelerated aging scenario.

10. Unscheduled DNA synthesis (UDS) was tow in Down patients at all the ages compared to age- and sex matched controls. Also, the decline in the UDS with advancing age was more precipitous in Down patients.

11. A similar pattern of results was obtained even after the lymphocytes were challenged with either UV (at 2 doses - 20J/m^2 and 40J/m^2) or with MNNG (50μM for 30 minutes).

12. While some induction in UDS was seen with the UV dose of 20J/m^2 in Down lymphocytes, 40J/m^2 dose failed to show any further induction, in contrast to that in normals, indicating that the capacity of Down patients to raise their level of DNA-repair against higher doses of UV is limited.

13. When the lymphocytes were treated with the methylating mutagen, MNNG, the induction in Down patients is quite comparable to that in controls suggesting that repair of the MNNG induced DNA-damage is spared in this syndrome.

14. A similar pattern of results was found in the case of activities UV and AP DNases, total DNA-polymerases and polymerase (3 with respect to UV and MNNG induction.

15. In the case of DNA-polymerase s, a recently discovered enzyme with a distinct role in short-patch DNA-repair, activity, both before and after induction with either UV or MNNG, was higher in Down subjects than in normals. Although there wss a decline in this activity with age, the values in Down patients were always higher. Furthermore, the percentage of e activity out of total DNA polymerase activity was about 17% in Down syndrome subjects while it was only about 5% ın normals

16. Since both (3 and ε polymerases are known to participate in short patch DNA-repair (e.g. MNNG induced repair), it is concluded that in Down syndrome, repair involving a short patch synthesis is not very adversely affected while the repair involving long patch synthesis (e.g. UV induced repair) is distinctly reduced

17. Decreased DNA-repair potential, particularly involving long patch synthesis may be an important causative factor for the accelerated aging symptoms seen in Down patients.

18. Preliminary studies on the Average Telomeric Length (ATL) showed that in aging humans, there is a reduction of ATL with age

19. When the subjects were grouped as LBMI and NBMI (3 in each group), the LBMI group showed slightly longer ATL as compared to NBMI indicating a delayed aging process.

20. Preliminary studies with Down syndrome subjects, showed that the ATL is shorter as compared to the age- and sex-matched controls - once again showing that Down syndrome patients are in a biologically advanced aging state.

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