Republic of Iraq
Ministry of Higher Education
And Scientific Research
Al-Nahrain University
College of Science
Department of Biotechnology

In vivo Study of Mitochondrial DNA and Cytogenetic Changes in Mice Treated with *Mitomycin C* as a Mutagenic Agent

A Thesis
Submitted to the College of Science
Al-Nahrain University
In Partial Fulfillment of the Requirements for the Degree
of Master of Science in Biotechnology

By
Rawa Ali Zahid Bakr
B.Sc. Microbiology 1999
Baghdad University

November 2006 Shawal 1426

بِسْمِ اللهِ الرَّحْمنِ الرَّحِيمِ

{وَيَسْأَلُونَكَ عَنِ الرُّوحِ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُم مِّن الْعِلْمِ إِلاَّ قَلِيلاً }

صَدَقَ اللهُ العَظِيم

الإسراء٨٥

Certification

We certify that the preparation of this thesis entitled ‘‘***In vivo* Study of Mitochondrial DNA and Cytogenetic Changes in Mice Treated with Mitomycin C as a Mutagenic Agent’’** was made by **Rawa Ali Zahid Bakr,** under our direct supervision at the College of Science / Al-Nahrain University in partial fulfilment of the requirements for the degree of Master of Science in Biotechnology.

Signature:
Name**:Prof.Esmaeel K. Shubber**
(Supervisor)

Date: / /

Signature:
Name:**Assistant.Prof.Majed H.Al-Gelawi**
(Supervisor)

Date: / /

Signature:
Name: **Assistant Prof. Dr. Nabeel K. Al-Ani**
(Head of Department)

Date: / /

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Signature:
Name**:Prof.Esmaeel K. Shubber**
(Supervisor)

Date: / /

Signature:
Name:**Assistant.Prof.Majed H.Al-Gelawi**
(Supervisor)

Date: / /

Signature:
Name: **Assistant Prof. Dr. Nabeel K. Al-Ani**
(Head of Department)

Date: / /

To My Mother and Father, thank you for all and every thing that you are

To My precious Russul, the light in my life

To My beloved Bilal, I treasure you

And To all of those who stand against injustice

Rawa

2006

ACKNOWLEDGEMENTS

My supreme gratitude to the merciful creator **Allah** for without his mercy**,** I could not finish this work.

I would like to express my deep gratitude to my supervisors prof. Dr.Esmaeel K.Shubber and Dr.Majed Al-Gelawi for giving me the best possible introduction to the world of science. Their guidance, optimism and scientific support. I am particularly grateful for the opportunity to pursue a somewhat untraditional line of research during the years of the project.

I am deeply appreciative to Dr.Khulood Al-Samaraee for creating the chance for me to study at Al-Nahrain University.

The work was initiated at the department of molecular biology and genetic engineering, biotechnology research centre.

I also would like to thank Dr.Laith Alani Dean of the college and Dr.Khulood Al-Samaraee ,head of biotechnology research centre for providing the excellent research environment at the biotechnology department and biotechnology research centre .

Special thanks to my present and previous co-workers on the biotechnology research centre.

Deepest thanks to my fellow worker, my friend and my husband Bilal Kamil for helping me to overcome the difficulties I faced throughout the years of the project and who taught me how to reason with scientific data.

I especially thank Dr. Intesar Hussien and Dr.Dhafer H.Ghali for their invaluable assistance and technical tips on cytogenetic preparations.

I also acknowledge Dr. Hazim E.Abdalbari, Dr. Ibtihal H.Mahmood, Muhammed A.W.Alaskari and Muhammed A.W.Aladhami and Duha Sabti, wishing them all the good.

Most importantly, I owe a profound gratefulness to my family, my sister Russul for lifting me up in my darkest hours and for her sensible engraved advices. To my mother and father for their spiritual encouragement throughout all the years of the study.

Rawa A.ZAHID

2006

Summary

Six to eight weeks old white mice sharing the same mother and father were injected intraperitoneally with different dosages 0.1, 0.2 and 0.5mg/kg body weight (bw) of the chemotherapeutic antitumor drug Mitomycin C (MMC). The effect of the drug on mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) was investigated by molecular techniques and cytogenetic analyses were performed on spleen and bone marrow cells.

MtDNA was isolated from spleen tissues and molecular analysis was preformed by Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) assay and Specific PCR directed to the non coding control region or displacement loop (D-loop) in mtDNA of mice. Restriction endonuclease analysis was performed on the specific PCR product.

In RAPD-PCR, ten different decamer primers chosen randomly were employed to analyze mtDNA of mice treated with 0.5mg/kg bw MMC in addition to the control animals. RAPD-PCR assay revealed polymorphisms ranging between 7.7 -100%. The high percentage of polymorphism observed might be due to exposure to MMC.

The D-loop region of mice progeny from the same mother and father treated with different dosages of the drug MMC (0.1, 0.2 and 0.5mg/kg bw was amplified by the PCR using specific primers. The PCR product was then subjected to four restriction enzymes (*BamHI*, *HaeIII*, *HindIII* and *Sau3A*) to examine induced potential variation within the region. Restriction enzyme analysis of D-loop region amplified by PCR did not detect any DNA variation within this region between mice individuals treated with all of the three doses of MMC in comparison to the control animals.

The mitomycin C effect was tested cytogenetically in both spleen and bone marrow cell suspensions from the same animals. Direct short term culture was used for chromosomal preparation and examination for both

bone marrow and spleen cells in 0.1, 0.2 and 0.5mg/kg bw MMC treated animals and non treated control mice. Results showed that a negative relationship was observed between the mean values of mitotic index (MI) and blast index (BI) to higher doses of MMC in both bone marrow and spleen cells while a positive correlation was observed in total number of chromosomal aberrations (CA) and higher doses of MMC in both bone marrow and spleen cells.

Molecular technique of RAPD-PCR was able to detect mtDNA variations between 0.5mg/kg bw treated animal in comparison to the control. While analysis of the D-loop region of mice treated with 0.1, 0.2 and 0.5mg/kg bw by restriction analysis did not detect DNA alterations in reference to the control animals. The inhibition of proliferation rate and the increase in CA observed in both bone marrow and spleen cells, together with the inhibition of blast percentage in bone marrow cells confirm the positive correlation between MMC and DNA damage in mice somatic cells.

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List of Abbreviations

ADP……..	Adenosine diphosphate
AP-PCR…….....	Arbitrary primed polymerase chain reaction
ATP……..	Adenosine triphosphate
BI…….	Blast index
Bp…….	Base pair
bw…….	Body wieght
CA……..	Chromosomal aberrations
CAP……..	Chloramphenicol
CCP……..	Cell cycle progression
CD…….	Central domain
CNS……	Central nervous system
CPEO……	Chronic progressive external ophthalmoplasia
CsCl…….	Cesium chloride
CSB……	Conserved sequence block
D.W……	Distilled water
dNTPs……..	Deoxynuclioside triphosphate
dATP…….	Deoxy adenine triphosphate
dCTP…….	Deoxy cytosine triphosphate
dGTP…….	Deoxy guanine triphosphate
dTTP…….	Deoxy thymine triphosphate
D-loop……..	Displacement loop
DNA……	Deoxyribonucleic Acid
ET……	Electron transport
ETAS……	Extended termination-associated sequences
FISH……	Fluorescent *In Situ* Hybridization
Gpx…….	Glutathione peroxidase
G1phase……..	The first gap of the cell cycle
G2 phase…….....	The second gap of the cell cycle
H strand…….	Heavy strand
HCC…….	Hepatocellular carcinoma
HIV……..	Human immunodeficiency virus
HSP……..	Heavy strand transcription promoter
HVRs……	Hypervariable regions
IMS……..	Inter-membrane space
ip……..	Intraperitoneal injection
KSS……..	Kearns-Sayre syndrome
L strand…….....	Light strand
LHON……..	Leber's hereditary optic neuropathy
LSP……..	Light strand transcription promoter
M phase……	Mitotic phase of the cell cycle

MI…….....	Mitotic index
MMC……	Mitomycin C
MNGIE…….	Mitochondrial neurogastrointestinal encephalomyopathy
MnSOD…….....	Mitochondrial Mn superoxide dismutase
MtDNA…….....	Mitochondrial deoxyribonucleic acid
MTX……..	Methotrexate
NAD^+……	Nicotinamide adenine dinucleotide
nDNA……	Nuclear deoxyribonucleic acid
nt…….	Nucleotide
O_H…….....	Origin of H strand synthesis
O_L…….	Origin of L strand synthesis
OXPHOS…….....	Oxidative phosphorylation
PAGE…….	Polyacrylamide Gel Electrophoresis
RAPD-PCR…….....	Random amplified polymorphic DNA polymerase chain reaction
Rh123…….	Rhodamine 123
RI……	Replicative index
ROS……	Reactive oxygen species
RRF……	Ragged red fibers
rRNA…….	Ribosomal ribonucleic acid
S phase ……..	Synthesis phase of the cell cycle
SCE……	Sister chromatid exchange
SDS…….....	Sodium dodecyl sulfate
TBE……	Tris-borate buffer
TCA……..	Tricarboxylic acid cycle
Tfam……..	Mitochondrial transcription factor A
TP…….	Thymidine phosphorylase
tRNA…….	Transfer ribonucleic acid
TTGE ……	Temporal temperature gradient gel electrophoresis
URF……	Unidentified reading frame

Mitochondrial DNA small, beautiful and essential

Grivell, L.A.

CHAPTER ONE

INTRODUCTION AND AIMS OF THE STUDY

1.1. Introduction

Genotoxins are a health hazard, thus many methods are devised to detect these agents. Conventional tests utilized bacteria, fungi, plants and mammals. Mutagen detection methods should meet several criteria like accuracy, sensitivity and relevancy to human biology, not all methods meet these criteria and thus continuous developments are needed.

Mitochondria are double membranous organelles that generate energy for cellular processes by producing adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS). These organelles contain their own, extrachromosomal DNA. Human mtDNA is a double stranded, circular molecule that encodes for 13 protein subunits and 24 structural RNAs (2 ribosomal RNAs and 22 tRNAs) that are required for the intramitochondrial translation of the protein –coding units (Johns, 1995).

Mitochondrial defects have long been suspected to contribute to human pathologies related to some of the degenerative diseases, aging and cancer. Leber's hereditary optic neuropathy (LHON) was one of the first diseases tracked down to mitochondrial DNA missense mutation that was discovered in 1988, since then hundreds of diseases were attributed to mtDNA mutations like different myopathies, diabetes mellitus, Parkinson's and many others (Chinnery and Turnbull, 1997).

Point mutations, deletions and duplications of mtDNA were observed in various solid tumors and hematological malignancies. Mutations in the coding and non-coding regions have been identified. In mammals the non-coding region Displacement loop (D-loop) or control region is known as the sequence bounded by the genes for $tRNA^{Phe}$ and $tRNA^{Pro}$. The D-loop region is a mutational hot spot; mutations of mtDNA were reported in the D-loop region of different diseases like Alzheimer and Diabetes mellitus (Chinnery and Turnbull,

1997), and cancers such as in brain tumors, breast, colorectal carcinoma, lung, prostate and many others. It was reported that D-loop mutations could be used as clonal markers in hepatocellular carcinoma (HCC) (Nomto *et al.*, 2002).

Molecular genetic methods involving Polymerase Chain Reaction (PCR), Southern blot analysis were used to detect specific mtDNA and nuclear gene mutations. More developed methods involve direct sequencing of PCR products to detect novel mtDNA mutations (Larsson and Oldfors, 2001).

Mitochondrial DNA alterations are becoming an alternative or even a superior indicator for genotoxic effects. DNA damage may result from direct reaction between a genotoxic chemical and the target DNA molecule and maybe mediated by free radicals since mtDNA is in consistent exposure to reactive oxygen species produced as byproducts by the process of OXPHOS (Wallace, 1999). Additionally, differences between nuclear DNA (nDNA) and mtDNA may contribute to mitochondrial DNA mutation rate being higher in ten times. The nDNA is protected by histone and non-histone proteins while mtDNA lacks protective proteins, damage to nDNA is competently removed by DNA repair system located inside the nucleus, on the other hand mitochondria have inefficient DNA repair system. These differences rendered mtDNA more sensitive to genotoxic effects than nDNA (Clayton, 1982). MtDNA in mice was found to be more susceptible to mutations in *in vitro* culture conditions in comparison to *in vivo* (Hatif, 2006).

The many distinct differences in mitochondrial structure and function between normal cells and cancer cells offer a unique potential for the clinical use of mitochondria as markers for the early detection of cancer. The high copy number of mtDNA and the homoplasmic nature of mtDNA mutations provide distinctive advantage over nuclear genome based methods.

1.2. Aims of the present study

An *in vivo* system utilizing mtDNA in addition to chromosomal DNA was hypothesized in mice using the antitumor drug MMC as a mutagenic agent in order to:-

1. Study the ability of using the mtDNA molecule as a biomarker to detect damage resulted from the exposure to genotoxic compounds and cancer epidemiology.
2. Exploit RAPD-PCR to detect induced DNA damage within the mtDNA .
3. Detect possible induced DNA damage within the D-loop region amplified by PCR in mtDNA of mice using some of the restriction endonucleases.
4. Evaluate induced genetic DNA damage utilizing some of the cytogenetic parameters such as MI, BI and CA.

CHAPTER TWO

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1. Mitochondria

Mitochondria are extra nuclear organelles present in the cytoplasm of the majority of eukaryotes providing most of the energy needed by a cell. Mitochondria meaning thread like organelles were first observed by Altman in 1894, they are double membranous oval particles with 1-2 μM in length and 0.5-1 μM in width. The basic morphology of the organelle as observed by early electron microscopy shows the peripheral or outer membrane enclosing the entire contents of the mitochondrion and an inter- membrane space (IMS) between the outer and inner membrane which encloses the matrix forming a series of folds and invaginations called cristae (Figure 2-1 A). The cristae structures project inward towards the interior space of the organelle. The large surface area of the inner membrane serves as a location of the enzymes involved in the process of oxidative phosphorylation (OXPHOS), the surface area and number of cristae are generally correlated with the degree of metabolic activity exhibited by a cell (Modica-Napolitano and Singh, 2002).

The matrix contains the mitochondrial DNA (mtDNA) molecules, proteins for the replication and transcription of mtDNA, mitochondrial ribosomes for protein synthesis and enzymes carrying out metabolic functions (Figure 2-1B) such as the tricarboxylic acid cycle (TCA), β-oxidation of fatty acids (Modica-Napolitano and Singh, 2002). Structural insights into mitochondria and membrane topology are being revised using electron microscope tomography (Perkins and Frey, 2000).

Mitochondria constantly undergo fissions and fusions as a part of multiplication (Singh, 2000). Living cells stained with the lipophilic cation

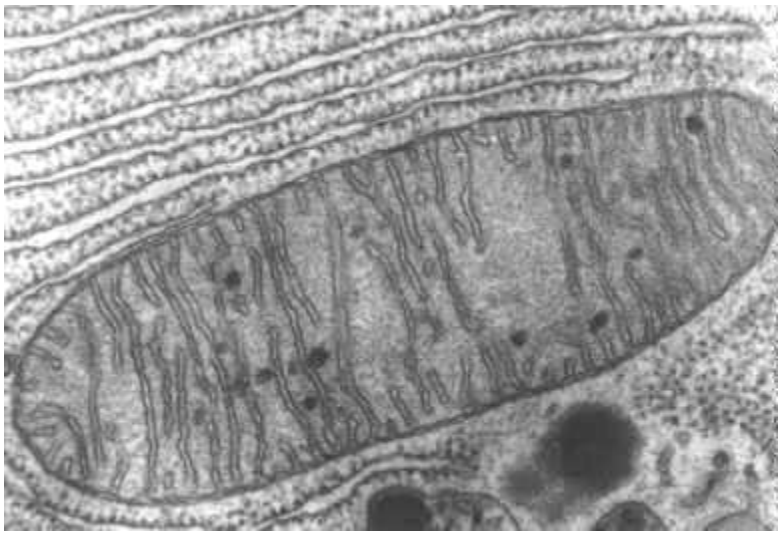

Figure (2-1A): Electron micrograph of the mitochondrion (from http://cellbio.utmb.edu/miroanatomy/)

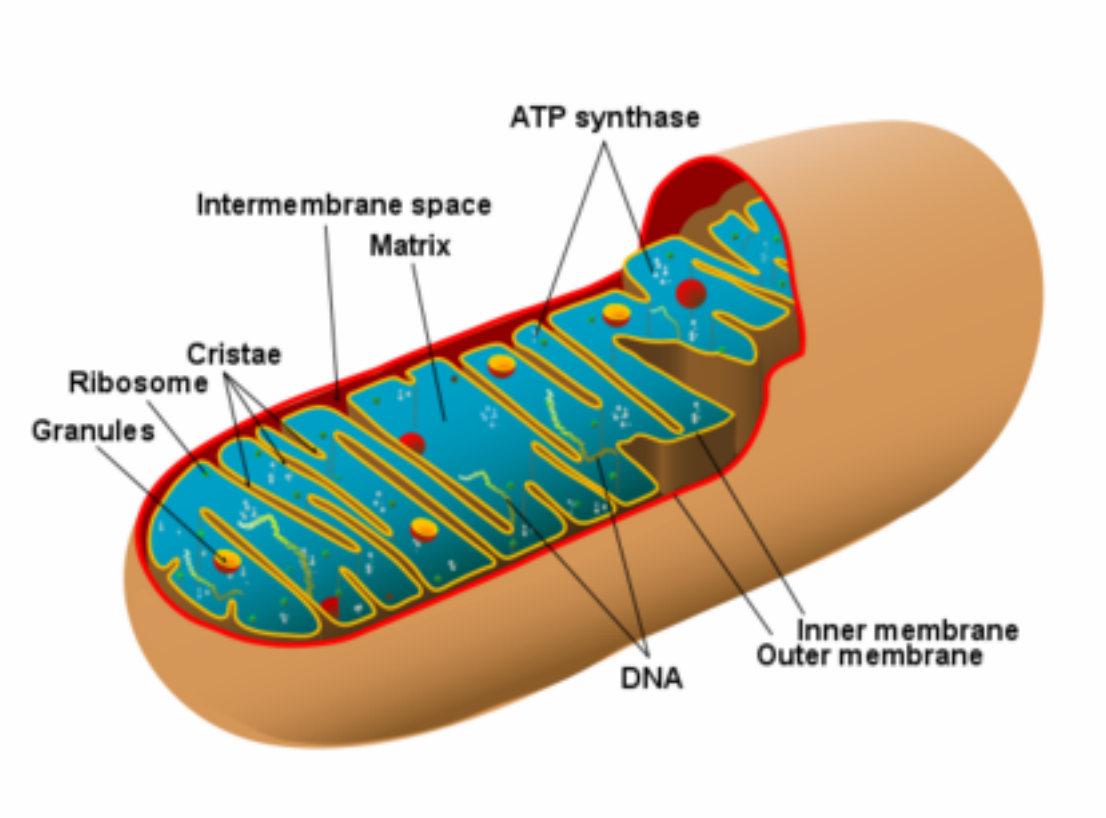

Figure (2-1B): Structural features of the mitochondrion (from http://en.wikipedia.org/wiki/Mitochondrion)

rhodamine 123 (Rh123) observed by fluorescence microscopy show mitochondria as a dynamic network of filamentous structures changing in form, size and location (Johnson *et al.,* 1980).

2.1.1. The Origin of Mitochondria

Theories about mitochondria origin were first postulated by Altman in 1894 when he observed these organelles and suggested that they were free living elementary organisms within the cell (Larsson and Oldfors, 2001).

Symbiotic origin of mitochondria is a model that was primarily attributed to L. Margulis in 1981 is now widely accepted, that is organelles such as mitochondria and chloroplasts were originally free living respiring bacteria and cyanobactria respectively (Tamarin, 1996). At first a eukaryotic cell without mitochondria evolved and this organism captured a protobacterium by endocytosis and a symbiotic relationship was established and thus these organelles retain some evolutionary similarities to other prokaryotes (Singh, 2000).

Some of the similarities with prokaryotes is that mitochondrial rRNA is more similar to prokaryotic rRNA than to eukaryotic, although the mtDNA is constructed of imported cellular proteins (translated in the cytoplasm) is sensitive to prokaryotic antibiotics such as streptomycin and chloramphenicol, and perhaps the most important fact that the organelle possess a genetic system different from the main mechanism found in the eukaryotic cell but similar to that seen in prokaryotes (Tamarin, 1996). However, in order to ensure transmission to daughter cells, the mitochondrion adapted to intracellular niche by reducing the size of its genome, it's presumed that this was accomplished by the deletion of non essential genes and the transfer of many essential ones to the nucleus where proteins are transcribed and translated in cytoplasmic ribosomes and selectively imported back into the mitochondrion (Wallace, 1999).

Sequence data of a wide variety of organisms support the endosymbiont hypothesis of mitochondrial origin (Lang *et al*., 1997).

2.1.2. Functions of Mitochondria

Mitochondria are semi-autonomous organelles that participate in essential functions such as cellular metabolism and regulation of cell death or apoptosis besides other important functions. Perhaps the most characterized function of mitochondria is the production of the common energy form adenosine triphosphate (ATP) required for different metabolic processes in the cell through the process of OXPHOS which is a unique biochemical pathway achieved by a well coordinated effort of the protein products from two separate genomes (nuclear and mitochondrial) working together in the same cell (Carew and Huang, 2002).

The respiratory chain consists of five enzyme complexes and contains around 87 different protein subunits or polypeptides 13 of which are encoded by mtDNA, thus the majority of the respiratory chain components are nuclear encoded, translated in the cytoplasm and imported into the mitochondria (Carew and Huang, 2002).

Proteins targeted to mitochondria are synthesized as precursors carrying an N-terminal extension as a leader sequence that directs the protein to mitochondria. Import into mitochondria is carried out by a complex ATP dependent transport system and inside the mitochondria a series of post translational modifications take place to produce the mature or functional protein (Zeviani and Antozzi, 1997).

Catabolism of carbohydrates begins with the transport of pyruvate from the cytoplasm into the mitochondria. Pyruvate is converted to acetyl CoA by a soluble multi-enzyme pyruvate dehydrogenase complex located in the mitochondrial matrix. Acetyl CoA is produced by oxidative decarboxylation

and further oxidized by a cyclic process known as the tricarboxylic acid cycle (TCA) or citric acid cycle. All but one of the enzymes involved in TCA cycle are soluble proteins located to the inner mitochondrial membrane.TCA cycle results in the production of different energetic molecules or respiratory substrates. OXPHOS is the next stage of aerobic metabolism that couples oxidation of respiratory enzyme complexes found on the inner mitochondrial membrane, these enzyme complexes are capable of accepting and denoting electrons in a specific sequence depending on their relative oxidation-reduction potentials and substrate specificity (Modica-Napolitano and Singh, 2002).

MtDNA encodes for 13 polypeptide subunits in four of the five respiratory chain enzyme complexes including seven subunits (ND1-ND6, ND4L) of complex I (NADH dehydrogenase), one subunit (cyt b) of complex III (cytochrome c reductase) , three subunits (COXI-COXIII) of complex IV (cytochrome c oxidase COX) and two subunits (ATPase6-ATPase8) of complex V (ATP synthase) (Moraes *et al.*, 2003). Complex II (succinate dehydrogenase SDH) is entirely nucleus encoded. Protons are pumped out of the matrix by complexes I, III, IV and the energy created from this electrochemical gradient is used by complex V to synthesize ATP from ADP adenosine diphosphate (Larsson and Oldfors, 2001).

Some electrons transported through the respiratory chain may escape from the electron transport complexes leading molecular oxygen to form super oxide radical (O_2^-), this occurs mainly at complexes I, III (Saybasili *et al.*, 2001). Certain mtDNA mutations may cause alterations in the components of respiratory chain leading to increase of super oxide radicals generation which in turn may be converted into other reactive oxygen species (ROS) (Carew and Huang, 2002).

In addition to the central role in cellular energy fulfillment, mitochondria participate in numerous functions. Such as programmed cell death or apoptosis,

as mitochondria may excite the cascade of intracellular events leading to apoptosis (Modica-Napolitano and Singh, 2002).

Fatty acid oxidation is a metabolic activity located in the mitochondria. Certain enzymes involved in urea cycle and gluconeogenesis are found in the mitochondrial matrix as well as the coenzyme nicotinamide adenine dinucleotide ($NAD^{+)}$ required for the phosphorylation in glycolysis. Also mitochondria have a role in intracellular homeostasis of inorganic ions such as calcium and phosphate (Modica-Napolitano and Singh, 2002).

2.2. Mitochondrial Inheritance

Depending on the organism and cell type, estimates of the number of mitochondria per cell ranges between ten and ten thousand, this is usually determined by serial sectioning of whole cells which is a tedious technique involving electron microscope (Tamarin, 1996).

Mitochondrial inheritance in animals is governed by two general patterns, First mtDNA is exclusively maternally inherited (Giles *et al.,* 1980), Copies of mitochondrial DNA in the egg cell is 10^3 fold greater than that in the sperm, when the two cells fuse to form the zygote , mtDNA is only contributed by the female gamete (Modica-Napolitano and Singh, 2002). However small amount of leakiness was found in mice, about one mitochondrion per thousand is of paternal origin. other exceptions to the rule of maternal inheritance is found in species such mussels where mitochondrial inheritance is biparental while in some gymnosperm plants like redwoods , only paternal mitochondria is contributed to the zygote i.e. paternal inheritance. Cytoplasmic or non-Mendelian inheritance is the term used to describe the maternal pattern of inheritance in organelles (Tamarin, 1996).

Due to the peculiar pattern of mtDNA inheritance, polymorphisms studied in mtDNA from groups of people through out the world show that human

populations arose from a common female ancestor or "Mitochondrial Eve" in the distant evolutionary past. By calculating how long it would take to accumulate the pattern of mutations observed in modern human it was concluded that the ancestor of all modern humans arose about 200,000 years ago, probably in Africa and thus its also known as "African Eve" (Johnson *et al*., 1983).

The second pattern of mitochondrial inheritance is represented by two terms, homoplasmy and heteroplasmy. Homoplasmy means the existence of a uniform population of mitochondria within an organism, i.e. all the mitochondria within an individual are genetically identical (Tamarin, 1996). Heteroplasmy refers to the polymorphism within an individual; an individual may carry several allelic forms of mtDNA available in different proportions in different tissues (Larsson and Clayton, 1995). Thus heteroplasmy means the coexistence of more than one type of mtDNA within a cell, a tissue, or an individual (Larsson and Oldfors, 2001).

The biparental inheritance and leakiness of paternal mitochondria are exceptions to the maternal inheritance rule leading to heterogeneity of mitochondria within an organism or heteroplasmy (Tamarin, 1996).

Mitochondria are randomly distributed to daughter cells during cell division, after many replication rounds and over time the proportion of mutants within a cell can be variable and may drift toward predominantly mutant or wild type in order to achieve homoplasmy and thus the severity of a mutation may vary according to the proportion of mutant mtDNA an individual carries, this effect contributes to the various phenotypes observed amongst family members carrying the same pathogenic mtDNA mutation (Carew and Huang, 2002). Heteroplasmy is considered as a frequent event, occurring in 10% to 20% of humans (Gibbons, 1998).

2.3. The Mammalian Mitochondrial Genome

Mitochondria contain their own genome along with their own transcription, translation and protein assembly machinery, mtDNA was first discovered in 1963 (Nass and Nass, 1963). The Mammalian mitochondrial genome is a closed-circular, double helical DNA molecule. Each mitochondrion contains 2-10 copies of its genome (Carew and Huang, 2002). In metazoan animal cells, mtDNA molecule is a circle between 15,000 – 18,000 base pair (bp) long with interesting similarity in small size and information content (Avise *et al.*, 1979).

In human, mtDNA molecule is composed of 16 569 bp, the nucleotide sequence and gene organization was fully elucidated in 1981 (Anderson *et al.,* 1981). Organisms such as yeasts, higher plants and others have mtDNA molecules with five to twenty five or more times larger than in animals (Tamarin, 1996).

In mammals mtDNA contains 37 genes encoding for two rRNA (12S and 16S), 22 tRNAs, required for protein synthesis in mitochondria and it codes for 13 polypeptides of 87 protein subunits required for the oxidative phosphorylation (Modica-Napolitano and Singh, 2002). Each of the 13 polypeptides is a highly hydrophobic subunit of one of four respiratory enzyme complexes sited in the inner mitochondrial membrane, other mitochondrial proteins involved in transcription and translation of mtDNA are all encoded by nuclear genes and transported into the mitochondria (Carew and Huang, 2002).

There are several unique features of mtDNA and mitochondrial genetics such as; the hallmark of mammalian mtDNA organization is the extreme economy of DNA sequence usage. Introns are absent in mtDNA and all the coding sequences are contiguous except for two non coding regions the displacement loop (D-loop) and a small region located about 11kb from the D-loop, includes the origin of replication of the L strand (Larizza *et al.*, 2002).

The genetic code of mtDNA differ from the universal nuclear genetic code, Also the rate of evolution of mtDNA is more than ten times higher than that of nuclear DNA due to the lack of protective histons and effective DNA repair system and a possible explanation is the continuous exposure of oxygen species produced as byproducts of OXPHOS (Larsson and Oldfors, 2001).

2.3.1. Mitochondrial Genome in Mice

Mouse mitochondrial genome is a 16 295 bp closed circular DNA which is proved to be a model system for studies of mtDNA replication (Bogenhagen *et al.*, 1981) and transcription (Van Etten *et al*., 1982). MtDNA of mouse L cell was fully sequenced by the chemical sequencing method of Maxam and Gilbert in 1981 (Bibb *et al.*, 1981). Sequence analysis revealed several notable features unique to mouse mtDNA and a surprising conservation of overall gene organization compared with the sequence of human mtDNA (Anderson *et al*., 1981).

Nucleotide 1 is the 5¯ terminus of $tRNA^{Phe}$, the numbering continues counter clockwise around the genome into rRNA and protein coding genes. MtDNA has two strands; a heavy (H) strand which codes for most of the mitochondrial genes (Figure 2-2). Most transcripts are therefore of light (L) strand sequence and are complementary to the H strand (Bibb *et al.,* 1981).

Of the 22tRNAs identified, 14 are coded in the H strand and 8 in the L strand. The genetic code in mouse mitochondria like that in human is different from the universal code in that UGA codes for tryptophan and not for termination and AUA codes for methionine and not isoleucine (Van Etten *et al.*, 1982).

Thirteen open reading frames for mitochondrial translation have been identified all but one of these open reading frames were identified for the three largest subunits of cytochrome c oxidase (COI, II, III).subunit 6 of ATPase6

complex and cytochrome b (cytb) of the cytochrome bc1 complex, identification was made by homology with the corresponding human genes and by comparison with amino acid and nucleotide sequence for mitochondrial proteins from bovine and yeast (Bibb *et al.*, 1981). Corresponding genes of human mtDNA are found in the same relative positions (Anderson *et al*., 1981).

The other 8 genes have not been identified and are known as unidentified reading frames (URFs) following Anderson terminology, there are three pairs of reading frames URFA6L and ATPase6, URF4L and URF4, URF5 and URF6 exist in the DNA sequence as overlapping reading frames. The same eight URFs exist in the corresponding map positions in the mitochondrial genome of human and bovine (Van Etten *et al.*, 1982).

Although mitochondrial genome tend to be as small as possible and is under a great selective pressure, the URFs are in an intact form in all three species of human, bovine and mouse suggesting that these URFs represent genes for mitochondrial proteins. Polyadenylated mitochondrial transcripts corresponding to these URFs in mouse and human genomes are a further support (Bibb *et al.*, 1981).

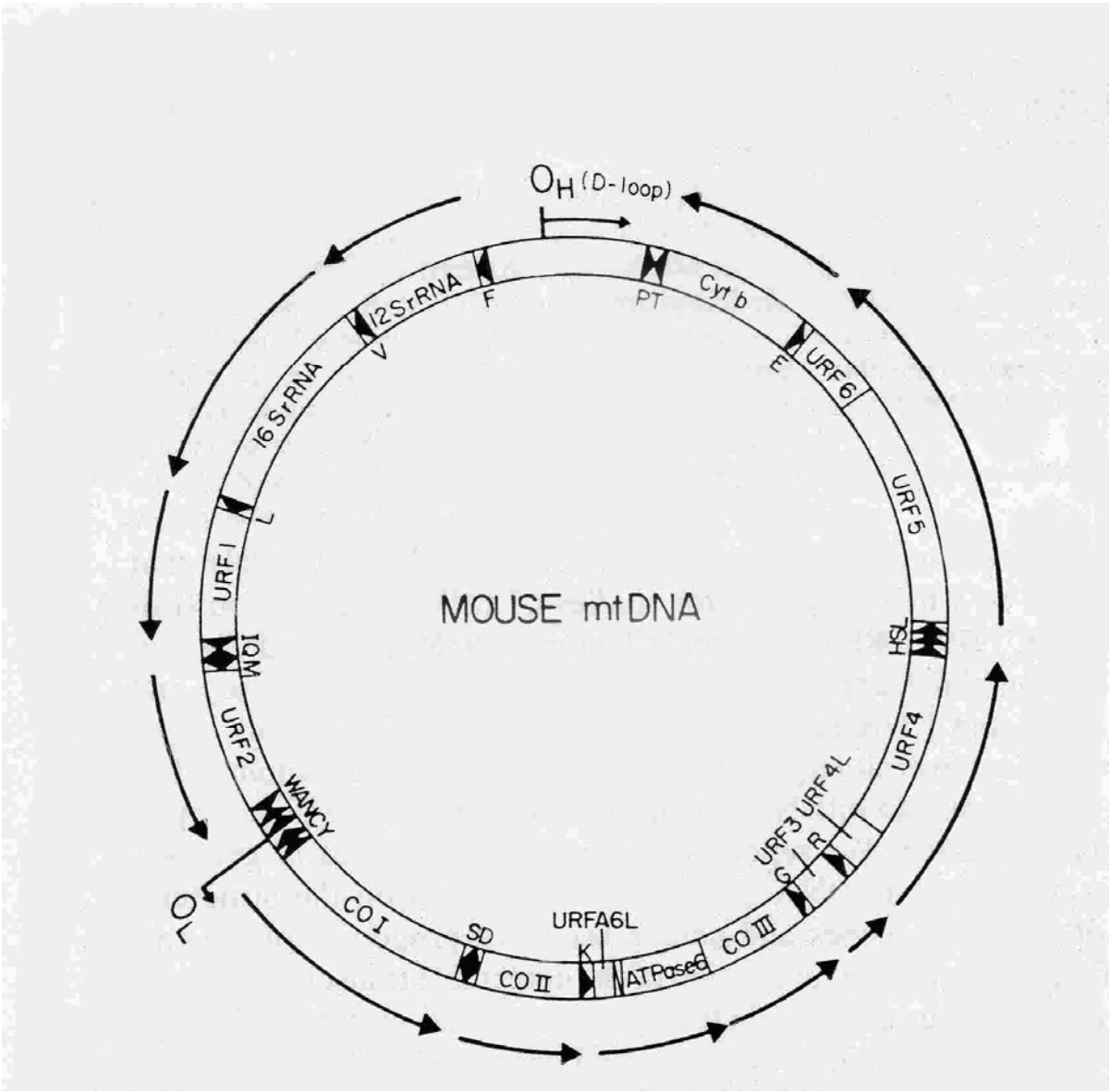

Figure (2-2): The Circular Genomic Map of Mouse mtDNA

The D-loop region of H-strand synthesis (O_H) and origin of L strand synthesis (O_L) are noted. Black triangles denote tRNA genes, which are assigned the one –letter universal code, and the polarity of each triangle indicates the direction of transcription of the tRNA gene. The outer arrows identify the mapped transcripts which correspond exactly to the reading frames of known genes and unidentified reading frames (URF) (taken with adaptation from Van Etten *et al.*, 1982).

2.3.2. The Displacement loop Region

The displacement loop (D-loop) region is the major non coding region, it contains a distinctive triple-stranded structure created by the nascent heavy H strand which displaces the parental H strand and hence the name displacements loop (Larizza *et al.*, 2002).

The rate of evolution in the D-loop region is higher compared with rRNA and protein coding genes of the mitochondrial genome (Matson and Baker, 2001). The analysis of 27 D-loop from ten mammalian orders have shown that three domains can be identified in this region, the extended termination-associated sequences (ETAS) domain, it spans from the $tRNA^{Pro}$ to the central domain, the central domain (CD), and the conserved sequence block (CSB) domain spanning from CD to the $tRNA^{Phe}$ (Larizza *et al.*, 2002). The CD is highly conserved in all species, the functional significance of this region is not clear (Matson and Baker, 2001). It was suggested that ETAS elements provide a termination signal for replication (Doda *et al.*, 1981); the ETAS domain contains two conserved blocks called ETAS1 and ETAS2 (Sbisá *et al.*, 1997). The domain CSB represents a functionally important region as it contains the replication origin of H strand, the promoters of H and L strands and three conserved blocks (CSBI, II, and III) which was suggested to be involved in the genesis of RNA primers for H-strand replication. The ETAS and CSB provide greatest variability for population analysis (Matson and Baker, 2001).

Studies of mammalian control regions have identified repeated sequences present in both the ETAS and CSB domain. These tandem repeats differ in length, copy number and base composition in different species (Larizza *et al.*, 2002).

In humans, the substitution rate of the non-coding control region (D-loop) has been estimated to be 2.8 to 5 times higher than the rate for the rest of the mitochondrial genome (Aquadro and Greenberg, 1982); and within this region,

the two peripheral domains (hypervariable region) evolve even faster (Pesole *et al*., 1999).

The mtDNA control region or D-loop can provide an appropriate region for examination for a number of different types of studies like population structure, phylogeographic structure. Heterogeneity within the control region has been used to study patterns of genetic variation at several different levels of divergence to construct phylogenetic trees in mammals (Matson and Baker, 2001). The fair degree of variation exhibited in this region between individuals in the peripheral hypervariable regions (HVRs) included within the ETAS and CSB is considered as a useful tool for human identity testing (Butler, 2001). In Iraq, mtDNA lineage and the diversity for forensic applications were studied using a part of the control region (Al-Zahery, 2000).

2.3.2.1. The D- Loop Region in Mice

Comparative analyses of complete rodent sequences revealed that ETAS, CD and CSB are present in all rodent species (Larizza *et al.*, 2002). Figure 2-3 shows the structure of the D-loop region in rodents as in agreement with previous surveys on mitochondrial control region on mammals. In mice, the D-loop region is 879 bp that contains the origin of H strand synthesis (O_H). It's known as the sequence bounded by the genes for $tRNA^{Phe}$ (nucleotide 1) and $tRNA^{Pro}$ (nucleotide 15.416) (Bibb *et al.*, 1981).

The origin of H strand synthesis is maintained as a triple-stranded structure due to repeated synthesis and degradation of a family of short, single stranded DNAs complementary to L strand forming D-loop (Bibb *et al*., 1981). The H strands of the D-loop are found with different lengths at the 5^{-}-3^{-} ends which were mapped by Doda *et al.* in 1981.

Only one member of this family is present in any single mtDNA molecule (Van Etten *et al.*, 1982). It's thought that these strands are too complex for a

single role in DNA replication (Bogenhagen and Clayton, 1978). But since D-loop region contains the transcription promoters of H and L strands it's possible that this region is essentially involved in control of gene expression (Bibb *et al.*, 1981). Consistent with that are the facts that no open reading frames of specific size exist in the D-loop region which makes it unlikely that this sequence has a coding function. Also no RNA species complementary to the region bounded by $tRNA^{Phe}$ and $tRNA^{Pro}$ were found (Van Etten *et al.*, 1982).

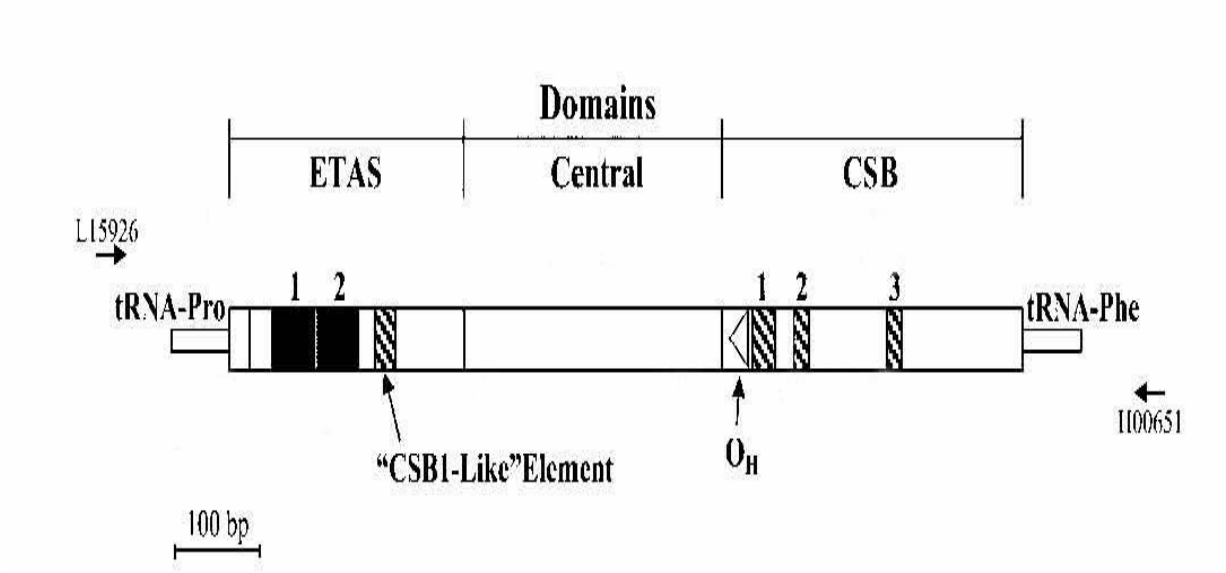

Figure (2-3): Schematic diagram of the mtDNA control region in rodents. Arrows identify the location and direction of amplification primers. Location of termination associated sequence (ETAS); central conserved and conserved sequence block (CSB) domains are also presented. All CSB (diagonal striped box) and ETAS (black box). The three stranded D-loop structure extends from the O_H in the CSB domain to near the ETAS elements, the entire central domain is within the D-loop (taken with adaptation from Matson and Baker, 2001).

2.3.3. Replication of Mitochondrial DNA

MtDNA replication is not synchronous with nuclear DNA replication which occurs during the S-phase, also not all mtDNAs replicate in synchrony, some may replicate twice during a cell cycle while others are not replicated at all (Singh, 2000). It was observed that mtDNA replicates in differentiated quiescent cells and that mitochondria found near the nucleus are more likely to replicate their mtDNA than mitochondria present in peripheral regions of the cell (Davis and Clayton, 1996).

Replication of mtDNA starts at one origin O_H with an RNA primer transcribed from L-strand promoter .The synthesis of the RNA transcript requires the mitochondrial RNA polymerase enzyme and a nuclear encoded transcription factor Tfam (Shadel and Clayton, 1997). The generation of a short transcript opens an R-loop. The transcript is processed by RNA processing enzymes inside the mitochondria to form a primer. The primer is elongated by mtDNA polymerase enzyme resulting in a relatively large D-loop (~1 Kb) of discrete size. DNA replication pauses at this point which made it recognizable by electron microscopy. H strand elongation proceeds two thirds of the way around the mtDNA, displacing the parental H stand until it reaches the L strand origin of replication O_L which is found between five tRNA genes (Singh, 2000).

Once exposed on the displaced H strand, O_L folds forming a stem loop structure and mtDNA primase is attracted for the synthesis of a second primer from which the L-stand synthesis is initiated proceeding back along the H strand template (Finnilä, 2000). Removal of the RNA primers and ligation of the whole DNA circles finishes the replication process (Shadel and Clayton, 1997). Consequently mtDNA replication is bidirectional however asynchronous (Clayton, 1982).

2.3.4. Transcription of Mitochondrial DNA

MtDNA transcription of the heavy and light strands starts from two transcription promoter sites (HSP, LSP). HSP and LSP are located in close proximity in the D-loop region of mammalian mtDNA. LSP short transcripts are either converted to primers for DNA replication or elongated to include the entire mtDNA circle (Singh, 2000).

Transcripts from both promoters proceed around the mtDNA circle to create a polycistronic RNA from which 22tRNAs, 12S and 16S rRNAs, and 13mRNAs are liberated from both primary transcripts by endonucleolytic cleavage (Singh, 2000). The mechanism of cleavage was described as the 'tRNA punctuation model of RNA processing' (Ojala *et al*., 1981).

2.4. Mitochondrial Disease and Mutation

One of the first indications that mitochondria may play a role in human pathogenesis was reported more than 40 years ago by Rolf Luft in 1962 in a patient with hypermetabolism whose skeletal muscle contained large numbers of abnormal mitochondria it was the first implication of respiratory chain dysfunction (Luft *et al*., 1962; Wallace, 1999; Larsson and Oldfors, 2001).

Biochemical and morphological studies of muscle tissues from patients with mainly encephalomyopathy revealed mitochondrial dysfunction (Singh, 2000). Then mtDNA was discovered in 1963 (Nass and Nass, 1963), sequenced and genes identified for man and mouse in 1981 (Anderson *et al.,* 1981; Bibb *et al.*, 1981).

In 1988 the identification of mtDNA mutation was reported, the maternally inherited Leber's hereditary optic neuropathy (LHON) a sudden onset blindness resulting from mtDNA missense mutation was the first disease identified by Wallace and colleagues (Wallace *et al.*, 1988).

The elucidation of genetic bases for a large number and variety of mitochondrial diseases in the late 1980s launched a new area of research as mitochondrial medicine (Singh, 2000). In addition to LHON, chronic progressive external ophthalmoplasia (CPEO) and Kearns-Sayre syndrome (KSS) were the first mitochondrial diseases defined at the genetic level and since then over 50 pathogenic mtDNA substitution mutations and hundreds of mtDNA rearrangement mutations (deletions and insertions) have been identified (Wallace, 1999).

The mode of mitochondrial inheritance can be variable, maternal, Menedelian, autosomal dominant or autosomal recessive and a combination of the two. MtDNA mutations associated with mitochondrial diseases may produce childhood and adult diseases and can occur spontaneously or acquired resulting from environmental exposure to biochemical toxins or exposure to various drugs including those used against human immunodeficiency virus (HIV) (Clay *et al.*, 2001).

Adding more complexity, the correlation between phenotype and genotype in mitochondrial disease is also variable and overlapping .each mtDNA mutation can produce a wide range of phenotypes due to many reasons; the versatile functions it performs in different tissues, the variability of normal or wild type to mutated mtDNA proportion and different aerobic dependencies in different tissues (Wallace, 1999).

Different tissues have different threshold requirements for oxidative phosphorylation, an idea proposed by Wallace, as the percentage of mutant mtDNAs increases, the cellular energy capacity declines until it falls below the bioenergetic threshold, the minimum energy needed by a cell or tissue in order to function normally, beyond this threshold the tissue behaves abnormally and symptoms appear. The brain, cardiac and skeletal muscles have the highest energy demand in general and thus oxidative phosphorylation deficiencies are

common with neuropathy, cardiopathy and myopathy often with defects in other organs (Wallace, 1999; Singh, 2000).

Clinical signs and symptoms in mitochondrial disorder commonly indicate multi-organ involvement. Morphological analysis on light microscopy examination of muscle biopsy specimen from patients with mitochondrial myopathy shows the typical finding of red ragged fibers (RRF) which is a muscle fiber transformation represented by the subsarcolammal accumulation of mitochondria that are stained red by Gomori trichome technique (Engel and Cunningham, 1963). On electron microscopy mitochondria appear abnormal with increased size, abnormal cristae and often contain paracrystalline inclusions with isoform of mitochondrial creatine kinase as the major constituent of these inclusions (Stadhouders *et al.,* 1994). A relationship between deficient oxidative phosphorylation and abnormal proliferation of mitochondria in RRF suggests that the latter is secondary to defective respiratory chain function (Larsson and Oldfors, 2001). However absence of RRF does not exclude disease (Clay *et al*., 2001).

Biochemical analysis follows morphological investigations to assess the metabolic capabilities of oxidative phosphorylation to identify any defect however morphological and biochemical data alone are unable to provide a systematic classification of mitochondrial disease (Zeviani and Antozzi, 1997).

MtDNA mutations result in a characteristic mosaic pattern of enzyme deficiency as a result of segregation where pathogenic mtDNA with mutated mtDNA proportion is variable in different cells while nuclear DNA mutations show generalized reduction in the activity of the affected respiratory chain enzyme complex (Larsson and Oldfors, 2001).

Cytoplasmic hybrid or cybrid is a standard technique used to define whether cellular defects related to mitochondrial disease are due to mtDNA mutation. A mtDNA mutation carrying resistance to the mitochondrial ribosome inhibitor chloramphenicol (CAP) is transformed from CAP- resistant (CAP^R) cells to

CAP-sensitive (CAP^S) by fusion of enucleated cytoplasmic fragments(Cytoplasts) (Wallace, 1999).

Since mitochondrial diseases follow different inheritance patterns and phenotypes of mitochondrial diseases can vary it's more productive to classify patients by genetic defect rather than by clinical manifestation.

Pathogenic mtDNA mutations include base substitution and rearrangements, base substitution can be subdivided into missense mutations that affect the 13 protein encoding genes and those that affect the rRNA and tRNA genes having global effects on mitochondrial protein synthesis. Diseases such as LHON, dystonia and Leigh's disease are good examples of the clinical variability accompanying a heteroplasmic missense mutation. MTND6*LDYT14459A mutation can cause LHON and dystonia. LHON occurs in midlife with sudden blindness caused by death of the optic nerve while dystonia present early with mental retardation, generalized movement disorder and impaired speech; degeneration of the brain's basal ganglia is also frequent in dystonia (Zeviani and Antozzi, 1997).

It is thought that lower percentage of mutant MtDNA it is associated with LHON while higher percentage is associated with dystonia. MTND6*LDY 14459A mutation converts the amino acid alanine into valine at codon 72 by the G-A transition in mitochondrial *ND6* gene which encodes a subunit of complex I , NADH dehydrogenase causing a substantial reduction in complex activity (Wallace, 1999).

A complex array of symptoms is produced when mutations occur in mitochondrial synthesis genes. Mutations are heteroplasmic in sever cases, frequently causing abnormalities in the central nervous system (CNS). The association of mitochondrial myopathy with RRFs is referred to as mitochondrial encephalomyopathy. MERRF mycolonic epilepsy and RRFs caused by MTTK*MERRF8344Gin the $tRNA^{leu}$ gene. MTTL1*MELAS 3243G a mutation in $tRNA^{leu}$ gene when present in a high percentage > 85% of

mtDNAs can cause MELAS a stroke activity and mitochondrial myopathy. The same mutation when present in low percentage 5-30% of mtDNAs was found to be associated with maternally inherited diabetes mellitus. Complex I, IV in patients harboring either of the mutations above have reduced levels of mitochondrial protein synthesis and complex I, IV activities. Milder mitochondrial mutations in protein synthesis genes can be homoplasmic affecting the CNS only. MTTQ*ADPD 4336C mutation in $tRNA^{Gln}$ gene, an example of such a mutation which was associated with late onset Alzheimer disease (Wallace, 1999).

MtDNA rearrangements can be either single MtDNA deletions or more rarely duplications. mutations are heteroplasmic and mostly found in patients with Kearns- Sayre syndrome (KKS), chronic progressive external ophthalmoplasia (CPEO), Pearson's marrow-pancreas syndrome, a fatal disease associated with pediatric pancytopenia (loss of all blood cells) and maternally inherited diabetes mellitus (Zeviani and Antozzi, 1997).

Nuclear DNA mutations could affect mtDNA replication and repair, transcription, protein synthesis in the matrix, protein import and certain other properties of mitochondria, an example is the mitochondrial transcription factor A (Tfam), a regulatory factor encoded by a nuclear gene if reduced might affect mtDNA replication, transcription and may lower the level of mtDNA and of transcripts or both (Singh, 2000).

Mitochondrial diseases resulting from mutations in nuclear OXPHOS genes follow Mendelian inheritance patterns and exhibit clinical features of mtDNA mutations. Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is a syndrome with mitochondrial myopathy. RRFs, abnormal mitochondria, decreased respiratory chain activity and multiple mtDNA abnormalities. MNGIE an autosomal recessive disease linked to mutations in *TP* which is a nuclear gene encoding thymidine phosphorylase. Destruction of mitochondria results in the appearance of clinical symptoms however inactivation of TP alters

thymidine pools that are crucial in mtDNA maintenance. MtDNA depletion syndrome is another disease, characterized by sever reductions in mtDNA levels in muscle, liver or kidney leading to organ failure and death .A nuclear mutation disrupting the regulation of mtDNA copy number during development results in random loss of the mtDNA is believed to be the cause of this disease (Wallace, 1999).

2.4.1. Mitochondrial DNA Damage, Repair and Carcinogenesis

The mtDNA damage is more extensive and persistent than nDNA damage, persistence of mtDNA damage could be due to continued reactive oxygen species (ROS) production and /or damage within the electron transport (ET) system. Alterations in mtDNA physiology may contribute to a cellular stress response, cell growth arrest and subsequent apoptosis (Yakes and Van Houten, 1997). ROS are a natural byproduct of respiration. OXPHOS is the major endogenous source of the ROS including super oxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) (Wallace, 1999).The cell has evolved a two step mechanism to disable oxygen free radicals: in the first step, mitochondrial manganese superoxide dismutase (MnSOD) dismutases (reduce and oxidize) the superoxide free radical to form hydrogen peroxide and oxygen (reaction 1 below) (Internet 1). H_2O_2 is converted to H_2O by glutathione peroxidase (GPx) (reaction 2 below). H_2O_2 can also be converted to highly reactive hydroxyl radical by the Fenton reaction in the presence of reduced transition metals for example ferrous iron Fe^{2+}(reaction 3 below) (Wallace, 1999).

$$2O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad \text{(1)}$$

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \quad \text{(2)}$$

$$2H_2O_2 + Fe^{2+} \longrightarrow {}^{\cdot}OH + OH^- + Fe^{3+} \quad \text{(3)}$$

Superoxide and hydrogen peroxide are not thought to be the major causes of mutations in the mitochondrial genome; the most mutagenic of the reactive oxygen species is the hydroxyl free radicals which react with all types of biologically important molecules like nucleic acids, proteins, sugars and lipids producing radicals that undergo further reactions. DNA radicals can react with protein radicals (in histons) to form cross links interfering with chromatin unfolding, DNA repair, replication and transcription (Internet 1).

DNA damage induced by ROS includes, single and double strand breaks, abasic sites and base damages. Studies indicate that mtDNA damage contains elevated basal level of base damage when compared to nDNA (Richter *et al*., 1988; Yakes and Van Houten, 1997).Cellular exposure to certain compounds was shown to induce significant mtDNA damage such as alloxan (Yakes and Van Houten, 1997).

Oxidative mtDNA damage has been linked to the initiation of specific human diseases such as neuronal degeneration and cardiovascular diseases (Richter *et al*., 1988).aging and cancer (Zeviani and Antozzi, 1997; Modica-Napolitano and Singh, 2002).

The repair of ROS induced damage in both mtDNA and nDNA occur through a base-excision repair pathway that generally involves removal of damaged bases by the action of damage specific DNA glycosylases (Yakes and Van Houten, 1997). Concerning mtDNA repair machinery, it was observed that mitochondrial enzymes are capable of performing base excision repair, however

there is no clear evidence that they are capable of repairing bulky DNA adducts resulted from carcinogens. Many chemical carcinogens have been shown to target mtDNA 50 to 100 times more actively than nDNA, the resulted bulky adducts that are not successfully repaired can serve as blocks to DNA replication promoting increased frequency of deletion in mtDNA (Bogenhagen, 1999). Quantitative PCR was used to measure H_2O_2 induced DNA damage and repair in a 17.7Kb fragment upstream of the nuclear ß-globin gene and 16.2kb fragment mitochondrial genome and revealed that mtDNA is more sensitive than nDNA to H_2O_2 induced damage and protracted treatment leads to persistent mtDNA damage and loss of mtDNA function (Yakes and Van Houten, 1997).

There are several reasons for the high steady state level of oxidation in mtDNA, one of which is that mitochondria consume >90% of the cells oxygen and OXPHOS is the source of continuous oxygen radicals. Another reason is that mtDNA is not covered extensively by proteins like for example histons and thus mtDNA may be more susceptible to attack by excited oxygen than nDNA besides the fact that mitochondria have an inefficient repair system (Richter *et al*., 1988).

The increasing mitochondrial oxidative stress caused by changing the cellular capacities of reactive oxygen species may lead to the impairment of mtDNA, or the disruption of the apoptotic process induced by mitochondria which may contribute to neoplastic transformation, Frequent mtDNA alterations around position 310, a region contributing to persistent RNA-DNA formation, leading to initiation of mtDNA replication suggesting that sever alterations in this region might be involved in giving growth advantage of tumor cells (Okoci *et al.,* 2002). Consistent with mitochondrial ROS being important in tumor formation, MnSOD was found to be reduced in many types of tumors such as in prostate cancer (Petros *et al.*, 2005).

MtDNA mutations were investigated in bladder cancer and in a carcinogen induced rat bladder cancer model. Results showed that mtDNA exhibits a high rate of mutations in both human and rat bladder cancer and that the high incidence of mtDNA mutations could play an essential role in the process of carcinogenesis and that mtDNA mutations could be valuable as a marker for the early detection of bladder cancer diagnosis (Chen *et al*., 2004). In mice, the D-loop region was found to be a hot spot and may contribute to the carcinogenesis of murine tumors (Dai *et al.*, 2004). The unique single stranded structure of mtDNA D-loop region is more easily damaged by various genotoxic mechanisms and so this region maybe a natural hot spot for experimentally induced DNA damage (Singh, 1992)

2.4.2. Cancer and Mitochondrial DNA

Mitochondrial defects have long been suspected to play a role in the development and progression of cancer. Research concerning mitochondrial respiration alteration in the context of cancer was pioneered by Warburg over more than 70 years ago (Warburg, 1930).

Several distinct differences were identified on the microscopic, metabolic, biochemical, molecular and genetic levels between the mitochondria of normal versus cancer cells. Cancer cells have altered metabolism such as higher rate of glycolysis, increased rate of glucose transport and gluconeogenesis and reduced pyruvate oxidation besides alterations in other vital processes. Alterations in the molecular composition of the inner membrane of tumor mitochondria were also identified regarding polypeptide and lipid composition, protein synthesis rates, organelle turnover and the capacity to accumulate and retain calcium were found to be different in mitochondria of normal cells and transformed cells Various tumor cell lines showed differences in the number, size and shape of their mitochondria in comparison to normal controls. Rapidly growing tumors

tend to have mitochondria that are fewer in number, smaller in size and have less cristae than that found in normal cells. Remarkably, benign oncocytoma of thyroid, kidney, salivary gland, parathyroid and breast share some features found in mitochondrial encephalomyopathy when large numbers of abnormal mitochondria aggregate as resolved by ultra structural studies. Amounts and forms of DNA were also found to be different in the mitochondria of normal versus transformed cells (Modica-Napolitano and Singh, 2002).

Mutations in mtDNA have been reported in a variety of human malignancies including breast, ovarian, colorectal, gastric, hepatic, esophageal, pancreatic, renal, prostate, brain, thyroid, bladder, head, neck, lung and leukemia. Examination of mtDNA from matched normal and malignant tissue samples and cancer cell lines revealed the presence of different types of mtDNA mutations ranging from point mutations, deletions and duplications in different mtDNA genes like 16SrRNA, 12SrRNA, ND1, ND2, ND5, ATPase6 and cytochrom b, the D-loop region seems to be the most frequent site of somatic mutations across most tumor types (Carew and Huang, 2002).

Both homoplasmic and heteroplasmic mutations have been found in cancer cells (Modica-Napolitano and Singh, 2002). But most tumors contain homoplasmic (100%) mutant mtDNA because of the clonal nature of cancers (Carew and Huang, 2002). Polyak *et al.* 1998 provided one of the first examples of homoplasmic mtDNA mutations in tumor cells where he showed that mitochondria can rapidly become homogenous in colorectal cancer cells using cell fusions. The complete mitochondrial genome of ten human colorectal cell lines was sequenced, mutations were found in seven (70%) cell lines. Most of the mutations were transitions at purines consistent with an ROS-related derivation and were somatic occurring in the primary tumor from which the cell lines were derived. The majority of mutations were also homoplasmic which is an indication that the mutant genome was dominant at the intracellular and intercellular levels.

Achieving homoplasmy of tumor cells or mitochondrial dominance was the focus of many researchers. Previous cell fusion experiments have indicated that mitochondria from tumor cells can selectively proliferate when such cells are fused to normal cells and that mitochondrial dominance is nonrandom (Shay and Ishii, 1990). Consistent with the previous experiments, Polyak *et al.* 1998 through fusion experiments clearly indicated that tumor mitochondria of one type can have significant replicative advantage over other types, that is a single cell with a mutant mitochondrial genome acquired selective growth advantage during tumor evolution allowing it to become the predominant cell type in tumor population, whether the selection was strictly by mitochondrial control or a combination of nuclear and mitochondrial factors was not determined.

In another study, another possibility was considered for mitochondrial dominance that is the observed homoplasmy arose completely by chance in tumor progenitor cells through unbiased DNA replication and sorting during cell division without selection for physiological advantage or tumoriogenic requirement. An extensive computer modeling was developed to investigate that there is a sufficient opportunity that somatic mtDNA mutations may be driven entirely by random processes. However, some mitochondrial point mutations may provide a selective advantage or disadvantage for the carrier mtDNA or host cell, a phenomenon may occur in some tumors but it is not necessary a general rule. The selective advantage in some of the mutations identified in tumors is unlikely (Coller *et al.*, 2001). Among the mtDNA mutations known as homoplasmic in tumors are nine instances of silent substitutions in amino acid coding regions and there are 33 different alterations in the length of a C-tract that is polymorphic in healthy individuals (Fliss *et al.*, 2000).

The first mtDNA alterations in hematological malignancies were discovered by Clayton *et al*. in 1967 using cesium chloride- ethidium density centrifugation and electron microscopy, they discovered alternate mtDNA structures.

Although these alternate structures can also exist in normal cells, an unusually high percentage of them were found in leukemic cells compared to normal controls.

Malignant glioma is perhaps the best characterized type of brain tumor in the context of mtDNA alterations concerning high frequency of mtDNA copy number changes. In a study involving 45 glioma specimens it was found that mtDNA was highly amplified in 87% of cases (Liang and Hays, 1996).

D-loop region, 16SrRNA, and NADH subunits were sequenced in matched premalignant prostate tumor and lymphocytes in 16 patients, mutations were found in three patients occurring early in disease progression and were detectable in bodily fluids during early stages of disease (Jeronimo *et al.*, 2001).

In breast cancer, comprehensive studies using a combination of temporal temperature gel electrophoresis and direct DNA sequencing screening the complete mitochondrial genome in 19 sets of paired normal and tumor tissues from the same patient revealed the identification of somatic mutations in 14 of the 19 patients (74%). most of the mutations (81.5%) were in the D-loop region however mutations were also found in the 16SrRNA, ND2, and ATPase6 gene (Tan *et al.*, 2002).

An investigation of 15 pancreatic cancer cell lines and xenografts revealed the presence of mtDNA mutation in nearly every case. Mutations were identified in different genes such as the 12SrRNA, 16SrRNA, ND1, ND2, ATPase, Cytochrom b, non coding D-loop region and in many other genes. A 6-8 fold increase in the mtDNA mass in tumor cells compared to normal pancreatic cells was also found. The high frequency of mtDNA mutation and mtDNA contents increase in pancreatic cancer cells make mtDNA a potential diagnostic tool (Johns *et al.*, 2001).

An analysis of 19 specimens from hepatocellular carcinoma (HCC) patients indicated that the D-loop mutations is a frequent event and maybe used as a molecular tool for the determination of clonality (Nomoto *et al.*, 2002).

2.5. Molecular Analysis

2.5.1. Polymerase Chain Reaction (PCR)

PCR is a powerful technique in DNA profiling that has revolutionized the course of molecular biology for its ability to synthesize millions of copies of a specific DNA sequence in a matter of only few hours, the first publication of PCR appeared in 1985 by Kary Mullis who received the Nobel Prize in chemistry in 1993 (McPherson and Møller, 2001).

PCR is a sensitive, rapid and is not limited by the quality of DNA and therefore utilized in different fields of biological sciences like in biomedical research, identity testing, detection of infectious agents and genetic defects, the study of molecular evolution, medical epidemiology and forensic medicine besides environmental monitoring (Ou, 1990).

Inside every cell DNA replication is carried out by the enzyme DNA polymerase and hence the name PCR which can be defined as an *in vitro* method of enzymatic synthesis of defined sequences of DNA (McPherson and Møller, 2001).

The reaction utilizes two oligonucleotide primers ranging from 20 to 30 bp in length which depends on the application; they hybridize to complementary sequences on opposite strands flanking the DNA sequence to be amplified. Elongation of the hybridized primers is catalyzed through a heat stable DNA polymerase in the presence of the four DNA building blocks, deoxynucleoside triphosphates (dNTPs), the repetitive series of cycles comprising template denaturation, primer annealing and extension of annealed primers results in the exponential accumulation of a specific DNA fragment (Figure 2-4), The ends of the fragment are defined by 5^{-} end of the primer (Saiki *et al.*, 1985). The primer extension products will serve as a template in the next cycle doubling the

number of target DNA copies and thus 20 cycles of PCR approximately generate a million copies of target DNA (McPherson and Møller, 2001).

Typically a PCR amplification reaction consists of 30 - 40 cycles using a thermal cycler where temperature is controlled automatically, each cycle starts with heating of samples 93- 95˚C to achieve full denaturation of double stranded template DNA. Complete separation of the two strands is sufficient for a successful PCR (Saiki, 1989). The second step starts when the samples are cooled down to 50-70˚C to allow primers to anneal to denatuered DNA, following annealing, the temperature increases to 70-75˚C in which synthesis of new copies of the template sequences by DNA polymerase occurs. 74ºC which is the optimum temperature in which Taq DNA polymerase functions efficiently for primer extension adding dNTPs to 3^- (OH) end of the primer (McPherson and Møller, 2001). Taq DNA polymerase is a widely used DNA polymerase isolated from hot springs habitat bacteria *Thermus aquticus* and remains the enzyme of choice for routine amplification of small DNA fragments (Ou, 1990).

A number of factors may affect a successful PCR reaction involving physical and chemical factors. Physical factors such as the thermal cycler used, cycle number and length and temperature. Chemical factors comprise all of the components contained in the reaction like Magnesium chloride ions ($MgCl^{2+}$) which is a critical factor that exists as dNTPs- $MgCl^{2+}$ complexes that interact with sugar phosphate backbone of nucleic acids influencing the activity of the Taq DNA polymerase. In addition to other ions contained in the PCR reaction buffer. Concentration of primers, dNTPs and Taq DNA polymerase influence in relatively different extents the reaction specificity (Sambrook and Russell, 2001).

Careful standardization of parameters, cautious employment of laboratory procedures is usually followed to achieve optimal results. Setting of control reactions in parallel with test samples to indicate any contamination problems

which are very imminent since PCR is capable of amplifying sequences from minute amounts of DNA even the DNA from a single cell (Li *et al*., 1988)

In general DNA sequences amplified by PCR have typically been in the size 0.1-5 Kb and it becomes exceedingly difficult to obtain efficient amplification as the desired product length increases (Strachan and Read, 1999) however amplification of sequences up to 22 Kb was reported (Cheng *et al.*, 1994). Fragments produced by PCR are usually resolved by electrophoresis in agarose gels and detected by staining the gel with ethidium bromide (McPherson and Møller, 2001).

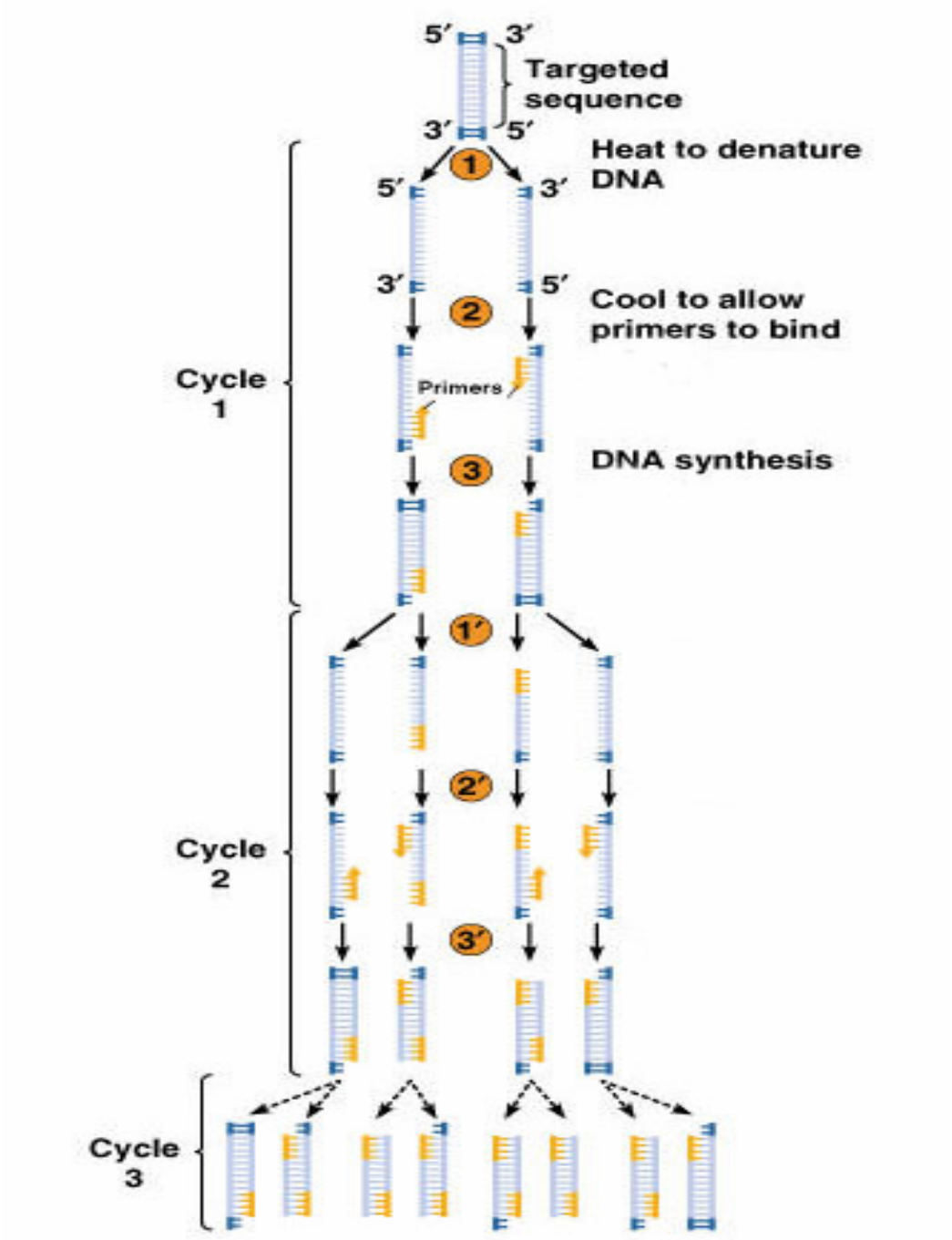

Figure (2-4): General scheme of the Polymerase Chain Reaction (PCR) (from http://www.ucfv.bc/biology/terry /202/ labs/ lab1).

2.5.1.1 Random Amplified Polymorphic DNA-PCR (RAPD-PCR)

Random Amplified Polymorphic DNA (RAPD) is a PCR based molecular technique also known as arbitrary primed PCR (AP-PCR) that was first described by Williams *et al.* in 1990 and is based on the amplification of DNA using short single primers usually 10 bases of arbitrary nucleotide sequence to reproducibly amplify segments of DNA from a wide variety of species. RAPD-PCR is based on arbitrary amplification under low stringency conditions, short primers and low annealing temperature 36˚C insures the amplification of various genomic regions simultaneously.

The number of amplified fragments depends on primer sequence, template DNA and size of the genome. In general, the number of generated fragments ranges between 5-10 and may reach to 21 with different molecular weights ranging from 200-4000 bp. Genetic variations between individuals appear when primers match to complementary sequences resulting in different patterns of amplified DNA fragments with different molecular sizes, amplification products can be detected through examination of an ethidium bromide-stained agarose gels (McPherson and Møller, 2001).

RAPD-PCR became a particularly popular technique for many reasons; its performed on any organism without the need for prior knowledge of DNA sequence information and it works effectively with tiny amounts of DNA and thus was applied in genetic mapping, for plant and animal breeding applications, DNA fingerprinting, study of population genetics, epidemiological studies and typing of micro-organisms and for the identification of pathogenic strains of bacteria and fungi (Williams *et al.*, 1990).

The approach is also often utilized for the detection of abnormal DNA sequences in human cancers. Genomic fingerprints of DNA from normal and tumor tissue can be used to compare deleted or generated DNA sequences in

cancer cells (McPherson and Møller, 2001). RAPD was used to detect genetic alterations in human tumors (Arribas *et al.*, 1997), genetic instability in tumors (Luceri *et al.*, 2000).

Several studies investigated the capability of RAPD markers as a tool to detect genomic DNA alterations caused by different environmental and mutagenic agents, they were used in the discrimination of abnormal bird phenotype (Ali, 2003), and detection of X-ray induced DNA damage (Shimada and Shima, 1998).The chemotherapeutic mitomycin C (Becerril *et al*., 1999). Copper (Atienzar *et al.*, 2001), and genetic alterations induced by benzo[a] pyrene (Atienzar *et al.,* 2002).

2.5.1.2. Specific PCR

PCR is designed to achieve selective amplification of a specific target DNA sequence within heterogeneous collection of DNA sequences. To construct specific oligonucleotide primers for a particular DNA amplification, the DNA region of interest must have been partially characterized previously with 15-25 nucleotides long that bind specifically to complementary DNA sequences at the target site in the presence of a suitable DNA polymerase and dNTPs (Berg *et al*., 2002).

The specificity of amplification is determined by the ability of primers to recognize and bind to sequences other than the intended target DNA. Two primers of 20 nt long are usually sufficient for amplification from complex DNA sources such as total genomic DNA from mammalian cells because the chance of an accidental perfect match in a different site in the genome for either one of the primers is extremely low and for both sequences to occur by chance in close proximity in the specified direction is normally exceedingly low (Strachan and Read, 1999). PCR high specificity comes from the stringency of hybridization at high temperatures (Sambrook and Russell, 2001).

PCR was utilized to amplify entire mtDNA using two or more overlapping pieces (Torroni *et al.*, 1992). In another study nine overlapping fragments of entire mtDNA in Finns and Tuscans were amplified by PCR and restriction analysis was preformed to examine sequence mtDNA variation (Torroni *et al.*, 1996).

2.5.1.3. Restriction analysis of PCR Products

Restriction endonucleases are enzymes mainly isolated from prokaryotes. They are classified into three types, Type II are the most widely used enzymes in molecular cloning; they cleave DNA within or near to their particular recognition sequence (Sambrook *et al.*, 1989). These enzymes recognize specific oligonucleotide sequences, either four, five or six base pairs in length and cleave within a double stranded DNA (Avise *et al.*, 1979).

The restriction enzyme analysis of mtDNA proved as valuable approach to a number of important problems. The existence of restriction endonuclease mtDNA site polymorphisms was exploited to test the hypothesis of maternal inheritance of mtDNA in human, in the rat *Rattus norvegicus* (Giles *et al.,* 1980), and in the white footed mouse *Peromyscus polionotus* (Avise *et al.*, 1979). The high degree of intraspecific mtDNA variation in many studied species particularly *Rattus rattus* suggests a rapid rate of evolution which was previously hypothesized (Castora *et al*., 1980).

PCR is further utilized by combining it with restriction endonuclease analysis. PCR products subjected to direct restriction analysis is useful for verifying site specific mutations that introduce or remove a restriction site from a PCR product (McPherson and Møller, 2001).

The application of valid restriction endonuclease studies is based on the discrimination between variant mtDNAs by the restriction enzymes resulting from single base changes rather than from deletions, insertions, or

rearrangements and that the discrimination is based on nucleotide sequence differences rather than on differential methylation (Castora *et al*., 1980). Many studies have previously reported that the mtDNA within an individual appears homogenous. Homogeneity of mtDNA within individuals was proved using restriction enzymes in peromyscus (Avise *et al.*, 1979), and in different species (Brown and Vinograd, 1974; Potter *et al*., 1975).

2.6. Cytogenetic Analysis

Cytogenetics is the study of chromosomes and their abnormalities. Cytogenetic analysis is a widely used indicator system for the evaluation of physically, chemically and biologically induced mutations allowing objective evaluation of alterations in the genetic material and permitting direct visual analysis of chromosomes (Nakanishi and Schnieder, 1979; Gebhart, 1981; Juma, 1993).

Humans are exposed to a wide range of natural and synthetic genetically toxic substances including physical agents such as ultra violate light, cosmic rays, heat and chemicals present in the diet and environment, such factors may have the ability to modify the genetic material DNA and induce mutations. Thus it's imperative to identify any potential genetic hazard associated with these factors and to estimate their biological impact. Common tests used to detect mutagens utilize different organisms such as bacteria (Ames *et al.*, 1975), yeasts (Zimmerman *et al*., 1975); other tests utilized Drosophila, plants and mammals.

Cytogenetic tests proved to be efficient and reliable short term indicators of potential mutagens and can be applied *in viv*o (Nakanishi and Schneider, 1979), *in vitro (*Gebhart, 1981), *in utero* (Kram, 1982) and *in ovo* (Bloom, 1984). *In vivo* analyses mimic *in vivo* exposure of humans with the ability to detect agents requiring metabolic activation in order to be converted into active forms

utilizing normal cell populations. *In vitro* analyses are simpler, more rapid and may have the ability to detect chromosomal abnormalities at lower compound concentrations (Schneider and Lewis, 1982).

Most of the assays used to identify potential mutagens and carcinogens are performed in cells, in tissue cultures or in animals. Several animal models are used in cytogenetic analysis, but mice are the most frequently used because of the fast reproduction, small size, easy handling and it represents a typical mammalian system (Tice *et al.*, 1989). Different somatic and germ tissues have been analyzed in *in vivo* studies including spermatogonial cells (Allen and Latt., 1976), bone marrow cells (Kram *et al*., 1979), and spleen cells (Nath *et al.*, 1988), in addition to other cell types.

Several parameters are used in cytogenetic analysis including the Mitotic Index (MI), Blast Index (BI), Cell Cycle Progression (CCP), Replicative Index (RI), Sister Chromatid Exchange (SCE), and Chromosomal Aberrations (CA).

2.6.1. Mitosis and Mitotic Index (MI):

Mitosis is the type of cell division in somatic cells whereby a single cell gives rise to two daughter cells. Mitosis is vital in tissue formation and maintenance as it occurs in all embryonic tissues except mature neurons, skeletal muscle cells and red blood cells or what are known as the post mitotic tissues where cells do not divide at all (Sandberg, 1992).

The most vital activities in preparation for cell division occur during the growth phase of the cell cycle, known as interphase such as the synthesis of RNA, mRNA, and rRNA types which are responsible for the synthesis of cellular proteins and most importantly the chromosomal deoxyribonucleic acid (DNA) duplication which occurs during the distinct S phase (Synthesis phase) of the cell cycle. The M phase is another phase of the cell cycle in which nuclear division (mitosis) and cytoplasmic division (cytokinesis) take place. The period between M phase and the S phase is called G1 phase (gap phase)

while the period between the completion of DNA synthesis and the M phase is called G2 phase and thus the interphase of cell cycle consists of successive G1,S and G2 phases constituting 90% or more of the total cell cycle time. The average time is about 16 hours in human and other mammalian cells. Mitotic abnormalities have been recognized for many years as a common occurrence in malignant tumors. Mitotic abnormalities maybe quantitative, qualitative or both (Sandberg, 1992).

The mitotic index is counted as the ratio of nuclei in the mitotic stage to interphase nuclei in a thousand cells. This parameter is used to detect the effect of physical and chemical genotoxicity in living cells (Shubber *et al.*, 2003). The MI is considered as a useful indicator of cellular proliferation in tumor cells (Al-Abasi, 2001).

2.6.2. The Blastogenic Index (BI):

Blast cells are immature cells usually large in size that become progressively smaller as they mature. Blast cells are the progenitors of different types of cells sited in the bone marrow. Blastogenic index is used to describe the morphological changes of small resting lymphocytes into a blast-like cell due to the exposure to a specific mitogen. The transformation of lymphocytes is considered as a sign of immune recognition (Al-Duliemy**,** 2005).

Cells stimulated by a specific mitogen are committed to increase in size and start synthesizing DNA (Sharma and Sharma, 1980).The blastogenic index determined by the ratio of blast cells in a one thousand cells. It was used in the evaluation of immune response in cancer patients (Al-Abasi, 2001).

2.6.3. The Chromosomal Aberrations (CA):

Chromosomal abnormalities are defined as changes resulting in a visible alteration of the chromosome. CA have significant genetic, evolutionary and medical consequences and have become a distinctive biological method for the estimation of whole body dose. Types of chromosomal abnormalities include numerical and structural chromosome abnormalities. Numerical abnormalities

involve the gain or loss of complete chromosomes such as in Down syndrome in which three copies of the chromosome 21 exist known as trisomy 21. Monosomy in Turners syndrom is another numerical abnormality in which one copy of the 21 or 22 chromosome exists (Tamarin, 1996).

Structural chromosomal abnormalities occur when breaks arise and repaired incorrectly. Chromosomes can break due to exposure to ionizing radiation, physical stress or chemical compounds, breaks can occur at either the chromatid or chromosomal level. Chromosomal breaks occurring during the S phase of the cell cycle before DNA replication result in chromosome level break involving both sister chromatids at the same point while any breaks that occur after the S phase are in single chromatids that is they are chromatid level breaks, chromosomal abnormalities are summarized as follows:

I. Noncentromeric breaks
   - A. Single breaks (chromatid)
     1. Restitution
     2. Deletion
   - B. Single breaks (chromosomal): Dicentric bridge
   - C. Two breaks (same Chromosome)
     1. deletion
     2. Inversion
   - D. Two breaks (non homologous chromosomes)

II. Centromeric breaks
   - A. Fission
   - B. Fusion

(Tamarin, 1996).

Cytogenetic analyses were used in the assessment of a large number of physical, chemical and biological agents. In blood lymphocytes exposed to X-

rays the MI was found to be decreased, CCP inhibited, SCE frequency increased and CA is significantly higher than the non-exposed controls (Shubber and Salih, 1988). Infants with Down's syndrome together with their parents were shown to have high MI (Shubber *et al.*, 1991).

Cancerous tissues are always characterized by distinct cytological features. The frequency of mitosis and chromosomal abnormalities especially numerical variations were found to be conspicuous features of cancer cells (Sharma and Sharma, 1980). The most well- known chromosomal aberrations and SCE are strongly correlated to other genetic changes such as mutation, neoplastic transformation and teratogenesis (Shubber and Juma, 1999).

2.7. Mitomycin C and DNA

Mitomycin C (MMC) is an antitumor antibiotic ($C_{15}\,H_{18}\,N_4\,O_5$) produced by soil actinomycetes *Streptomyces caespitosus*, it interrupts DNA replication and inhibits mitosis (Costa *et al.*, 1993). MMC is a clinically active antineoplastic agent used to treat a wide variety of tumor cells in the head, neck, cervix, breast and others (Holtz *et al.*, 2003).

MMC induces genetic damage after being metabolized into its active form (Shubber *et al.*, 1985). It's metabolized by cellular reductases via bioreductive drug activation process (Phillips *et al.,* 2000). The activated form will bind and cross link DNA (Pan *et al*., 1984). It inhibits DNA replication but transcription is not affected for a certain period, it acts possibly by linking two polynucleotide strands of DNA. On activation, a mitomycin C molecule loses a methanol and the ethylamine group acts as an alkalyting agent, linking it with one of the DNA strands (Sharma and Sharma, 1980). Both monofunctional and bifunctional adducts are formed between MMC and DNA (Holtz *et al.*, 2003). MMC is a potent inducer of SCE both *in vivo* and *in vitro* (Tice *et al.,* 1989). An inhibitor of CCP both *in vivo* and *in vitro* (Shubber and Salih, 1988). It also

induces SCE and chromosomal aberrations in human lymphocytes in a dose dependent pattern (Kosyakova and Chebotare, 2003).

Most studies concerning MMC toxicity focused on nuclear DNA regarding it as the primary cellular target for MMC, but a study preformed by Pritsos *et al.* 1997 provided conclusive evidence that mtDNA is a target for MMC eliciting biochemical consequences that could prove toxic. *In vivo* and *in vitro* effect of MMC on mtDNA was tested using treated mice and treated EMT6 mouse mammary carcinoma cells revealed a dose dependent relationship between different MMC dosages and mtDNA integrity also livers of MMC treated mice showed lowered mitochondrial integrity.

CHAPTER THREE

MATERIALS AND METODS

3. Materials and Methods

3.1. Apparatus:

Agarose Electrophoresis unit- Consort/Belgium

Analytical Balance- AE/UK

Autoclave- Express/Japan

Autovortex- Stuart Scientific /UK

Cold microfuge- Eppendorff/Germany

Deep Freeze-Sanyo/Japan

Gel documentation system-Consort/Belgium

Hot plate magnetic stirrer-IKA/USA

Incubator-Shellaba/ Belgium

Laminar air flow-Iraqi Agency of Atomic Energy/Iraq

Laminar air flow-Biotechne/France

Master cycler gradient-Eppendorff/Germany

Microscope-Olympus/Japan

Oven- Memmert/Germany

pH-Meter-Jenway/ UK

Spectrophotometer-Cecil/France

UV-Transilluminator system-Consort/Belgium

Water bath-Memmert/Germany

3.2. Materials

Agarose-Sigma

Boric acid-Riedel-de Haën

Bromophenol Blue-Xylene cyanole dye solution-Sigma

Chloroform-BDH

Colchicine-Ibn Hayyan

Ethanol alkohol-Sigma

Ether-Sigma

Ethylene diamine tetra acetic acid (EDTA)-Sigma

Formamide-Sigma

Giemsa's stain-BDH

Glacial acetic acid- Fischer Scientific international company

Glycerin-BDH

Isoamyl alcohol-Sigma

Lauryl sulfate (Sodium dodecyl sulfate)-Sigma

Methanol-Merck

Mitomycin (MMC)- Kyowa Hakko Kogyo Co.Ltd.

Pancreatic RNAase-Sigma

Phenol-Sigma

Phosphate buffer saline (PBS)-Sigma

Potassium Acetate-Sigma

Potassium Chloride-Sigma

Sodium bicarbonate-BDH

Sodium chloride (NaCl)-Fischer Scientific international company

Sodium hydroxide (NaOH)-Fluka

Sucrose-Fluka

Trizma base (Tris. [hydroxymethyl] aminomethane)-Sigma

3.3. Stock Solutions:-

3.3.1. Mitomycin C (MMC)

Five milliliters of sterile distilled waster (D.W.) was added to 10 mg ampoule of MMC (Kyowa Hakko Kogyo). 0.1, 0.2 and 0.5 mg/kg body weight (bw) was calculated according to the weight of each animal for every experiment. This solution was stored at 4 $^{\circ}$C in the dark (Juma, 1993).

3.3.2. Tris-HCl pH 8 (1M)

It was prepared by dissolving 121.1 gm of Tris-base in 800 ml of distilled water (D.W). pH was adjusted to 8.0 by adding concentrated HCl and volume completed with D.W. to 1 liter. Sterilized by autoclaving (Maniatis *et al.*, 1982).

3.3.3. EDTA pH 8 (0.5M)

The amount of 186.1 gm was dissolved in 800 ml of D.W. and stirred using a hot magnetic stirrer. Few NaOH tablets were added to assist dissolving; pH adjusted to 8.0 using NaOH (1N), volume was completed to 1 liter with D.W. and sterilized by autoclaving (Maniatis *et al.*, 1982).

3.3.4. NaCl (5M)

It was prepared by dissolving 292.2 of NaCl in 800 ml D.W. and volume completed to 1 liter by D.W. and sterilized using the autoclave (Maniatis *et al.*, 1982).

3.3.5. Tris-borate buffer (TBE)

TBE 5 X solution was prepared by dissolving the following 54gm of Tris – base, 27.5 gm of Boric acid, 20ml of EDTA (0.5 M) (pH=8.0) in an appropriate amount of D.W., pH was adjusted to 7.8 and volume completed to 1 liter with D.W., The solution was sterilized by autoclaving for 15 min at121º C at 15 psi and stored at room temperature (Sambrook *et al.*, 1989).

3.3.6. Potassium Acetate pH 5

It was prepared by adding 11.5 ml glacial acetic acid to 60 ml of 5M potassium acetate, the solution was completed to 100 ml using D.W., the resulting solution is 3M with respect to potassium and 5M with respect to acetate (Sambrook *et al.*, 1989).

3.3.7. Sodium dodecyl sulfate (SDS) 10%

One hundred grams (100 gm) of SDS was dissolved in 900 ml of D.W. heated to 68C˚to assist dissolution; pH was adjusted to 7.2 by adding concentrated HCl and volume adjusted to 1 liter (Maniatis *et al.*, 1982).

3.3.8. NaOH (1N)

Four grams (4 gm) of NaOH was dissolved in 80 ml of D.W. and the volume adjusted to 100 ml using D.W. (Maniatis *et al.*, 1982).

3.3.9. Buffer A

Was prepared with final concentrations of the following 0.25M of sucrose, 10mM of EDTA and 30mM of Tris-HCl pH 7.5, volume was adjusted with D.W. and sterilized by autoclave (Tamura and Aotsuka, 1988).

3.3.10. Buffer B

This buffer was prepared with final concentrations of the following 0.15 M of NaCl, 10mM of EDTA and 10mM of Tris-EDTA pH 8.0. Volume was adjusted with D.W. and sterilized by autoclave (Tamura and Aotsuka, 1988).

3.3.11. Modified Buffer B

Was prepared with the final concentrations of the following 0.15M of NaCl, 10mM of EDTA, volume adjusted with D.W. and sterilized using autoclave (Internet 2).

3.3.12. Phenol:-

Phenol is removed from the freezer, allowed to warm to room temperature and melted at 68C˚ using water bath. 250 ml of the melted phenol was then extracted several times with an equal volume of 1.0 M Tris pH 8.0 followed by 0.1MTris pH 8.0 and 0.2% mercaptoethanol. Until the pH of the aqueous phase was 7.6. The solution was kept under equilibration buffer at 4C˚ (Maniatis *et al.*, 1982).

3.3.13. Chloroform: Isoamylalcohol (24:1)

To prepare 25 ml, 1 ml of isoamyl alcohol was added to 24 ml of chloroform (Maniatis *et al.*, 1982).

3.3.14. Phenol: Chloroform

One part buffer saturated phenol and one part of chloroform : isoamyl alcohol 24:1 (Maniatis *et al.*, 1982).

3.3.15. Tris-EDTA (TE) pH 8.0:-

Was prepared by mixing Tris-HCl pH 8.0 to a final concentration of 10 mM and EDTA pH 8.0 to a final concentration of 1mM. Volume was completed with D.W. and sterilized by autoclaving (Sambrook *et al.,* 1989).

3.3.16. Pancreatic RNAase A 10mg/ml

It was prepared by dissolving 0.010 gm of the enzyme powder in sterile 1 ml of 10mM Tris- HCl pH 7.5, 15mMNaCl.heated to 100C˚ for 15 min and allowed to cool slowly to room temperature, dispensed into aliquots and stored at -20C˚ (Sambrook *et al.*, 1989).

3.3.17. Loading Dye

To prepare a stock solution, 5 ml of Bromophenol blue- Xylene cyanole dye solution supplied by sigma was mixed with an equal volume of D.W. To load DNA samples an aliquot was made by mixing 1 volume of the above solution with four volumes of formamide. Preparations were done according to instructions attached to the pack.

3.3.18. Ethidium Bromide 10mg/ml

One gm dye was dissolved in 100 ml of a sterile D.W. and the bottle was kept in a dark. Ethidium bromide is a powerful mutagen; gloves and a mask were worn during weighing and through all steps of handling. (Maniatis *et al., l*982).

3.3.19. Molecular weight markers:

In order to estimate the molecular size of the DNA bands, four molecular weight markers were used:-

A- LAMBDA (λ) DNA/EcoRI+HindIII: The DNA marker was prepared according to the manufacturer instructions by adding 1µl (0.5µg) of the DNA marker to 1µl of 6X loading dye solution and 4 µl of deionized water, heated for 5min at 65ºC and cooled on ice for 3 min. The total of prepared amount (6 µl) was applied on agarose gel. The DNA marker contains the following 13 discrete fragments (in base pairs): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564 and 125. The marker was provided by Cinngen (Iran).

B- DNA LADDER 123 bp: The DNA ladder was prepared by adding 0.5-2.0µg of 123 bp ladder to 10µl of TE buffer and 2.5µl of gel loading solution provided in the same patch. The molecular weight marker produced 34 bands ranging in size from 123- 4182 bp or increments 123 bp. The marker was provided by Sigma (USA).

C- ***LAMBDA (λ) DNA PstI Digest:*** This DNA marker was prepared according to the manufacturer instruction by adding 10 µg of Lambda DNA PstI digest to 0.3 volume of gel loading solution provided in the same patch. This marker gives a total number of 15 fragments ranging from 15-11,497 bp. The marker was provided by Sigma (USA).

3.3.20. Colchicine (0.5mg)

The solution was freshly prepared by diluting 1 tablet (0.5mg) in 1 ml D.W. and 0.250 ml was used to inject each animal intraperitoneally (ip) (Al-Duliemy, 2005).

3.3.21. Hypotonic Solution

Potassium chloride 0.075M was prepared by dissolving 5.75 gm of potassium chloride salt in 1000 ml of D.W. (Sharma and Sharma, 1980).

3.3.22. Fixative solution

The fixative solution was freshly prepared each time by mixing three parts of absolute methanol with one part of glacial acetic acid (Sharma and Sharma, 1980).

3.3.23. Giemsa's stain

The stock solution was prepared by dissolving 1 gm of the Giemsa's powder in 33 ml of glycerin and was put for two hours in 60C˚water bath shaker. 60 ml methanol was added to the mixture after cooling at room temperature. Mixed well and filtered using filter paper. The stock was kept in a dark bottle at room temperature (Al-Attar, 1982).

3.3.24. Sodium bicarbonate (0.5%)

The solution was made by dissolving 0.5gm in 100 ml of distilled water and the solution was sterilized in the autoclave.

3.4. Laboratory Animals

Mice used in all experiments were supplied by the Biotechnology Research Center/ AL-Nahrain University, housed and maintained in their animal facility. Each experiment contained animals in the same age and sharing the same mother and father being male or female was not crucial to the experiment, as long as they shared the same parents. Mice were kept in plastic cages. Food and drinking water was supplied regularly in clean wares, wood shavings were used as bedding and changed every week to minimize the risk of potentially harmful micro-organisms (Foster and Small, 1981).

Six to eight weeks old mice were used and weighed between 17 and 23gm. Different dosages of MMC 0.1, 0.2 and 0.5mg/kg of body weight (bw) were injected via (ip) route for 24 hours before sacrifice of the animals except for the

control animal. Two hours prior to sacrifice the solution of freshly prepared colchicine was also injected via ip route. Mice were anesthetized with ether for 1-2 min and animals fixed upwardly to a mobile plate and the abdominal region was wiped with 70% alcohol. Sterile forceps, blades and scissors were used to dissect mice in order to obtain the bone marrow and spleen for molecular and cytogenetic analyses.

3.5. Molecular Analysis

3.5.1. MtDNA Extraction

MtDNA was isolated from spleen tissues under aseptic conditions according to the method "Rapid isolation method of animal mitochondrial DNA by the alkaline lysis procedure" described by Tamura and Aotsuka, 1988.

Procedure:-

1. Spleen tissues were sliced and cut into very small pieces using sterilized surgical blades and scissors and then homogenized in buffer A.
2. Transferred to 1.5 ml eppendorff tube and centrifuged at 1000 g for 1 min at 4˚C in order to pellet nuclei and cellular debris.
3. The supernatant was recovered and recentrifuged at 12,000 g for ten min at 4˚C to pellet mitochondria.
4. The supernatant was resuspended in buffer B to a final volume of 50µl.
5. One hundred micoliters (100µl) of freshly prepared 0.18N NaOH containing 1% sodium dodecyl sulfate (SDS). The mixture was vortexed briefly and tubes kept on ice for 5 min.
6. Seventy five micoliters (75 µl) of an ice cold solution of potassium acetate (3M potassium and 5M acetate), gently vortexed and kept on ice for additional 5 min.

7. The mixture was centrifuged at 12,000g for 5 min at 4ºC and the supernatant was recovered. An equal volume of phenol-chloroform was added and mixed thoroughly using a vortex.
8. The mixture was centrifuged at 12,000g for 2 min at room temperature, the aqueous phase was transferred to a fresh tube and two volumes of absolute ethanol was added, mixed by vortexing and let stand at room temperature for 15 min.
9. Tubes were centrifuged at 12,000 g at room temperature. The resulted pellet of mtDNA was washed with 1 ml of 70% ethanol and then dried briefly.
10. An appropriate volume of 10mM Tris-HCl buffer pH 8 containing 1 mM EDTA and 20µg/ml of DNase free RNAase and samples of mtDNA were kept in deep freeze (-70˚C).

3.5.1.1. Modified mtDNA Extraction Protocol

The same protocol was used with modifications in temperature, centrifugation speed and time (Internet 2). After the spleen tissues were homogenized in the same buffer A, eppendorff tubes were centrifuged at the 500g for 2 minutes at 4ºC cold microfuge. The supernatant was transformed to fresh tubes and centrifuged at 20,000 g for 10 minutes. The pellet was resuspended in 50µl of modified buffer B. Tubes were gently vortexed inverted and kept on ice for 5 minutes after the addition of 18M NaOH /1% SDS and once more after the addition of Potassium acetate. Tubes were centrifuged at 20,000 g for 5 minutes and then standard phenol extraction and DNA precipitation was followed.

DNA precipitate was dissolved in sterile deionized distilled water which is more preferable since samples were further to be processed by PCR (McPherson and Møller, 2001).

3.5.1.1. Spectrophotometer Determination of nucleic acids

Ten micro liters (µl) of each mtDNA sample were added to 490 µl of distilled water and mixed well. A spectrophotometer was used to measure the optical density (O.D.) at wave lengths of 260nm and 280nm. An O.D. of 1 corresponds to approximately 50µg/ml for double stranded DNA. The concentration of DNA was calculated according to the formula described in Sambrook *et al.*, 1989.

DNA concentration (µg/ml) = O.D. 260nm×50×Dilution factor

3.5.1.2. Agarose Gel Electrophoresis

To separate DNA fragments, Agarose gels in different concentrations were used 0.8-1.5% for extracted mtDNA and RAPD analysis, 2% for visual checking of specific PCR product and 2.5% for restriction digests. Gels were run horizontally in 0.5X TBE buffer. Electrophoretic buffer was added to cover the gel. Samples of DNA were mixed with 1/10 volume of the loading buffer and loaded into the wells and gels were run at 5V/cm. Agarose gels were stained with ethidium bromide 0.5µg/ml for 30-45 minutes. DNA bands were visualized by U.V. transilluminator at 365 nm wavelength described by Maniatis *et al.*, 1982. A gel documentation system was used to document the observed bands.

3.5.2. RAPD-PCR reactions

The following materials were used:

1-Ready mix "REDTAQ"PCR reaction mix: is a ready to use mixture that contains Taq DNA polymerase, pure deoxynucleotides and reaction buffer in a 2X concentrate provided by Sigma.

2- A series of ten random sequence decamer primers were used provided by Operon Technologies in a lyophilized form that was dissolved in sterile distilled

water to achieve 5pmol/µl. These primers had a G+C content of 60-70%. The primers used and their sequences are listed in table (3-1). For each reaction, 150 ng of template DNA and 10 pmol of all random primers were added to 12.5 of "REDTAQ"PCR reaction mix and sterile distilled water was used to achieve a total volume of 25 µl. Master mixes were preformed in order to achieve homogeneity of reagents and reduce the risk of contamination. Negative control reactions were run in parallel. All amplifications were performed on ice in aseptic conditions using a laminar air flow hood.

The program used for RAPD-PCR was denaturation for 2min at 94ºC and 40 cycles of 1min at 92ºC, 1min at 36ºC and 2min at 72ºC and final 72ºC for 10min.

Table (3-1): The names of the primers used in this study and their sequences

No	**primer**	**Sequence 5^{-}---3^{-}**
1	OPA.18	AGGTGACCGT
2	OPB.10	CTGCTGGGAC
3	OPC.19	GTTGCCAGCC
4	OPE.2	GGTGCGGGAA
5	OPE.13	CCCGATTCGG
6	OPN.7	CAGCCCAGAG
7	OPN.16	AAGCGACCTG
8	OPO.16	TCGGCGGTTC
9	OPO.20	ACACCAGCTG
10	OPAB.9	GGGCGACTAC

3.5.3. Specific PCR

PCR reactions were performed using the following:-

1-Specific Primers: a pair of primers specific for the D-loop region of mice were provided by Alpha DNA. The primers used were as follows:

H00651 (12SrRNA) 5¯-ATTAATTATAAGGCCAGGACCAAACCT-3¯

L15926 (tRNAThr) 5¯-ATAAACATTACTCTGGTCTTGTAAACC-3¯

Provided in a lyophilized form and were dissolved in sterile distilled water to give a final concentration of 10pmol/µl.

2-Thermophilic DNA polymerase: the enzyme in a storage buffer supplied with a 10X buffer from Promega.

3-dNTPs mixture: a 10mM concentration of all of the four deoxynucleoside phosphates provided by Sigma.

Amplification was performed on ice in aseptic conditions in a laminar air flow using 0.5 ml tight cap eppendorff tubes. 50µl of a solution containing 50mMKCl, 10mMTris-HCl (pH 9 at 25˚C), 1.5 mM $MgCl_2$ and 0.1% Triton-X100, 200µM of dNTPs mixture, 25pmol of each primer, 250ng of mtDNA and 1.5 units of Taq DNA polymerase.

In order to achieve homogeneity of reagents and reduce the risk of contamination a master mix for all samples was prepared containing all the components of the reaction except for DNA, mixed gently with sterile distilled water to achieve the appropriate volume. 45µl of the master mix was dispensed into PCR tubes and 5µl of each DNA sample was added. PCR was preformed with a protocol consisting of 40 cycles of denaturation at 93˚C for 1min, hybridization at 50˚C for 1 min and 72˚C for 2 min (Kocher *et al*., 1989).

A negative control reaction in each PCR experiment was set up containing all components of the reaction but template DNA so that any contaminating DNA present in the reaction would be amplified and detected on agarose gel. 5µl of PCR product was submitted to electrophoresis in 2% agarose gels to determine

how successful the PCR was by visual observation of the single target band with the expected size of the D-loop region.

3.5.4. Restriction Endonuclease Analysis

All restriction digestion reactions were preformed on ice, in a total volume of 25µl by using reaction conditions of incubation time and temperature and buffers provided by the manufacturer (Table 3-2). One unit of each restriction enzyme was used per 1 µl of PCR product. EDTA of pH 8 was added to a final concentration of 10 mM to inactivate the restriction reactions. Digestion products were run on 2.5% agarose gel electrophoresis. All restriction endonucleases were supplied from Sigma

Table (3-2): The restriction endonucleases used in the study, recognition sequences indicated by the black arrows and their incubation temperatures.

Restriction enzyme	**Recognition Sequence 5^{-}---3^{-}**	**Incubation temperature**
AvaI	**G▾PyCGPuG**	**37ºC**
BamHI	**G▾GATCC**	**37ºC**
EcoRI	**G▾AATTC**	**37ºC**
HaeII	**PuGCGC▾Py**	**37ºC**
HaeIII	**GG▾CC**	**37ºC**
HindIII	**A▾AGCTT**	**55ºC**
HpaI	**GTT▾AAC**	**37ºC**
PstI	**CTGCA▾G**	**24 ºC**
Sau3A	**▾GATC**	**37ºC**
SmaI	**CCC▾GGG**	**37ºC**

3.5.5. Molecular Weight Estimation

The molecular weight of DNA fragments was done by using a computer software M.W. Detection program photocapture from Consort.

3.6. Cytogenetic Analysis

Analysis of chromosomes, MI, BI and CA on bone marrow and spleen cells were performed *in vivo* and were modified by scaled down into 1.5ml eppendorff tubes.

3.6.1. Bone marrow cells recovery:

Bone marrow cells were recovered as described by Shubber *et al.*, 1985 under aseptic conditions, femurs of each individual were removed, muscles and tissues cut out, bone heads cut with scissors to insert an insulin syringe and 1.5ml PBS was flushed repeatedly into the cavity to obtain bone marrow cells. The supernatant was vortexed to assure dissociation of cells.

3.6.2. Spleen cells recovery:

The abdominal cavity of the same animal was opened and the spleen was recovered, a reddish–brown elongated organ lying adjacent to the greater curvature of the stomach (Foster and Small, 1981), the organ was cut out from other tissues and washed in PBS. The tip of the spleen was cut out and 3ml PBS was flushed through the organ using a syringe as described by Shubber *et al.*, 1985. Cells were collected in two tubes of 1.5ml eppendorff tubes under aseptic conditions. The spleen cell suspension was made prior to slicing of the tissue for mtDNA isolation.

3.6.3. Cells harvest:-

Bone marrow and spleen cell suspensions were harvested according to the method described by Juma, 1993.

1. The mixture of cells-PBS for both bone marrow and spleen was centrifuged at 2000rpm for 10min at 4ºC and the supernatant discarded.
2. One and a half milliliters (1.5ml) of hypotonic solution was gradually added to pellet cells and the suspension was mixed thoroughly. Cells were incubated in 37ºC water bath for 30 min for bone marrow and for 20 min for Spleen and inverted occasionally.
4. Tubes were recentrifuged at 2000rpm for 10 min, the supernatant was discarded and freshly prepared freezer-chilled fixative solution was added drop-wise with initial mixing to give total volumes of 1.5ml. Cells were gently resuspended and refrigerated a 4ºCfor 20 minutes.
5. Cells were centrifuged at 2000rpm for 10 minutes and supernatant discarded. 1.5 ml of fresh fixative solution was added resuspended and centrifuged. This step was repeated until a clear cell suspension was obtained.
6. After the last wash 1 ml of fixative solution was added resuspended and made ready for slide preparation

3.6.4. Slide preparation and Staining:

Slides were pre-cleaned and stained according to the method described by Juma, 1993. Cells were resuspended and dropped using a Pasteur pipette, onto precleaned slides dipped in cold distilled water and then slides were left for air drying in a warm place to assist metaphase spread.

Slides were stained with freshly prepared 2.5% Giemsa's solution for 20 minutes and rinsed briefly in distilled water and examined microscopically at 40X and photographed under oil immersion. The MI was determined as the ratio of mitotic cells to interphase nuclei in 1000 cells for bone marrow and spleen cells.

MI%= Mitotic Cells \ 1000 cells × 100

The BI was scored as the percentage of blast cells in 1000 cells and was examined in bone marrow cells.

BI%= Blast cells \ 1000 cells × 100

CA were scored in 100 metaphase for bone marrow and 25 metaphase for spleen cells under oil immersion at 100X. Well spread metaphases were chosen and structural chromosomal aberrations like ring chromosomes, dicentric, deletions, chromatid and chromosome gaps and breaks were scored.

3.6.5. Statistical Analysis

A Microsoft SPSS was used to estimate the significant variations in MI, BI and CA values.

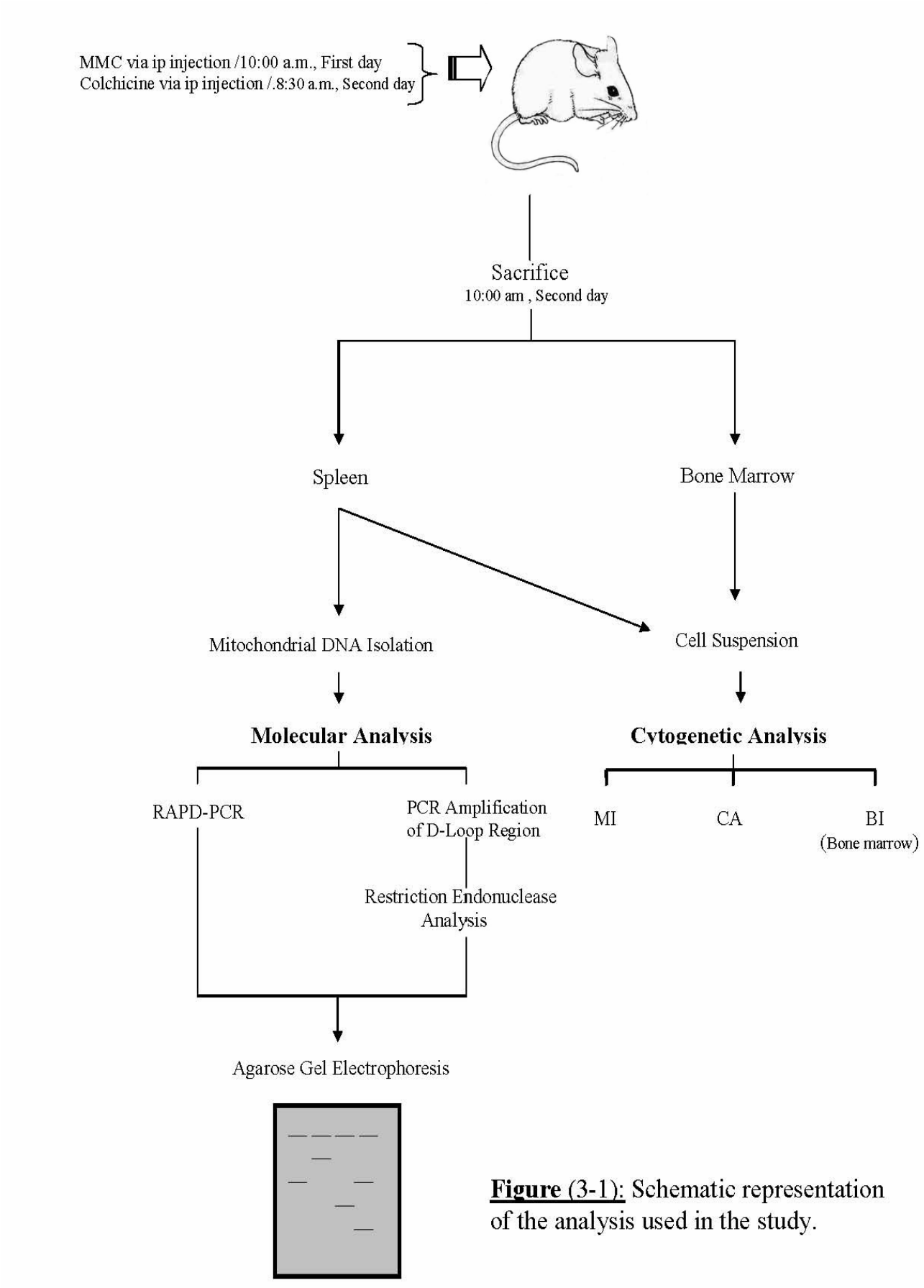

Figure (3-1): Schematic representation of the analysis used in the study.

CHAPTER FOUR

RESULTS AND DISCUSSION

4. Results and Discussion

4.1. Extraction of mtDNA

Standard methods of mtDNA isolation are relatively tedious, high cost and time consuming involving cesium chloride (CsCl) density centrifugation to obtain highly purified mtDNA preparations free of nuclear DNA and therefore impose limitations on the number of samples to be analyzed (Palva and Palva, 1985). Enhancement of mtDNA isolation methods became a necessity and thus many rapid and simple to perform methods were described avoiding complicated instrumentation with improved yields and quality of the isolated mtDNA (Welter *et al*., 1989).

Several mtDNA protocols were used in this study including the one described for plasmid DNA extraction from bacterial cells (Brinboim and Doly, 1979). The yield of this method continuously contained visible traces of chromosomal DNA with very low purity estimates. The use of hypotonic Potassium chloride (KCl) solution as a first step was suggested to make cells of spleen tissues swell and explode excluding nDNA in a later centrifugation step and then obtain mitochondrial pellet to proceed with the use of a described lysis buffer that was proved to produce large quantities of both nDNA and mtDNA (Longmire *et al.*, 1997). The use of this method yielded no detectable DNA on agarose gel.

The method used in this study was successfully used in extracting mtDNA from mouse tissues and proved to be useful in any organism as long as the condition of mtDNA is closed circular. The protocol involves two main procedures, preparation of mitochondrial pellet and mtDNA extraction by

alkaline lysis which is accomplished by exploiting the covalently closed circular or super coiled property of mtDNA. MtDNA is competently separated from linear form nDNA (Tamura and Aotsuka, 1988).

MtDNA was isolated more efficiently regarding purity and concentration using the same protocol but with some modifications with respect to temperature, centrifugation speed and time (Internet 2). The yield of mtDNA isolated from mice spleen tissues range was 0.5- 0.8 µg/µl with purity of 1.3-1.7. The bulk of mtDNA appeared as ~ 16 kb band in reference to the λ DNA /*EcoRI* + *HindIII* marker, however traces of chromosomal DNA were still evident (Figure 4-1) suggesting that pure extracts of mtDNA were only possible using CsCl density centrifugation method which is labor intensive using expensive and unfortunately unavailable equipments.

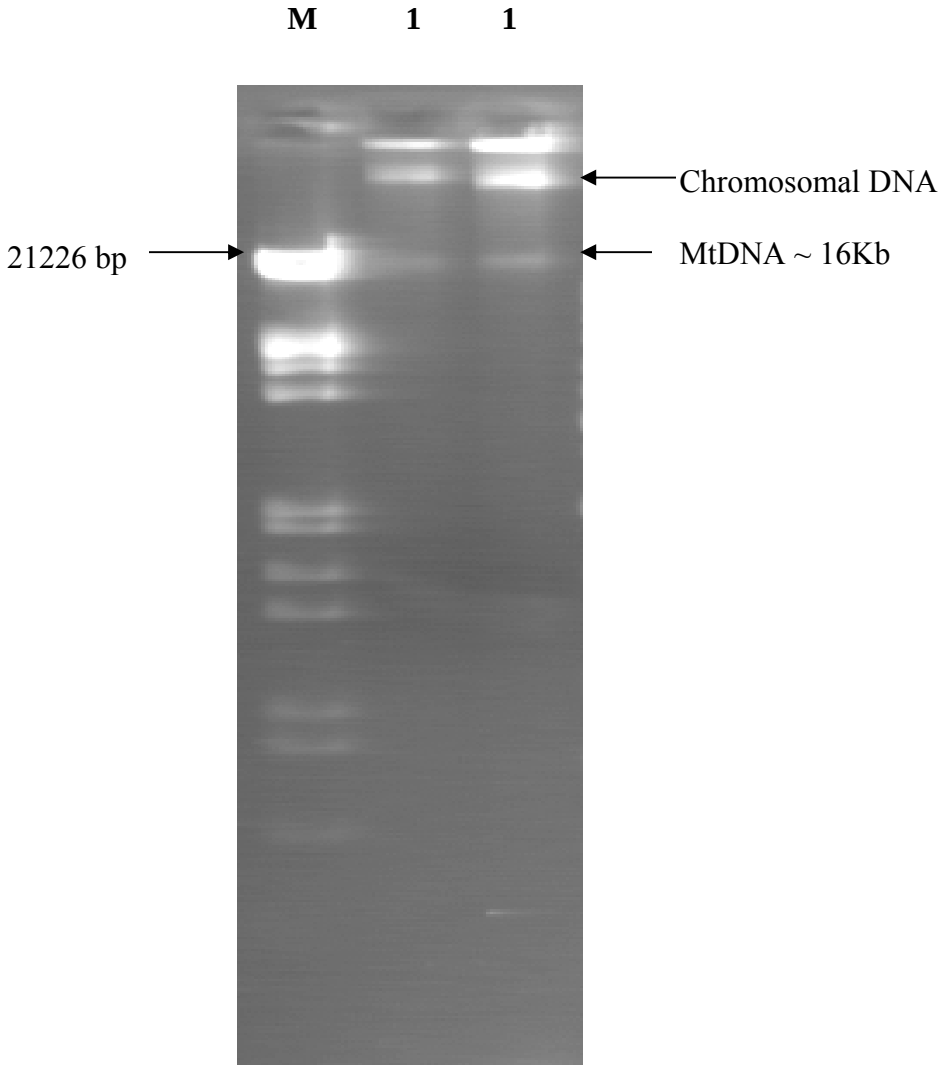

Figure (4-1): Agarose gel electrophoresis of mtDNA isolated from mice spleen. M: λ DNA /*EcoRI* + *HindIII* marker, 1: isolated mtDNA. Fragments were fractionated by electrophoresis on a 0.8% agarose gel (2hr., 5V/cm, Tris-borate buffer) and visualized by ethidium bromide staining.

4.2. RAPD Analysis

A successful PCR is dependent on the reaction conditions including reagents, temperature and preventing contamination. Many studies indicate that RAPD-PCR is sensitive to reaction conditions and that optimization of these conditions is necessary to reach the highest specificity and product yield (Williams *et al.*, 1990). Standard amplification conditions were applied in RAPD-PCR with ten primer sequences. Red Taq ready mix supplied in 2X concentrate reaction buffer, offered the standard concentrations of Taq DNA polymerase of 1.5 U and 200 μM of dNTPs in each reaction. 10 pmol of each primer were used, the primer concentration represents the optimal concentration, decrease in primer concentration leads to weak PCR product while increase could result in the formation of primer dimer artifact leading to misinterpretation of results (Saiki, 1989).

The PCR based techniques do not require highly purified DNA preparations as it works well with partially purified DNA samples (Edwards *et al.*, 1991; McPherson and Møller, 2001). However DNA extracts may contain inhibitory compounds like detergents used in cell lysis and protein denaturation in addition to other inhibitory compounds that could interfere with PCR leading to reaction failure (McPherson and Møller, 2001). 150 ng of mtDNA was used for RAPD amplification. Between 5-500 ng usually provide reliable results of RAPD-PCR (Lowe, 1996). Low DNA concentrations were reported to affect RAPD patterns (Williams *et al.*, 1990).

The RAPD-PCR programming used was a modified version of the program originally suggested by Williams *et al.* 1990 of temperature profile

of initial denaturation for 2 min at 94ºC and 40 cycles of 1min at 92ºC, 1min at 36ºC and 2min at 72ºC and final extension 72ºC for 10 min.

The annealing temperature in which primers hybridize to complementary sequences on the template DNA is perhaps the most critical in PCR programming, it is usually set at 36 ºC for decamer random primers (Innis *et al.*, 1990). The annealing temperature for RAPD primers is based on Tm calculations. Tm is the temperature at which half the primers are hybridized to the target sequences. Tm calculations for RAPD primers is based on guanine and cytosine (G+C) content which is constantly 60-70% in RAPD primers supplied by the manufacturer Operon company. The G+C content in an oligonucleotide of 10-mers should be 40% or greater in order to generate detectable amplified products (Williams *et al.*, 1990). The formula used in Tm calculation for primers up to 20 nucleotides in length reflects the fact that G/C base pairing is more stable than A/T due to their greater hydrogen bonding (McPherson and Møller, 2001), the annealing temperature is usually higher than the Tm in 2-12 ºC (Newton and Graham, 1997).

Length of PCR products is defined by positions of the PCR primers. Taq DNA polymerase used in this study is capable of amplifying products up to 2-4 Kb. The PCR ability to amplify minute quantities of template DNA is of great advantage for amplifying a target sequence of interest but it's disadvantageous because small amounts of contaminating DNA may also be amplified (McPherson and Møller, 2001). PCR negative controls containing no DNA were seen as empty gel lanes in agarose gels indicating that no contamination whether in PCR working area or reagents had occurred.

RAPD-PCR products were analyzed on agarose gel electrophoresis using the appropriate molecular size markers of 123 DNA ladder and λ DNA *PstI* digest to calculate the size of fragments produced by RAPD relative to

the migration of the fragments of the DNA markers. The estimation of RAPD bands on agarose cannot be reliably sized to anything better than 5% error rate (i.e. a band size of 100bp can vary between 95 and 105bp) (Lowe *et al.,* 1996).

The analysis of PCR amplified DNA fragments relies on several bases including the absence or presence of bands, differences in the molecular weight and intensity of amplified bands (Mayer *et al*., 1997).

Following PCR standard conditions of reagent concentrations and programming, detectable RAPD patterns were obtained using ten primers. Spleen mtDNA of 0.5 mg/kg bw MMC treated mice and non treated animals as controls were used in RAPD analysis. The primers used in this study were selected randomly and are classified into two categories according to results obtained.

In the first one, no amplified products were generated. This result was obtained with the primer OPE-13 (Figure 4-2A). The failure of this primer to generate amplification segments maybe attributed to the absence of primer complementary sequences on template DNA. Similar results were reported in different studies and a number of RAPD primers were scored as non amplification producing primers in wheat plant (Ahmed, 1999).

The second category is represented by primers that have generated reproducible bands including OPAB-9, OPC-19, OPN-7, OPO-20, OPA-18, OPB-10, OPE-2, OPN-16 and OPO-16 (Figures 4-2A, B). Some of which revealed no polymorphism in banding patterns between mtDNA of 0.5 mg/kg bw MMC treated mice and control animals like OPN-7, OPO-20, OPB-10 and OPE-2.

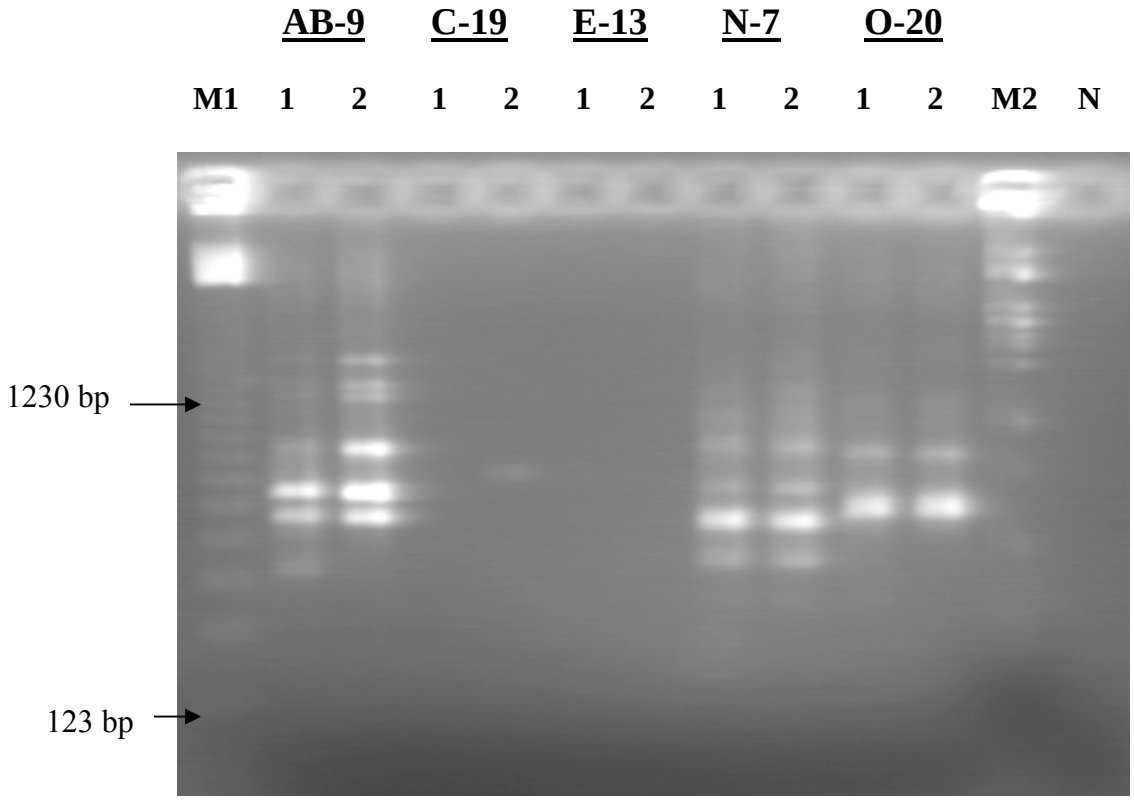

Figure (4-2A): Agarose gel electrophoresis of RAPD-PCR amplification of mice mtDNA of non treated (1) and 0.5 mg/kg bw MMC treated (2) animal with the following primers from the left to right **OPAB-9, OPC-19, OPE-13, OPN-7,** and **OPO-20**. M1:123 DNA ladder, M2: λ DNA *PstI* digest, N: PCR negative control. Fragments were resolved on a 1.5% agarose gel (2.5hr., 5V/cm, Tris-borate buffer) and visualized by ethidium bromide staining.

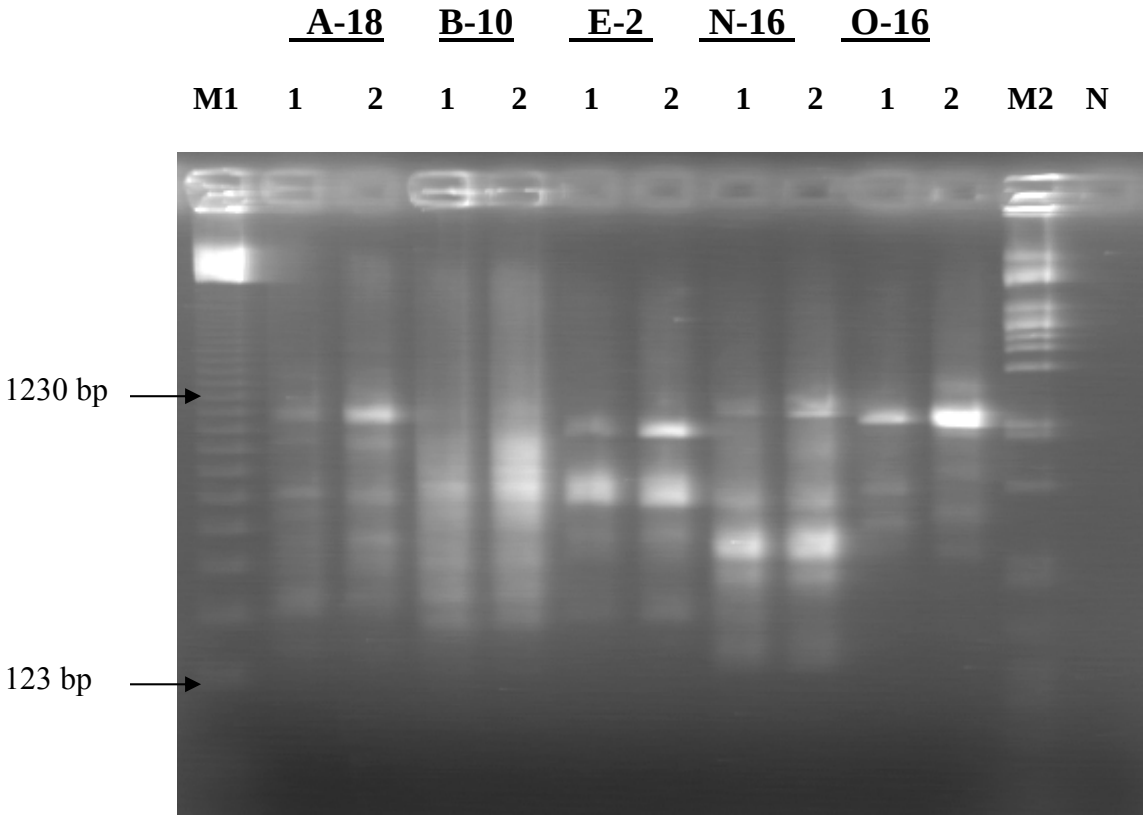

Figure (4-2B): Agarose gel electrophoresis of RAPD-PCR amplification of mice mtDNA of non treated (1) and 0.5 mg/kg bw MMC treated (2) animal with the following primers from the left to right **OPA-18, OPB-10, OPE-2, OPN-16,** and **OPO-16.** M1:123 DNA ladder, M2: λ DNA *PstI* digest, N: PCR negative control. Fragments were resolved on a 1.5% agarose gel (2.5hr., 5V/cm, Tris-borate buffer) and visualized by ethidium bromide staining.

The explanation for the observed pattern of homogeneity between MMC treated and control animals with these primers is that there could be no direct action by MMC in sequences complementary to the primers used and that each primer found sections where it can hybridize and thus the characteristic pattern was not altered. RAPD assay is believed to detect single base changes in DNA in some instances because a single base change in the genome of interest may prevent amplification by introducing a mismatch at just one end of DNA segment (Williams *et al*.,1990). However the technique is prone to problems with reproducibility, but since RAPD-PCR mechanism was further understood, such problems were reduced although careful amplification of protocols is needed for reproducible results (Lowe *et al.*, 1996).

The Primers used in this study which generated different levels of polymorphisms between MMC treated mice and control animals included OPAB-9, OPC-19, OPA-18, OPN-16 and OPO-16 (Figures 4-2A, B). Sources of DNA polymorphisms include deletions of a priming site, insertions rendering the priming sites too distant to support amplification or insertions that change the size of DNA segment without preventing its amplification in addition to base changes, as in experiments introduced by Williams *et al.* 1990 most of single nucleotide changes in primer sequence caused complete change in banding patterns of amplified DNA.

RAPD technique was used in assessing DNA alterations in cell populations exposed *in vitro* to genotoxins. Alterations of DNA in a number of cells were reflected as variations in the fingerprint obtained for the control population and were defined as band losses and / or gains as a well as alterations in the intensity of amplification of some of them. These alterations *in vivo* are considered mutations produced by changes to, deletions of or insertions into the pair bases (Muralidharan and Wakeland,

1993). Banding patterns observed with primers OPAB-9, OPC-19, OPA-18, OPN-16 and OPO-16 between 0.5 mg/kg bw MMC treated mice and the control animal included common and polymorphic bands. The common bands may represent a species DNA homology in mice .While polymorphic bands could be originated from heterogeneity at the genomespecies level. However species heterogeneity exists in low levels and hence yields low polymorphic DNA markers. It is also expected that more than 90% similarity exists between individuals of the same strain (Al-Bustan *et al.*, 2001). Besides the fact that majority of RAPD fragments are identical between individuals or strains (Williams *et al.*, 1990) and thus polymorphisms observed with primers were relatively low and could be interpreted as genetic heterogeneity at the genomespecies for example the primer OPAB-9 produced a polymorphic band in a total of 13 observed bands. The detected percent of polymorphism was rather low between the treated mice and control animals. Polymorphism of each primer was calculated as the percent of polymorphic bands to the number of total bands produced by the designated primer (Ali, 2003). (Table 4-1) shows the names of primers, total bands and polymorphic bands and the percent of polymorphism between MMC treated and non- treated mice for each primer.

Primers OPC-19 and OPO-16 produced the highest percent of polymorphisms. Primer OPC-19 produced a single band of 829bp observed in MMC treated animal while primer OPO-16 produced a total of 8 polymorphic bands. All primers produced polymorphic bands except for primer OPE-13, polymorphism ranged from 7.7- 100%. The high polymorphisms detected by RAPD-PCR maybe interpreted as MMC induced DNA alterations. These results are consistent with results obtained by Becerril *et al.*1999 in which 0.5µg/ml MMC induced DNA alterations on an established rain bow trout cell line (RTG-2) was detected by the use of RAPD.

Table (4-1): The names of primers, total generated bands, polymorphic bands and the percent of polymorphisms between MMC treated and non- treated mice for each primer.

Primers	**Total number of bands**	**Number of polymorphic bands**	**Polymorphism %**
OPAB-9	13	1	7.7
OPC-19	1	1	100
OPE-13	0	0	0
OPN-7	12	0	0
OPO-20	4	0	0
OPA-18	10	3	30
OPB-10	6	0	0
OPE-2	8	0	0
OPN-16	10	2	20
OPO-16	9	7	77.7

4.3. Specific PCR of D-loop Region in Mice MtDNA

PCR was utilized to amplify the D-loop or control region of mice mtDNA using a set of specific primers directed toward this segment. The design of these primers was based on comparative studies of mtDNA published sequences for mammals including human and mice in addition to other animals. The sequences of the primers follow, the letter L and H refer to the light and heavy strands; the number refers to the position of the 3¯ base of the primer in the complete human mtDNA.

H00651 (12SrRNA) 5¯-ATTAATTATAAGGCCAGGACCAAACCT-3¯
L15926 (tRNAThr) 5¯-ATAAACATTACTCTGGTCTTGTAAACC-3¯

(Kocher *et al.*, 1989)

MtDNA isolated from spleen tissues of MMC treated mice and the non treated control animal was subjected to PCR amplification of the D-loop region using the specific primers directed to this region in mice. Usually it's not necessary to purify mtDNA prior to amplification, a simple protocol for isolating genomic DNA is sufficient for using the PCR (Kocher *et al*., 1989; Matson and Baker, 2001). Primers directed to a specific region fish out their related sequences in a complex mixture of DNA and an mtDNA sequence is amplified several thousand folds. Moreover, tissue amounts needed to produce a sequence are usually small (Kocher *et al*., 1989), in which case very beneficial given that spleen tissues used in this study in 6-8 weeks old mice weighed between 0.22 and 0.56 gm.

Since restriction endonucleases analysis was further to be used in examining potential mtDNA variations in the D-loop region, mtDNA samples were used to minimize chromosomal restriction patterns and facilitate the comparison of results.

In setting PCR reactions for the first time with new template DNA, new primers, a new preparation of thermostable DNA polymerase enzyme, amplification is generally less than optimum. Achieving balance between reaction components is usually required to reduce non specific amplification and / or to enhance the yield of the desired DNA product (Sambrook and Russell, 2001).

Using primers H00651 (12SrRNA), L15926 (tRNAThr), a sufficient product of PCR with the expected size was obtained after several optimization experiments with conditions. A single discrete band of 1026 bp was generated. The region 879bp or D-loop region of interest is contained within the amplified fragment as primers anneal to regions flanking the targeted sequence determined by the number of 3^{-} in each primer taken into consideration that mtDNA molecule in mice is 16 295 bp (Bibb *et al.*, 1981).

In the first PCR experiment, standard concentrations of 25 pmol of each primer, 200µM of dNTPs mix and1.5 U of Taq DNA polymerase for each reaction were used, 250 ng of template DNA was added. The PCR yield was a band of the desired product but faint to non detectable. Increasing the number of cycles from 35 to 40 cycles resulted in a sharp intense band, all reaction components were kept at the same concentrations.

Although the second round of optimization yielded a significant increase of the desired product but, a generalized smear of amplified DNA was produced. In order to reduce the non specific products generated, the amount of template DNA was reduced to 200 ng instead of 250 ng. Concentrations of primers, dNTPs and Taq DNA polymerase were still the same figures 4-

3A, B show PCR product of the mice D-loop region before and after optimization. All optimization rounds were carried out with a PCR negative control to detect any contamination. Due to careful employment of laboratory techniques, all negative controls appeared as empty gel lanes through all experiments of optimization and the ones used in the study indicating that no contamination had occurred. Identification of sources to the problems faced in optimization of the specific PCR with D-loop directed primers was based on trouble shooting guides (McPherson and Møller, 2001; Sambrook and Russell, 2001).

The optimal conditions for this particular primer-template combination were determined after three rounds of optimization; primers worked efficiently and gave rise to unique product in large amounts. Optimal conditions were followed in mice treated with 0.1, 0.2 and 0.5mg/kg bw of MMC and control animal and D-loop region was successfully detected in all animals (Figure 4-3B).

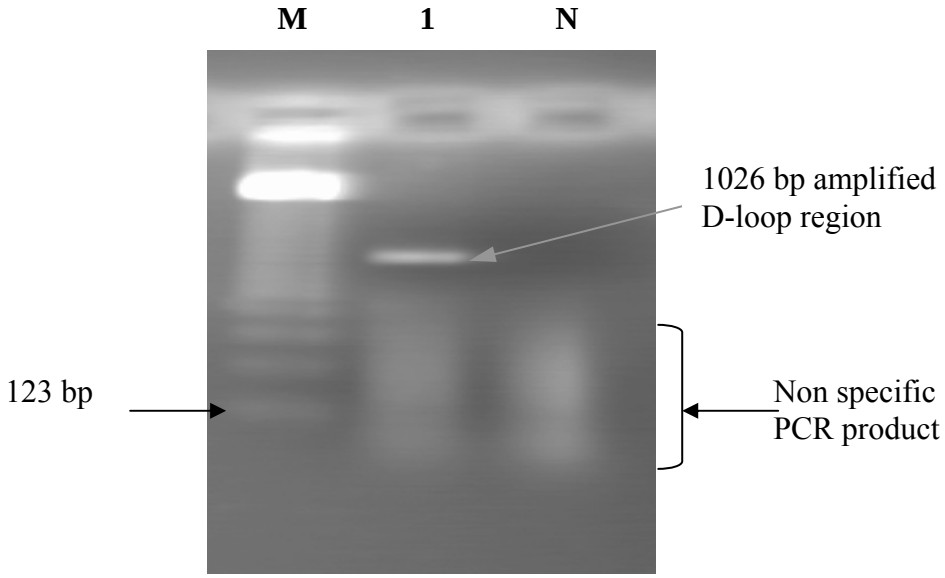

Figure (4-3A): Agarose gel electrophoresis of PCR amplification of D-loop region of mice mtDNA extracted from spleen. M: 123 bp DNA ladder, 1: amplified D-loop region (indicated by the arrow), N: negative control of PCR reaction. Non specific products appear in both lanes 1 and N. Fragments were resolved in a 2% agarose gel (45min, 5V/cm, Tris-borate buffer) visualized by staining with ethidium bromide

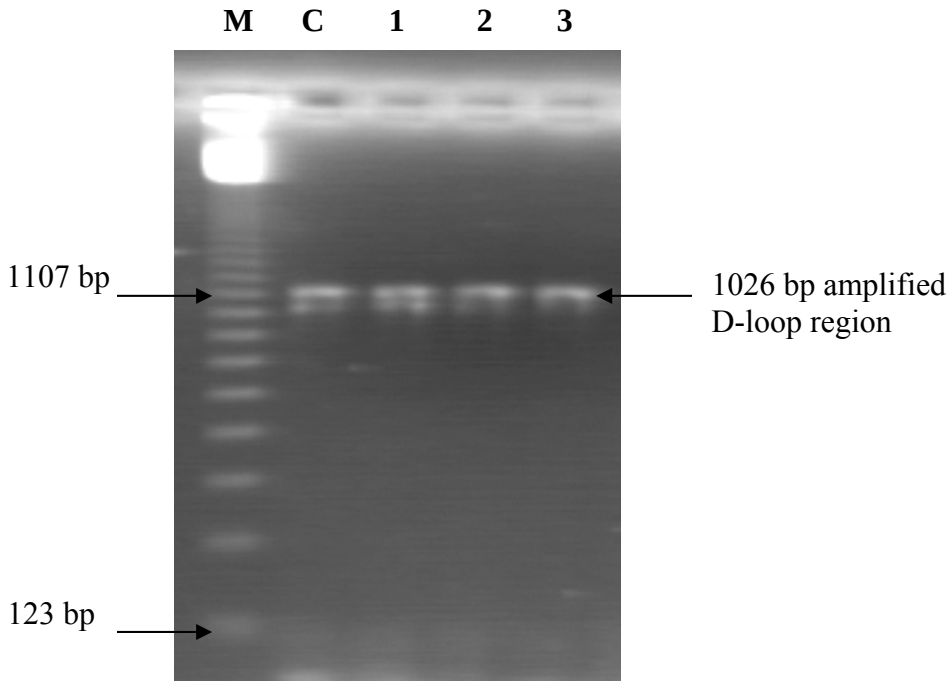

Figure (4-3B): Agarose gel electrophoresis showing the optimization of PCR amplification of the D-loop region in mice mtDNA extracted from spleen. M: 123 bp DNA ladder, C: non treated animal. 1, 2, 3: represent mice treated with 0.1, 0.2 and 0.5 mg/kg bw of mitomycin C Fragments were resolved in a 2% agarose gel (2.5hr., 5V/cm, Tris-borate buffer) visualized by staining with ethidium bromide

4.4. Restriction Analysis of PCR Product

Analysis of PCR products by restriction endonucleases is relatively a rapid, simple and efficient approach giving clear results (McPherson and Møller, 2001). The analysis of 1026 bp PCR product containing 879 bp D-loop region of mouse was subjected to eight restriction endonucleases and was preformed in two stages the first one was accomplished by surveying several enzymes searching for ones that cut within the mentioned region. In the second stage four enzymes were selected, two of which were found to have restriction pattern particular of the enzyme in the D-loop region. The other two, *BamHI* and *HindIII* were not engaged in the first stage but are known to cleave mtDNA in mice and are widely used enzymes. The second stage involved four mice individuals treated with different dosages 0.1, 0.2 and 0.5 mg/kg bw of the antitumor drug MMC in addition to the control animal. A pilot experiment was preformed in the beginning to determine the concentration of restriction enzyme in units to 1µl of PCR product.

EcoRI, AvaI, SmaI, PstI, HaeII, HpaI, HaeIII and *Sau3A* restriction endonucleases were selected and used to cleave the PCR amplified D-loop region of mouse. A control reaction was preformed in parallel with each restriction enzyme including all components of the reaction except for the enzyme, a D-loop restriction control (non restricted D-loop) for comparison purposes.

The restriction of the D-loop region with the enzyme *EcoRI* yielded no cleavage pattern and the D-loop fragment remained intact in comparison with D-loop restriction control (Figure 4-4). The specific sequence of GAATTC recognized by *EcoRI* was not found within this region and thus no cleavage was observed. A possible explanation could be that these recognition sequences are found in the mtDNA outside from the D-loop region. As this enzyme was reported to give rise to two fragments in mtDNA in the mouse cell line LA9 (Brown and Vinograd, 1974).

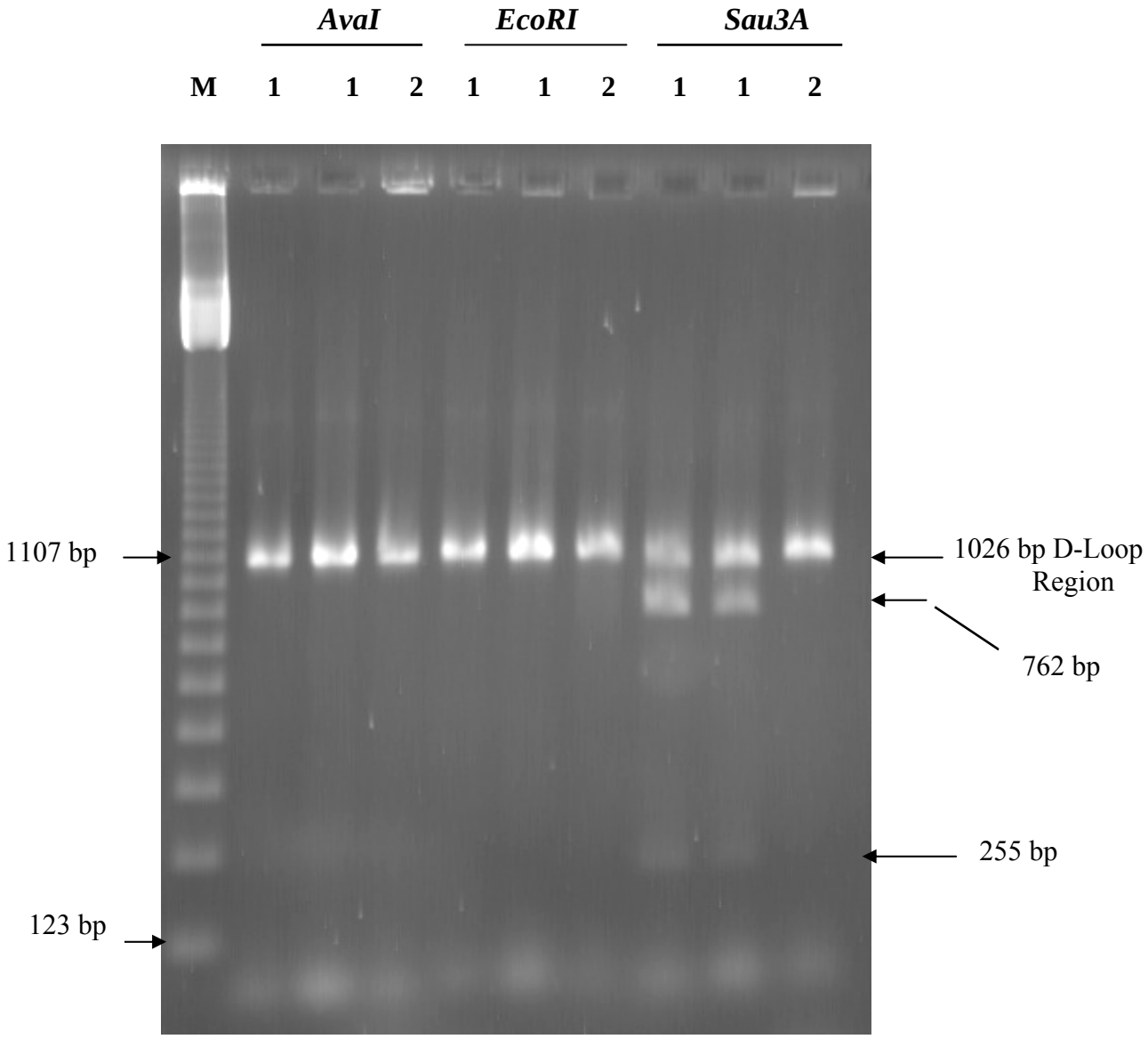

Figure (4-4): Restriction analysis of PCR amplified D-loop region of mice mtDNA by ***AvaI, EcoRI,*** and ***Sau3A***. M: 123 DNA ladder, 1: PCR product of D-loop region, 2: control or non restricted D-loop. Fragments were resolved by electrophoresis in a 2.5% agarose gel (3hr., 5V/cm, Tris-borate buffer) and visualized by ethidium bromide staining.

The same results were obtained with enzymes *AvaI, SmaI, PstI, HaeII, HpaI.* D-loop fragment appeared in the same molecular size a in the D-loop restriction control relative to 123bp DNA ladder (Figures 4-4, 5, 6). Since *PstI* enzyme was used to produce restriction patterns of mtDNA in BALB/c mice (Tamura and Aotsuka, 1989) and was reported to have two recognition sequences within mtDNA of mice (Srivastava and Moraes, 2001). Interpretation of the results obtained with the enzyme *EcoRI* could apply in the case of *PstI.*

Using *AvaI, SmaI, HaeII* and *HpaI* enzymes revealed no digestion pattern particular to any of these enzymes. Probably the D-loop region of mice lacks the presence of cleavage sites recognized by any of these enzymes. Results with these enzymes were observed reproducibly however they could recognize cleavage sites in the mtDNA outside the D-loop region of mice. No references were found concerning these enzymes with respect to mtDNA in mice.

A restriction pattern was obtained in the D-loop region of mice using *HaeIII* restriction endonuclease producing a fragment of~ 500 bp with a smear of DNA fragments of molecular size less than 123 bp relative to the DNA ladder. The enzyme *HaeIII* recognizes a 4bp sequence GGCC and cleaves random DNA sequences roughly once in 256bp (Sambrook *et al.*, 1989). This endonuclease was used in natural population studies of mtDNA in peromyscus and reported to produce a large number of fragments (Avise *et al.*, 1979), it was also used in the comparison of restriction phenotypes of house mouse *Mus musculus* (Ryan *et al*., 2000). The production of a band with a molecular size that roughly equaled half the designated D-loop region means that a number of recognition sequences specific for *HaeIII* are available and that this enzyme could have the potential to recognize base

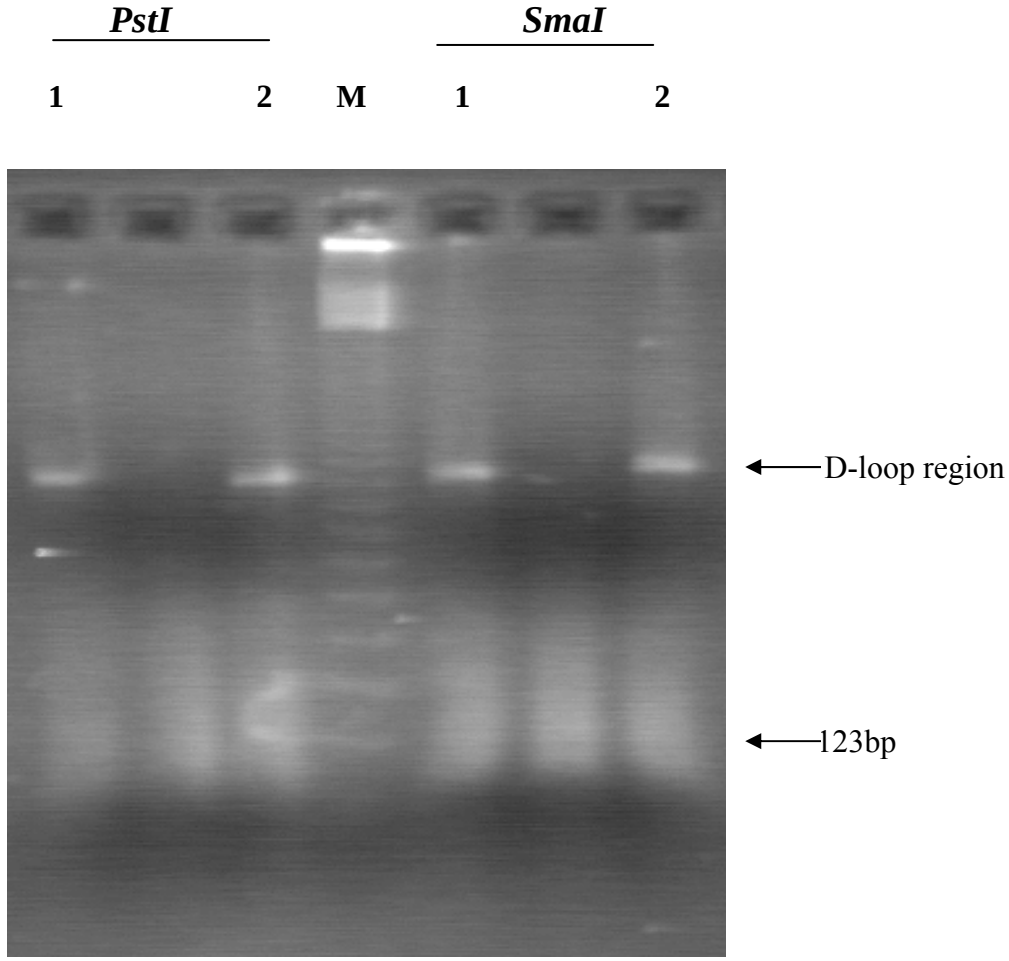

Figure (4-5): Restriction analysis of PCR amplified D-loop region of mice mtDNA by ***Pst1*** and ***SmaI.*** M: 123 DNA ladder, 1 PCR product of D-loop region, 2: control or non restricted D-loop region. Fragments were resolved by electrophoresis in a 2.5% agarose gel (2hr., 5V/cm, Tris-borate buffer) and visualized by ethidium bromide staining.

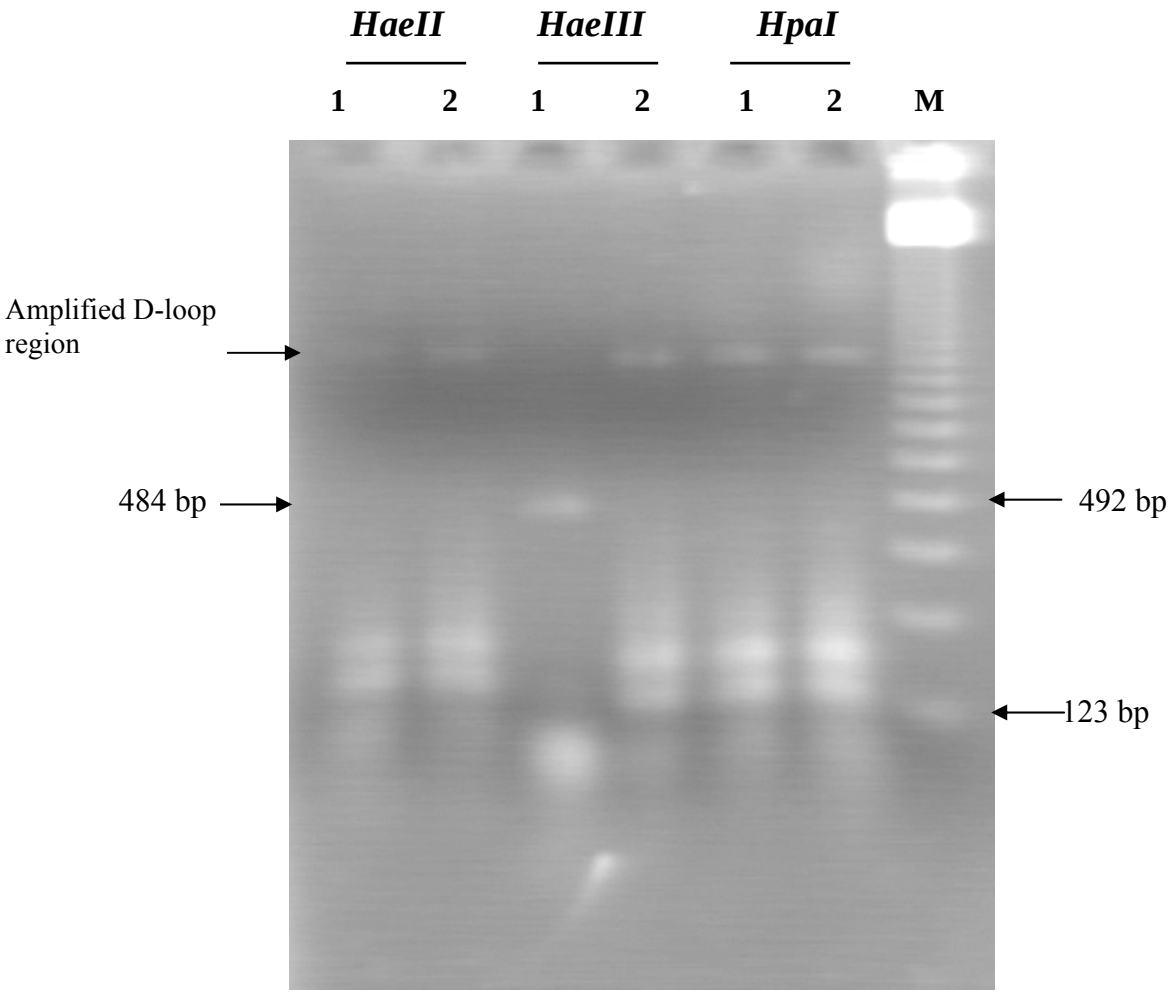

Figure (4-6): Restriction analysis of D-loop region amplified by PCR using enzymes ***HaeII, HaeIII*** *and* ***HpaI*****.** M: 123 DNA ladder, 1: PCR product of D-loop region, 2: control or non restricted D-loop region. Fragments were resolved by electrophoresis in a 2.5% agarose gel (2hr., 5V/cm, Tris-borate buffer) and visualized by ethidium bromide staining.

changes in target sequences in the D-loop region of mice mtDNA if any occurred when animals were exposed to the drug MMC due to abundant cleavage sites. The smear observed in the digestion pattern could be a large number of small DNA fragments.

Sau3A is another restriction endonuclease recognizing a 4bp DNA sequence of GATC. *Sau3A* profile for mouse D-loop region was obtained producing two intense fragments with molecular sizes estimated as 1026 and 762 bp in addition to another very faint band of 255 bp estimated in reference to the 123 bp DNA ladder (Figure 4-4).

When a homogenous mtDNA sequence is digested to completion, the florescence of each band in the digestion pattern should be proportional to the molecular weight of the fragment and the total of the molecular weight of the observed fragments should be equal to the expected genome size of mtDNA (Brown and Vinograd, 1974). The bands produced by *Sau3A* exceeded total size of the D-loop fragment amplified by PCR. Patterns with bands exceeding the total size of a specific fragment subjected to restriction are either a result of incomplete digestion or from heterogeneity in DNA content (Avise *et al.*, 1979).

To test the first suggestion the same quantity of amplified D-loop was subjected to an increasing series of *Sau3A* concentrations with extended incubation at 37ºC in water bath for overnight instead of one hour in order to digest PCR product to completion. The same digestion pattern was obtained in different concentrations of the enzyme (Figures 4-7). Thus these results could be explained as mtDNA composition heterogeneity and that individual mice do not possess a single mtDNA sequence in the D-loop region.

Sau3A with the 4bp recognition sequence recognized two types of mtDNA the first; a type that did not contain the target sequence specific for *Sau3A* in the D-loop region which in agarose gel appeared in the same level as the D-loop restriction control while the second type contained the enzyme

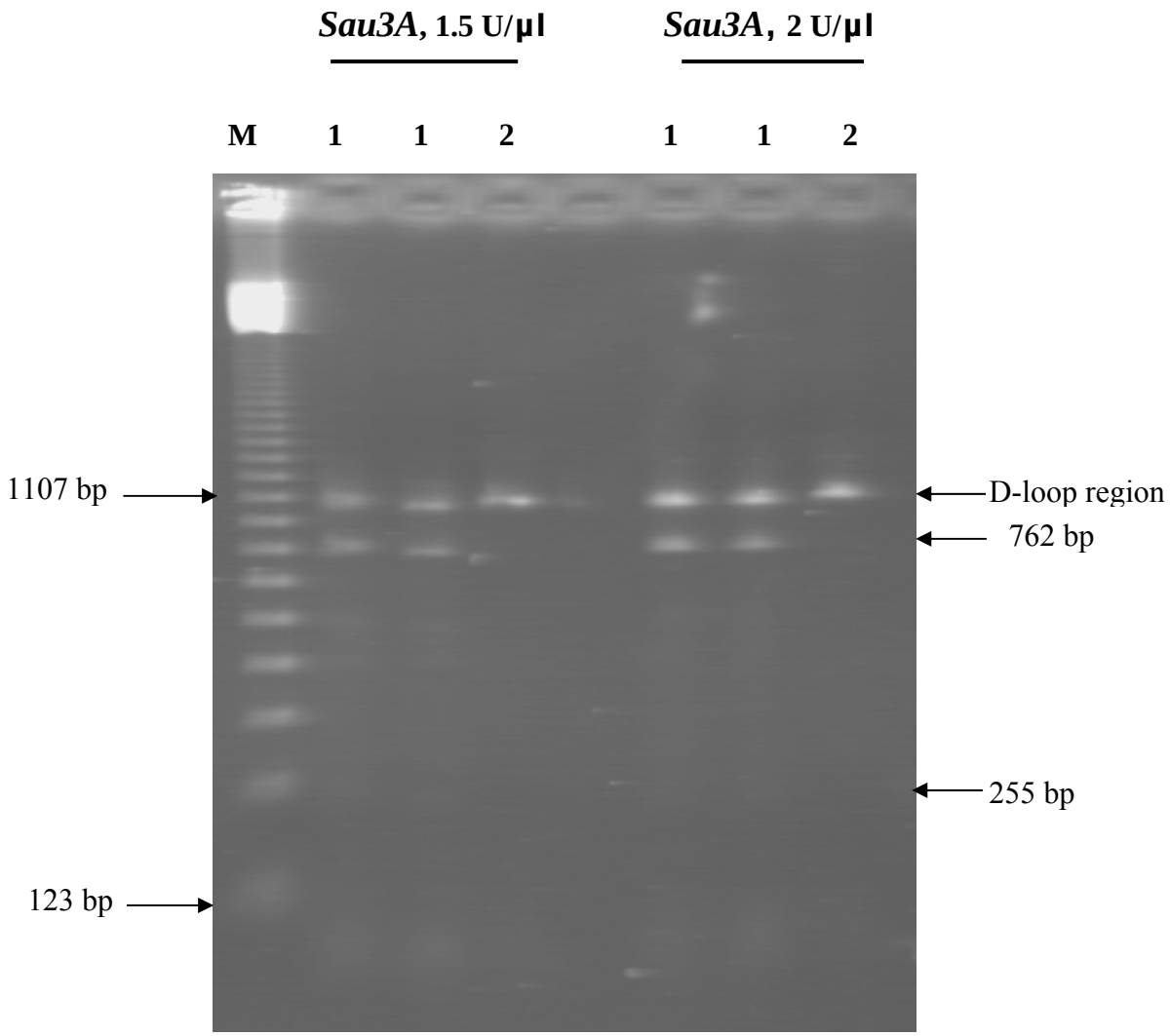

Figure (4-7): Restriction analysis of PCR amplified D-loop region of mice mtDNA using different concentrations of ***Sau3A*** enzyme. M: 123 DNA ladder, 1: PCR of D-loop region, 2: control or non restricted D-loop region. Fragments were resolved by electrophoresis in a 2.5% agarose gel (2.5hr., 5V/cm, Tris-borate buffer) and visualized by ethidium bromide staining.

recognition sequence within the D-loop region and was observed as two bands, a heavy intense one estimated as 762 bp in size and a 255 bp faint band the sum of these two bands almost equals the total size of the amplified D-loop.

The uniparental or maternal inheritance was proved in different species including mice (Avise *et al.*, 1979), also in human and rat indicating that parental mtDNA could be detected if present at a level of 4% (Giles *et al.*, 1980). However paternal mtDNA was detected in mice with contribution of 0.01-0.1% using more sensitive methods (Gyllensten *et al.*, 1991). In another study paternal transmission of mtDNA was detected through out development from pronucleus stage to neonates with elimination of the parental mitochondria afterwards, the researchers concluded that mammalian genome transmission is uniparental in interaspecific crosses and that leakage of paternal mtDNA is limited to interspecific crosses which rarely occur in nature (Kaneda *et al.*, 1995).

Since mice posses a single mtDNA sequence (i.e. mtDNA within individuals appear to be homogenous) (Brown and Vinograd, 1974). Results obtained with *Sau3A* indicate the presence of two sources for the types of mtDNA observed. A paternal leakage reading is a possibility however further studies involving sensitive methods and backcrosses are needed to decide whether this enzyme can recognize paternal mtDNA.

In the second stage of this study, restriction enzymes were used in the amplified D-loop region of mice as diagnostic markers for DNA base changes and the major objective was to identify restriction polymorphism distinguishing between the treated and non-treated mice within the region mentioned above.

BamHI, HaeIII, HindIII and *Sau3A* restriction endonucleases were selected to analyze the D-loop region of four mice sharing the same parents

and injected with 0.1, 0.2, 0.5 mg/kg bw of MMC in addition to a non treated individual used as control animals.

The enzymes *BamHI* and *HindIII* were used because they are commonly used and plentiful. They recognize hexanucleotide sequences in DNA and are not single site hitting enzymes; *BamHI* was reported to produce a particular restriction pattern in the mtDNA of BALB/c mice (Tamura and Aotsuka, 1988). *HindIII* gives rise to three fragments in mtDNA of mouse cell line LA9. As for enzymes *HaeIII* and *Sau3A,* they both recognize tetranucleotide sequences and were the only enzymes that were found to have target sites within the D-loop region of mice mtDNA in the first stage of restriction enzyme survey.

The D-loop fragment amplified by PCR remained intact and no digestion pattern was observed using *BamHI* in all of the four individuals, those treated with different dosages of MMC and the control animal (Figure 4-8). *BamHI* enzyme could not recognize a restriction site specific for the enzyme in the D-loop region of control animal because it was absent in this region although it was reported to have restriction sites within mtDNA of mice (Tamura and Aotsuka, 1988). These *BamHI* specific sites maybe found in the mtDNA but outside from the D-loop region. Treatment with MMC did not induce DNA base changes and could not create a novel restriction site that is detectable by *BamHI.*

Treatment with *HindIII* enzyme resulted in similar results as in *BamHI* (Figure 4-8), no digestion pattern was obtained in the D-loop region of MMC treated mice and control animal and thus the same interpretation of results obtained with *BamHI* could apply in this case.

HaeIII enzyme produced the same digestion pattern in all of MMC treated animals and the control animal. Recognition sequences specific for *HaeIII* were available in all of the individuals and base changes within this recognition sequences were not induced by exposure to MMC in any of the

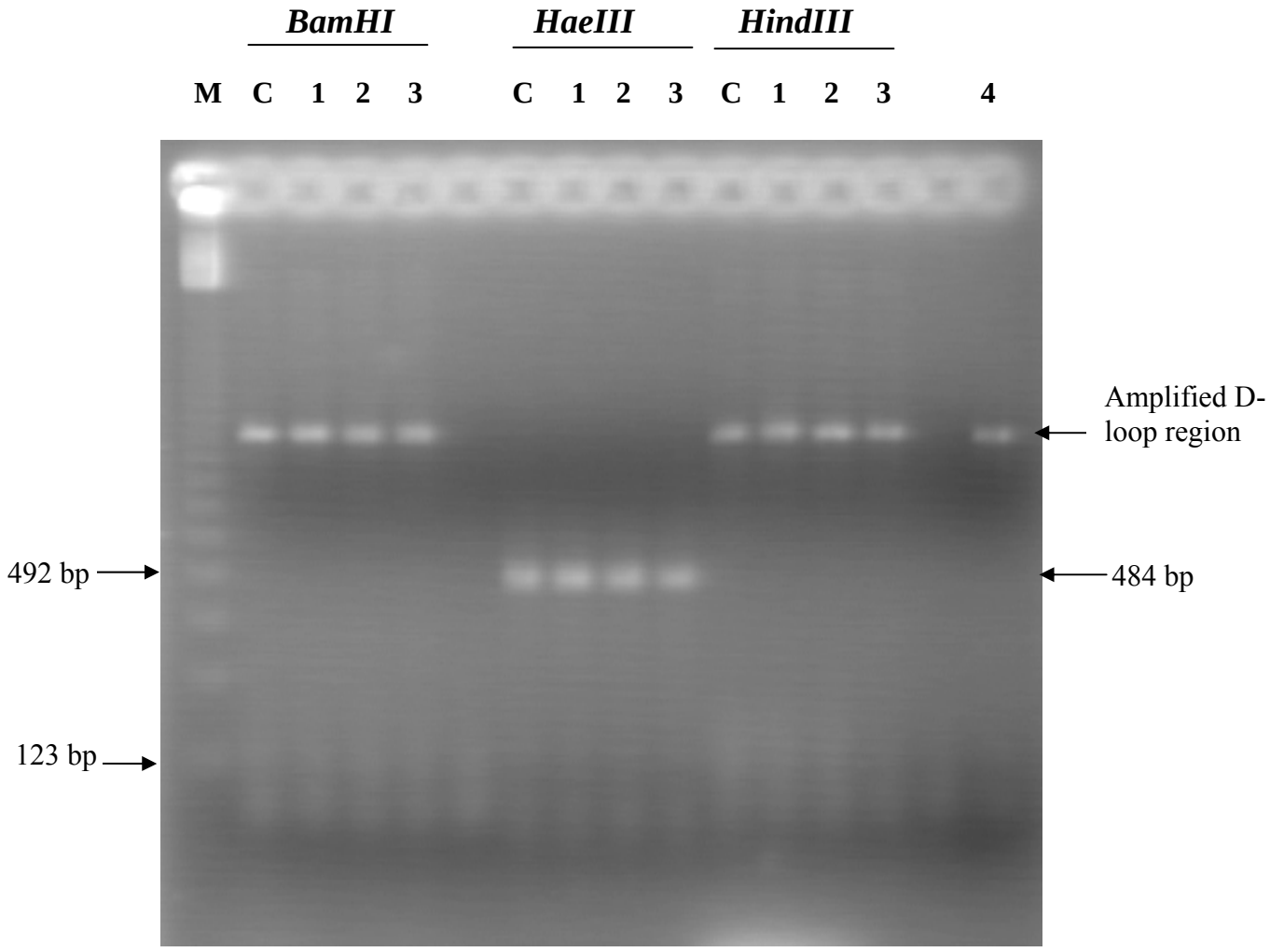

Figure (4-8): Restriction analysis of PCR amplified D-loop region of mice mtDNA treated with different dosages of MMC and non treated control animal with enzymes ***BamHI, HaeIII*** *and* ***HindIII***. M: 123 DNA ladder, C: amplified D-loop of non treated animal, lanes 1, 2, 3: D-loop of 0.1, 0.2, 0.5 mg/kg bw MMC treated mice respectively, 4: control or non restricted D-loop region. Fragments were resolved by electrophoresis in a 2.5% agarose gel (2.5hr., 5V/cm, Tris-borate buffer) and visualized by ethidium bromide staining.

0.1, 0.2, 0.5mg/kg bw doses (Figure 4-8). Induced base changes within *HaeIII* target sequences would have abolished these sites resulting in polymorphic restriction pattern which was not observed.

Sau3A cleavage pattern was also identical in all of the animals and the control mice. The generation of two intense bands and a faint one was obtained (Figure 4-9). *Sau3A* was a good candidate for producing potential polymorphism within MMC treated animals because earlier it generated two types of mtDNA and perhaps one of them was more sensitive to base changes distinguished by the enzyme than the other but both types remained intact in all the treated animals and the control mice.

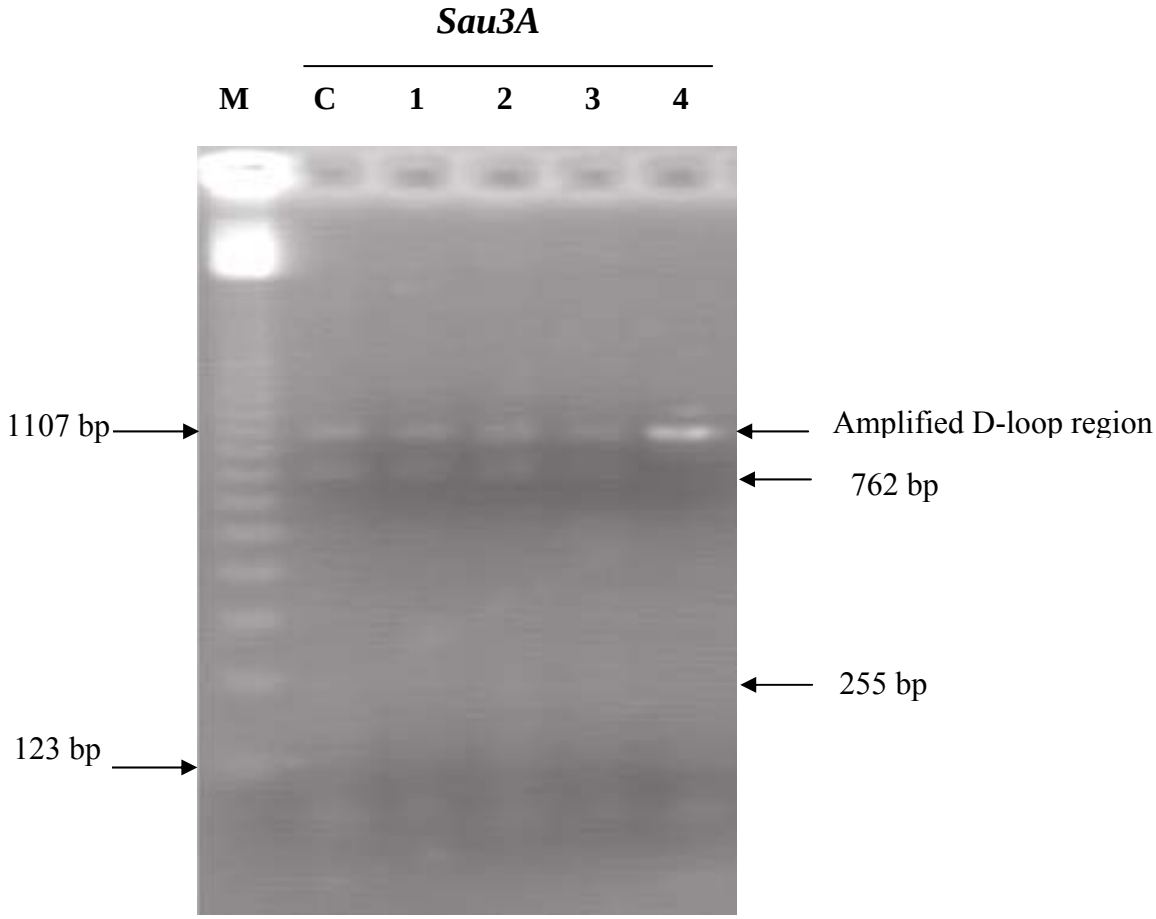

Figure (4-9): Restriction analysis of PCR amplified D-loop region of mice treated with different dosages of MMC and non treated control animal with ***Sau3A***. M: 123 DNA ladder, C: amplified D-loop of non treated animal, lanes 1, 2, 3: D-loop of 0.1, 0.2 and 0.5 mg/kg bw MMC treated mice respectively, 4: control or non restricted D-loop region. Fragments were resolved by electrophoresis in a 2.5% agarose gel (2hr., 5V/cm, Tris-borate buffer) and visualized by ethidium bromide staining.

4.5. Cytogenetic Analysis

In bone marrow cells, the MI mean value for 0.1mg/kg bw MMC treated mice (6.7% ± 0.13) was significantly (P< 0.05) lower compared to control animals where the mean value was (9.5% ± 0.33). MI decrease followed a dose dependent manner, higher doses of 0.2 and 0.5 mg/kg bw of MMC resulted in corresponding decrease in MI mean values (5.7% ± 0.25) and (5.2% ± 0.09) in comparison to the values observed in the control animals (Table 4-2).

In the spleen cells, the MI mean value was (1.8% ± 0.05) of 0.1mg/kg MMC treated animals compared to the controls where the mean value was (2.4% ± 0.09), the difference was found not to be significant (P > 0.05), however significant differences where observed (P< 0.05) between MI mean values of 0.2 and 0.5 mg/kg bw treated animals (1.3% ± 0.11) and (0.5% ± 0.13) respectively, in comparison to the control animals (Table 4-3). The MI decline relative to higher doses of MMC treated animals in both bone marrow and spleen cells is consistent with previous results (Al-Duliemy**,** 2005).

In bone marrow cells preformed *in vivo*, we took into account the percentage of marrow blasts which may represent another marker for the effect induced by MMC on bone marrow cells (Figure 4-10A). Low marrow blast percentage was observed in animals treated with 0.5mg/kg bw MMC (4.7%±1.06) compared to the blast percentage in control animals (7.8%±2.06) (Table 4-2).

The mean number of total CA in both bone marrow and spleen cells for MMC treated with 0.1mg/kg bw (44 ± 5.26) and was found to be significantly higher (P < 0.05) than the control animals (17 ± 1.92) (Table 4-2). Total CA in both bone marrow and spleen cells varied with different doses of MMC as shown in tables 4-2, 3. A significant increase in total CA was observed in a dose dependent manner in both types of cells.

Table (4-2): Cytogenetic analysis of bone marrow cells of mice treated with different doses of mitomycin C and the non treated controls

Treatment		MI% +SE	BI% +SE	Chromosomal Aberrations (CA) in 100 metaphase + SE							
				Ring Chromosome	Dicentric	Chromatid Gap	Chromatid Break	Chromosome Gap	chromosome Break	Deletion	Total no. of CA
Negative Control		a 9.5 ±0.33	a 7.8 ±2.06	a 2 ±0.33	a 5 ±0.66	a 5 ±0.31	a 1 ±0.31	a -	a -	a 4 ±0.31	17 ±1.92
Mitomycin C	**0.1mg/Kg**	b 6.7 ±0.13	ab 6.4 ±0.35	b 11 ±1.2	a 10 ±1.45	ab 7 ±0.53	ab 7 ±1.45	a -	a 1 ±0.00	a 8 ±0.63	44 ±5.26
	0.2mg/Kg	a 5.7 ±0.25	ab 6.1 ±0.2	b 14 ±0.66	a 12 ±0.66	b 9 ±0.31	ab 8 0.33±	a -	a 1 ±0.31	a 9 ±0.63	53 ±2.9
	0.5mg/Kg	b 5.2 ±0.09	b 4.7 ±1.06	c 23 ±1	a 11 ±0.88	b 9 ±0.31	b 12 ±0.63	b 3 ±0.53	a -	a 9 ±1.12	67 ±4.47

Differences a, b, c are significant (P< 0.05)
SE: Standard error

Table (4-3): Cytogenetic analysis of spleen cells of mice treated with different doses of mitomycin C and the non treated controls.

Treatment		MI% +SE	Chromosomal Aberrations (CA) in 25 metaphase + SE							
			Ring Chromosome	Dicentric	Chromatid Gap	Chromatid Break	Chromosome Gap	chromosome Break	Deletion	Total no. of CA
Negative control		a 2.4 ±0.09	a 1 ±0.53	a 4 ±0.53	a 1 ±0.00	a 1 ±0.00	a -	a -	a 4 ±0.53	11 ±1.39
Mitomycin C	**0.1mg/Kg**	ab 1.8 ±0.05	a 1 ±0.33	a 5 ±1.2	a 2 ±0.33	a 2 ±0.53	a -	a -	a 7 ±0.33	17 ±2.72
	0.2mg/Kg	bc 1.3 ±0.11	a 1 ±0.00	a 3 ±0.66	a 3 ±0.33	a 3 ±0.53	a -	a 1 ±0.33	a 6 ±0.83	17 ±2.68
	0.5mg/Kg	c 0.5 ±0.13	a 2 ±0.33	a 4 ±0.83	a 4 ±1.2	a 1 ±0.33	b 1 ±0.00	a -	a 10 ±1.80	22 ±4.49

Differences a, b, c are significant (P< 0.05)
SE: Standard error

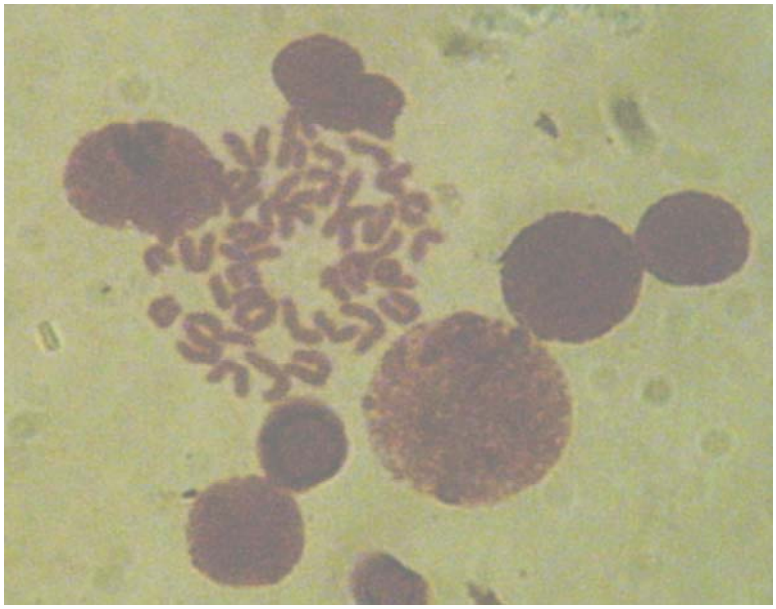

Figure (4-10A): Bone marrow cells with metaphase and a blast cell. Magnification power 100X.

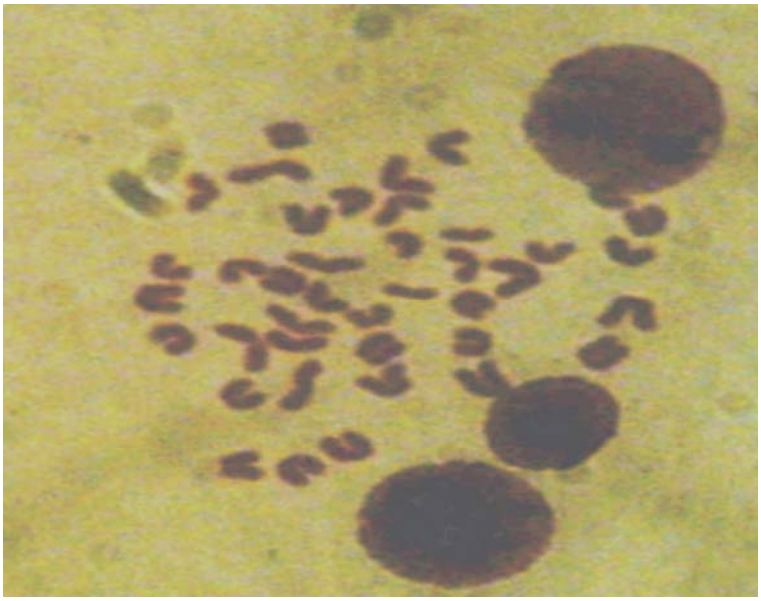

Figure (4-10B): Normal metaphase of bone marrow cells showing the forty intact chromosomes of mice. Magnification power 100X.

MMC is a well known mutagenic and clastogenic agent, base substitutions, sister chromatid exchanges, chromosomal aberrations and micronuclei, both *in vivo* and *in vitro* are genetic effects that can be observed as a function of dose and exposure period (Pelt *et al*., 1991; Salvadori *et al.*, 1994; Becerril, 1999).

Results obtained in this study came in consistence with the previously mentioned studies (Shubber *et al*., 1985; Juma, 1993). MMC was found to affect the cellular proliferation rate for the mean value of MI in treated animals was significantly lower in comparison to the control animals in both bone marrow and spleen cells. Determination of proliferation was used over the years in intent of predicting clinical aggressiveness such as in human breast cancer (Thor *et al.*, 1999). The mean value of blast index was significantly lower in bone marrow of treated animals. Significant reduction in both MI and BI due to the exposure of antitumor drugs was reported in colon cancer patients treated with the anticancer drug methotrexate (MTX) (Al-Abasi, 2001).

Structural chromosomal abnormalities involve changes in the structure of one or more chromosomes, they are extremely complex. Some unique chromosomal aberrations were used in the diagnosis of certain types of cancer for example Philadelphia chromosome is a characteristic abnormality in patients with leukemia (Sharma and Sharma, 1980). In this study, different types of CA were observed and scored in both bone marrow (Figure 4-10B, C) and spleen cells (Figure 4-11A, B) like ring chromosome which is caused by two chromatid breaks in the same chromosome and adhesion of the produced sticky ends (Strachan and Read, 1999), dicentric chromosomes that have two centromeres formed by breakage and reunion involving two chromosomes (Sandberg, 1992). Chromosome gaps and breaks which take place before DNA replication were also scored and chromatid gaps and breaks which take place after the replication of DNA.

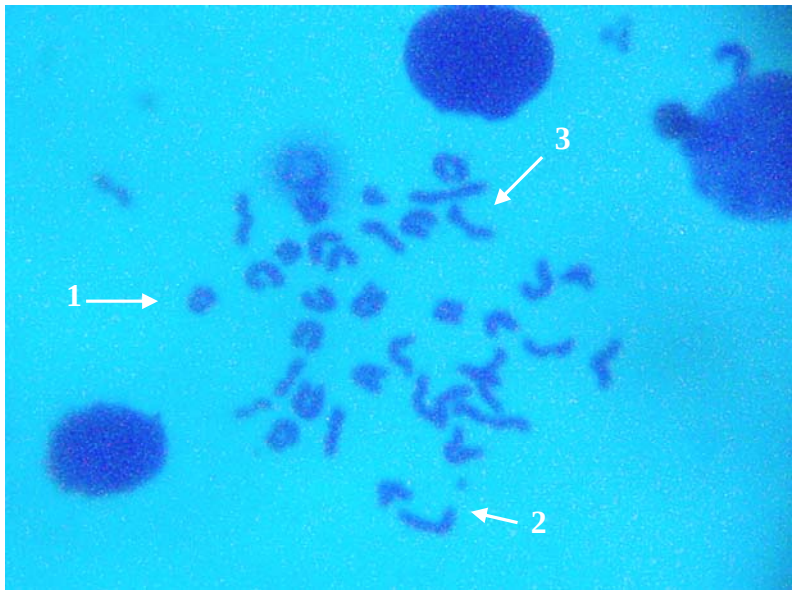

Figure (4-10C): Metaphase of bone marrow cells showing chromosomal aberrations, 1: Ring chromosome, 2: Deletion. 3: Chromatid gap. Magnification power 100X.

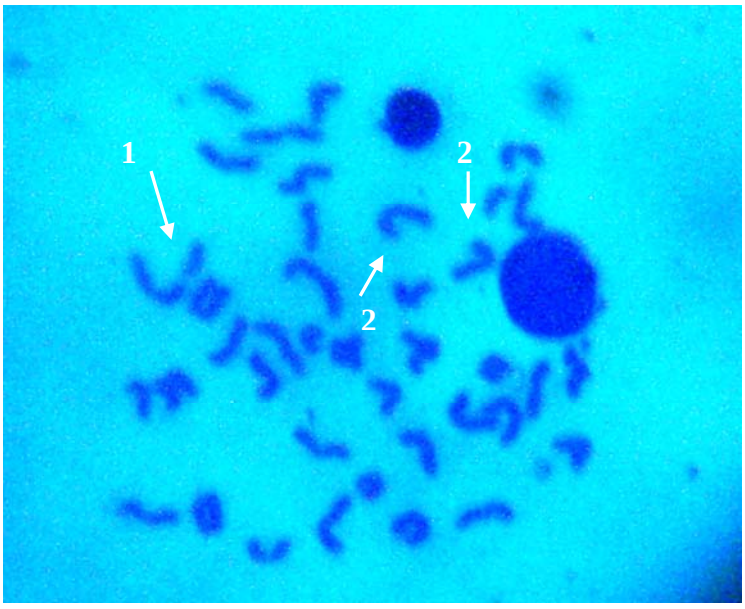

Figure (4-10D): Metaphase of bone marrow cells showing chromosomal aberrations, 1: Chromatid break, 2: Deletion. Magnification power 100X.

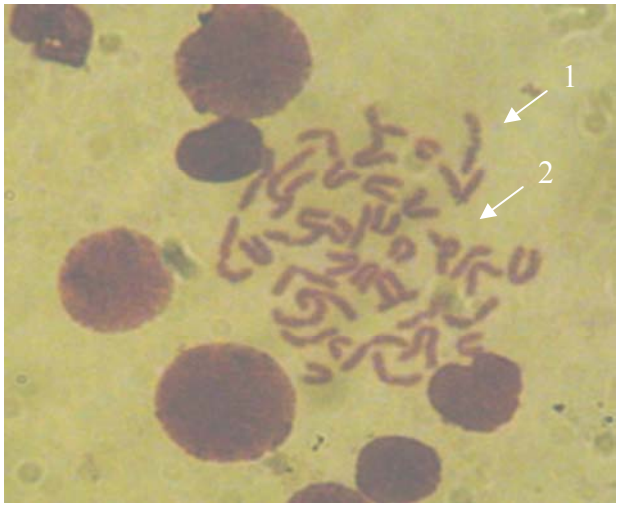

Figure (4-11A): Metaphase of spleen cells showing chromosomal aberrations, 1: chromatid gap, 2: Dicentric. Magnification power 100X.

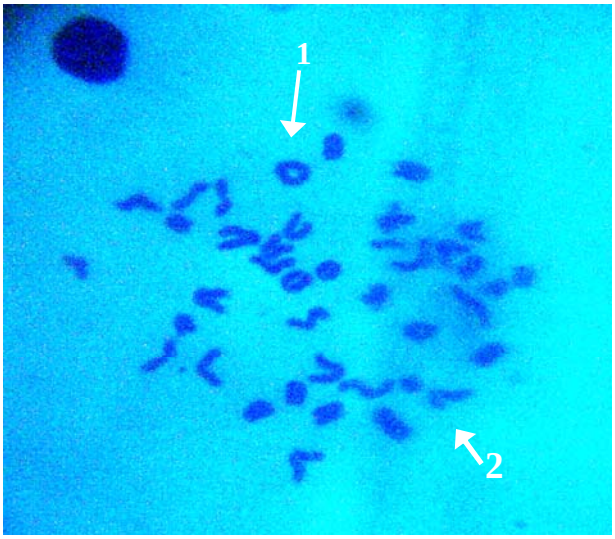

Figure (4-11B): Metaphase of spleen cells showing chromosomal aberrations, 1: Ring chromosome, 2: Deletion. Magnification power 100X.

Deletion is another type of chromosomal abnormality and is known as the loss of a chromosomal segment as a result of a single breakage in a chromatid and the formation of two centric and acentric fragments, the latter one is lost afterwards and eventually degraded. Abnormal chromosomes do not segregate stably in mitosis; cells that receive abnormal chromosomes may show several defects (Tamarin, 1996). Chromosomal aberrations are produced by specifically chromosomal mechanisms, most CA are produced by misrepair of broken chromosomes, by improper recombination or by malsegregation of chromosomes during mitosis or meiosis (Strachan and Read, 1999).

CA were scored in a good quality spreads, chromosomes are neither condensed or elongated nor overlapping. In bone marrow cells ring chromosomes appeared more frequent than other CA. Ring chromosomes may appear as a result of exposure to chemical and physical mutagens, MMC was reported to induce ring chromosome abnormality (Al-Duliemy, 2005). However all types of CA were significantly multiplied ($P<0.05$) for example in the bone marrow cells of 0.5mg/kg bw treated MMC animals CA were highly increased (67±4.47) in comparison to corresponding CA in control animals (17±1.92) (Table 4-2).

CA in bone marrow were scored in 100 metaphase, obtaining 100 good spread metaphase in spleen cells was rather a difficult task for the numbers of metaphase were very low, this is may be related to MMC treatment as several drugs were reported to have the possibility of causing failure in getting dividing cells in metaphase stage (Sharma and Sharma, 1980). Another reason for this difference between bone marrow and spleen cells may be the fact that this organ consists of masses of lymphoid tissue that function in the destruction of old red blood cells while bone marrow is the place where new blood cells are produced and thus cells are generated

continuously (Internet 3,4). CA in spleen cells were scored in 25 well spread metaphase with deletion aberration relatively being the most frequent type compared to other observed CA with ring chromosome and chromatid gap in the second grade. Total CA in spleen cells in 0.5mg/kg bw MMC treated animals were significantly ($P< 0.05$) higher compared to total CA in control animals (Table 4-3). The inhibition of proliferation rate in both bone marrow cells, the decrease in bone marrow percentage and the increase in CA observed in both bone marrow and spleen cells confirm the positive correlation between MMC and DNA damage in mice somatic cells.

The creation of animal models of mitochondrial disease provides new insights in the understanding of human mitochondrial disorder. Rodent model systems were used in mitochondrial research for example the rodent Clethrionomys was utilized in genotoxicity testing to assess genetic damage in areas near Chornobyl nuclear power station (Baker *et al.*, 1999). Mouse models of mitochondrial disease were used by creating for example mouse lines with inactivating mutations in nuclear genes encoding mitochondrial proteins (Wallace, 1999).

In this study the mouse animal was used to investigate potential mtDNA changes induced by MMC. The dosages of the antitumor drug MMC for this work was selected in accordance with previous studies inducing cytotoxicty and various genotoxic endpoints including chromosomal aberrations (Shubber *et al.,* 1985; Shubber and Juma, 1999). MMC is capable of inducing oxidative DNA damage (Ochs *et al*., 1999). Superoxide radicals generated by MMC may be mutagenic (Nikawa *et al.*, 2001). Several reductase enzymes have been shown to catalyze the reduction of MMC to cytotoxic metabolites. NADH Cytochrome b_5 reductase is an enzyme which predominantly localized in the mitochondria is capable of converting the

prodrug MMC into a DNA alkalyting agent and generates oxygen radicals (Holtz *et al.*, 2003).

RAPD-PCR technique was used to reveal DNA polymorphisms in mtDNA contaminated with nDNA due to exposure to MMC in mice animals treated with 0.5mg/kg bw and the control non treated animal. The high percent of polymorphisms obtained with primers OPC-19 and OPO-16 were interpreted as DNA changes induced by the exposure to MMC since the fact that the majority of RAPD fragments produced are identical between individuals of the same strain (Williams *et al.*, 1990). The mice used in this study belonged to the same strain and thus high percent polymorphisms could be due to MMC exposure. RAPD-PCR was used in assessing DNA variations of abnormal phenotype in broiler chicken (Ali, 2003).

Restriction analysis of the D-loop or control region of mice amplified by PCR using specific primers directed to this region, revealed no DNA alterations between animals treated with different dosages of MMC in comparison to the control animals. The enzymes used recognize different nucleotide sequences in DNA. Mutations within restriction sites change the sizes of restricted fragments and hence the positions of bands (McPherson and Møller, 2001). Also distinction between variant DNA by restriction enzymes is based on differences in nucleotide sequence rather than methylation (Castora *et al.*, 1980). However a change in the restricted fragments was not observed in the amplified D-loop region between animals treated with different dosages of MMC in comparison to the control animals.

The D-loop region was chosen in this study for many reasons of functional and structural significance. The rate of evolution in this region is faster as compared with the rRNA and protein coding genes of the mitochondrial genome (Matson and Baker, 2001). Regulation of replication

and transcription of mtDNA is controlled through this region (Coskun *et al.*, 2003). The D-loop region is an essential hypervariable area representing somatic mutational hot spot. MtDNA mutations in this region are a frequent event in many different cancers providing a potential molecular tool for diagnosis (Okochi *et al.*, 2002).

In the search for restriction enzymes that distinguish recognition sequences and cleave within them in D-loop region of mice, eight enzymes were engaged. Only two endonucleases were found to have recognition sites, *Sau3A* and *HaeIII*. Both enzymes recognize a 4bp recognition sequence. The D-loop digestion with four restriction endonucleases including *Sau3A, HaeIII, BamHI* and *HindIII* of mice animals treated with MMC in different dosages in addition to control animals revealed no change in despite of the D-loop region may be a natural hot spot for experimentally induced DNA damage for the D-loop structure contains a single stranded DNA that is more easily damaged by various genotoxic mechanisms (Singh *et al.*, 1992).

Conceivably, MMC did not produce DNA alterations in mice D-loop region in sites that are detectable by the restriction enzymes used in this study or another possible explanation could be that the drug did not induce DNA alterations to a detectable level after being metabolized into its active form. Because of the high copy number of mitochondria in a cell and multiple DNA copies inside the organelle (Ziviani and Antozzi, 1997; Modica-Napolitano and Singh, 2002). An mtDNA mutation may become physiologically relevant for a given cell only if the mutant fraction in that particular cell exceeds a certain threshold (Khrapko *et al.*, 1999). One of the obstacles that have long been hindering definitive molecular diagnosis of mitochondrial disorders is the state of heteroplasmy (Wong *et al.*, 2002) (Moraes *et al.*, 2003). As a consequence it is possible to miss DNA alterations which levels fall below the lower limit of detection necessitating

the need for more accurate and quantitative methods in the search for mtDNA mutations whether naturally occurring or induced in the laboratory. The temporal temperature gradient gel electrophoresis (TTGE) is one of the methods used to detect low proportions of mutant mtDNA and distinguishes heteroplasmic from homoplasmic variation (Wong *et al.*, 2002).

Conclusions

From this study we concluded the following**:-**

1- The ability to extract mtDNA using simple methods with limited efficiency.

2- The use of RAPD-PCR technique was useful to give apparent genetic variations due to exposure to the drug used in this study.

3- The ability to effectively amplify a specific region of mtDNA that has a high mutation rate, the D-loop region to study genetic variations or mutations known to be related to certain types of cancer.

4- Only restriction endonucleases with four base pair recognition sequences were able to cleave within the PCR amplified D-loop region of white mice. *Sau3A* and *HaeIII* were the only enzymes out of ten that have recognized cleavage sequences in the mentioned region.

5- *Sau3A* endonuclease could be used as a molecular tool in phylogenetic studies concerning mtDNA and may prove helpful in the understanding and recognition of paternal inheritance or maternal inheritance leakage in mice.

6- The dose dependent manner of MI inhibition and CA high frequency with higher MMC administered doses in comparison to control animals in both spleen and bone marrow cells together with the decrease in blast percentage in bone marrow cells is an indicator of successful induction of genetic variation in experimental animals.

7- The mice model used in this study is a reliable animal model in the evaluation of genetic variation by the RAPD-PCR technique.

Recommendations

For the future works in this field and according to our findings we recommended the following:

1- The use of more accurate methods in obtaining nDNA free mtDNA extract is needed.

2- Inclusion of more mutagens and/or carcinogens in studies on laboratory animals in search for more genetic variations and larger effect.

3- The use of more sensitive and accurate molecular methods in studying potential affected mtDNA regions such as DNA sequencing to identify base pair changes and to use Fluorescent *In Situ* Hybridization (FISH) technique to detect any chromosomal changes.

4- More studies using *Sau3A* restriction endonuclease are needed to predict its role in finding recognition sequences within the D-loop region of mtDNA in mice using Polyacrylamide Gel Electrophoresis (PAGE) or by creating probes of the fragment resulted continuously.

5- To include other restriction endonucleases in the search for mtDNA variants within the PCR amplified D-loop region in mice treated with MMC and to include mtDNA coding regions.

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ا لخلا صة

استخدمت لهذة الدراسة مجموعة من الفئران البيض بعمر٦- ٨ أ سابيع تعود لنفس الام و الاب حيث حقنت في الغشاء تحت الجنبي بجرع مختلفة ٠،٠١و٠،٠٢و ٠،٠٥ملغم/كغم من وزن الجسم من العقار المضاد للسرطان المايتومايسين سي Mitomycin C . تم التحري عن التأثيرالسمي الوراثي لهذا العقار على كل من الدنا المايتوكوندیري و الدنا النووي باستخدام الطرق الوراثية الجزيئية و التحليلات الخلوية الوراثيـة علىكل من خلايا الطحال و نخاع العظم.

عزل الدنا المايتوكوندیري من نسيج الطحال و تم اجراء التحليلات الوراثية الجزيئية باستخدام تفاعل البلمرة المتسلسل (PCR) باستخدام بادئات عشوائية (RAPD-PCR) مرة و بادئات متخصـصة مـرة اخرى لتكثير منطقة محددة غير مشفرة او ما يعرف بمنطقة السيطرة و تسمى كذلك D-Loop region في الدنا المايتوكوندیري ثم استخدمت بعض انزيمات التقييد في تقطيع و تحليل ناتج البلمرة المتسلسل باستخدام البادئات الخاصة بمنطقة الـD-Loop لدنا المايتوكوندريا في الفئران.

في تفاعل RAPD-PCR تم و بطريقة عشوائية انتقاء عشرة بادئات مختلفة بطـول عـشرة قواعـد نايتروجينية لكل بادئ لتطبيقها على الـدنا المايتوكونـديري لحيوانـات معاملـة بعقـار المايتومايـسين سي٠،٠٥ملغم/كغم من وزن الجسم، اظهرت النتائج وجود تباينات وراثية تراوحت نـسبها ٧,٧ – ١٠٠ % بين الحيوانات المعاملة وحيوانات السيطرة وعزيت نسب التبـاين الـوراثي العاليـة كنتيجـة للتعـرض للمايتومايسين سي.

استخدمت كذلك منطقة D-loop لفئران معاملة بجرع مختلفة من العقار ٠،٠١و٠،٠٢و ٠،٠٥ملغم/كغـم من وزن الجسم اضافةالى حيوانات السيطرة كهدف للتكثير بتفاعل البلمرة المتسلسل با سـتخدام بادئـات متخصصة ثم عرض ناتج التكثير الذي تم الحصول عليه الى الهضم بأربعة انزيمـات تقييـد مختلفـة و هي *HindIII, BamHI, HaeIII, Sau3A* لغرض التحري عن اي اختلاف محتمل ناتج عـن المعاملـة بالتراكيز المختلفة من العقار. بينت نتائج تجارب التحليل بانزيمات التقييدعدم وجـود اي فروقـات بـين الحيوانات المعاملة و حيوانات السيطرة.

اجريت كذلك بعض الفحوصات الوراثية الخلوية على خلايا الطحال و نخاع العظم لنفس الحيوانـات حيث استخدمت طريقة الزرع المباشر لهذه الخلايا لغرض اجراء فحوصات الا نقسام الخلـوي و تحليـل الكروموسومات لكلا النوعين المذكورين من الخلايا، كما و درس معامل الخلايا الارومية لخلايـا نخـاع العظم فقط بالتراكيز المذكورة و حيوانات السيطرة. اظهرت النتائج ان معامل الانقسام في كل مـن خلايـا الطحال و نخاع العظم و كذلك معامل الخلايا الارومية في خلايا نخاع العظم فقط قد تناسـبا عكـسيا و

بصورة معنوية مع التراكيز العالية من العقار في حين تناسبت التشوهات الكروموسومية طرديا و بصورة معنوية ايضا مع تركيز العقار لكلا النوعين من الخلايا .

ان التحليلات الوراثية الجزيئية بطريقة RAPD-PCR كان لها القابلية على التحري عن تباينات في الدنا المايتوكوندري للحيوانات المعاملة بـ ٠،٠٥ملغم/كغم من وزن الجسم مقارنة بحيوانات الـسيطرة فـي حين ان تحليل منطقة D-loop و المكثرة بتفاعل البلمرة المتسلسل للحيوانات المعاملة بـ ـ ٠،٠١و٠،٠٢و ٠،٠٥ملغم/كغم من وزن الجسم بانزيمات التقييد لم يؤدي الى التحري عن تباينات وراثيـة بالمقارنـة مـع حيوانات السيطرة. بينما يعد انخفاض معدلات الانقسام الخلوي و زيادة معدلات التشوهات الكروموسـومية في خلايا الطحال و نخاع العظم يرافقه الانخفاض في معامل الخلايا الارومية في خلايا نخاع العظم فقـط كدليل على التاثير السمي الوراثي للمايتومايسين سي على دنا الخلايا الجسمية في الفئران .

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قسم التقانة الاحيائية

دراسة الدنا المايتكوندیري و التغايرات الوراثية الخلوية في الفئران المعاملة بالمايتومايسين سي كعامل مطفر داخل الجسم الحي

رسالة مقدمة الى كلية العلوم

في جامعة النهرين كمتطلب جزئي من متطلبات نيل درجة

ماجستير علوم في التقانة الاحيائية

من قبل

رواء علي زاهد بكر

بكالوريوس احياء مجهرية ٩٩ ١٩

جامعة بغداد

تشرين الاول 2006 شوال 1427