Evaluation of Genotoxic Effects of Chromium on Tannery Workers by Various Cytogenetic Assays

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Seminar

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Dedication

Dedicated to my dear parents.

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Abstract

Chromosomal aberration is an important biomarker for carcinogenesis. The study of DNA damage at the chromosomal level is an essential part of genetic toxicology. Different Chromium compounds are used in tanneries. Hexavalent chromium, Cr (VI) is considered as a human lung carcinogen with potentially widespread exposure. This study was designed specifically to evaluate cytogenotoxicity of chromium compounds used in leather industries on human peripheral blood lymphocytes by using a series of cytogenotoxic assays such as micronucleus test, chromosome aberration test and alkaline Comet assay. These tests are proved to be extremely useful techniques in cytogenotoxicity measurements. The micronucleus test assesses chromosomal damage by the formation of micronucleus on once-divided binucleatetd cells, whereas various types of structural chromosome aberrations and extent of DNA damage are detected by chromosome aberration and comet assay respectively. Different types of structural damages like Break, Gap and dicentric chromosomes were detected by Chromosome aberration test. Micronucleus test also revealed significant chromosomal damage in chromium exposed human blood cells. In line with results of Micronucleus test and Chromosome aberration test, significant DNA damage was also detected by Comet assay. The results of Comet assay indicate that chromium compounds induced significant increase in the comet tail DNA (%), tail moment and Olive tail moment; the parameters used to determine genotoxicity. These results from all three tests provide evidence that exposure to chromium and chromium compounds in human peripheral lymphocytes results in induction of DNA damage. Therefore the present study gives an indication of an association between chromium induced DNA damage and chromosomal instability.

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8-OHG	8- hydroxyguanine
ALS	alkaline labile sites
AASL	Atomic Absorption Spectrophotometric Laboratory
AP	apurinic/apyrimidinic
ANOVA	analysis of variance
A.U.	Arbitary Unit
BSA	bovine serum albumin
C	Celsius
CASP	Computer Assay Software Project
Cr	Chromium
Cr(III)	Trivalent chromium
Cr(VI)	Hexavalent chromium or Chromate
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNPH	2, 4-dinitrophenylhudrazine
DSB	double strand breaks
e.g.	For example
EDTA	Ethylenediaminetetraacetic acid
et al.	and others
Fapy G	2,6-diamno-4-hydroxy-5-formamidopyrimidine
Fig.	Figure
FISH	Fluorescence in situ hybridization
HCT	Head center threshold
HT	Head threshold
hr	hour
i.e.	that is

IARC	International Agency for Research on Cancer
LMPA	low melting point agarose
M	Molar
mg	milligram
Min	Minute
ml	milliliter
mM	millimolar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
No./no.	Number
NMPA	normal melting point agarose
OTM	Olive tail moment
PBS	Phosphate Buffer Saline
pH	Negative logarithm of hydrogen ion concentration
rpm	Revolution per minute
RT	Room temperatute
ROS	reactive oxygen species
SDS	Sodium dodecyl sulphate
SCGE	Single-cell gel electrophoresis
SCG	single cell gel
SSB	single strand breaks
Sec	Second
SEM	Standard error of mean
SH	Sulfhydryl
TT	Tail threshold
UV	Ultra violet
vol.	Volume
WHO	World Health Organization

WHO	World Health Organization
µg	Micro gram
µl	Micro liter

Chapter One

Introduction

Chapter 1
Introduction

Metals are stable and persistent environmental contaminants. Many metals have the potential to cause genetic alterations in the target tissues of exposed humans. Such alterations, if they occur in tumour suppressor genes, may lead to the development of cancer in the target organs (Keshava and Ong, 1999). Chromium is one of the heavy metal which causes genetic alterations in the target tissue. Epidemiological studies carried out in the Federal Republic of Germany, Italy, Japan, the UK and the USA of workers in the chromate production industry have consistently shown excess risks for lung cancer. Similarly, studies carried out in the Federal Republic of Germany, France, the Netherlands, Norway, the UK and the USA of workers in the production of chromate pigments have also consistently shown excess risks for lung cancer. Workers in this industry are exposed to chromates, not only in the pigments themselves but also from soluble chromium (VI) compounds in the raw materials used in their production. Excess risk for lung cancer has been clearly established in facilities where zinc chromate was produced, although other chromium pigments were also generally made in these plants. In two limited reports from the UK and in a small Italian study, excesses of lung cancer were reported in workers in the chromium plating industry. Cases of sinonasal cancer were reported in epidemiological studies of primary chromate production workers in Japan, the UK and the USA, of chromate pigment production workers in Norway and of chromium platers in the UK, indicating a pattern of excess risk for these rare tumours.

Chromium (VI) compounds may cause adverse effects to the skin, the respiratory tract and, to a lesser degree, the kidneys in humans, while chromium (III) is less toxic. Although chromium (III) compounds were generally even more reactive than chromium (VI) compounds with purified DNA and isolated nuclei, 12 compounds of various solubilities (chromic chloride, chromic acetate, chromic nitrate, chromic sulfate, chromic potassium sulfate, chromium alum, neochromium, chromic hydroxide, chromic phosphate, chromic oxide, chromite ore and cupric chromite) gave positive results in only a minority of studies using cellular test systems, often under particular treatment conditions or at very high concentrations, which were generally orders

of magnitude higher than those needed to obtain the same effects with chromium (VI) compounds.

1.1. Chromium

Chromium is found in the environment in two major forms: reduced Cr (III) and Cr (VI), or chromate. It is a steely-gray, lustrous, hard metal that takes a high polish and has a high melting point. It is also odorless, tasteless, and malleable. Chromate, the most biologically active species, is readily taken up by living cells and reduced intracellularly, via reactive intermediates, to stable Cr (III) species. Cr (III), the most abundant form of chromium in the environment, does not readily cross cell membranes and is relatively inactive in vivo. However, intracellular Cr (III) can react slowly with both nucleic acids and proteins and can be genotoxic. (E T Snow). With other metallic elements, e.g., lead and potassium, together with oxygen, chromic anhydride forms the chromates and dichromates. These compounds are salts of chromic acid and are used as pigments in paints, in dyeing, and in the tanning of leather.

Chrome yellow, a pigment, consists largely of lead chromate. Other chrome colors are black, red, orange, and green. In the chrome process for tanning leather, a dichromate is used, and chromium hydroxide, a basic compound of chromium, hydrogen, and oxygen, is precipitated and held in the leather.

1. Chrome alum or Chromium (III) potassium sulfate: $KCr(SO_4)_2$, $KCr(SO_4)_2{\cdot}12(H_2O)$
2. Chrome Orange: lead (II) chromate and lead (II) oxide- ($PbCrO_4 + PbO$).
3. Chromium carbonyl or chromium hexacarbonyl: $Cr(CO)_6$ C_6CrO_6
4. Chromium trioxide: CrO_3
5. Chromium (III) sulfate: $Cr_2(SO_4)_3 \bullet 12(H_2O)$.

1.1.1. Oxidation states of chromium

Oxidation states	Compound
-2	$Na_2[Cr(CO)_5]$
-1	$Na_2[Cr_2(CO)_{10}]$
0	$Cr(C_6H_6)_2$
+1	$K_3[Cr(CN)_5NO]$
+2	$CrCl_2$
+3	$CrCl_3$
+4	K_2CrF_6
+5	K_3CrO_8
+6	K_2CrO_4

Table 1.1: Compounds of different oxidation state of Chromium

1.1.2. Chromium exposure

1.1.2.1. Toxicity of Chromium

Chromium toxicity depends on its oxidizing status. The hazardous effect decreases with the reduction of Cr (VI) to Cr (III). There are various chemical reducers of Cr (VI), including sulfides, dissolved organic substance, aqueous Fe (II) and minerals enriched in Fe (II), and Fe (0) (Yu. N. Vodyanitskii, 2009).

1.1.2.2. Toxic effect

Water insoluble chromium (III) compounds and chromium metal are not considered a health hazard, while the toxicity and carcinogenic properties of chromium (VI) have been known for a long time. Because of the specific transport mechanisms, only limited amounts of chromium (III) enter the cells. Several in vitro studies indicated that high concentrations of chromium (III) in the cell can lead to DNA damage (Eastmond et al, 2008). Acute oral toxicity ranges between 1500 and 3300 µg/kg (Katz et al, 1992). World Health Organization recommended maximum allowable concentration in drinking water for chromium (VI) is 0.05 milligrams per liter. Hexavalent chromium is also one of the substances whose use is restricted by the European Restriction of Hazardous Substances Directive.

The chromate ion is transferred into the cell by a transport mechanism. The acute toxicity of chromium (VI) is due to its strong oxidation properties. After it reaches the blood stream, it damages the kidneys, the liver and blood cells through oxidation reactions. Hemolysis, renal and liver failure are the results of these damages. Aggressive dialysis can improve the situation (Dayan, 2001). The carcinogenity of chromate dust is known for a long time, and in 1890 the first publication described the elevated cancer risk of workers in a chromate dye company (Newman, 1890; Langard, 1990). Chromium salts (chromates) are also the cause of allergic reactions in some people. Contact with products containing chromates can lead to allergic contact dermatitis and irritant dermatitis, resulting in ulceration of the skin, sometimes referred to as "chrome ulcers". This condition is often found in workers that have been exposed to strong chromate solutions in electroplating, tanning and chrome-producing manufacturers (Basketter, 2000). In some parts of Russia, pentavalent chromium was reported as one of the causes of premature dementia (Pierre R. Roberge et al, 2009).

1.1.3. Mechanism of Genotoxicity

Chromates enter cells more readily than chromium (III) compounds and are reduced ultimately to chromium (III). The reduction process and the subsequent intracellular activity of reduced chromium species are important for the mechanism of toxicity and carcinogenicity of chromium (VI). Particulate chromium (III) compounds can also enter cells by phagocytosis. Chromium (VI) compounds cross the placental barrier in greater amounts than chromium (III) compounds. Chromium trioxide increased fetal death rate, caused growth retardation and increased the frequency of skeletal deformities and of cleft palate in rodents. Developmental effects have also been reported in mice exposed to chromic chloride.

Three mechanisms have been proposed to describe the genotoxicity of chromium (VI):

The first mechanism includes highly reactive hydroxyl radicals and other reactive radicals which are byproducts of the reduction of chromium (VI) to chromium (III). The second process includes the direct binding of chromium (V), produced by reduction in the cell, and chromium (IV) compounds to the DNA. The last mechanism attributed the genotoxicity to the binding to the DNA of the end product of the chromium (III) reduction (Cohen et al, 1993).

The heaviest metal exposure occurs in the workplace among occupationally exposed groups. A person spends, on average, one-third of his life at his workplace and therefore the environment in which he works can be a major factor in determining health (K. Danadevi, Roya Rozati, 2004).

Leather, a traditional export item in Bangladesh, enjoys a good reputation world wide for its quality. This sector plays a significant role in the economy of Bangladesh in terms of its contribution to export and domestic market. In south-western part of Dhaka city, there is a tannery area occupying 25 hectares of land at Hazaribagh, where about 90% of tannery industries of Bangladesh are located. The tanning industries of Hazaribagh process some 220t of hide per day with an associated release of 600-1000 Kg of tanned skin-cut waste (SCW) per ton processed raw hide (Zahid, *et al.,* 2004). One of the major concerns of processing of hides is the heavy metals, especially, chromium used in the tanning processes. Large amounts of chrome powder and chrome liqueur are used during tanning process (UNIDO, 2000).

There are many environmental hazards associated with the chemicals used in the tanning processes. But the hazards can come out in several ways. One is through the waters of canals and rivers after mixing with effluents. Substantial work has been done to study on this. The entrance of harmful chemicals into the food chain through the use of SCW as feed staff, which is only recently been studied by Hossain *et al.* (2007), professional exposure of different chromium compounds for long period of time increases cancer risk (A. Hilali *et al*, 2007).

1.2 Cytogenotoxic Assays

Cytogenotoxicity describes a deleterious action on a cell's genetic material affecting its integrity. Genotoxic substances are known to be potentially mutagenic or carcinogenic, specifically those capable of causing genetic mutation and of contributing to the development of tumors. This includes both certain chemical compounds and certain types of radiation. Genotoxins affecting sperm and eggs can pass genetic changes down to descendants who have never been exposed to the genotoxin. So far various approaches have been made to measure the cytogenotoxicity of various potential carcinogens. Among the biological approaches, the Ames assay for mutagenicity using his(-) mutants of *Salmonella typhimurium* and the sister chromatid exchange assay (SCE) for cytogenotoxicity using mammalian cells are probably the most popular, although they are not satisfactory in terms of sensitivity (Shin'ichi Nito et al., 2000). In recent times Micronucleus test, Chromosome Aberration test and Comet Assay have been proved to be extremely useful in cytogenotoxicity measurement. These tests are proved to be very sensitive and at the same time accurate in terms of measuring cytogenotoxicity. So in this study these newer techniques have been applied to measure the cytogenotoxic effects, induced by Chromium.

1.3 Micronucleus Test

The Micronucleus test is one of the most promising methodologies at the present time, which detects chromosomal damage on once-divided binucleatetd cells. A micronucleus (MN) is formed during the metaphase/anaphase transition of mitosis (cell division). It may arise from a whole lagging chromosome (aneugenic event leading to chromosome loss) or an acentric chromosome fragment detaching from a chromosome after breakage (clastogenic event) which do not integrate in the daughter nuclei (Fenech M et al., 2003). These micronuclei are the erratic nuclei that arise from chromosomal fragments or chromosomes that are not passed to the daughter nuclei during cell division. The test is based on the formation of "micronuclei" from particles of chromatin material which, due to chromosome breakage or spindle dysfunction, do not migrate to the poles during anaphase and are not incorporated into the telophase nuclei of the dividing cell. Such chromatin fragments, or even whole chromosomes in the case of chromosome lag, result in the formation of one or more small satellite nuclei in the cytoplasm of the daughter cells (Robert C. Miller, 1973).

1.3.1. Development

During the 1930s and 1940s, a number of pioneering cytogeneticists described the occurrence of micronuclei following the X-irradiation of diverse materials, including grasshopper neuroblasts (Carlson, 1938), onion root-tip meristems (Sax, 1941) and *Tradescantia* pollen grains (Koller, 1943), and rightly attributed their formation to the presence of chromatin bridges and fragments at anaphase and the resultant exclusion of these fragments from the daughter nuclei at telophase. In 1954 micronuclei were first used to quantify chromosomal damage by H.J. Evans et al., in root tips of the Broad Bean, *Vicia faba*. The first in vitro study was carried out by Heddle and colleagues (Countryman and Heddle, 1976) aimed at analyzing micronuclei in cultures of human peripheral blood lymphocytes exposed to mutagens in vitro. One of the early problems with the analysis micronuclei in human peripheral blood lymphocytes was the fragility of the cells during preparation and slide making, which often resulted in the displacement of micronuclei from the

cells in which they originated. This problem was readily overcome by the development of modified hypotonic and preparative procedures which preserved the cytoplasm and retained the micronuclei within the parent cell membrane (Iskander, 1979, Hogstedt, 1984). Another, and more important problem, was the variable response of the lymphocytes to mitogenic stimulation and the presence of cells in culture that had not undergone a division and therefore could not have given rise to any micronuclei. Such cells of course reduce the frequency of micronuclei. One way around this problem was to label cells with a specific marker to identify those cells that had proceeded through a DNA synthesis phase post mutagen treatment. This was achieved by the use of radioactive isotopically marked DNA precursors, detected by autoradiography, or through the use of non-isotopic or non-radioactive substances that were incorporated into DNA during replication and which resulted in altering staining properties (Pincu et al., 1984). The major breakthrough was based on a discovery by Carter in 1967. Carter had shown that when culterd mouse cells were exposed to low concentrations of cytochalasin B, the substance inhibited cytokinesis without blocking mitosis. Cells that had gone through a proliferative cycle in the presence of this substance therefore ended up by becoming binucleate. Michael Fenech and Alec Morley realized that the use of cytochalasin B offered a simple and effective method to mark cells that had proceeded through a mitotic cycle in studies on mutagen-induced chromosome damage (Fenech and Morley, 1985). Thus the cytochalasin block method, as it was described had arrived, which is now known as cytokinesis block micronucleus (MN) assay (CBMN).

1.3.2. Principles of detection

The micronucleus test affords a procedure for the detection of aberrations involving anaphase chromosome behavior utilizing a particularly useful cell type (Robert C. Miller, 1973). To analyze the induction of micronuclei it is essential that nuclear division has occurred in both treated and untreated cultures. The most informative stage for scoring micronuclei is in cells that have completed one mitosis during or after treatment with the test substance. Cell cultures of human or rodent origin are exposed to the test substances both with and without an exogenous source of metabolic activation unless cells with an adequate metabolizing capability are used. Concurrent solvent/vehicle and positive controls are included in all tests (Scott, D et al., 1991).

During or after exposure to the test substance, the cells are grown for a period sufficient to allow chromosome or spindle damage to lead to the formation of micronuclei in interphase cells. For induction of aneuploidy, the test substance should be present during mitosis. Harvested and stained interphase cells are analysed for the presence of micronuclei. Ideally, micronuclei should only be scored in those cells that have completed mitosis during exposure to the test substance or during the post-exposure period, if one is used (Morita et al., 1992). In cultures that have been treated with a cytokinesis blocker, this is achieved by scoring only binucleate cells.

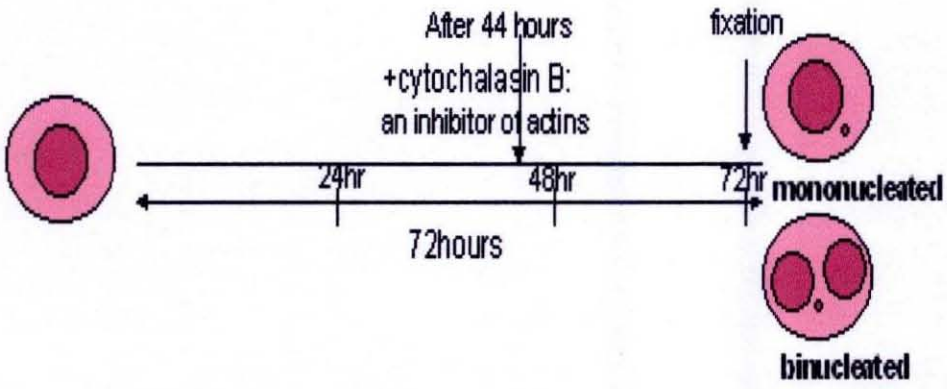

Figure 1.1: Formation of binucleated cells by cytokinesis blocker, cytochalasin B in Micronucleus test

In the absence of a cytokinesis blocker, it is important to demonstrate that the cells analysed are likely to have undergone cell division during or after exposure to the test substance. For all protocols, it is important to demonstrate that cell proliferation has occurred in both the control and treated cultures, and the extent of test substance-induced cytotoxicity or cytostasis should be assessed in the cultures scored for micronuclei (Fenech, M. and Morley, A.A., 1985).

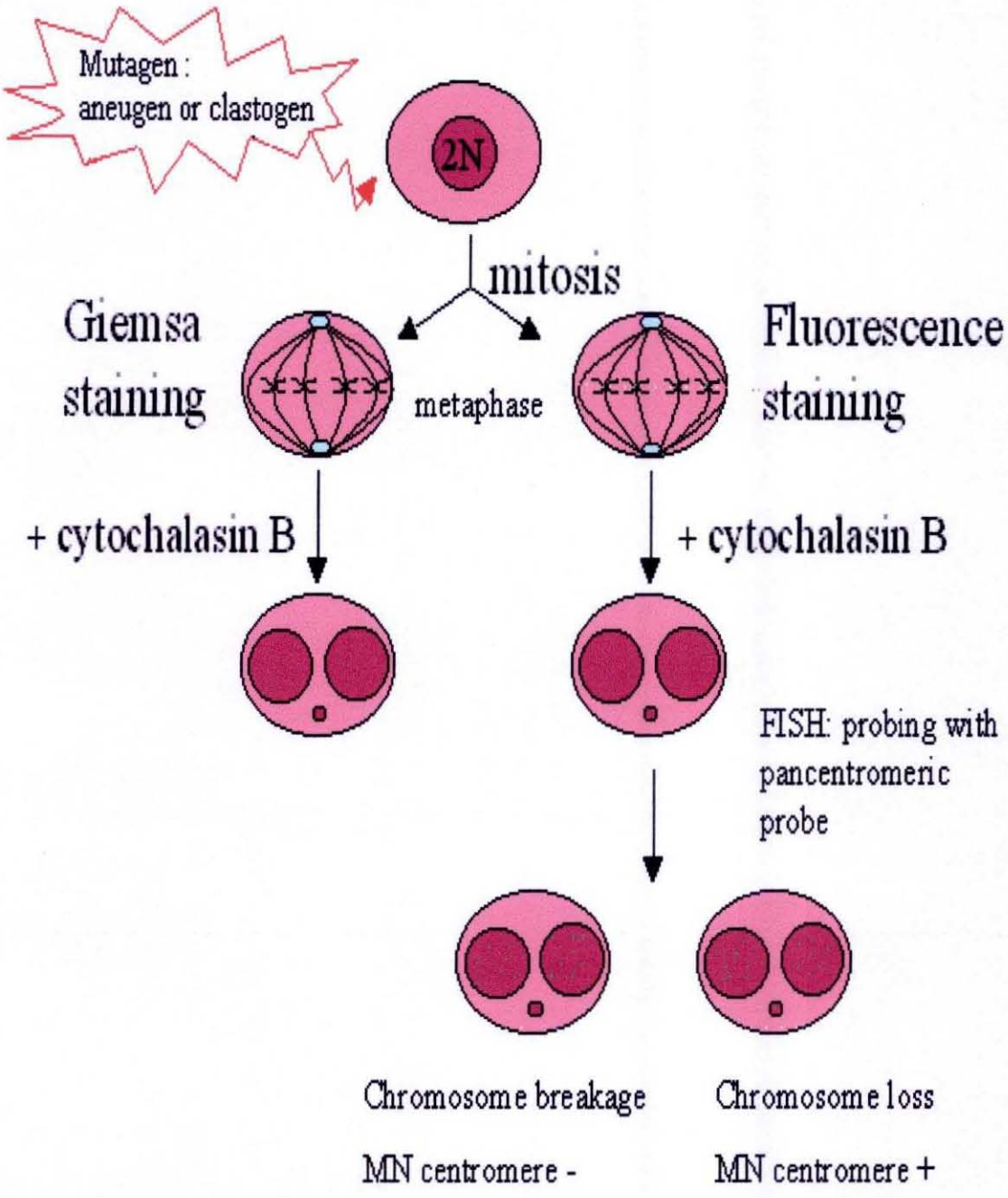

Figure 1.2: General schematic for Micronucleus test protocol

1.3.3. Applications

1.3.3.1. Genotoxicity measurement

The in vitro micronucleus assay is a genotoxicity test for the detection of micronuclei (MN) in the cytoplasm of interphase cells. The assay detects the activity of clastogenic and aneugenic chemicals in cells that have undergone cell division during or after exposure to the test substance. The addition of the cytoB prior to the targeted mitosis, allows for the identification and selective analysis of micronucleus frequency in cells that have completed one mitosis because such cells are binucleate (Fenech M. et al., 2003). The frequency of micronucleus formed, can be used as a parameter for measuring genotoxicity.

1.3.3.2. Identifying carcinogenic chemicals

The micronucleus assay is an *in vitro* method that uses cultured human or rodent cells. It provides a comprehensive basis for investigating chromosome damaging potential *in vitro* because both aneugens and clastogens can be detected. In addition to using the micronucleus assay to identify chemicals that induce MN, the use of a cytokinesis block, immunochemical labelling of kinetochores, or hybridisation with centromeric/telomeric probes (fluorescence *in situ* hybridisation; FISH), also can provide information on the mechanisms of chromosome damage and micronucleus formation (Fenech, M. and Morley, A.A., 1985).

1.3.3.3. Biomonitoring

Human biomonitoring of early genetic effects requires accurate, sensitive and, if possible easy and not too time-consuming methodologies to assess mutations. It is becoming increasingly evident that an increased rate of DNA damage and chromosome breakage or loss is an important risk factor for an elevated risk of cancer (Hagmar et al., 1994) and possibly other ageing degenerative diseases such as Alzheimer's disease (Migliore et al., 1999). One of the most promising methodologies at the present time for assessing DNA damage is the cytokinesis block

micronucleus (MN) assay (CBMN), which detects both chromosome and genome mutations in binucleated cells. The micronucleus assay in human biomonitoring is mainly applied to peripheral blood lymphocytes and to a lesser extent in epithelial cells (Micheline Kirsch-Volders et al., 2001).

1.3.4. Advantages/ Disadvantages

MN-assay	***Advantages***	**Disadvantages**
With/without cyto-B	▪ Cell/cell approach. ▪ Simultaneous detection of chromosome + genome mutations. ▪ Discrimination between clastogen/ aneugen. ▪ Possible co-detection of apoptosis/necrosis. ▪ Applicable on many cell types. ▪ Rapidity. ▪ Cheap. ▪ Simplicity. ▪ Potential for automation. ▪ Statistical power.	▪ Does not detect all structural chromosome aberrations (only acentric fragments). ▪ Requires cell division for expression of MN.
With cyto-B	▪ Discrimination between cells which underwent nuclear division and cells which did not. ▪ Enables detection of dicentric bridges as nucleoplasmic bridges. ▪ Assessment of cell proliferation (& percent; binucleated cells).	▪ Possible interference of cyto-B with test chemical; like spindle poisons. ▪ Possible interference with other inhibitors of cytokinesis. ▪ Cytoxicity of cytochalasin B itself varies between cell types and sometimes even between subtypes of the same cell type.

Table 1.2: Advantages and disadvantages of Micronucleus test

1.4. Chromosome Aberration Test

The chromosome aberration test is an extremely useful technique to detect various types of structural chromosome damage. The purpose of the *in vitro* chromosomal aberration test is to identify agents that cause structural chromosome aberrations in cultured mammalian cells. Structural aberrations may be of two types, chromosome type aberration or chromatid type aberration. With the majority of chemical mutagens, induced aberrations are of the chromatid type, but chromosome-type aberrations also occur (Evans, H.J., 1976). Structural chromosome aberrations may be induced via DNA breaks by various types of mutagens. Such DNA breaks may either rejoin such that the chromosome is restored to its original state, rejoin incorrectly or not rejoin at all. These last two cases may be observable on microscopic preparations of metaphase cells. However, many of these gross changes probably will not allow cell survival after division, but they serve as indicators for the induction of smaller, not readily observable changes, which do allow cell survival but may have deleterious consequences for the organism (Ishidate, M.Jr. et al, 1985).

1.4.1. Development

In vitro metaphase tests for chromosomal aberrations (CA) have undergone considerable evolutionary changes over the last 20 yr. Treatment and sampling times have been a particular focus of attention as an attempt to develop protocols that detect weak genotoxins. Different approaches evolved in different parts of the world and led to a need to harmonise (David Kirkland, 1998).

Earlier studies in human lymphocytes where cytogeneticists relied on the inherent metabolic activity of the cells to activate polycyclic hydrocarbons such as benz(a)pyrene and were able to demonstrate that CA were induced (D.J. Kirldand et al., 1981). One of the key elements missing from these earliest studies was, therefore, any form of exogenous metabolic activation.

By 1983, there was a recommendation for the use of S9 in the first OECD guidelines [OECD, Guidelines for Testing of Chemicals, Guideline 473, Paris, 1983]. For the first time, as well,

there were recommendations about the important issues of defining an upper exposure limit for testing (in terms of concentration and toxicity) and timing (how long to treat and when to sample). These, more than any other aspects of protocol design, have been on-going sources of debate for most of the last 20 years. Shortly after, in 1985, Galloway et al. described their ideal protocol based on a collaborative investigation of 22 compounds in 2 different laboratories. Treatments were continuous until harvest (at the first mitosis) in the absence of S9 and for 2 h in the presence of S9. Interestingly they discovered the lack of reproducibility from one day to another in the estimation of toxicity, and incorporated measures of toxicity concurrently in the main CA assay, a feature, which was only to be established as an international recommendation many years later (David Kirkland, 1998).

1.4.2. Principles of detection

The chromosome aberration test is most often performed on human peripheral blood lymphocytes in presence of test substance. As peripheral lymphocytes are in the resting G_0 stage of the cell cycle, they have to be stimulated to divide by an aspecific antigen, like phytohaemagglutinin. After 46.5 hours just before fixation (at 48 hours) a spindle inhibitor like colcemid or colchichine is added to block the cells in the (pro) metaphase of the first mitosis (Figure 1.3).

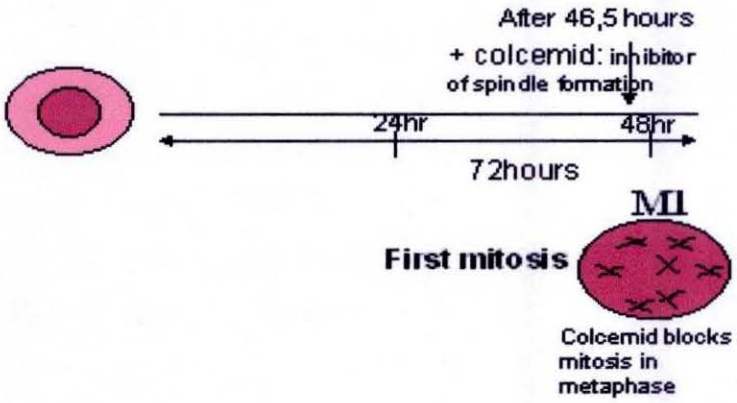

Figure 1.3: Blocking of mitosis in metaphase stage by colchichine or colcemid treatment.

After they are being treated with a metaphase-arresting substance (e.g. Colcemid or colchicine), the cells are harvested, stained and metaphase cells are analysed microscopically for the presence of chromosome aberrations.

Induced chromosomal aberrations can be divided into two main classes: chromosome-type aberrations, involving both chromatids of a chromosome, and chromatid-type aberrations involving only one of the two chromatids. Chromosome-type aberrations (symmetric aberrations), like dicentrics, inversions, ring chromosomes, are produced in the G0 or G1 stage of the cell cycle (i.e. prior to replication), while chromatid type aberrations (asymmetric aberrations), like breaks and gaps, are produced during the S or G2 stage (i.e. during or after replication). Most chemical mutagens are S-dependent clastogens and therefore produce chromatid-type aberrations. Several types of Structural aberrations can be distinguished:

1. Gaps

2. Breaks

3. Dicentric chromosomes

4. Ring chromosomes

1.4.3. Applications

1.4.3.1. Screening of mutagens and carcinogens

This test is used to screen for possible mammalian mutagens and carcinogens. Many compounds that are positive in this test are mammalian carcinogens; however, there is not a perfect correlation between this test and carcinogenicity. Correlation is dependent on chemical class and there is increasing evidence that there are carcinogens that are not detected by this test because they appear to act through mechanisms other than direct DNA damage.

1.4.3.2. Biomarker of effect

This test is highly relevant for cancer risk assessment. The number and types of aberrations provide valuable data on the possible health risks associated with radiation exposure such as cancer induction and for the application of particle beams in cancer therapy (Evans H.J. 1976).

1.4.3.3. Genotoxicity measurement

Treatment of cells with DNA-damaging agents can result in unrepairable lesions in both strands of DNA. This leads to chromosome breakage, a phenomena that can be visually detected with the help of a microscope in metaphase cells. There are five basic premises that guide genetic toxicity testing for the identification of presumptive carcinogens: (1) the Salmonella mutation test (SAL) is a necessary component of testing schemes for the identification of carcinogens; (2) an *in vitro* chromosome aberration, or chromosome damage, test is needed in addition to a gene mutation test; (3) a cultured mammalian cell mutagenicity test is needed to "confirm" or "complement" the Salmonella test; (4) an *in vivo* test is needed to confirm a positive *in vitro* test; and (5) results from test batteries have higher predictive value and provide a greater assurance than results from the individual component tests of the battery (Errol Zeiger, 1997).

1.4.4. Advantages/ Disadvantages

Advantages	Disadvantages
▪ Cell/ cell approach. ▪ Accurate identification of all the different chromosome mutation types. ▪ Possible co-detection of mitotic indices. ▪ No full automatic but interactive scoring possible.	▪ Requires in vitro cell cultivation ▪ Labor intensive ▪ Requires highly qualified skills and experience

Table 1.3: Advantages and disadvantages of Chromosome Aberration Test

1.5. Comet Assay

Single-cell gel electrophoresis (SCGE) or the comet assay can be used for detection of DNA damage and repair at the single-cell level and provides a unique opportunity to investigate intercellular differences in any eukaryotic cell population (Collins et al., 1995; Rojas–et al., 1999). The test has widespread applications in genotoxicity testing, DNA damage and repair studies, environmental biomonitoring, and human population monitoring. The comet assay has been used to detect DNA damage in a variety of animal and human cell types. One of these cell types is lymphocyte in whole blood samples. Many epidemiological studies have been published on Chromium (K.Danadevi, Roya Rozati, 2004). And there are many have been published relating the effects of chromium on DNA damage by using lymphocytes by the comet assay.

1.5.1. Development

The comet assay, also called the single-cell gel electrophoresis (SCGE) was first introduced by Ostling and Johanson in 1984 as a microelectrophoretic technique for the direct visualization of DNA damage in individual cells (Stling et al., 1984). A small number of irradiated cells suspended in a thin agarose gel on a microscope slide were lysed, electrophoresed, and stained with a fluorescent DNA binding dye. The electric current pulled the charged DNA from the nucleus such that relaxed and broken DNA fragments migrated further. The resulting images, which were subsequently named for their appearance as 'comets', were measured to determine the extent of DNA damage.

The original neutral method of Ostling and Johanson appeared to be sensitive to the effect of single-strand breaks on DNA supercoiling. The lysis conditions used by these authors were likely to be ineffective in removing all proteins, so that the major influence of radiation appeared to be to the release of a 'halo' of DNA by loss of DNA supercoiling, creating a sensitive assay for the presence of single-strand breaks. More stringent lysis conditions resulted in loss of more than 95% of the cellular proteins which allowed broken duplex molecules to migrate. This adaptation provides the basis for the neutral method which detects only DNA double-strand breaks. Two laboratories independently modified the method about five years ago by applying denaturing conditions to measure DNA single-strand breaks (Fig. 1.5). The first group (Stling O. et al., 1984) concentrated on maximizing sensitivity for measurement of low numbers of strand breaks, while the second laboratory directed initial efforts towards detection of subpopulations varying in drug or radiation sensitivity (Olive et al., 1989).

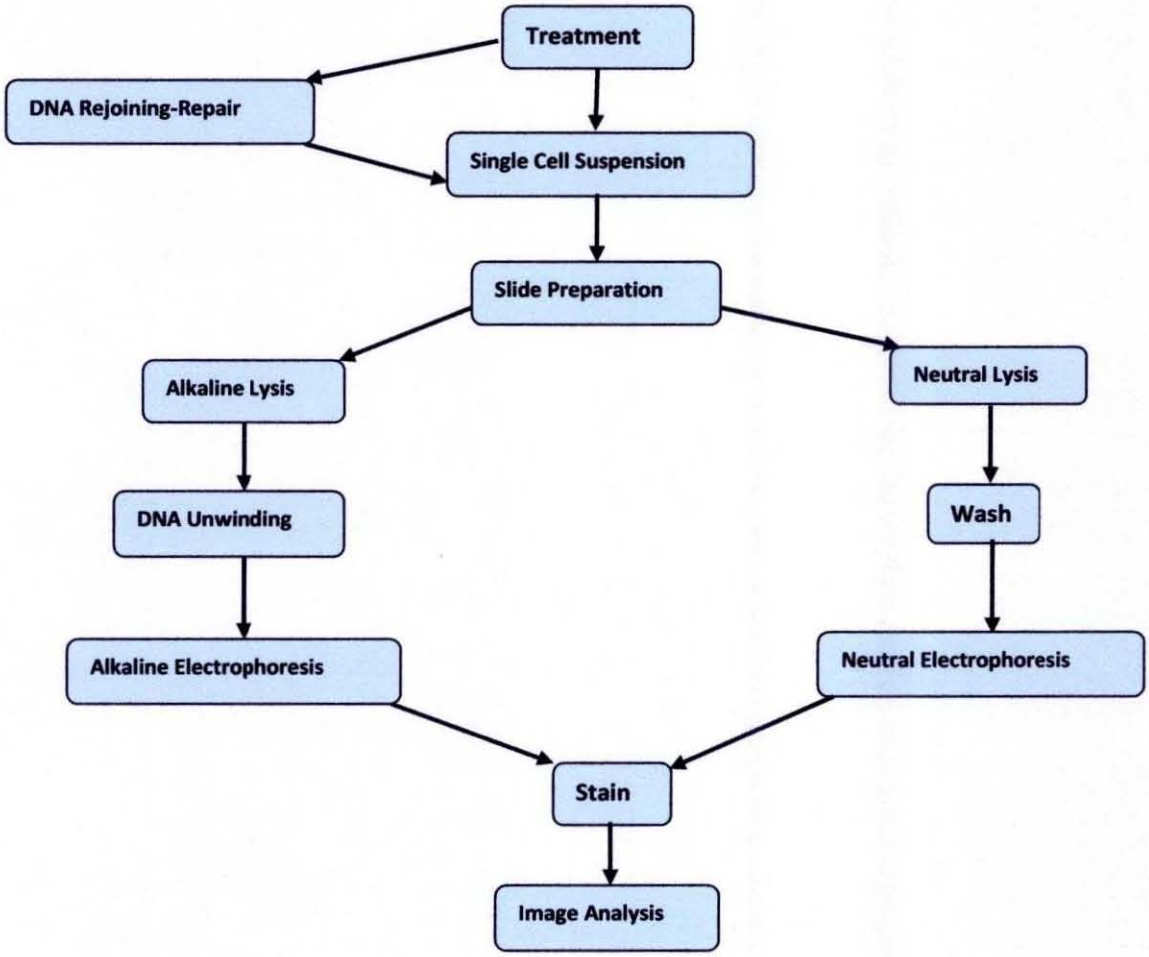

Figure 1.4: General schematic of the comet assay protocol. A general protocol for both the alkaline and neutral assays is presented.

There has been increasing interest in the comet assay in the past two to three years. Because the unique design of the comet assay provides direct determination of the extent of DNA damage in individual cells, it is possible to determine whether all cells within a population demonstrate the same degree of damage. This technique may prove to be valuable in the dissections of the mechanisms of genotoxicity and DNA repair.

1.5.2. Principles of detection

Procedures for measuring DNA strand breaks are generally based upon the principle that strand breaking agents reduce the size of the large duplex DNA molecule. In addition, DNA single and double-strand breaks can have dramatic effects on higher-order chromatin structure because of its supercoiling and tight packaging within the nucleus. Assays that measure DNA single strand breaks generally require unwinding of the double stranded DNA molecule for sensitive detection. A high pH (> 12.3) is generally utilized to facilitate denaturation, unwinding, and expression of single strand breaks as well as DNA breaks that only become apparent after exposure to alkali (so-called alkali labile lesions). Gel electrophoresis performed under denaturing conditions using constant or pulsed fields embraces principles of DNA migration under electrophoretic conditions in a conventional sense (Olive et al., 1990).

The detection of DNA single-strand breaks in individual cells was first described by Rydberg and Johanson (Rydberg et al., 1978). Cells were embedded in agarose on microscope slides and lysed under mildly alkaline conditions in order to allow a partial unwinding of the DNA. The slides were neutralized and stained for analysis using acridine orange. A ratio of green to red fluorescence was determined, representing the ratio of double- to single-stranded DNA respectively. A flow cytometry method was also introduced in which cells, embedded in agarose, were allowed to unwind in alkali before staining and analysis of fluorescence using a flow cytometer (Rydberg et al., 1984). In order to increase the sensitivity for damage detection, Ostling and Johanson subsequently modified these procedures, and included an electrophoresis step for a short period. This allowed for further discrimination of DNA damage (Stling et al., 1984).

Comets form as broken ends of the negatively charged DNA molecule become free to migrate in the electric field towards the anode. Two main principles are believed to determine the pattern of comet formation. The ability of DNA to migrate is a function of both the size of the DNA and the number of broken 'ends' which may be attached to larger pieces of DNA but which can still migrate a short distance from the comet head. Tail length initially increases with damage but

reaches a maximum that is largely defined by the electrophoresis conditions, not the size of the fragments.

With increasing numbers of breaks, DNA pieces migrate freely into the tail of the comet, and at the extreme (the apoptotic cell); the head and tail are well separated. These two concepts, stretching and migration of separated strands are generally accepted to explain the DNA migration patterns observed in the comet assay.

1.5.3. Different types of comet assay

In order to achieve various objectives, various modifications of the comet assay have been developed. A brief description of the various modifications of comet assay procedures is summarized and given in table 1.4.

1.5.3.1. Neutral comet assay

This type of comet assay was first introduced by Ostling and Johanson (Welch et al., 2000) who used what is termed a 'neutral' pH of 9.5 for lysis and electrophoresis. This pH is below the limit for DNA unwinding, and was reported by Singh et al (Berg et al.,2001) to detect only double strand breaks (DSB), with more strongly alkaline conditions (pH 10 or above) needed for unwinding and detection of single strand breaks (SSB). The comet tails are less pronounced at neutral, and this lessens the sensitivity, or DNA damage score gradient, of the assay (DPHE. 2000; Waychunas et al., 1993). This can be an advantage when a less sensitive method is needed (Waychunas et al., 1993).

1.5.3.2. Alkaline comet assay

Singh and co-workers (1988) presented the alkaline version of the comet assay, in which DNA is allowed to unwind at pH >13. The strongly alkaline conditions make for clearer images, and a steeper gradient of response. Besides SSBs, other types of DNA damage, such as alkaline labile sites (ALS), can be detected at strongly alkaline conditions (UNICEF. 2000). SSBs are formed from alkali-labile sites at pH >13, revealing otherwise hidden damage. Employing milder alkaline (pH = 12.3) conditions prevents conversion of alkaline labile sites into breaks (Mueller et al., 2001). Therefore, by modifying the pH of the lysis/unwinding and/or electrophoresis steps over the range 9.5-13.5, comet assay models of different sensitivity (but of similar limits of detection) can be applied (Nikolaidis et al., 2004; Waychunas et al., 1993).

Version	Description/Characteristics	Advantages/Application
Neutral comet assay	Lysis and electrophoresis performed at pH 9.5; less DNA unwinding and less pronounced comet tails; detects single strand and double strand reaks/similar limit of detection but less sensitive than alkaline version	Useful in situations where less sensitivity is needed, e.g., when background damage or induced damage is high
Alkaline comet assay	Lysis/unwinding/electrophoresis performed at more strongly alkaline conditions; at pH >13, alkaline labile sites are converted to single strand breaks; altering pH over the range 9.5-13 alters sensitivity	Clearer comet images are obtained; greater response to damage seen compared to neutral version; commonly used 'standard' comet assay model; usually adopted when investigating possible protection against damage inducers, e.g. H_2O_2

Enzyme assisted comet assay	Specific enzymes used immediately after lysis to transform susceptible sites to single strand breaks; enzymes used include Endo III (reveals oxidized pyrimidines), FPG (reveals oxidized purines), uvrABC (reveals UV damage); assay has increased sensitivity, and enhanced specificity for particular types of DNA lesions	Can detect specific types of damage; has enhanced sensitivity; is useful for looking at differences in basal damage after supplementation
Fluorescent in situ hybridization comet assay (FISH comet assay)	Uses gene 'tagging' with fluorescent markers and so can be used to visualize a specific gene in the three-dimensional chromosomal structure	Enables domain-specific DNA repair to be investigated; a specific gene in the three-dimensional chromosomal structure can be located; different rates of repair of damage in specific genes relative to the genome overall can be measured
Lysed cell or partially lysed (nucleus intact) comet assay	Lysis of cell and nuclear membranes or cell membrane only (leaving nucleus intact) prior to exposure to test agent, thus exposing 'naked' DNA or nucleus to the agent	In conjunction with the whole cell, 'standard' version, this version can be applied in assessment of direct versus indirect mechanisms of action of genoprotective agents

Table 1.4: Overview of different approaches used in the comet assay and their application

1.5.3.3 Enzyme-linked comet assay

The use of enzymes can produce a comet assay model of greater sensitivity and, in addition, a more specific assay can be developed. In addition to SSBs, DSBs and apurinic sites, other types of damage, such as oxidized bases or UV-induced dimers, which do not cause strand breaks, exist. These types of DNA damage cannot be detected unless lesion-specific enzymes are added (at the post-lysis stage) to create breaks at the sites of damage. Enzymes that have been used to date include Endonuclease III, which detects oxidized pyrimidines, formamidopyrimidine glycosylase (FPG), which detects oxidized purines (Andersen JK et al., 2004) uvrABC, an enzyme complex that can be used to detect UV damage on bulky lesions (Andersen JK et al., 2004).

1.5.3.4. Fluorescence in situ hybridization–comet assay (FISH-comet)

Fluorescence in situ hybridization (FISH) can be used to identify chromosomes, or to detect a particular gene, or damage to this, in the comet image. The combination of the comet assay and FISH combination also provides the opportunity to investigate domain-specific DNA repair, and can be used to locate a specific gene in the three-dimensional chromosomal structure (Oliver et al., 1987).

1.5.3.5. Lysed cell comet assay

In the original version of the comet assay, treatment of cells with the potential genoprotective agent of interest comes before the lysis step. Several groups have modified the comet assay procedure by lysing embedded cells before treatment with testing agents (Levine et al., 1983, 1990; Pratico et al., 2000). This allows direct contact of 'naked' nuclear DNA with the agent under study, and removes the possibility of cellular response or adaptation. Another modification is lysing the embedded cells by briefly immersing the gel in 1% Triton X-100 detergent. This dissolves the cell membrane but not the nuclear membrane (Reznick et al., 1994).

1.5.4. Applications

Application of the comet assay allows these possibilities to observe complete rejoining of strand breaks during the repair period and moreover, it is possible to analyze the response of cells in different phases of the cell cycle, or the responses of cells with different ploidy (e.g., tetraploid tumor cells versus diploid normal cells).

1.5.4.1. Genotoxicity measurement

Mutagenic and carcinogenic effects resulting from DNA damage are the long term sequelae of genotoxicity. Genotoxic agents can be defined functionally as having the ability to alter DNA replication and genetic transmission. The major endpoints of short-term genotoxicity assays include DNA damage, point mutations, and chromosomal aberrations (Combes et al., 1992). The exquisite sensitivity of the comet assay, and the ability it provides to measure DNA damage in individual cells, has destined it to become a tool in rapidly predicting the genotoxicity of compounds of interest. The first practical use of the comet assay in genotoxicity testing was an evaluation of the mutagenic potencies of agents generated by the treatment of potassium permanganate with acidic solutions (M. Laget et al., 1995). DNA damage determined by the comet assay was used as an endpoint to predict the presence of genotoxic metabolites in specific organs.

1.5.4.2. Detection of apoptosis

In contrast with necrotic cells, apoptotic cells maintain cell membrane integrity following the nuclear fragmentation event, whereas necrotic ceils demonstrate signs of plasma membrane permeability and integrity loss prior to late DNA degradation. Fragmentation of nuclear DNA into nucleosomal sizes makes this event an obvious endpoint for evaluation using the comet assay. Because apoptotic DNA fragmentation is characterized by the generation of double stranded breaks, both the neutral and alkaline assays can be used with equal efficiency for breaks detection. DNA is extensively degraded allowing most of the comet head to migrate when subjected to electrophoresis.

Under standard alkaline assay conditions, apoptotic cellular DNA migrates several times the length of the original undamaged comet head. The most obvious aspect of apoptotic comets is the movement of most of the DNA from the head into the tail of the comet, which cannot be accounted for by tail length alone (Olive et al., 1993).

1.5.4.3. Biomonitoring

Many human biomonitoring studies have been carried out by using comet assay to investigate the genotoxicity of different chemical compounds as well as environmental pollutants and carcinogens. This technique shows some advantages for its use in human monitoring studies, such as, it is a rapid, simple and sensitive methodology, it requires a small number of cells and can be applied to proliferating and non-proliferating cells and allows the evaluation of DNA damage and repair at the level of single cell (Rojas et al,1999). Many investigations have been performed employing lymphocytes and leukocytes, exfoliated bladder cells, nasal and buccal epithelial cells, sperm cells and exfoliated tear duct epithelial cells for human monitoring purpose (Rojas et al., 1999). Comet assay is suitable to conduct studies with a large population group with accuracy. There are about 75 works in human biomonitoring purpose by applying the comet assay, principally in 3 different areas a): the evaluation of dietary protective factors, b): the assessment of clinical exposures and c): the appraisement of occupational, environmental and lifestyle exposures.

1.5.4.4 Detecting excisable DNA damage

Agents such as UV radiation that produce DNA lesions which do not form strand breaks directly can be examined using the single cell gel assay. Rather than detecting strand breaks produced by the irradiation, it is possible to detect strand breaks produced by the cell as it attempts to repair the lesion. Damaged cells allowed no repair time, demonstrate no detectable tail formation, much like negative control cells in other exposure systems. With repair time, comets appear and disappear in accordance with the formation of strand breaks and ligation corresponding to incision and rejoining (Gedik et al., 1992).

1.5.4.5. Detection of DNA crosslinks

Crosslinking induced by nitrogen mustard and c/s-platinum has been demonstrated using the comet assay (Olive et al, 1990b; 1992b). The functional principles upon which the experiment was based were that increasing numbers of crosslinks prevented the migration of DNA in a standard X-ray-induced strand break assay using the alkaline assay. Formaldehyde at very low concentrations can induce DNA single strand breaks measurable using the alkaline single cell gel assay. At higher concentrations, DNA migration in the system is reduced as interstrand crosslinks contribute to the increased complexity of the migrating molecules. In addition to DNA interstrand crosslinks, some drugs (e.g., etoposide) also create protein-linked strand breaks. Such breaks are present after alkali lysis, but may only be revealed after addition of proteinase K when the assay is conducted under neutral conditions (Olive et al., 1993a).

1.6. Rationale of the Present Study

The study of DNA damage at the chromosome level is an essential part of genetic toxicology because chromosomal mutation is an important event in carcinogenesis. Chromosome mutations and related events are the cause of many human genetic diseases and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumour suppressor genes of somatic cells are involved in cancer induction in humans and experimental animals. Trivalent chromium [Cr (III)] is recognized as an essential nutrient, and is widely used as a nutritional supplement for humans and animals. Recent reports of the induction of genetic damage in cultured cells exposed to Cr (III) compounds in vitro have heightened the concern that Cr (III) compounds may exert genotoxic effects under certain conditions, raising the question of the relative benefit versus risk of dietary and feed supplementation practices (David A. Eastmond , James T. MacGregor and Ronald S. Slesinski, 2008). Hexavalent chromium [(Cr (VI)] is used in a wide range of industries. Cr-VI from chromate industries and atmospheric emissions contribute to the Cr contamination in the environment. Cr is a reproductive metal toxicant that can traverse the placental barrier and cause a wide range of fetal effects including ovotoxicity (Sakhila K. Banu, Jawahar B. Samuel, Joe A. Arosh, Robert C. Burghardt and Michael M. Aruldhas, 2008). So far no sensitive genotoxic assays have been developed for the detection of chromium induced chromosomal instability.

The present study focuses on the health risk of tannery workers, whose are chronically exposed to chromium. The measurement of chromium induced DNA damage by in vitro micronucleus test, chromosome aberration test and comet assay is of much importance because DNA damage can lead to cancer and other diseases. It is well known that animal models are not a good predictor of human carcinogenesis for chromium, suggesting that the mechanisms for metabolism and toxicity of chromium in humans differ from those in animals. In this study all the tests were performed on cultured peripheral blood exposed to chromium compounds for a long period of time. Data from the present study will help us to gain further insight into the genotoxic effects of chromium on public health and take proper measures to be protected from this danger.

1.6.1. Aims and Objective of the Study

The human body is constantly under attack by endogenous and exogenous agents, which may cause DNA damage. The potential role of chromium as carcinogen should be of great concern. Therefore, it is important to have a method of monitoring individuals who may be at risk of DNA damage by chromium and the ensuing process of carcinogenesis. In this study the in vitro micronucleus test, chromosome aberration test and comet assay were applied to examine the effect of chromium on chromosomal instability in human peripheral blood. The in vitro micronucleus assays have emerged as one of the preferred methods for assessing chromosome damage because they enable both chromosome loss and chromosome breakage to be measured reliably. The chromosome aberration test also has been proved to be an effective method to detect chromosome damage. The purpose of the chromosome aberration test is to identify agents that cause structural chromosome aberrations in cultured mammalian cells. Structural aberrations may be of two types, chromosome or chromatid. The comet assay also provides this method of assessing DNA damage that includes single strand breaks, double strand breaks, sites of base loss [apurinic/ apyrimidinic (AP) sites] and base lesions (Collins et al., 1995). Whole blood is the source of different types of cells and provides the toxicant environment.

Therefore in the present study, a concerted effort has been made to investigate the genotoxic effect of chromium in cultured human blood by in vitro micronucleus test, in vitro chromosome aberration test and comet assay. We also sought to elucidate the possible co-relations between these different tests for measurement of genotoxicity.

1.6.2. Specific Objectives

(a) Examine the chromium induced genotoxicity in human by the micronucleus test to assess the micronuclei that are formed due to chromosomal damage.

(b) Investigate the effect of chromium on structural chromosomal damage by chromosome aberration test.

(c) Analyze the chromium induced DNA damage in lymphocytes by Comet assay.

(d) Validate that the Micronucleus test, chromosome aberration test and Comet assay are as accurate as established methods for determining chromium induced DNA damage.

(e) Correlate the chromium expose and the cancer risk of the tannery workers and general people.

Chapter 2

Materials and Methods

Chapter 2
Materials and Methods

This chapter is a summary of the materials and method used in this study. For further details in particular of analytical procedures the reader is referred to the individual papers.

2.1. Instruments

The following instruments were used during the present experimental work:

Instrument name	Company name
Carbon Dioxide Incubator	JENCONS-PLS (Germany).
Centrifuge machine	MSE micro centaur (SANYO, Japan).
Centrifuge machine	Eppendorf centrifuge 58101 (Germany)
Computer software (for comet analysis)	CASP, version 1.2.2.
Computer software (for statistical analysis)	GRAPHPAD PRISM 4 (USA).
EC 150 Power Supply	E-C Apparatus Corporation; MAXICELL-EC360M.
Electrophoresis Chamber	E-C Apparatus Corporation.
Flat bottomed Cell Culture Plate	Iwaki microplate; ASAHI Technoglass, Japan.
Haemocytometer (Neubauer improved)	Superior Marienfield, Germany
Laminar flow chamber	CRP Bayshore New York.
Light Microscope	Motic-BA200.
Magnetic stirrer	Stuart (Bibby Sterilin Ltd., UK).
Oven	Galanz microwave oven (China).
p^H meter	Hanna Instruments HI 8417 (France).
Vortex mixer	Digisystem Lab. Instrument Inc. (Taiwan).
Water bath	Clifton Shaking Bath NE5-28D (England).

2.2 Materials

The following chemicals of analytical grade were used during the present experimental work:

Chemicals	Company name
Agarose (LMPA, NMPA)	Sigma Chemical Co. (U.S.A.)
Colchicine	Sigma Chemical Co. (Germany)
Cytochalasin B	Sigma Chemical Co. (Germany)
Dimethyl Sulfoxide	LOBA Chemie (India)
EDTA	Sigma Chemical Co. (USA)
Fetal Bovine Serum	Sigma Chemical Co. (Germany)
Formaldehyde	Sigma Chemical Co. (U.S.A.)
Giemsa Stain	Sigma Chemical Co. (Japan)
Glacial Acetic Acid	MERCK (India)
Glycerol	Sigma Chemical Co. (U.S.A.)
KH_2PO_4	MERCK (India)
Methanol	Sigma Chemical Co. (Germany)
Mitomycin C (MMC)	Sigma Chemical Co. (Germany)
Na_2EDTA	Sigma Chemical Co. (U.S.A.)
$Na_2HPO_4.2H_2O$	MERCK (India)
NH_4NO_3	Sigma Chemical Co. (Germany)
Penicillin	Sigma Chemical Co. (Germany)
Potassium Chloride	MERCK (India)
SDS	LOBA-CHEMIE (India)
Silver nitrate	Sigma Chemical Co. (Japan)
Sodium Carbonate	MERCK (India)
Sodium Lauryl Sarcosinate	Sigma Chemical Co. (U.S.A.)
Streptomycin	Sigma Chemical Co. (Germany)
TCA	MERCK (India)
Trizma Base	Sigma Chemical Co. (Germany)
Tungstosilicic acid	BDH (England)
$ZnSO_4.7H_2O$	Sigma Chemical Co. (Germany)

2.3. Study Design

2.3.1. Subjects selection

Subject selection was done on basis of the following criteria:

(i) Subjects were aged within 21 to 50 years (Mean 34.07 ± 0.4522 yrs).

(ii) The subjects were from tannery industries and were exposed to chromium by different chemicals.

(iii) The control were healthy at the time of blood collection, without having any infectious disease.

(iv) The BMI ratios of the subjects were within normal range and they were used to have balanced diet.

2.3.2. Sample collection

Venous blood samples (4-5 ml) were collected from the study participants in EDTA containing sterile tubes, aluminium foil coated, and brought to the laboratory in ice packed containers and immediately processed for further analysis.

2.4. Methods of Assessments of Chromium induced Genotoxicity

2.4.1. Micronucleus test

The isolated human peripheral lymphocytes were then cultured in the following way for micronucleus test. The culture was incubated for 72 hours in CO_2 incubator.

2.4.1.1. Lymphocyte Culture for Micronucleus test

In our experiment, we followed the technique used by Michael Fenech, with some modification. At first in the cell culture plate, the followings were added:

- 4.125 ml RPMI 1640 medium
- 500 µl Fetal Bovine Serum (FBS) [10%]
- 50 µl Phytohemagglutinin A (PHA) [10 µg/ml]
- 20 µl L-glutamine (freshly prepared) [2 mM]
- 40 µl Streptomycin [100 µg/ml]
- 40 µl Penicillin [100 IU]

Finally 500 µl of whole blood was added to the culture plate and mixed well by gently pipetting. The culture plate was wrapped with aluminum foil and incubated at 37° C in dark condition for 24 hour in a humidified CO_2 (5%) incubator. After 24 hours of incubation, Mitomycin C (for final conc. of 0.1µg/ml and 0.2µg/ml) was added into two wells of culture plate as positive control and distilled water was added into one well of culture plate as negetive control and mixed well by gently pipetting. Then the culture was incubated for another 20 hours. After 20 hour (44 hour after the initiation of culture) Cytochalacin B (for final conc. of 6.0µg/ml) was added to the culture and mixed well by gently pipetting and then the culture was incubated for another 28 hour.

2.4.1.2. Cell Harvest and staining for Micronucleus test:

After 28 hour (72 hour after the initiation of culture) of incubation, the culture media was transferred into a 15.0 ml Falcon tube and centrifuged at 200 X g for 10 min at room temperature. The supernatant was discarded and the pellet was dissolved in 10.0 ml of 0.075 M KCl solution. The tubes were incubated at 37° C for 20 min. Then the tubes were centrifuged at

200 X g for 10 min at room temperature. The supernatant was discarded and the pellet was resuspend in 10.0 ml freshly prepared fixative solution (Methanol: Acetic acid – 3: 1). The centrifugation and fixation steps were repeated for another 3 times. At the last step, the pellet was dissolved in 0.5 ml of supernatant. Two drops of 20 µl cell suspension was pipetted on a clean slide and smeared with another slide and then the slides were left in laminar flow to dry for 1.0 – 2.0 hour. Then the slides were stained with 5% Giemsa solution for 10.0 – 15.0 min at room temperature. After staining, slides were dried in laminar hood for 1.0-2.0 hour and observed under light microscope at 100 X magnifications.

2.4.2. Chromosome Aberration Test

The isolated human peripheral lymphocytes were cultured in the following way for Chromosome aberration test. The culture was incubated for **48** hours in CO_2 incubator

2.4.2.1. Lymphocyte Culture for Chromosome Aberration test

In our experiment, we followed the technique used by Hossein Mozdarani and Hamid Gourabi, with some modification. At first in the cell culture plate, the followings were added:

- 4.125 ml RPMI 1640 medium
- 500 µl Fetal Bovine Serum (FBS) [10%]
- 50 µl Phytohemagglutinin A (PHA) [10 µg/ml]
- 20 µl L-glutamine (freshly prepared) [2 mM]
- 40 µl Streptomycin [100 µg/ml]
- 40 µl Penicillin [100 IU]

Finally 500 µl of whole blood was added to the culture plate and mixed well by gently pipetting. The culture plate was wrapped with aluminum foil and incubated at 37° C in dark condition for 24 hour in a humidified CO_2 (5%) incubator. After 24 hours of incubation, Mitomycin C (for final conc. of 0.1µg/ml and 0.2µg/ml) was added into two wells of culture plate as positive control and distilled water was added into one well of culture plate as negetive control and mixed well by gently pipetting. Then the culture was incubated for another 22 hours. After 22 hours of incubation (46 hour after the initiation of culture), Colchicine (for final conc. of 0.2 µg/ml) was

added to the culture and mixed well by gently pipetting and then the culture was incubated for another 2 hour.

2.4.2.2. Cell Harvest and staining for Chromosome Aberration test:

After 2 hour (48 hour after the initiation of culture) of incubation, the culture media was transferred into a 15.0 ml Falcon tube and centrifuged at 200 X g for 10 min at room temperature. The supernatant was discarded and the pellet was dissolved in 10.0 ml of 0.075 M KCl solution. The tubes were incubated at 37° C for 20 min. Then the tubes were centrifuged at 200 X g for 10 min at room temperature. The supernatant was discarded and the pellet was resuspended in 10.0 ml freshly prepared fixative solution (Methanol: Acetic acid – 3: 1). The centrifugation and fixation steps were repeated for another 3 times. At the last step, the pellet was dissolved in 0.5 ml of supernatant. Two drops of 20 µl cell suspension was pipetted on a clean slide and smeared with another slide and then the slides were left in laminar flow to dry for 1.0 – 2.0 hour. Then the slides were stained with 5 % Giemsa solution for 10.0 – 15.0 min at room temperature. After staining, slides were dried in laminar hood for 1.0-2.0 hour and observed under light microscope at 100 X magnifications.

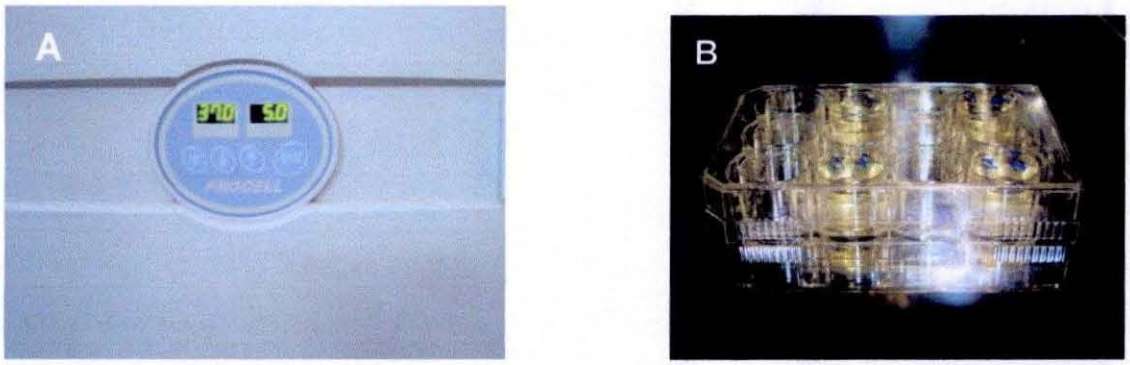

Figure 2.1: **(A)** The Carbon (5%) Dioxide incubator at 37° C. **(B)** A culture plate inside the Carbon Dioxide incubator at dark condition.

2.4.3. Comet Assay

The basis of the comet assay involves embedding a suspension of single cells in low melting point (LMP) agarose on a microscope slide, thus creating a mini DNA agarose gel. The cell membrane and nuclear membrane are lysed and the DNA is electrophoresed. After staining, the cells are observed under microscope and compared with control. Since DNA is negatively charged, nucleoids with damage display migration of DNA towards anode during electrophoresis, forming a 'comet' like image. The 'comet' like structure consists with a distinct head, comprising of intact DNA and a tail, consisting of damaged or broken pieces of DNA.

2.4.3.1. Cell culture preparation for Comet Assay

The whole blood were cultured in the tissue culture plate with 4.125 ml RPMI 1640 medium, 500 µl Fetal Bovine Serum (FBS) [10%], 50 µl Phytohemagglutinin A (PHA) [10 µg/ml], 20 µl L-glutamine (freshly prepared) [2 mM], 40 µl Streptomycin [100 µg/ml], 40 µl Penicillin [100 IU]. Finally 500 µl of whole blood was added to the culture plate and mixed well by gentle pipetting. The culture plate was wrapped with aluminum foil and incubated at 37° C in dark condition for 24 hour in a humidified CO_2 (5%) incubator. After 24 hours of incubation, Mitomycin C (for final conc. of 0.1µg/ml and 0.2µg/ml) was added into two wells of culture plate as positive control and distilled water was added into one well of culture plate as negetive control and mixed well by gently pipetting. Then the culture was incubated for another 24 hours. After 24 hours (48 hours after the initiation of culture) of incubation, the culture media was transferred into a 15.0 ml Falcon tube and centrifuged at 200 X g for 10 min at room temperature. The supernatant was discarded and the pellet was dissolved in 1.5 ml of Phosphate Buffer Saline (PBS) solution. This cell culture was immediately used for the comet assay.

2.4.3.2. Slide Preparation

In our experiment, we followed the technique used by Alok Dhawan, with some modification. At first, the slides were diped in methanol over night and burnt them over a blue flame to remove the machine oil and dust. Two third of the slides were dip in 1% normal melting point agarose (NMPA) for 40 minutes while continuous heating was applied to retain the agarose in liquid state. After 40 minutes, the slides were gently taken out from the agarose containing beaker,

wiped underside of the slides to remove excess agarose and allowed to dry in room temperature. The slides can be stored at room temperature until needed (not more than 3 days) avoiding high humidity conditions. Generally we prepared slides the day before use. At least two slides were prepared for each experimental sample.

2.4.3.3. Sample Loading

0.7% low melting point agarose (LMPA) was melted and kept in 37 °C waterbath for at least 20 minutes to adjust temperature. 10 µl of lymphocyte was taken in an eppendorf tube containing 100 µl of 0.7% LMPA and mixed well. 110 µl of this suspension was added on one end of the agarose precoated slide and gently covered it with another coverslide (25.4 x 76.2 mm; 0.75 mm thick) to make a microgel (avoiding bubble formation). Then the gel was allowed to solidify in an icebox for 10 minutes. After solidification, the coverslide was removed gently by sliding. Then the third agarose layer (110 µl of 0.7% NMPA) was added on the slide and covered it by the same coverslide similarly. The gel was again allowed to solidify in icebox for 10 minutes. After solidification, the coverslide was removed gently by sliding and the slides were subjected to lysis by lysis solution.

2.4.3.4. Alkaline Lysis

The slides were placed at 4°C for 75 minutes in lysis solution consisting of high salts and detergents. Lysis solution consisted of 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma base, 200 mM NaOH, 1.0% N-lauroylsarcosine. 10% DMSO and 1% Triton X-100 were added to lysis solution before use. The working lysis solution was kept at 4°C for 20 minutes before use.

2.4.3.5. Unwinding, Electrophoresis and Neutralization

After lysis, the slides were placed side by side (avoiding space between them) in a horizontal electrophoretic chamber containing freshly prepared cold alkaline electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH; pH 13.0) for 20 minutes at 4°C to produce single-stranded DNA and to express alkali-labile sites as single-strand breaks. Then the power supply pack was switched on and electrophoresis was carried out at 24 V and 300 mA for 40 minutes at 4°C. All of the above steps-from sample preparation to electrophoresis were carried out under dim light to

avoid additional DNA damage. After electrophoresis, the slides were gently transferred into a box containing cold neutralization buffer (0.4 M Trizma base; pH 7.5) and washed three times with deionized water (each of 5 minutes).

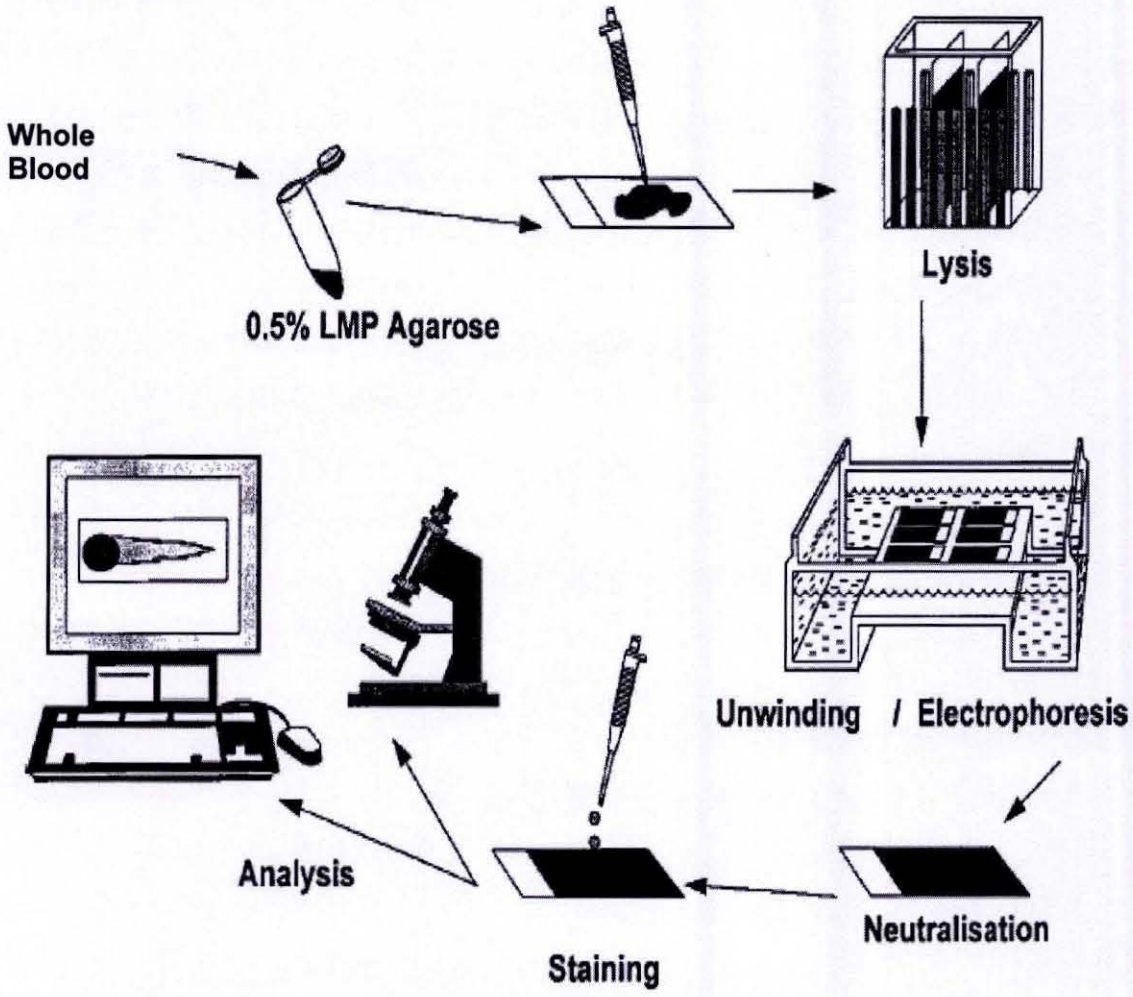

Figure 2.2: Schematic diagram for the performance of the comet assay

2.4.3.6. Silver Staining

After neutralization, modified version of silver staining was used as described by Cerda et al. (1997) and Nadin et al. (2001). Following washing for 5 minute slides were dried (usually 1-1.5 hr) and then fixed for 10 min in fixative solution A (15% TCA, 5% $ZnSO_4.7H_2O$ and 5% Glycerol), washed three times with deionized water and dried overnight at room temperature. Slides were re-hydrated for 5 min in deionized water and submerged in a horizontal staining jar containing freshly prepared staining solution D [132 ml of Staining solution B (5% Na_2CO_3) and 150 ml of staining solution C (0.1% NH_4NO_3, 0.1% $AgNO_3$, 0.25% tungstosilicic acid and 0.15% formaldehyde)]. Staining was carried out for 35 min in dark and gentle shaking condition. Slides were brought out from the staining jar and washed two times (20 min each) with deionized water on shaker (gentle shaking). Then slides were immersed in a stopping solution E (1% glacial acetic acid) for 5 min and air-dried.

2.4.3.7. Image Analysis

The slides were examined under conventional light microscope (Motic-BA200) (400X magnification) equipped with CCD camera (Nikon Cool Pix 99F). 100 cells for per slides were randomly examined. The comet images were captured by the camera, transferred into computer and analyzed. For comparing the extent of DNA damage in different samples, comet images were analyzed using a software–Computer Assay Software Project (CASP, version 1.2.2), developed by Konca et al. (2003). The CASP software was downloaded from http://www.casp.of.pl. Threshold values of CASP parameters were adjusted to obtain the optimal values for our staining protocol. The selected parameters were: Head center threshold (HCT) = 0.999, Comet thresholds (CT) = 0.005, Head threshold (HT) = 0.05, Tail threshold (TT) = 0.05 and profile 1.The following comet parameters were analyzed by CASP: Length of head and tail of comet and total comet length (in pixels), % of DNA in the comet's head and in the tail, the tail moment (arbitrary units) and olive tail moment (arbitrary units). The Olive tail moment (OTM) is [percent of DNA in the tail] x [distance between the center of gravity of DNA in the tail and that of center of gravity of DNA in the head in x-direction].

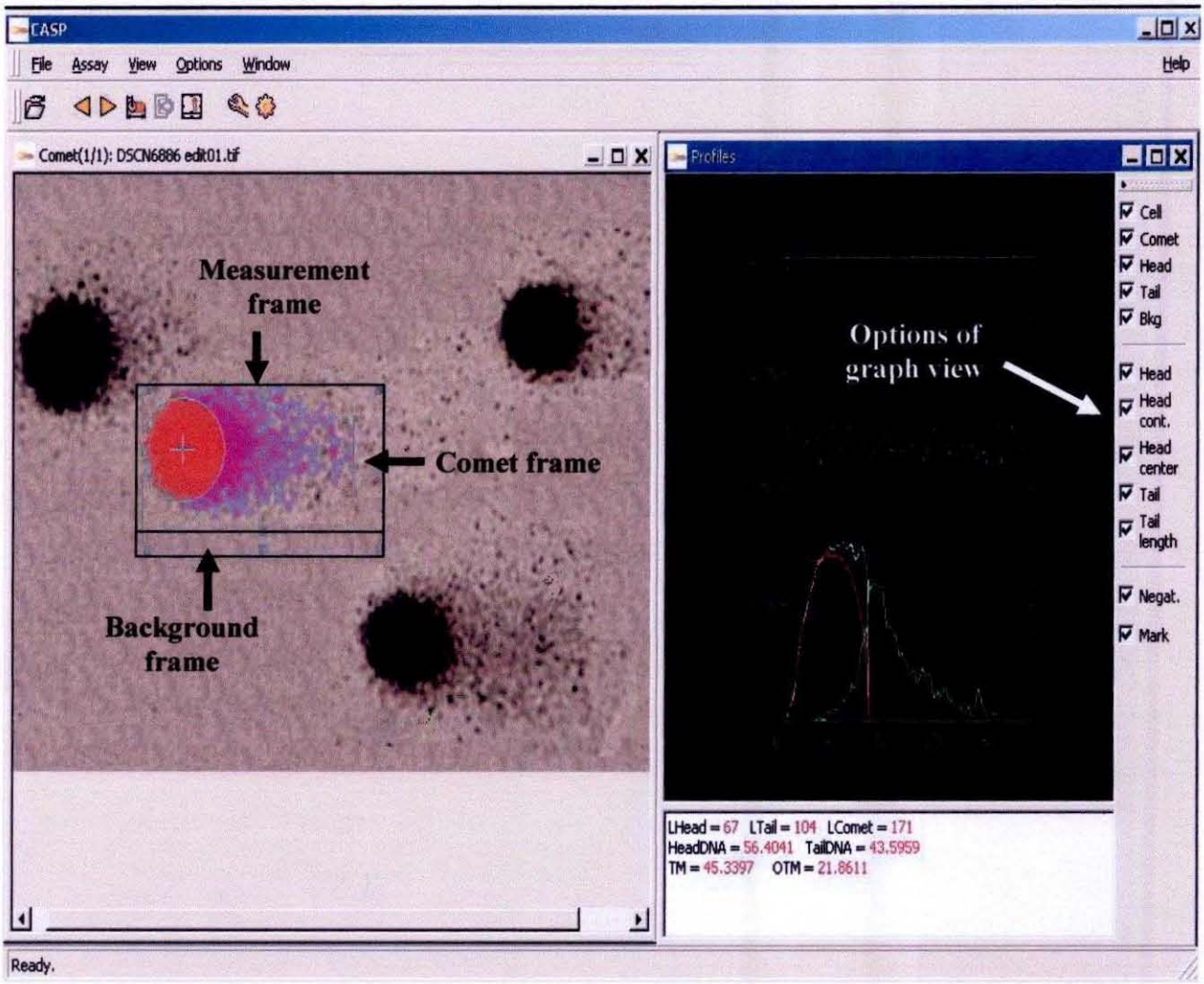

Figure 2.3: Comet image analysis by CASP 1.2.2 software showing comet head and tail.

2.5. Statistical analysis

The results are expressed as mean ± SEM (Standard error of mean). All parameters for inter-group differences were analyzed by one way ANOVA (analysis of variance) followed by Bonferroni test. The statistical program used was GRAPHPAD PRISM 5 (USA). The values of $P<0.05$ was considered statistically significant.

Chapter 3

Results

Table 3.1 shows the characteristics of the study subjects. The study subjects (25) are tannery workers and exposed to Chromium via different chemicals used in Leather industries and control subjects (15) are never exposed to chromium.

Characteristic	No.
Sex	
Male	**28**
Female	**12**
Age (years)	
21–30	**16**
31-50	**24**
Tobacco smoking	
Nonsmokers	**17**
Smokers	**23**
Eat meat or dairy	
Never	**0**
Occasionally[a]	**28**
Often[b]	**12**
Eat freshwater fish	
Never	**0**
Occasionally	**22**
Often	**18**
Eat green vegetables	
Never	**0**
Occasionally	**10**
Often	**30**
Skin hyperkeratosis	
Yes	**7**
No	**33**
Skin hyperpigmentation	
Yes	**0**
No	**40**

Table 3.1: Study subjects' characteristics

The total number of subjects is 40. [a]One to three times per month. [b]More than three times per month.

adult men and women. BMI categories are regularly regarded as a satisfactory tool for measuring whether sedentary individuals are underweight, Normal weight, overweight or obese.

$$BMI = \frac{\text{Weight (kg)}}{\text{Height}^2\ (m^2)}$$

BMI Categories:

- Underweight = <18.5
- Normal weight = 18.5-24.9
- Overweight = 25-29.9
- Obesity = BMI of 30 or greater

The average BMI of the 40 subjects were = 20.515±0.3925 (mean ± S.E.M), which indicates that they belonged to normal body weight range.

3.2.1. Chromium induced Micronuclei formation in damaged DNA

The Micronucleus test revealed that Chromium causes damage to peripheral blood lymphocyte nuclear DNA collected from 25 different individuals. Figure 3.1 is the representative picture of the Micronucleus test. As shown in Figure 3.1A that no micronucleus formed in negative control (distilled water treat instead of MMC). Micronuclei were formed due to Chromium exposure.

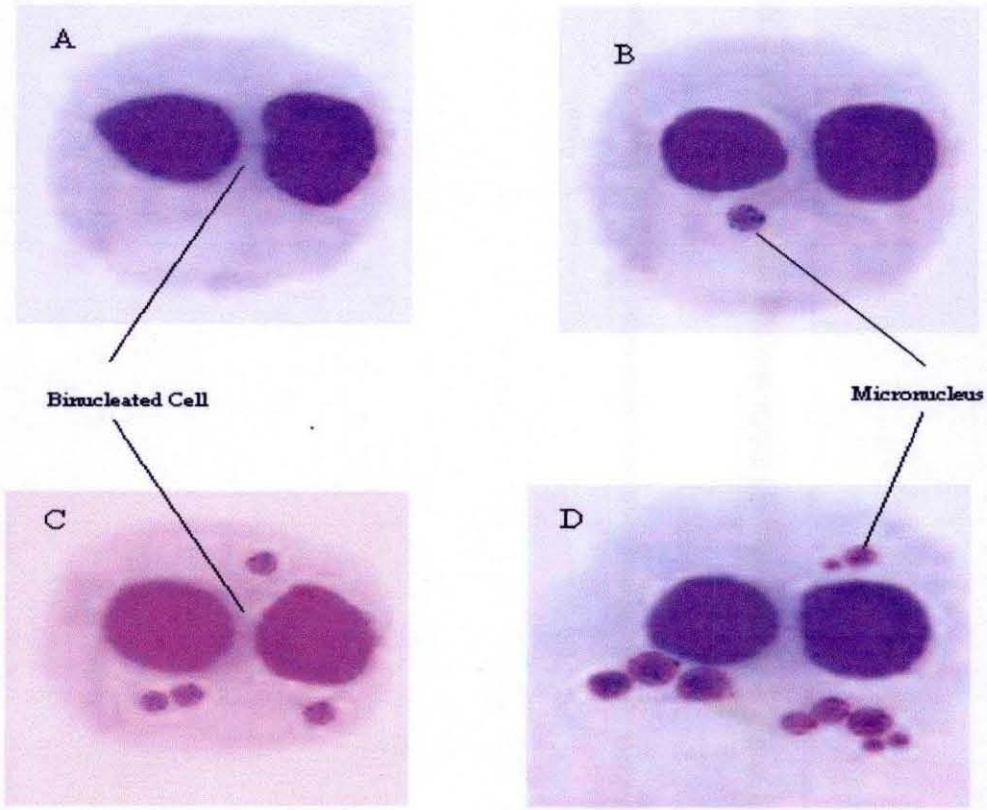

Figure 3.1: Micronucleus test depicting the genotoxic effect of Chromium to lymphocytes. 3.1A represent the negative control. 3.1B, 3.1C and 3.1D represent the Micronuclei that were formed due to expose of chromium or chromium compounds of different subjects for different period of time.

index (NDI), which was calculated from 500 cells according to the formula.

$$NDI = \frac{\{M1 + 2(M2) + 3(M3) + 4(M4)\}}{N}$$

Where, **M1-M4** represent the number of cells with one to four nuclei and **N** is the total number of viable cells scored.

Group	Number of BN[a] cells scored	MN[b] Distribution					Total MN	*MN %*	NDI[c]
		0	1	2	3	4			
Negative control (n=15)	500	494	5	1	0	0	07	1.4	1.014
Chromium exposed (n=10) Age (21-30)	500	448	36	11	4	1	74	14.8	1.148
Chromium exposed (n=15) Age (31-50)	500	416	57	18	6	3	123	24.6	1.246
Positive control, MMC (0.2µg/ml) exposed (n=15)	500	379	82	23	10	6	182	36.4	1.364

Table 3.2: Frequencies and distribution of MN in lymphocytes

The total number of subjects is n=40; [a]Binucleated cells; [b]Micronuclei; [c]Nuclear Division Index

formation frequency compared to the non exposed group (negative control group). It is apparent from the data presented in Figure 3.2 that MNi formation were significantly ($p < 0.0001$) increased in lymphocytes that were exposed for long period of time (Age > 30y). Due to long time (>20y) exposure the MNi formation increase 26 fold in the Age gr2 compared to negative control group. The lymphocytes exposed to chromium (Age Gr1) $_{and}$ MMC (positive control) also showed a significantly increased value of MNi.

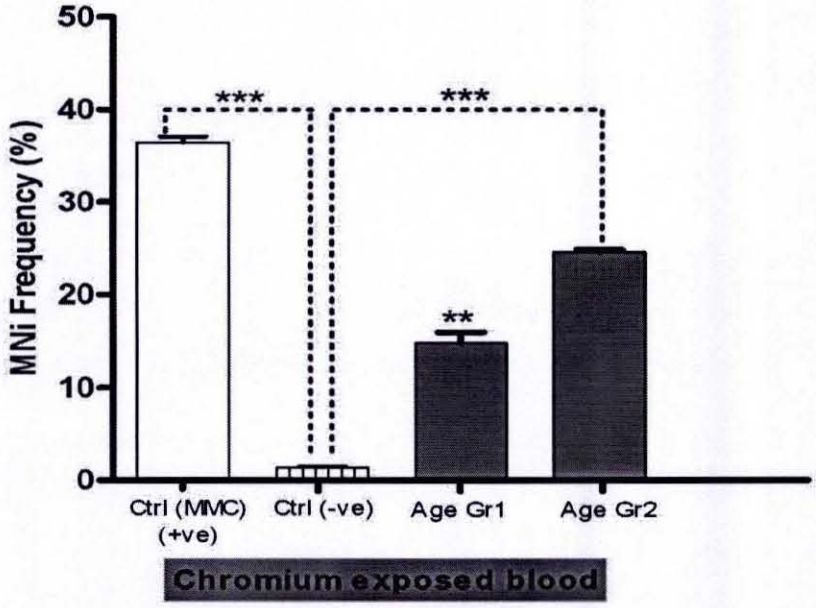

Figure 3.2: MNi Frequency (%) measurement in control and sample (Chromium exposed) lymphocytes. Here, the MNi frequency was determined in 500 cells per group.

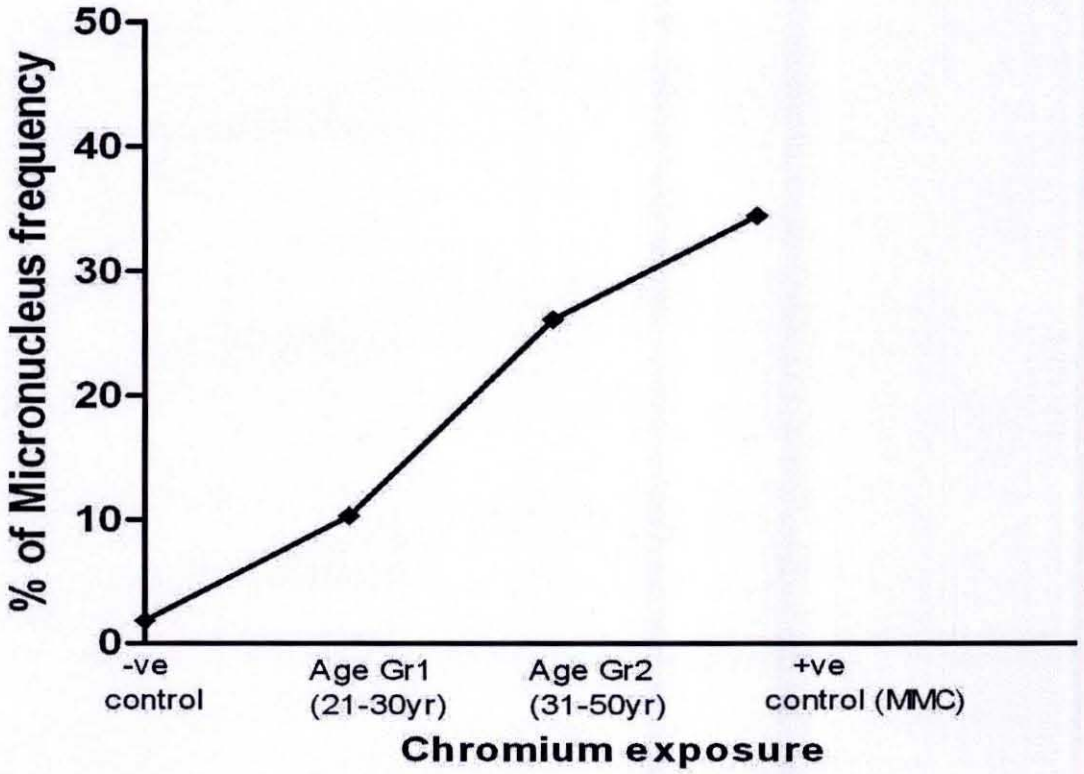

Figure 3.3: Increase in MNi frequency (%) with increased time period of chromium exposure.

3.3. Evaluation of DNA damage by Chromosome Aberration test

3.3.1. Chromium induced Chromosomal Aberration in Lymphocytes

The Chromium induced DNA damage was measure by Chromosome Aberration test of 40 different blood lymphocytes among them 25 are tannery workers(exposed group) and 15 are healthy control (non exposed group) . Figure 3.4 shows images of Chromosome Aberration test. Figure 3.4A indicates that no damage was occurred in negative control. In contrast, various types of chromosome aberration found due to different period of exposure as evident from.

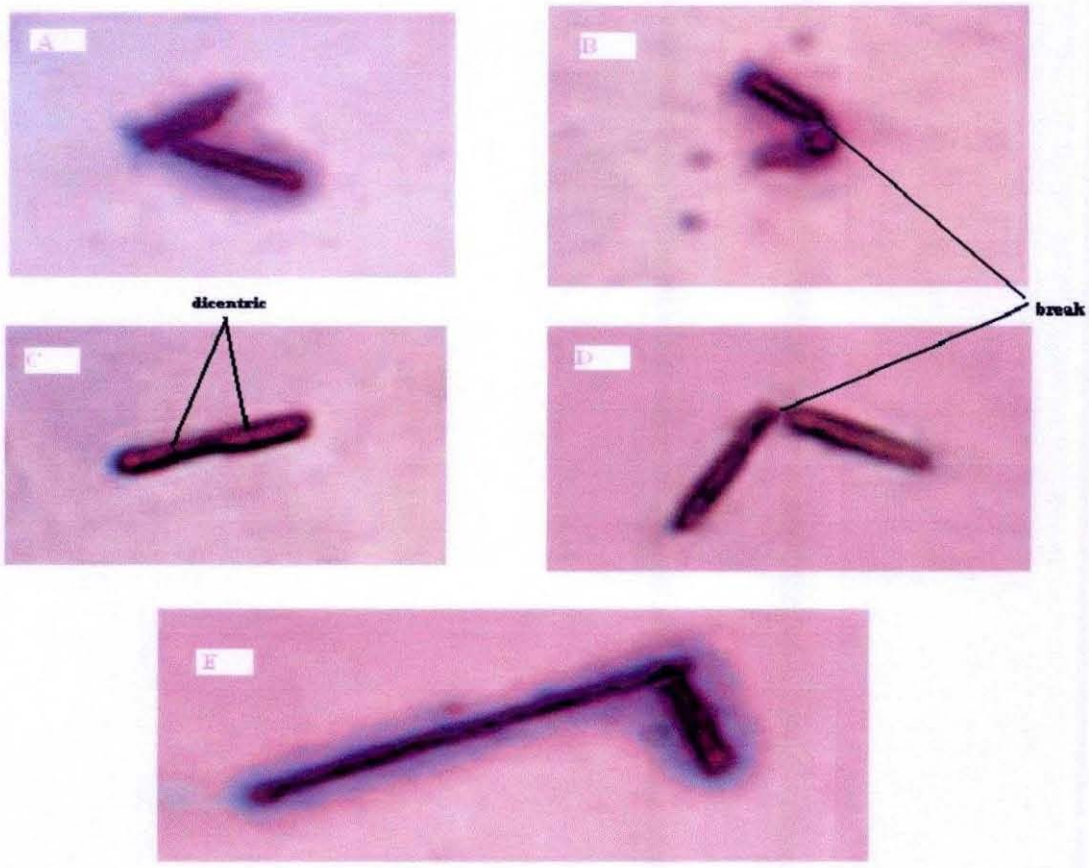

Figure 3.4: Chromosome Aberration test depicting the genotoxic effect of chromium to lymphocytes. 3.4A represent the negative control. 3.4B, 3.4C and 3.4D represent the various types of aberrated chromosomes that were formed due to different period of chromium exposure.

Group	No. of cells analyzed	Chromosome Aberration Types				Total Number of Aberrations	% Of cells with Chromosome Aberration
		Break	Gap	Centric Fusion	Ring		
Negative control (n=15) Age (21-30)	500	2	1	1	0	4	0.8
Chromium exposed (n=10) Age (21-30)	500	13	22	15	3	53	10.6
Chromium exposed (n=15) Age (31-50)	500	18	33	21	6	78	15.6
Positive control, MMC (0.2μg/ml) exposed (n=15)	500	25	46	29	11	111	22.2

Table 3.3: Frequency and distribution of Chromosome Aberrations in lymphocytes

The total number of subjects is n = 40

Chromium. It is quite obvious from the data presented in Figure 3.5 that chromosome aberration were significantly ($p < 0.0001$) increased in lymphocytes that were exposed for long period of time (Age > 30y). Due to long time (>20y) exposure the MNi formation increase 20 fold in the Age Gr2 compared to negative control group. The lymphocytes exposed to chromium (Age Gr1) and MMC (positive control) also showed a significantly increased value of chromosome aberration.

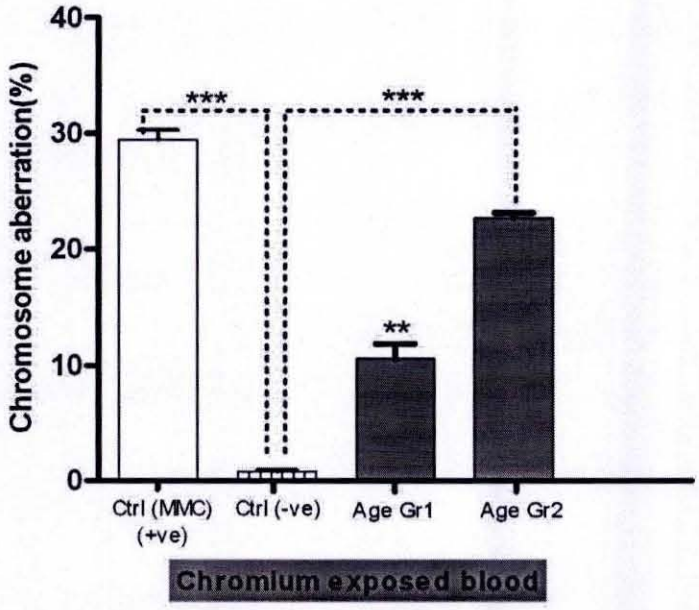

Figure 3.5: Chromosome aberration (%) measurement in control and Chromium exposed sample.

Here, the chromosome aberration (%) was determined in 500 cells per group.

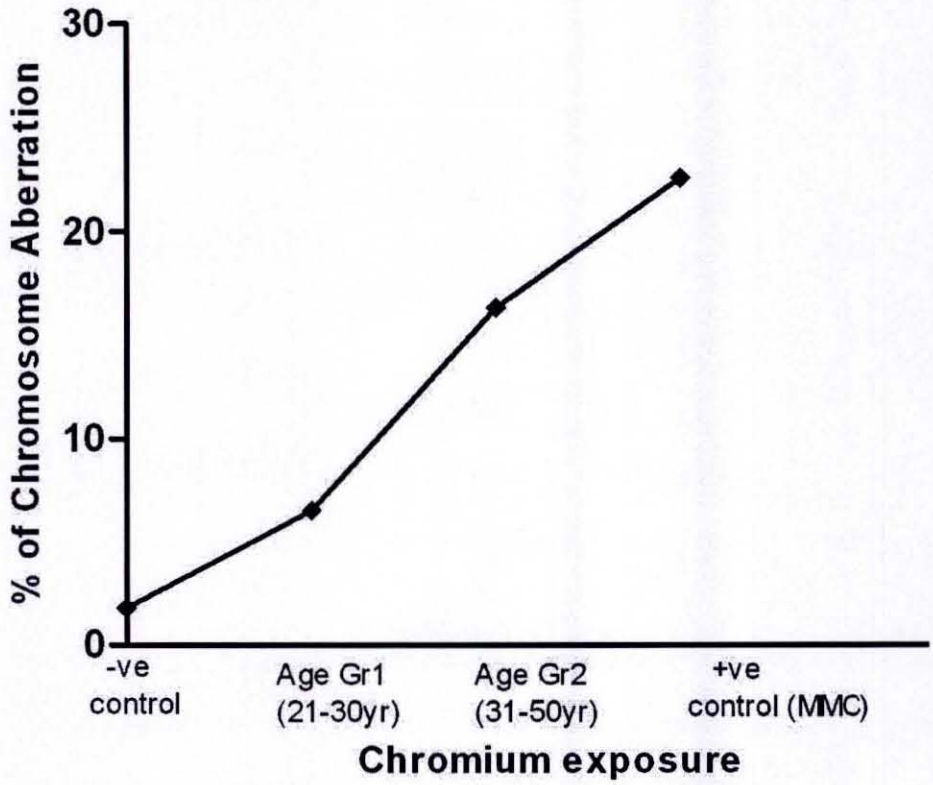

Figure 3.6: Increase in chromosome aberration (%) with increased time period of chromium exposure.

3.4.1. Chromium-induced genotoxicity

The alkaline comet assay or single cell gel electrophoresis revealed that Chromium causes severe damage to the cells' nuclear DNA. Figure 3.7 is the representative picture of comet assay for the negative control and Chromium exposed lymphocytes. As evident from the figure that the nuclear DNA of negative control cells is perfectly round (Fig. 3.7A). Almost all DNA were concentrated inside the head region of comet since no DNA damage was occurred. In contrast, the nuclear DNA of different age groups lymphocytes is fragmented. Figure 3.7 [B-D] shows the increase in comet tail length due to DNA damage induced by Chromium or chromium compounds.

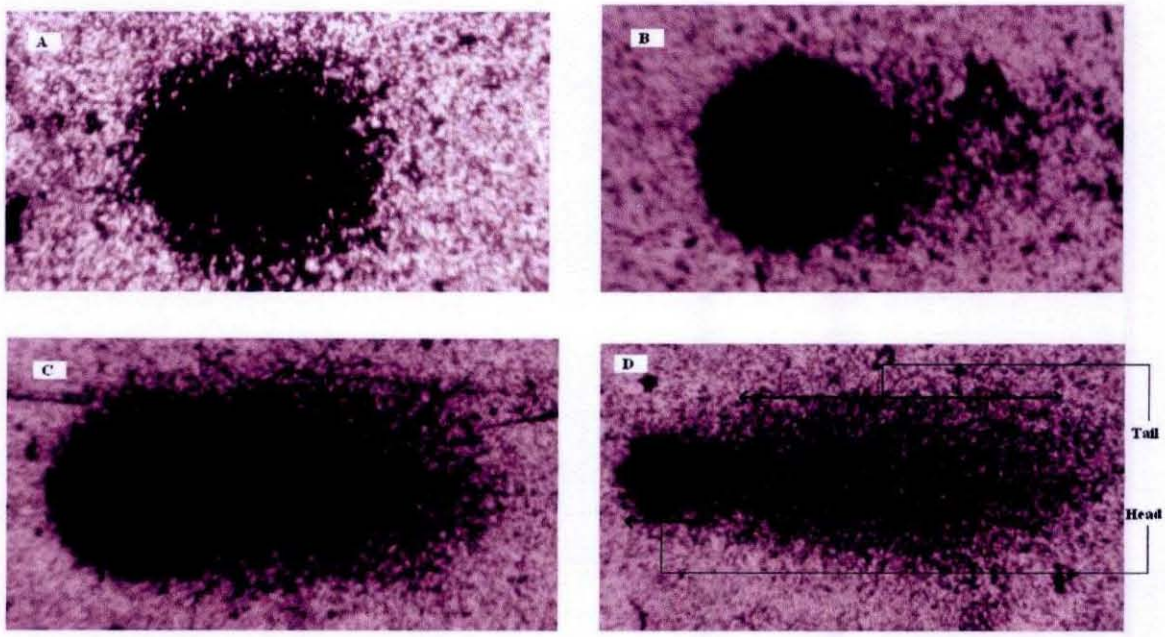

Figure 3.7: Representative comet images of lymphocytes, depicting the genotoxic effect of chromium. 3.7A represent the negative control. 3.7B, 3.7C and 3.7D represent comet images of damaged DNA that were formed due to chromium or chromium compounds exposure to the tannery workers for different periods of time.

study, we considered (a) the percent of DNA fragmentation (percent of DNA in the Comet tail and Comet head), (b) Comet tail moment and (c) Olive tail moment. The higher the percent of DNA fragmentation, the more severe is the damage. Similarly, the longer the comet tails, the higher the DNA fragment, the more severe is the damage.

3.4.2.1. Head DNA percentage (%)

As shown in Figure 3.8 most of the Chromium exposed lymphocytes cells were severely damaged and had lower DNA percentage in their head region. Since DNA in lymphocytes of negative control was intact, all most all DNA remained in the head region therefore the cells from the negative control group had higher DNA percentage in head region. From Figure 3.8 it is found that percentage of head DNA was significantly ($p < 0.001$) decreased in lymphocytes that were exposed for a long period of time (>30y) with chromium or chromium compounds.

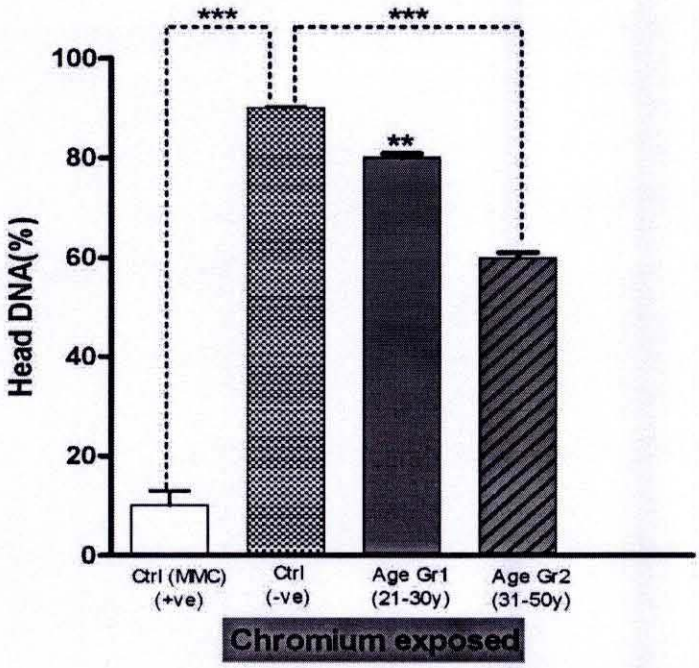

Figure 3.8: Head DNA (%) measurement in control and Chromium exposed lymphocytes. Here the head DNA (%) was determined in ~100 cells per group. Each value represents the mean ± SEM. Here, ***, $p < 0.001$ significantly different from its respective control.

and had higher DNA percentage in their tail region. The tail region of these cells contained more DNA compared to intact cell. From Figure 3.9 it is found that percentage of tail DNA was significantly (p<0.001) increased in lymphocytes that were exposed for a long period of time (>30y) with chromium or chromium compounds.

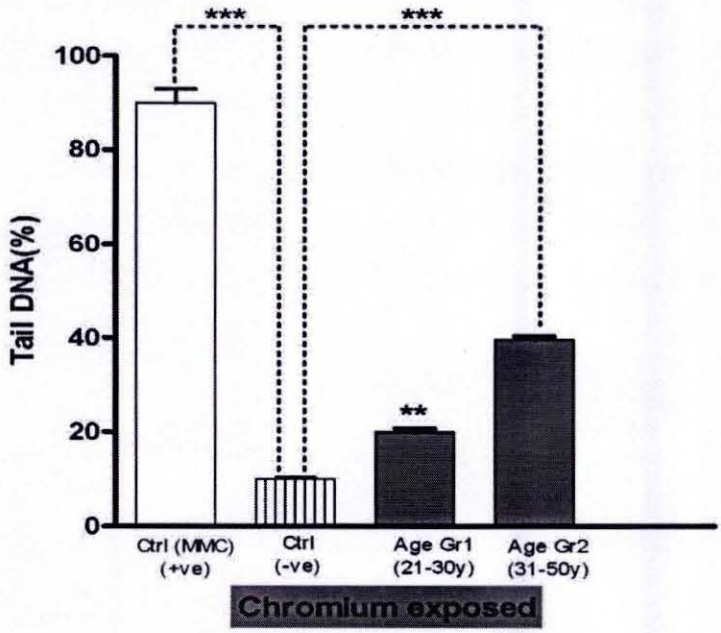

Figure 3.9: Tail DNA (%) measurement in control and chromium exposed lymphocytes. Here, the tail DNA (%) was determined in ~100 cells per group. Each value represents the mean ± SEM. Here, ***, $p < 0.001$ significantly different from its respective control.

figure 3.10, a significantly ($p<0.001$) increased value of tail moment was found in the Chromium exposed lymphocytes. Cr exposure (Age Gr2) caused 2.6-fold increase in comet tail moment value compared to negative control groups.

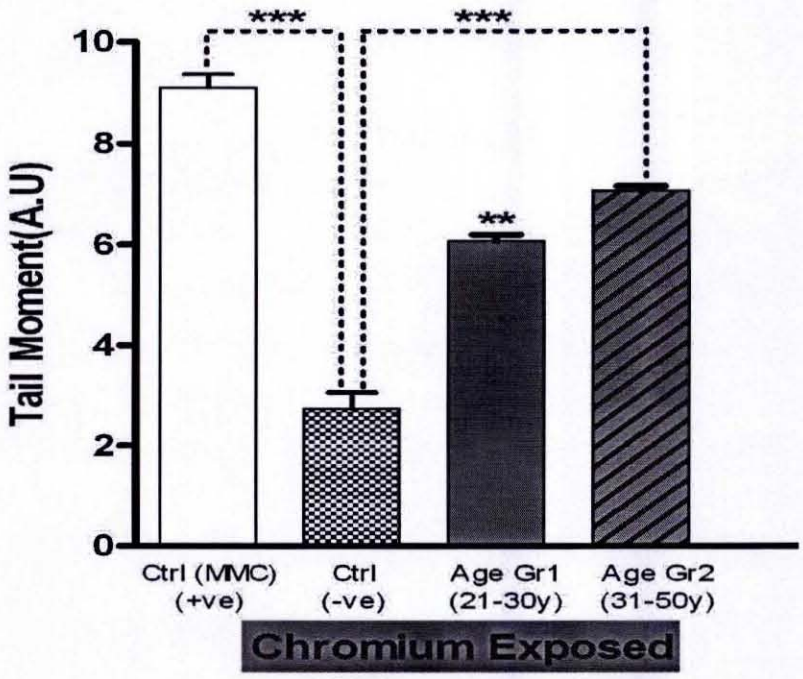

Figure 3.10: Tail moment measurement in control and Chromium exposed lymphocytes. Here the tail moment was determined in ~100 cells per group.

Each value represents the mean ± SEM. Here, ***, $p < 0.001$ significantly different from its respective control. The tail moment is the product of tail length and DNA content in the tail. Tail moment is expressed in arbitrary unit (A.U.).

lymphocytes. From figure 3.11 it is found that in Chromium exposed (Age Gr2), OTM value increased significantly ($p<0.001$) in DNA of lymphocytes compared to negative control group.

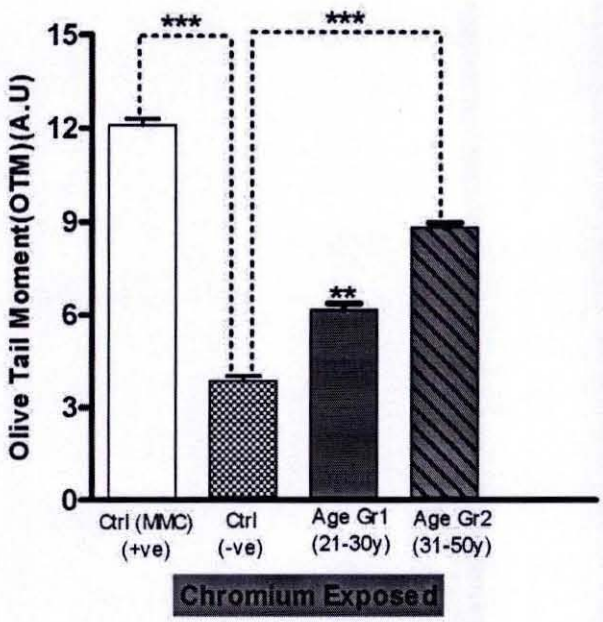

Figure 3.11: Olive Tail Moment measurement in control and Chromium exposed lymphocytes. Here the olive tail moment was determined in ~100 cells per group.

According to Olive et al. the Olive tail moment (OTM) is [percent of DNA in the tail] x [distance between the center of gravity of DNA in the tail and that of center of gravity of DNA in the head in x-direction]. Olive Tail moment is expressed in arbitrary unit (A.U.).

Each value represents the mean ± SEM. Here, ***, $p < 0.001$ significantly different from its respective control.

Chapter 4

Discussion

Leather, a traditional export item in Bangladesh, enjoys a good reputation worldwide for its quality. This sector plays a significant role in the economy of Bangladesh in terms of its contribution to export and domestic market. In south-western part of Dhaka city, there is a tannery area occupying 25 hectares of land at Hazaribagh, where about 90% of tannery industries of Bangladesh are located. The tanning industries of Hazaribagh process some 220t of hide per day with an associated release of 600-1000 kg of tanned skin-cut waste (SCW) per ton processed raw hide (Zahid, *et al.,* 2004). One of the major concerns of processing of hides is the heavy metals, especially, chromium used in the tanning processes. Large amounts of chrome powder and chrome liqueur are used during tanning process (UNIDO, 2000).

There are many environmental hazards associated with the chemicals used in the tanning processes. The hazards can come out in several ways. One is through the waters of canals and rivers after mixing with effluents. The entrance of harmful chemicals into the food chain through the use of SCW as feed staff, which is only recently been studied by Hossain *et al.* (2007), professional exposure of different chromium compounds for long period of time increases cancer risk (A. Hilali *et al*, 2007).

The aim of the present study was to develop some sensitive assays to measure the genotoxicity of chromium. In the present study the cytogenotoxic effect of Chromium was evaluated in vitro. The study was carried out in cultured human peripheral blood, because a high level of chromosomal aberration in peripheral blood lymphocytes may be an early biomarker of cancer risk (Paolo Boffetta et al., 2006). Three different cytogenotoxic assays were used in the present study to assess the cytogenotoxic effects of chromium. These are Micronucleus test, Chromosome aberration test and Comet assay. The study subjects were working for different period of time in different tanneries, they were healthy at the time of blood collection, without having any infectious disease and the control subjects were non-exposed to Chromium and chromium compounds by any kind of sources. The BMI ratios of the subjects were within normal range and they were used to have balanced diet, which indicated sound conditions of

mechanisms. Chromium (IV) can participate in fenton like reaction, which causes DNA damage. Electron spin resonance measurements demonstrated that in the presence of H_2O_2, synthesized Cr (IV)-ester acts as a potent Fenton-like reagent and generate OH radical and Cr (V) species. Comparative studies show that Cr (IV) is more potent than Cr (V) in generating hydroxyl radical. DNA damage assays demonstrate that Cr (IV) and the Cr (IV)/ H_2O_2 -generated OH radical can cause DNA strand breaks. This is the first direct evidence for DNA damage by Cr (IV) and Cr (IV)-mediated compounds (H Luo, Y Lu, 1996).

$$Cr\ (IV) + H_2O_2 \text{----------} \rightarrow Cr\ (V) + OH^{\cdot} + OH^{-}$$

Pentavalent Chromium, Cr (V) also DNA damage by different mechanism. Single-stranded DNA suffered extensive damage due to oxidation of the ribose moiety. The primary oxidation product was characterized as 5-methylene-2-furanone. Although all four bases (A, C, G and T) were released during the oxidation process, the concentration of guanine exceeds the other three. DNA oxidation is shown to proceed through a Cr (V)-DNA intermediate in which chromium(V) is coordinated through the phosphodiester moiety. Alternative mechanisms for DNA oxidation by oxochromate(V) are proposed to account for formation of 5-methylene-2-furanone, based on hydrogen abstraction or hydride transfer from the C1' site of the ribose followed by hydration and two successive [beta]-eliminations. (Rathindra N. Bose*, B. Stephen Fonkeng, 1998).

Hexavalent chromium compounds are genotoxic carcinogens. Chronic inhalation of hexavalent chromium compounds increases risk of lung cancer (lungs are especially vulnerable, followed by fine capillaries in kidneys and intestine). It appears that the mechanism of genotoxicity relies on pentavalent or trivalent chromium. According to some researchers, the damage is caused by hydroxyl radicals, produced during reoxidation of pentavalent chromium by hydrogen peroxide molecules present in the cell. Strontium chromate is the strongest carcinogen of the chromates used in industry. Soluble compounds, like chromic acid, are much weaker carcinogens (Salnikow, K et al, 2008).

complexes cannot be ruled out (T.C. Lee et al., 1989). Hydroxyl radicals tend to react with DNA bases not by electron transfer but instead to form adducts, for example by addition to carbon-carbon double bonds (i.e. the 5, 6-bond in pyrimidines). Hydroxyl radical adducts of the bases in DNA can lead to formation of strand breaks. The lesions may have deleterious consequences for cells, such as mutagenesis, carcinogenesis, aging, and apoptosis or lethality (Toby G. Rossman, 2003).

Keeping the above findings in mind we decided to evaluate chromium induced genotoxicity in human peripheral lymphocytes by the Micronucleus test, Chromosome aberration test and Comet assay. Micronucleus test detects chromosomal damage on once-divided binucleatetd cells when micronuclei (MNi) are formed during the metaphase/anaphase transition of mitosis (cell division). In the present study, cytochalasin-B (which inhibits the cytokinesis step) blocked micronucleus assay has been performed to monitor Chromium induced DNA damage. Results obtained from Micronucleus test revealed that the percent of frequency of MNi in binucleated cells were significantly higher in Chromium exposed lymphocytes. We found a MN frequency of 14.8% in the workers (Age Gr 1) and 24.6% (Age Gr 2). 1.4% in the negative control group (Table 3.3). These findings imply that damage in lymphocytes due to Chromium exposure for longer period of time resulted in increased frequencies of cells, missing whole chromosomes or fragments of chromosomes from the nucleus and thus forming micronuclei.

Chromosome aberration test detects various types of structural chromosome damage. Chromosomal aberrations are usually considered to derive from unrepaired or misrepaired DNA lesions induced by DNA damaging agents. We found various types of chromosome aberrations such as Gap chromosomes, Break chromosomes, Ring chromosomes and Dicentric chromosomes. A Gap chromosome is a localized area of thinning in a chromatid or chromosome. Gap chromosome is created by a microscopically evident loss of part of a chromosome, which may simulate a complete break and eventually make a Break chromosome. A Ring chromosome is created through breaks in the chromosome arms and fusion of the proximal broken ends, leading to loss of distal material. Dicentric chromosomes are formed when two chromosome

between the Chromium exposed groups and negative control group (nonexposed). We found 10.6% and 15.6% cells with aberrated DNA in the exposed group (Age Gr1 and Age Gr2 respectively) (Table 3.3). Particular types of aberrations such as dicentric chromosomes also increased with increasing period of chromium exposure. It was found by comparing Micronucleus test and chromosome aberration test that the percent of MNi was less doses compared to CA. The reason is that, there is a greater probability of two or more aberrations being enclosed in a single micronuclear membrane (Hossein Mozdarani et al., 2002).

DNA damage was also evaluated in terms of single strand breaks by comet assay. One of the powerful methods for detecting cell specific DNA damage is single-cell gel electrophoresis (SCGE) or the comet assay. In this technique DNA migration in an electric field is considered to be proportional to strand breakage, which gives an estimation of genotoxicity. DNA damage in living cells takes the forms of single strand breaks (SSBs), double strand breaks (DSBs) and alkali-labile sites as well as base damage, and comet assay is able to detect these forms of damage. In this context, using the comet assay, we applied the alkaline treatment, which aids in the unwinding and denaturation of DNA molecules, thus allowing for the sensitive detection of single-strand damage. We observed that there was considerable loss in the integrity of DNA in comet head region due to single strand breaks, as evident from figure 3.7. Moreover, the comet head DNA percentage (Fig. 3.8), tail DNA percentage (Fig. 3.9), tail moment (Fig. 3.10) and Olive tail moment (Fig. 3.11) as measured here, not only provided an indication of the extent of DNA damage but also was an effective indicator of the genotoxic effect of Chromium towards DNA damage.

In conclusion, it may be postulated that chromium is capable of inducing genotoxicity in cultured human peripheral blood lymphocytes, as evident by the increase of-

- Micronuclei formation due to chromosome damage, assessed by Micronucleus test.
- Various types of structural chromosome damage, investigated by Chromosome Aberration test. And
- DNA strand breakage, analyzed by Comet assay.

These cytogenotoxic assays in cultured human peripheral blood lymphocytes reflect the occurrence of similar events in the cells of target tissues involved in carcinogenic process. Moreover, chromosomal aberration in high level, in peripheral blood lymphocytes may be an early biomarker of cancer risk. Therefore, considering the data obtained from the present study, it can be concluded that chromium induces DNA damage and consequently chromosomal instability, which may lead to cancer to the chromium or chromium compound exposed human.

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