# ASSESSMENT OF DNA DAMAGE IN PULMONARY TUBERCULOSIS PATIENTS BY SINGLE CELL GEL ELECTROPHORESIS / COMET ASSAY

**A Dissertation submitted to**

**THE TAMILNADU Dr.M.G.R. MEDICAL UNIVERSITY**
**Chennai-600096**

**In partial fulfillment of the requirements for the award of degree of**

**MASTER OF PHARMACY**
**IN**
**PHARMACY PRACTICE**


**Submitted by**
**REG. NO: 26105588**


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## CERTIFICATE

This is to certify that the Dissertation entitled **"Assessment of DNA damage in pulmonary Tuberculosis patients by Single cell gel electrophoresis / Comet assay"** submitted to The Tamilnadu Dr. M.G.R. Medical University, Chennai, is a bonafide project work of **Mrs. Ushananthini.A.S. (Reg No:26105588),** carried out in the Department of Pharmacy Practice, Swamy Vivekanandha College of Pharmacy, Tiruchengode for the partial fulfillment for the degree of Master of Pharmacy under the guidance of **Dr.M.P.NARMADHA, M.Pharm, Ph.D,** Swamy Vivekanandha College of Pharmacy, Tiruchengode. This work is original and has not been submitted earlier for the award of any other degree or diploma of this or any other university.

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**Signature of H.O.D & Guide**

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**Ushananthini. A.S.**

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## CONTENTS

# ABSTRACT

**Aim and objective:** To determine the extent of cell damage in Pulmonary Tuberculosis patients with the help of comet assay.

**Study type:** Prospective study.

**Place and duration of study:** Tiruchengodu Govt. Hospital from September 2011 to April 2012.

**Patients and Method:** Blood samples were collected from 40 subjects [Control (Go), newly diagnosed TB patients (G1), Three months treated TB patients (G2), Six months treated TB patients (G3). n=10 subjects in each group]. Peripheral blood samples were collected, embedded in agrose gel, lysed in high ionic strength solution with Triton X-100, and then electrophoresed at pH>13, Ethidium bromide stained were then subjected to analysis under Fluorescent microscope.

**Result:** Subjects of newly diagnosed TB (G1) patients did not show any remarkable cell damage as compared to control (G0). Extent of cell damage in Three months treated TB patients (G2) and in Six months treated TB patients (G3) was extremely significant ($p < 0.0001$). From quantitative comet metrics, comet length which shows extremely significant difference in males compared with females. P value is <0.0003.

**Discussion*:*** Extent of cell damage was observed significantly high in Anti-tubercular drug treatment patients (ie) three months treated and six months treated TB patients. Due to their social habits also DNA damage was significantly increased in tuberculosis patients.

**Conclusion:** Dependence of drug treatment and social habits seems to be the major contributor of the cell damage.

# 1. INRTODUCTION

Tuberculosis (TB) is a highly contagious infection caused by the bacterium called Mycobacterium tuberculosis. TB can affect anyone of any age. People with weakened immune systems are at increased risk. TB can persist for decades in infected individuals in the latent state as an asymptomatic disease and can emerge to cause active disease at a later stage. There was an evidence that M.tuberculosis cells are exposed to DNA damaging agents such as reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) generated by host macrophages. Thus, DNA repair pathways and the mechanisms that are involved in the maintenance of genome integrity appear to be an important factor for M.tuberculosis pathogenesis and persistence in the host. Helicases are known to play an important role in DNA replication, repair and recombination. Thus, helicases could be a potential drug target against M.tuberculosis. Understanding the role of these proteins could provide new insights into pathogenesis of M.tuberculosis in humans[1]. The repair of DNA damage is expected to be particularly important to intracellular pathogens such as M.tuberculosis, and so it is of interest to examine the response of M. tuberculosis to DNA damage. The expression of recA, a key component in DNA repair and recombination, is induced by DNA damage in M. tuberculosis[2].

DNA damage accumulates with age, and genetic defects in DNA repair and DNA damage signaling pathways lead to symptoms of premature aging in humans. When DNA is damaged, our cells attempt to repair the damage, making use of several different types of DNA repair. Importantly, DNA damage and repair also lead to extensive chromatin reorganization. These chromatin changes can occur as far as a megabase from some forms of DNA damage, meaning that some types of DNA damage could affect an extended region of chromatin containing multiple genes[3].

## 1.1) Pulmonary tuberculosis

Pulmonary tuberculosis that affects the lungs. An infected person may at first feel vaguely unwell or develop a cough blamed on smoking or a cold. A small amount of greenish or yellow sputum may be coughed up when the person gets up in the morning. In time, more sputum is produced that is streaked with blood. Persons with pulmonary TB do not run a high fever, but they often have a low-grade one. They may wake up in the night drenched with cold sweat when the fever breaks. The patient often loses interest in food and may lose weight, chest pain may present. If the infection allows air to escape from the lungs into the chest cavity (pneumothorax) or if fluid collects in the pleural space (pleural effusion), the patient may have difficulty in breathing. If a young adult develops a pleural effusion, the chance of tubercular infection being the cause is very high. The TB bacilli may travel from the lungs to lymph nodes in the sides and back of the neck. Infection in these areas can break through the skin and discharge pus. Before the development of effective antibiotics, many patients became chronically ill with increasingly severe lung symptoms. They lost a great deal of weight and developed a wasted appearance. This outcome is uncommon today, at least where modern treatment methods are available[4].

In pulmonary tuberculosis patients, little is known about peripheral DNA damage, although increased oxidative stress is a well documented entity. Therefore, we aimed to investigate DNA damage in pulmonary tuberculosis patients. DNA damage was assessed by Comet assay[5].

The importance of DNA damage in clinical studies would demand an accurate, fast and sensitive method to detect DNA damages which is also capable of monitoring DNA repair as well. There are several different methods to detect DNA damage including: alkaline elution, clonogenic cell survival assay, single-cell gel electrophoresis (SCGE) and others[6]. The SCGE, also

known as comet assay, is one of the recent methods established in order to detect different types of DNA damage. The comet assay has been established as a simple, rapid, cheap, flexible and, most importantly, sensitive method to detect DNA damage, which is also able to detect DNA damage in individual cells. The concept of detecting DNA by cellular lysis was introduced in 1970s; however, the current and most commonly used variation of the comet assay (the alkaline comet assay) was developed in 1998 by Singh et al. Since 1998, modifications have been introduced to the assay to increase its sensitivity[7] .The comet assay is currently used in different areas of biological sciences to detect DNA damage.

The alkaline single cell gel electrophoresis (SCG) technique is a useful tool for assessing DNA damage in human populations. This method has several advantages: a high sensitivity for detecting DNA damage expressed as single strand breaks and alkaline-labile sites, data from individual cells are obtained, the amount of blood required for this method is very small (µl), it is relatively easy to perform, and results are obtained in a short time .

It has since gained in popularity as a standard technique for evaluation of DNA damage/repair, biomonitoring and genotoxicity testing. It involves the encapsulation of cells in a low-melting-point agarose suspension, lysis of the cells in neutral or alkaline (pH>13) conditions, and electrophoresis of the suspended lysed cells. This is followed by visual analysis with staining of DNA and calculating fluorescence to determine the extent of DNA damage[8,9].

DNA strand breaks allow DNA to extend from lysed and salt-extracted nuclei, nucleoids, to form a comet like tail on alkaline electrophoresis. Cells undergoing active cell death or apoptosis demonstrate highly fragmented DNA. Progression of cell death results in the extensive formation of double strand breaks and is readily detected using alkaline electrophoretic conditions[10].

The aim of this work is to study qualitatively the development of comet images evaluating extent of cellular damage and quantify different comet metrics.

# 2. LITERATURE REVIEW

## 2.1) DEFINITION

Tuberculosis (TB) is a potentially fatal contagious disease. It is caused by a bacterial micro organism, the tubercle bacillus or Mycobacterium tuberculosis**,** that affects especially the lungs but may spread to other areas (as the kidney or spinal column), and that is characterized by fever, cough, difficulty in breathing, formation of tubercles, caseation, pleural effusions, and fibrosis[11] .

## 2.2) ETIOLOGY

TB properly refers only to disease caused by Mycobacterium tuberculosis. Similar disease occasionally results from the closely related Mycobacteria, M.bovis, M.africanum, and M.microti.

TB results almost exclusively from inhalation of airborne particles (droplet nuclei) containing M.tuberculosis. They disperse primarily through coughing, sneezing and whose sputum contains a significant number of organisms (typically enough to render the smear positive). People with pulmonary cavitary lesions are especially infectious. Droplet nuclei containing tubercle bacilli may remain suspended in room air currents for several hours, increasing the chance of spread. However, once these droplets land on a surface, it is difficult to resuspend the organisms (eg, by sweeping the floor) as respirable particles. Although such actions can resuspend dust particles containing tubercle bacilli, these particles are far too large to reach the alveolar surfaces necessary to initiate infection[12].

## 2.3) EPIDEMIOLOGY

About one third of the world's population is infected. Of these, perhaps only 15 million have active disease at any given time. In 2006, an estimated 9.2 million new TB cases occurred worldwide (139/100,000). Of these, Africa and Southeast Asia each accounted for about 3 million cases, and the Western Pacific region for about 2 million. Case rates vary very widely by country, age, race, sex, and socioeconomic status. India and China reported the largest numbers of new cases, but South Africa has the largest case rate: 940/100,000. Epidemiological data on tuberculosis, detect risk factors and other epidemiological issues involved in its transmission in some parts of Abia State, Nigeria. Sputum samples were collected aseptically from patients in some Public hospitals in Abia State. Out of 998 sputum examined, Mycobacterium tuberculosis was isolated from 198 (16.83%). Males had higher prevalence of infection in each year of study than females. Infection rates remained higher in dry season than rainy season in both 2005 and 2006. Traders, public and civil servants, police officers, Timber workers, teachers, drivers, students and pupils are among the population at risk in Abia State. Out of 198 positive sputum samples, 95 (47.97%) were new cases in 2006. This calls for the attention of public health workers, governmental and non-government agencies for concerted efforts against the disease in this region.

The average prevalence of all forms of tuberculosis in India is estimated to be 5.05 per thousand, prevalence of smear-positive cases 2.27 per thousand and average annual incidence of smear-positive cases at 84 per 1,00,000 annually. The Governmental efforts at intervention through Revised National Tuberculosis Control Programme (RNTCP) and at monitoring the epidemiology of intervention through organising routine reporting are highlighted, and data are presented and evaluated on these. RNTCP needs to be used as an effective instrument to bring a change in epidemiological situation, through fast expansion and achievement of global target[12].

An increasing morbidity and mortality from tuberculosis (TB) in the near future is forecast for the world at large, with the number of newly occurring cases predicted to increase from 7.5 million a year in 1990 to 8.8, 10.2 and 11.9 million in the years 1995, 2002 and 2005 respectively; an increase amounting to 58.6 per cent over a 15-yr period.

Global epidemiology

•8 million new cases of disease per year

•2 million deaths per year

•80% of new cases live in 22 high burden countries

•Global health emergency declared by WHO in 1993

The present review describes the global tuberculosis situation, and views it in the context of the goal of the antituberculosis intervention activities. It presents the epidemiological situation in India, comments on the current trend and discusses the efforts taken towards making projections on the likely burden of disease in India over time.

## 2.3.1) Numbers of notifications in England and Wales 1992- 2002

| | 1992 | 1993 | 1994 | 1995 | 1996 | 1997 | 1998 | 1999 | 2000 | 2001 | 2002# |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Northern & Yorkshire | 636 | 697 | 568 | 624 | 605 | 617 | 642 | 611 | 593 | 646 | 600 |
| Trent | 438 | 535 | 469 | 520 | 544 | 484 | 498 | 497 | 545 | 564 | 563 |
| Eastern | 290 | 270 | 245 | 267 | 260 | 232 | 268 | 228 | 254 | 326 | 343 |
| London | 2003 | 1959 | 2077 | 2042 | 2190 | 2411 | 2444 | 2493 | 2834 | 2886 | 2850 |
| South East | 393 | 467 | 457 | 484 | 457 | 483 | 503 | 495 | 553 | 598 | 700 |
| South West | 199 | 211 | 203 | 202 | 201 | 212 | 213 | 209 | 226 | 198 | 248 |
| West Midlands | 869 | 788 | 730 | 654 | 655 | 610 | 673 | 712 | 723 | 692 | 806 |
| North West | 769 | 794 | 660 | 633 | 581 | 615 | 674 | 693 | 651 | 661 | 650 |
| Wales | 201 | 199 | 181 | 180 | 161 | 195 | 172 | 205 | 193 | 143 | 131 |
| TOTAL | 5798 | 5920 | 5590 | 5606 | 5654 | 5859 | 6087 | 6143 | 6572 | 6714 | 6891 |

## 2.4) SIGNS AND SYMPTOMS

- A bad cough that lasts 3 weeks or longer
- Pain in the chest
- Coughing up blood or sputum

**2.4.1)** Other symptoms of active TB disease are,

- Weakness or fatigue
- Weight loss
- Loss of appetite
- Chills
- Fever
- Sweating at night.

## 2.5) RISK FACTORS

There are number of factors that make people more susceptible to TB infections. Worldwide the most important of these is HIV with co-infection present in about 13% of cases[13]. Tuberculosis is closely linked to both overcrowding and malnutrition making it one of the principal diseases of poverty. Chronic lung disease is a risk factor with smoking more than 20 cigarettes a day increasing the risk by two to four times and silicosis increasing the risk about 30 fold. Other disease states that increase the risk of developing tuberculosis include alcoholism and diabetes mellitus (threefold increase). Certain medications such as corticosteroids and Infliximab (an anti-αTNF monoclonal antibody) are becoming increasingly important risk factors, especially in the developed world. There is also a genetic susceptibility for which overall importance is still undefined[14].

## 2.6) TRANSMISSION

When people with active pulmonary TB cough, sneeze, speak, sing, or spit, they expel infectious aerosol droplets 0.5 to 5 μm in diameter. A single sneeze can release up to 40,000 droplets. Each one of these droplets may transmit the disease, since the infectious dose of tuberculosis is very low and inhaling fewer than ten bacteria may cause an infection[15].

People with prolonged, frequent, or intense contact are at particularly high risk of becoming infected, with an estimated 22% infection rate. A person with active but untreated tuberculosis can infect 10 to 15 other people per year. Others at risk include people in areas where TB is common, people who inject illicit drugs, residents and employees of high-risk congregate settings, medically under-served and low-income populations, high-risk racial or ethnic minority populations, children exposed to adults in high-risk categories, those who are immunocompromised by conditions such as HIV/AIDS, people who take immunosuppressant drugs, and health care workers serving these high-risk clients[16].

Transmission can only occur from people with active not latent TB. The probability of transmission from one person to another depends upon the number of infectious droplets expelled by a carrier, the effectiveness of ventilation, the duration of exposure, and the virulence of the M.tuberculosis strain. The chain of transmission can be broken by isolating people with active disease and starting effective anti-tuberculous therapy. After two weeks of such treatment, people with non-resistant active TB generally cease to be contagious. If someone does become infected, then it will take three to four weeks before the newly infected person can transmit the disease to others[17].

## 2.7) PATHOGENESIS

About 90% of those infected with M.tuberculosis have asymptomatic, latent TB infection (sometimes called LTBI), with only a 10% lifetime chance that a latent infection will progress to TB disease. However, if untreated, the death rate for these active TB cases is more than 50%.

TB infection begins when the mycobacteria reach the pulmonary alveoli, where they invade and replicate within the endosomes of alveolar macrophages. The primary site of infection in the lungs is called the Ghon focus, and is generally located in either the upper part of the lower lobe, or the lower part of the upper lobe. Simon foci may also be present. Bacteria are picked up by dendritic cells, which do not allow replication, although these cells can transport the bacilli to local (mediastinal) lymph nodes. Further spread is through the bloodstream to other tissues and organs where secondary TB lesions can develop in other parts of the lung (particularly the apex of the upper lobes), peripheral lymph nodes, kidneys, brain, and bone. All parts of the body can be affected by the disease, though it rarely affects the heart, skeletal muscles, pancreas and thyroid[18].

Tuberculosis is classified as one of the granulomatous inflammatory conditions. Macrophages, T lymphocytes, B lymphocytes and fibroblasts are among the cells that aggregate to form granulomas, with lymphocytes surrounding the infected macrophages. The granuloma prevents dissemination of the mycobacteria and provides a local environment for interaction of cells of the immune system. Bacteria inside the granuloma can become dormant, resulting in a latent infection. Another feature of the granulomas of human tuberculosis is the development of abnormal cell death (necrosis) in the center of tubercles. To the naked eye this has the texture of soft white cheese and is termed caseous necrosis[18].

If TB bacteria gain entry to the bloodstream from an area of damaged tissue they spread through the body and set up many foci of infection, all appearing as tiny white tubercles in the tissues. This severe form of TB disease is most common in infants and the elderly and is called miliary tuberculosis. People with this disseminated TB have a fatality rate near 100% if untreated. However, if treated early, the fatality rate is reduced to about 10%[19].

In many people the infection waxes and wanes. Tissue destruction and necrosis are balanced by healing and fibrosis. Affected tissue is replaced by scarring and cavities filled with cheese-like white necrotic material. During active disease, some of these cavities are joined to the air passages bronchi and this material can be coughed up. It contains living bacteria and can therefore spread the infection. Treatment with appropriate antibiotics kills bacteria and allows healing to take place. Upon cure, affected areas are eventually replaced by scar tissue[18].

## 2.8) DISEASE SIMILAR TO TUBERCULOSIS

There are many forms of mycobacteria other than M.tuberculosis, the tubercle bacillus. Some cause infections that may closely resemble tuberculosis, but they usually do so only when an infected person's immune system is defective. People who are HIV-positive are a prime example. The most common mycobacteria that infect AIDS patients are a group known as Mycobacterium avium complex (MAC). People infected by MAC are not contagious, but they may develop a serious lung infection that is highly resistant to antibiotics. MAC infections typically start with the patient coughing up mucus. The infection progresses slowly, but eventually blood is brought up and the patient has trouble in breathing. In AIDS patients, MAC disease can spread throughout the body, with anemia, diarrhea**,** and stomach pain as common features. Often these patients die unless their immune system can be strengthened. Other mycobacteria grow in swimming pools and may

cause skin infection. Some of them infect wounds and artificial body parts such as a breast implant or mechanical heart valve[20].

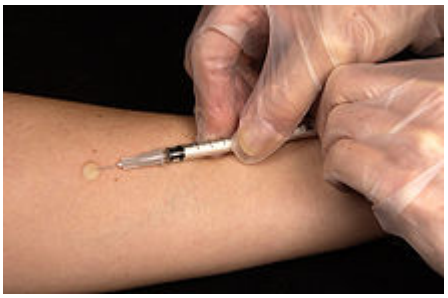
## 2.9) DIAGNOSIS

Mycobacterium tuberculosis (stained red) in sputum

Tuberculosis is diagnosed definitively by identifying the causative organism (M.tuberculosis) in a clinical sample (for example, sputum or pus). When this is not possible, a probable although sometimes inconclusive diagnosis may be made using imaging (X-rays or scans), a tuberculin skin test (Mantoux test), or a, Interferon Gamma Release Assay (IGRA).The main problem with tuberculosis diagnosis is the difficulty in culturing this slow-growing organism in the laboratory (it may take 4 to 12 weeks for blood or sputum culture). A complete medical evaluation for TB must include a medical history, a physical examination, a chest X-ray, microbiological smears, and cultures. It may also include a tuberculin skin test, a serological test. The interpretation of the tuberculin skin test depends upon the person's risk factors for infection and progression to TB disease, such as exposure to other cases of TB or immunosuppression[21].

New TB tests have been developed that are fast and accurate. These include polymerase chain reaction assays for the detection of bacterial DNA[22]. One such molecular diagnostics test gives results in 100 minutes and is currently being offered to 116 low- and middle-income countries at a discount with support from WHO and the Bill and Melinda Gates foundation. Another such test, which was approved by the FDA in 1996, is the amplified mycobacterium tuberculosis direct test (MTD, Gen-Probe). This test yields results in 2.5 to 3.5 hours, and it is highly sensitive and specific when used to test smears positive for acid-fast bacilli (AFB) [23].

## 2.10) SCREENING



Mantoux tuberculin skin test

Mantoux tuberculin skin tests are often used for routine screening of high risk individuals[24]. Currently, latent infection is diagnosed in a non-immunized person by a tuberculin skin test, which yields a delayed hypersensitivity type response to an extract made from M.tuberculosis. Those immunized for TB or with past-cleared infection will respond with delayed hypersensitivity parallel to those currently in a state of infection, so the test must be used with caution, particularly with regard to persons from countries where TB immunization is common[25]. Tuberculin tests have the disadvantage of producing false negatives, especially when the person is co-morbid with sarcoidosis, Hodgkin's lymphoma, malnutrition, or most notably active tuberculosis disease. The newer interferon release assays (IGRAs) such as T-

SPOT.TB and QuantiFERON-TB Gold .In Tube overcome many of these problems. IGRAs are in vitro blood tests that are more specific than the skin test. IGRAs detect the release of interferon gamma in response to mycobacterial proteins such as ESAT-6.These is not affected by immunization or environmental mycobacteria, so generate fewer false positive results. There is also evidence that IGRAs are more sensitive than the skin test[26].

## 2.11) TREATMENT

Treatment for TB uses antibiotics to kill the bacteria. Effective TB treatment is difficult, due to the unusual structure and chemical composition of the mycobacterial cell wall, which makes many antibiotics ineffective and hinders the entry of drugs[27]. The two antibiotics most commonly used are isoniazid and rifampicin and treatments can be prolonged. Latent TB treatment usually uses a single antibiotic, while active TB disease is best treated with combinations of several antibiotics, to reduce the risk of the bacteria developing antibiotic resistance[28]. People with latent infections are treated to prevent them from progressing to active TB disease later in life.

With the advent of effective Antituberculous chemotherapy, it became apparent that patients no longer needed sanatorium treatment. Results with regimens containing Isoniazid together with P-aminosalicylate (PAS) or Ethambutol, and sometimes Streptomycin, gave excellent results. Treatment was required for 18-24 months if relapse was to be prevented. The availability of Pyrazinamide and, more importantly, Rifampicin made shorter courses of treatment possibility. Most regimens in the developed world now contain Isoniazid, Rifampicin, and Pyrazinamide, possibly with another agent such as Ethambutol.

## 2.12) TREATMENT OF PULMONARY TUBERCULOSIS

The Joint Tuberculosis Committee recommends a 6 month regimen consisting of Rifampicin, Isoniazid, Pyrazinamide and Ethambutol for initial 2 months followed by further 4 months of Rifampicin, and Isoniazid. The fourth drug (Ethambutol) may be omitted in patients with a low risk of resistance to Isoniazid.

### 2.12.1) Dosing of First-Line Anti-TB Drugs*

| **Drug** | **Adults or Children** | **Daily**[†] | **Once/wk** | **2 Times/wk** | **3 Times/wk** |
|---|---|---|---|---|---|
| Isoniazid INH | Adults (maximum) | 5 mg/kg (300 mg) | 15 mg/kg (900 mg) | 15 mg/kg (900 mg) | 15 mg/kg (900 mg) |
| | Children (maximum) | 10–15 mg/kg (300 mg) | N/A | 20–30 mg/kg (900 mg) | N/A |
| Rifampicin | Adults (maximum) | 10 mg/kg (600 mg) | N/A | 10 mg/kg (600 mg) | 10 mg/kg (600 mg) |
| | Children (maximum) | 10–20 mg/kg (600 mg) | N/A | 10–20 mg/kg (600 mg) | N/A |
| Pyrazinamide | Adults (whole tablets): | | | | |
| | 40–55 kg | 1 g | N/A | 2 g | 1.5 g |
| | 56–75 kg | 1.5 g | N/A | 3 g | 2.5 g |
| | ≥ 76 kg[§] | 2 g | N/A | 4 g | 3 g |
| | Children (maximum) | 15–30 mg/kg (2 g) | N/A | 50 mg/kg (2 g) | N/A |
| Ethambutol | Adults (whole tablets): | | | | |
| | 40–55 kg | 800 mg | N/A | 2000 mg | 1200 mg |
| | 56–75 kg | 1200 mg | N/A | 2800 mg | 2000 mg |
| | ≥ 76 kg[§] | 1600 mg | N/A | 4000 mg | 2400 mg |
| | Children (maximum) | 15–20 mg/kg (1 g) | N/A | 50 mg/kg (2.5 g) | N/A |
| *Specific regimens are discussed in text. | | | | | |
| [†]Daily is considered either 5 or 7 days/wk. All dosing < 7 days/wk must be given as | | | | | |

| |
|---|
| directly observed therapy. |
| [‡]Continuation phase only. |
| [§]Maximum dose. |
| N/A = not applicable. |

**Isoniazid** is the cheapest and one of the most effective antituberculous drugs. Its bactericidal potency is equalled only by rifampacin. It acts on the tubercle bacilli multiplying in the walls of the cavities, as well as those potent than other antituberculous drugs in preventing drug resistance to other drugs.

Mechanism of action: INH is a prodrug. It is activates by the mycobacterial catalase-peroxidase to an active compound which inhibits the synthesis of mycolic acid, an important constituent of the mycobacterial cell wall. INH inhibits the same catalase-peroxidase and makes the organisms susceptible to oxidative mechanisms[29].

**Rifampicin** is the semisynthetic derivative of rifamycin B, important property is its effectiveness against Mycobacterium tuberculosis and Mycobacterium leprae. The former organism is inhibited by doses of the same magnitude as with INH but the sterlizing action is faster than that of INH.

Mechanism of action: Rifampicin acts by inhibiting bacterial DNA dependent RNA polymerase, thus stopping the expression of bacterial genes. Human RNA polymerase is not inhibited. Rifampicin is Bactericidal and acts against both intra and extra-cellular organisms. Effective against tubercle bacilli resistant to other standard drugs and against some of the atypical mycobacteria. The only drug which acts on the persisters[29].

**Pyrazinamide** is chemically related to thiosemicarbazones and nicotinamide, is effective orally against Mycobacterium tuberculosis of human type resistant to streptomycin and INH but is ineffective against the bovine and atypical forms of tubercle bacilli. In combination with streptomycin, INH and rifampicin, pyrazinamide exerts a potent sterilizing effect on the tuberculous lesions during the first two months of therapy.

Mechanism of action: Pyrazinamide is not definitely known. It is a prodrug and is converted to the active compound pyrazinoic acid. Like INH, it inhibits the bacterial synthesis of mycolic acid[29].

**Ethambutol** is effective orally against mycobacteria resistant to INH, Streptomycin and Ethionamide as well as against many atypical mycobacteria. Primary resistance to this drug has not been reported. When it is used along with other drugs, resistance to ethambutol develops slowly, and the development of resistance to the other drugs is greatly delayed.

Mechanism of action: It acts mainly against rapidly multiplying organisms in the walls of the cavities. It inhibits the synthesis of bacterial cell wall arabinosyl transferase[29].

## 2.13) MEDICATION RESISTANCE

Primary resistance occurs in persons infected with a resistant strain of TB. A person with fully susceptible TB develops secondary resistance (acquired resistance) during TB therapy because of inadequate treatment, not taking the prescribed regimen appropriately, or using low-quality medication[30]. Drug-resistant TB is a public health issue in many developing countries, as treatment is longer and requires more expensive drugs. Multi-drug-resistant tuberculosis (MDR-TB) is defined as resistance to the two most effective first-line TB drugs: rifampicin and isoniazid. Extensively drug-resistant TB (XDR-TB) is also resistant to three or more of the six classes of second-line drugs. Totally

drug-resistance TB (TDR-TB), which was first observed in 2003 in Italy, but not widely reported until 2012, is resistant to all currently-used drugs[31].

## 2.14) ADVERSE EFFECTS OF ANTITUBERCULAR DRUGS

Tuberculosis is an infectious disease which can be totally cured by combining antitubercular drugs. Current therapeutic regimens with isoniazid, rifampicin, pyrazinamide, ethambutol, and streptomycin have proved successful in treating tuberculosis. However, they are associated to a high rate of adverse effects that can lead to therapeutic failure. Understanding the nature and the severity of these adverse effects allows for their appropriate management. Toxic neuropathy and hepatitis are the most common adverse reactions to isoniazid. Rifampicin is generally well tolerated but some severe immuno-allergic reactions may occur in case of intermittent regimen. Pyrazinamide-induced liver injury is rare but sometimes lethal. Joint affections, usually due to hyperuricemia, are more frequent but easily manageable. The major adverse effect related to ethambutol is ocular optic neuropathy. It occurs dose-dependently and can be irreversible. Finally, administration of streptomycin is potentially associated with renal and cochleo-vestibular toxicity that might be milder than when induced by other aminoglycosides. The management of antituberculosis-induced adverse effects depends on parameters related to the adverse effect itself and to the administrated drug[32].

## 2.15) Supporting Literatures

McArt DG et al (2009), reported that the single-cell gel electrophoresis technique or comet assay is widely regarded as a quick and reliable method of analyzing DNA damage in individual cells[33].

Kumaravel TS et al (2006), reported that the length of DNA migration is directly related to the loops of released DNA or the size of DNA fragments, and is proportional to the amounts of strand breaks and alkali-labile sites[34].

Collins AR et al (1997), reported that the 'Percentage DNA in the tail' is the second primary comet measurement on which other derived units are based. The percentage of DNA in tail is directly proportional to the amount of damaged DNA[35].

Gonzalez C et al (2002), reported that certain drugs are known to induce DNA damage in healthy cells and potentiate the oxidative stress generated during cellular events. Induction of DNA damage in lymphocytes by various antibiotics, anaesthetic agents and oral contraceptives have been studied with the help of comet assay[36].

Nima V. Thakkar et al (2011), reported that the presence of significant cellular damage in diabetic population and diabetic hypothyroid complications as compared to hypothyroid patients and normal healthy individuals, which is suggestive of detrimental effects of diabetes. The DNA damaging effects of drug treatment like sulphonamides can not be ruled out as patients receiving glibenclamide showed high Olive Tail Moment (OTM) measurements indicating greater possibility of cellular damage. The high level of concordance of the results obtained in the comet assay showed that the comet assay is not only sensitive enough to detect low levels of DNA damage in human lymphocytes, but it is also highly specific and give an idea of diabetes control in T2DM individuals[37].

Kassie F et al (2000), stated that DNA strand breaks allow DNA to extend from lysed and salt-extracted nuclei, or nucleoids to form a comet like tail on alkaline electrophoresis. The comet assay, a technique, capable of detecting DNA damage and repair in individual cells, is a valuable approach for human biomonitoring studies[38].

Nima V. Thakkar et al (2010), concluded that cellular damage depends on age and duration of disease condition, although dependence of disease conditions seems to be a major contributor of the cell damage as indicated by increase in the percentage of apoptotic cells in diabetic subjects[39].

De Boeck et al (2000), said that the comet assay has been widely used for genotoxic studies and cell biological studies, and even in human biomonitoring studies. Among manyparameters of Comet features, the tail parameters that are the most frequently used are those of the tail moment, the tail DNA, and the tail length[40].

Olive et al (1990a, 1992), introduced that the tail moment and the percentage of DNA in the tail (the tail DNA) and these parameters have been used by many researchers for genotoxic studies[41].

Bocker et al (1997), reported that the tail moment and tail DNA showed more sensitivity than the tail length on the basis of an X-irradiation dose-response experiment. Correlation between the tail moment and the tail length of human lymphocytes was relatively lower than between the tail moment and the head-tail ratio, both in normal subjects and in ataxia telangiectasia patients[42].

Eunil Lee et al (2004), stated that the tail moment provides the most stable estimates for DNA damage because it has a large degree of uniformity in quantile dispersions. To study high degrees of damage from toxic exposure using B cells or G cells, however, the tail DNA showed more significant discrepancies than the other parameters, in terms of both the mean differences and the graphical differences between the two groups[43].

Semra Sardasa et al (2000), Diabetes patients often show increased production of reactive oxidative species (ROS) together with vascular complications. The presence of these ROS may lead to increased DNA damage in peripheral blood lymphocytes that may be revealed by the comet assay[44].

P Dandona et al (1996), said that the production of reactive oxygen species and lipid peroxidation are increased in diabetic patients, especially in those with poor diabetic control and hypertriglyceridamia. Mechanisms that contribute to the formation of free radicals in diabetics may include not only increased non-enzymatic and auto oxidative glycoxylation, but also metabolic stress resulting from changes in energy metabolism, the levels of inflammatory mediators and the status of antioxidant defense[45].

Semra Sardasa et al (2000), study provide evidence of oxidative damage in diabetic patients when evaluated by alkaline comet assay. Difference could be detected in IDDM and NIDDM patients groups in terms of extent of DNA migration. Clinical findings of increased serum levels of lipid peroxides, differences in creatinine levels and glycosylated haemoglobin-HbAic values in all patients suffering from diabetes mellitus supports the hypothesis that enhanced lipid peroxidation contributes to an increased formation of free radicals in diabetes mellitus. Cells with migration were at a lower level in non-smoker patients and a slightly lower level in antioxidant supplemented patients[46].

Ostling O et al (1987), said that the ability to measure heterogeneity in response to DNA damaging agents was first tested on cells exposed to the cancer chemotherapeutic drug, Bleomycin[47].

Peggy L Olive et al (2006), said that the idea was to combine DNA gel electrophoresis with Fluorescence microscopy to visualize migration of DNA strands from individual agarose-embedded cells. If the negatively charged DNA contained breaks, DNA supercoils were relaxed and broken ends were

able to migrate towards the anode during a brief electrophoresis. If the DNA was undamaged, the lack of free ends and large size of the fragments prevented migration. Determination of the relative amount of DNA that migrated provided a simple way to measure the number of DNA breaks in an individual cells[48].

Olive P.L et al (1990), said that the Comet head containing the high-molecular-weight DNA and the comet tail containing the leading ends of migrating fragments were measured in real time from digitized images using software developed for this purpose[49].

K Kalaiselvi et al (2002), said that with the aim of assessing possible genetic damage in leprosy patients, we carried out a cytogenetic study involving leprosy patients and age-matched controls living in the same area. Peripheral blood lymphocytes of the patients and control subjects were analyzed for the presence of DNA damage, using single cell gel electrophoresis (the Comet assay), and for cytogenetic parameters [chromosomal aberrations (CA) and micronucleus (MN) frequency]. The use of CA and MN as markers of early biological effects is well established in genotoxicity studies, while the Comet assay is a more innovative technique[50].

Guillermo T. Saez et al (1993), reported that the incubation of calf thymus DNA in the presence of rifamycin SV induces a decrease in the absorbance of DNA at 260 nm. The effect was found to be proportional to the antibiotic concentration and enhanced by copper (II) ions. In the presence of rifamycin SV and copper (II), a significant increase in thiobarbituric acid-reactive (TBA-reactive) material is also observed. This effect is inhibited to different degrees by the following antioxidants: catalase 77%; thiourea 72%; glutathione (GSH) 62%; ethanol 52%; and DMSO 34%, suggesting that both hydrogen peroxide ($H_2O_2$) and hydroxyl radicals ($OH\cdot$) are involved in DNA damage. Rifamycin SV-copper (II) mixtures were also found to induce the production of peroxidation material from deoxyribose and, in this case, glutathione and

ethanol were the most effective antioxidant substrates with inhibition rates of 91% and 88% respectively. Electrophoretic studies show that calf thymus DNA becomes damaged after 20 min. Normal DNA electrophoretic pattern was found to be preserved by catalase and GSH at physiological concentrations and by thiourea. No protection is observed in the presence of ethanol or DMSO. The results obtained indicate the involvement of different reactive species in the degradation process of DNA due to rifamycin SV-copper (II) complex and emphasize the role of reduced glutathione as an oxygen free radical scavenger[51].

Madhuri Jaju et al (2005), discussed that the genetic consequences of drug interaction in tuberculosis patients. Blood from tuberculosis patients was cultured before, during, and after withdrawal of therapy involving five different drug combinations of isoniazid (INH), thiacetazone (TAZ), para-aminosalicylic acid (PAS), and streptomycin (SM). The approaches used to detect DNA damage were chromosome aberrations and sister chromatid exchanges (SCEs). A total of 179 subjects were analyzed. In combination these drugs showed synergistic, additive, and antagonistic effects, though they were found to be nonclastogenic individually. Four of the drug combinations, INH+TAZ, INH+PAS, INH+TAZ+SM, and INH + PAS + SM, induced a significant increase in the frequency of aberrations, whereas INH+SM did not induce aberrations. In fact, SM appeared to reduce the frequency of aberrations. SCEs were increased in only two patients: one treated with INH + TAZ and the other with INH + PAS. The frequency of aberrations after withdrawal of therapy was decreased; it was slightly higher than the controls, though it was insignificant. Though the drug-induced aberrations do not persist after withdrawal of therapy, the chromosome damaging combinations of drugs should be used with caution, because the possibility of meiotic chromosome damage in germ cells (during therapy), which might be passed on to the next generation, cannot be ruled out[52].

C. Gonzalez et al (2002), reported that the infectious disease and malnutrition in children are public health problems in developing countries. Malnutrition is associated with higher levels of DNA damage, and this increased damage could be due to different factors, including the possibility that cells from malnourished children could be more susceptible to environmental damage. The aim of this study was to evaluate the susceptibility of lymphocytes from malnourished children to DNA damage induced by antibiotics by using the comet assay. The same group of malnourished infected children were studied before and after a treatment period, and compared to a group of well-nourished infected children. Results showed that before and after drug treatment, tail length migration was two times greater in malnourished than in well-nourished children. The proportion of cells with high damage was also increased in malnourished children. Additionally in well-nourished and malnourished children, a cell subpopulation (non-damaged cells) more resistant to DNA damage induced by antibiotics was observed; this was more prevalent in the well-nourished children. Meanwhile, in malnourished children, a cell population seems to be more susceptible and reaches higher levels of DNA damage. This might help explain the impaired immune response observed in malnourished children. The increased DNA migration and the increased proportion of cells with higher levels of damage seem to indicate that malnourished children are more susceptible to DNA damage induced by drugs[53].

# 3. AIM & OBJECTIVE

**AIM**

To Assess the DNA damage in Pulmonary Tuberculosis patients by Comet assay.

**OBJECTIVE**

- To assess the DNA fragmentations,
    - In newly diagnosed Tuberculosis patients
    - In 3 months treated Tuberculosis patients
    - In 6 months treated Tuberculosis patients, then
- To determine the extent of cell damage in Tuberculosis patients with the help of comet assay.
- To study the influence of Tuberculosis on DNA damage.
- To assess the influence of duration of drug treatment on DNA damage.

# 4. PLAN OF WORK

The proposed study was designed as given below,

- Identification of scope of work
- Literature survey
- Preparation of study protocol
- Designing the proforma
- Obtaining approval from the ethical committee
- Obtaining consent from the Hospital Authority
- Data collection
    - Selection of patients
    - Collection of patients details
    - Collection of blood sample from patient
- Analysis of data.

# 5. METHODOLOGY

**5.1) Study type:**

Prospective study

**5.2) Study site:**

Tiruchengodu Govt. Hospital

**5.3) Sample size:**

40 subjects, Total numbers of patients included in the study were 30. These 30 patients were selected after screening of all TB patients who were visiting TB out patient clinic. All those patients who met with the set inclusion criteria constituted the study population. Among these 30 patients, 10 were newly diagnosed TB patients (G1), 10 were TB patients treated with anti TB drugs for 3 months (G2), and 10 were TB patients treated with anti TB drugs for 6 months (G3). In order to compare the influence the disease state on cell damage, 10 Healthy volunteers (G0) of age and sex match were included to assess the cell damage.

**5.4) Study duration:**

8 months

**5.5) Ethical Clearance:**

The proposal was submitted to the Institutional Ethical Committee (IEC) of Swamy Vivekanandha College of Pharmacy. The IEC permitted to carry out the study dated on 21-09-2011 (IEC/ Sep/2011/07). The copy of approval was given in annexure 2.

**5.6) Study population**:

Newly diagnosed Patients with Tuberculosis based on sputum positivity and Tuberculosis patients taking Anti-tubercular drugs and Healthy volunteers.

### 5.6.1) Inclusion criteria:

- Patients taking Anti-tubercular drugs both in intensive and continuation phase.
- Adult TB male / female patients.

### 5.6.2) Exclusion criteria:

- Severely ill patients,
- TB Patients with liver disease,
- TB Pregnant and lactating,
- TB Diabetic / cardio vascular disease patients,
- TB Patients with other comorbid conditions,
- TB patients with HIV.

### 5.7) Initial Assessment:

Patient data entry form was used to record the patient details such as name, age, sex, educational status, occupation, social history, family history, dietary habits, and treatment regimen for TB.

### 5.8) Blood Sample collection:

2 ml Blood samples were collected from healthy volunteers and newly diagnosed TB patients, Three months treated TB patients, and Six months treated TB patients by venipuncture.

**5.9) Parameters to be measured:**

Analysing DNA Damage from blood samples.

Comet Length, Comet Height, Comet Area, Comet Intensity, Comet Mean Intensity, Head Diameter, Head Area, Head Intensity, Head Mean Intensity, %DNA in Head, Tail Length, Tail Area, Tail Intensity, Tail Mean Intensity, %DNA in Tail, Tail Moment, Olive Tail Moment.

**5.10) Single cell gel electrophoresis / Comet assay**

The single cell gel electrophoresis (SCGE)/ comet assay, developed by N.P. Singh[1], combines the simplicity of biochemical techniques for detecting DNA single strand breaks (frank strand breaks and incomplete excision repair sites), alkali-labile sites and cross linking with the single cell approach typical of cytogenetic assays. The advantages of the SCGE technique include:

(1) The collection of data at the level of the individual cell, allowing for more robust types of statistical analyses;

(2) The need for small numbers of cells per sample (<10,000);

(3) Its sensitivity for detecting DNA damage; and

(4) That virtually any eukaryotic cell population is amenable to analysis.

**I. Preparation of Reagents**

| Materials: | Supplier (Catalogue Number): |
|---|---|
| Dimethylsulfoxide (DMSO) | - Qualigens (CPW59) |
| Disodium EDTA | - HiMedia (RM1370) |
| Ethidium Bromide | - Sigma (E-8751) |
| Histopaque | - Sigma (1077-1) |
| Phosphate Buffered Saline (PBS) (Ca++, Mg++ free) | - HiMedia (TS1006) |
| Sodium Chloride (NaCl) | - Ranbaxy Rankem (S0160) |
| Sodium Hydroxide (NaOH) | - BDH-Merck (89021) |
| Triton X-100 | - HiMedia (RM 845) |

Trizma Base - Spectrochem (042061)

**Procedure:**

1. PBS (Ca++, Mg++ free): Dulbecco's PBS - 1 L packet: add 990 mL dH□O, adjust pH to 7.4, q.s.(quantity sufficient) to 1000 mL, store at room temperature.
2. Lysing Solution: Ingredients per 1000 mL:

| | |
|---|---|
| 2.5 M NaCl | 146.1 gm |
| 100 mM EDTA | 37.2 gm |
| 10 mM Trizma base | 1.2 gm |

Add ingredients to about 700 mL $dH_2O$ and begin stirring the mixture. Add ~8 gm NaOH and allow the mixture to dissolve (about 20 min). Adjust the pH to 10.0 using concentrated HCl or NaOH. q.s. to 890 mL with $dH_2O$ (the Triton X-100 and DMSO will increase the Volume to the correct amount), store at room temperature.

Final lysing solution: add fresh 1% Triton X-100 and 10% DMSO, and then refrigerate forat least 30 minutes prior to slide addition.

**NOTE:** The purpose of the DMSO in the lysing solution is to scavenge radicals generated by the iron released from hemoglobin when blood or animal tissues are used. It is not needed for other situations or where the slides will be kept in lysing for a brief time only.

3. Electrophoresis Buffer (300 mM NaOH / 1 mM EDTA):

Prepare from stock solutions: 1. 10 N NaOH (200 g/500 mL $dH_2O$)
2. 200 mM EDTA (14.89 g/200 mL $dH_2O$, pH 10)

Store both at room temperature. We prepare the NaOH and EDTA stock solutions every ~2 weeks.

For 1X Buffer (made fresh before each electrophoresis run): per liter, add 30 mL NaOH and 5.0 mL EDTA, q.s. to 1000 mL, mix well. The total volume depends on the gel box capacity.
Prior to use, measure the pH of the buffer to ensure >13.

4. Neutralization Buffer: 0.4 M Tris - 48.5 gm added to ~800 mL $dH_2O$, adjust pH to 7.5 with concentrated (>10 M) HCl: q.s. to 1000 mL with $dH_2O$, store at room temperature.

5. Staining Solution: Ethidium Bromide (EtBr; 10X Stock - 20 µg/mL): add 10 mg in 50mL $dH_2O$, store at room temperature. For 1X stock - mix 1 mL with 9 mL $dH_2O$.

**CAUTION:** Handle EtBr with adequate precaution as it is known carcinogen.

## II. Preparation of Slides for the SCGE/Comet Assay

| Materials: (Catalogue Number): | Supplier |
|---|---|
| Normal Melting Agarose (NMA) (RM273) | - HiMedia |
| Low Melting Point Agarose (LMPA) | - Sigma (A9414) |
| Methanol | - Qualigens |
| Coverslips (No. 1, 24 x 60 mm) | - Blue Star |
| Microcentrifuge Tubes (500010) | - Tarsons |
| Micropipettor and Tips | - Tarsons |
| Microscope Slides, Conventional / Micro gel electrophoresis (MGE) slides | - Blue Label or Es Em Inc (NS 0001) |
| Coplin jars (opaque) | - Tarsons (480000) |
| Horizontal Gel Electrophoresis Apparatus | -GIBCO BRL, Life Technologies |
| Electrophoresis Power Supply | - Techno Source |

**Preparation of base slides**

1. Prepare 1% (500 mg per 50ml PBS) and 0.5% LMPA (250 mg per 50 ml PBS) and 1.0% NMA (500 mg per 50 ml in Milli Q water). Microwave or heat until near boiling and the agarose dissolves. For LMPA, aliquot 5 mL samples into scintillation vials (or other suitable containers) and refrigerate until needed. When needed, briefly melt agarose in microwave or by another appropriate method. Place LMPA vial in a 37ºC dry/water bath to cool and stabilize the temperature.

2. Dip the slides in methanol and burn them over a blue flame to remove the machine oil and dust.

3. While NMA agarose is hot, dip conventional slides up to one-third the frosted area and gently remove (2). Wipe underside of slide to remove agarose and lay the slide in a tray on a flat surface to dry. The slides may be air dried or warmed at 50ºC for quicker drying. Store the slides at room temperature until needed; avoid high humidity conditions. We generally
prepare slides the day before use.

**NOTE:** Slides should be labeled before storage.

**III. Separation of Lymphocytes and treatment *in vitro*:**

1. 1 ml of blood is taken from a healthy donor by venipuncture. Lymphocytes are separated from it using Histopaque-1077. Briefly, blood is diluted 1:1 with PBS or RPMI (without FBS) and layered over 600 ul Histopaque and centrifuged at 800 X g for 20 minutes. The 'buffy' coat is aspirated into 3-5 ml of PBS/ RPMI and centrifuged at 250 X g for 10
minutes to pellet the lymphocytes. The pellet is resuspended in ~1 ml of IRPMI and counted over a Haemocytometer. Nearly 2 X 104 cells per 100 µL of medium are taken for each dose of the test material.

2. One ml of each dose is made in medium (without FBS) and lymphocytes added to it. The eppendorff is inverted to mix the cells and test material.

3. The ependorff-tubes are properly wiped with alcohol and kept in the incubator at 37ºC for 3 hours.

4. After the treatment, the cells are centrifuged at 3000 rpm for 5 minutes to pellet the lymphocytes. The test substance is aspirated and discarded. The pellet is then resuspended in 100 ml of PBS & 10 ml removed for trypan blue viability test.

5. 100 ml of 1% LMPA is added & 80 ml of the suspension layered onto the base slides, and a coverslip is placed on it. Put the slides on ice packs until Agarose layer hardens (~5 to 10 min).

6. Gently slide off coverslip and add a third agarose layer (90 μL LMPA) to the slide. Replace coverslip and return to the slide tray until the agarose layer hardens (~5 to 10 minutes).

7. Remove coverslip and slowly lower slide into cold, freshly made Lysing Solution. Protect from light and refrigerate for a minimum of 2 hours.

**IV.Electrophoresis of Microgel Slides**

The procedure described is for electrophoresis under pH>13 alkaline conditions.

1. After at least 2hour at ~4ºC, gently remove slides from the Lysing Solution. Place slides side by side on the horizontal gel box near one end, sliding them as close together as possible.

2. Fill the buffer reservoirs with freshly made pH>13 Electrophoresis Buffer until the liquid level completely covers the slides (avoid bubbles over the agarose).

3. Let slides sit in the alkaline buffer for 20 minutes to allow for unwinding of the DNA and the expression of alkali-labile damage.

**NOTE:** The longer the exposure to alkali, the greater the expression of alkali-labile damage.

4. Turn on power supply to 24 volts (~0.74 V/cm) and adjust the current to 300 milliamperes by raising or lowering the buffer level. Depending on the purpose of the study and on the extent of migration in control samples, electrophorese the slides for 30 minutes.

**NOTE:** The goal is to obtain migration among the control cells without it being excessive.
The optimal electrophoresis duration differs for different cell types. If crosslinking is one of the endpoints being assessed then having controls with about 25% migrated DNA is useful. A lower voltage, amperage and a longer electrophoresis time may allow for increased sensitivity. Different gel boxes will require different voltage settings to correct for the distance between the anode and the cathode.

5. Turn off the power. Gently lift the slides from the buffer and place on a drain tray. Drop wise coat the slides with Neutralization Buffer, let sit for at least 5 minutes. Drain slides and repeat two more times.

6. Slides may be stained with 80µL 1X Ethidium Bromide, leave for 5 min and then dipped in chilled distilled water to remove excess stain. The coverslip is then placed over it and the slides are scored immediately or dried before staining as in step 7.

7. Drain slides, keep them for 20 min in cold 100% ethanol or cold 100% methanol for dehydration. Air dry the slides and place them in an oven at 50ºC for 30 min. Store in a dry area.

8. When convenient, rehydrate the slides with chilled distilled water for 30 min and stain with EtBr as in step 6 and cover with a fresh coverslip. Before viewing slides, blot away excess liquid on the back and edges. After scoring, remove coverslip, rinse in 100% alcohol to remove stain, let dry, and store for archival purposes if needed.

**NOTE:** Perform steps 1- 4 under yellow / dimmed light. This is to prevent any DNA damage that may arise from fluorescent white light.

**V.Evaluation of DNA Damage**

1. For visualization of DNA damage, observations are made of EtBr-stained DNA using a 40x objective on a fluorescent microscope.

2. Although any image analysis system may be suitable for the quantitation of SCGE data, we use a Komet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK) linked to a CCD camera to assess the quantitative and qualitative extent of DNA damage in the cells by measuring the length of DNA migration and the percentage of migrated DNA. Finally, the program calculates tail moment.

3. The amount of migration per cell, the number of cells with increased migration, the extent of migration among damaged cells, and viability.

**5.11) Statistical Analysis:**

The statistical calculation were done using Graph pad Instat software version 3.01. The results are expressed as mean ± S.E.M. Difference between diseased (TB) and control subjects were assessed using one-way ANOVA followed by Tukey-Kramer Multiple Comparison Test. $P<0.05$ was considered as statistically significant.

# 6. RESULTS

Cells in living beings are affected or damaged due to various agents or factors such as environmental chemicals, radiations, alcohol, smoking, by producing free radicals. All free radicals are normally handled effectively by anti-oxidants / scavengers like glutathione in our body. However when the free radical production is more than the antioxidant in our body, it manifest as disease. So we can anticipate cell damage intern DNA damage in diseased condition. Various drugs are given in the management of diseases. Since most of the drugs are from synthetic source, these chemical substances might induce cell damage. Hence this study has been designed to investigate the extent of cell damage /DNA damage in tuberculosis patients and also to study anti-tubercular drug induced cell damage.

### 6.1) Demographic Details of Study population:

A total of 40 subjects were included in the study. Out of 40, 10 were healthy subjects (G0) and 30 were TB patients with 10 patients are newly diagnosed TB patients (G1), 10 patients is three months treatment (G2) and 10 patients is Six months treatment (G3). The TB Patients were administered the prescribed drugs as Directly Observed Therapy Short course in the hospital, after their breakfast.

**Table 1: Demographic details of study population.**

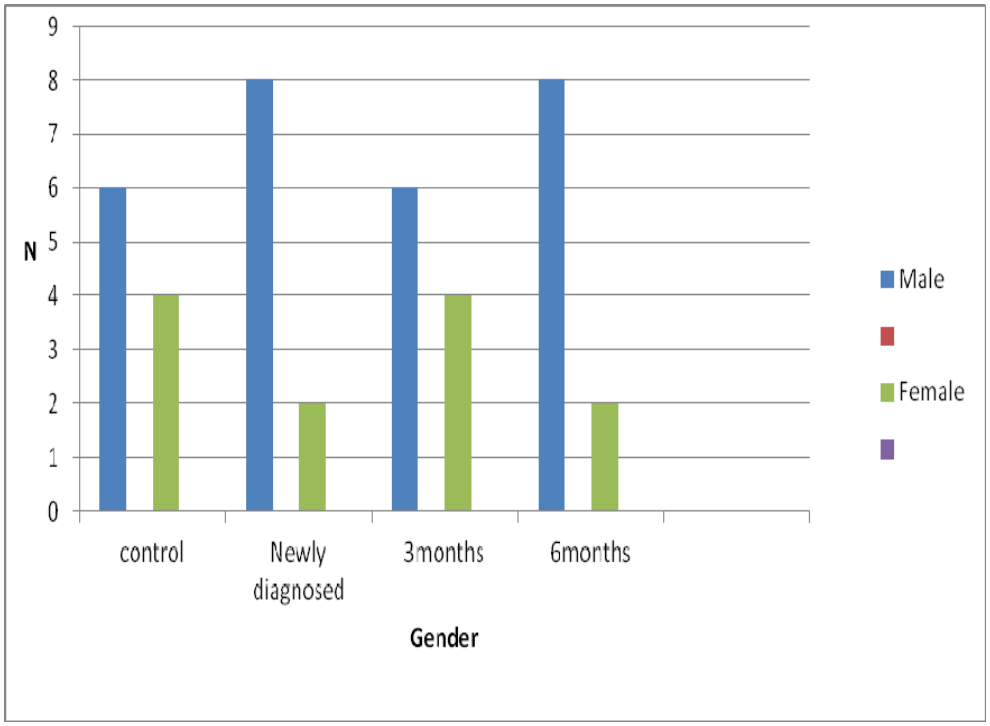
| **Parameters** | **No. of Subjects (%)** | | | | |
|---|---|---|---|---|---|
| | **G0 (n=10)** | **G1 (n=10)** | **G2 (n=10)** | **G3 (n=10)** | **Total (n=40)** |
| **Sex** | | | | | |
| Male | 6 (60%) | 8 (80%) | 6 (60%) | 8 (80%) | 28 (70%) |
| Female | 4 (40%) | 2 (20%) | 4 (40%) | 2 (20%) | 12 (30%) |
| **Age** | | | | | |
| 25-35 yrs | 4 (40%) | 4 (40%) | 4 (40%) | 2 (20%) | 14(35%) |
| 36-45 yrs | 4 (40%) | 2 (20%) | 4 (40%) | 4 (40%) | 14(35%) |
| 46-55 yrs | - | 2 (20%) | - | 2 (20%) | 4 (10%) |
| 56-65 yrs | 2 (20%) | 2 (20%) | 2 (20%) | 2 (20%) | 8 (20%) |
| **Education Status** | | | | | |
| Illiterate | - | 2 (20%) | - | 2 (20%) | 4 (10%) |
| Primary | 6 (60%) | 4 (40%) | 6 (60%) | 6 (60%) | 22 (55%) |
| SSLC | 4 (40%) | 4 (40%) | 2 (20%) | - | 10 (25%) |
| HSE | - | - | - | 2 (20%) | 2(5%) |
| UG | - | - | 2 (20%) | - | 2 (5%) |
| **Occupation** | | | | | |
| Coolie | 2 (20%) | 6 (60%) | 4(40%) | 4 (40%) | 16(40%) |
| Driver | 4 (40%) | - | 2 (20%) | - | 6 (15%) |
| Employee | - | 4 (40%) | 2 (20%) | 4(40%) | 10 (25%) |
| House wife | 4 (40%) | - | 2 (20%) | 2 (20%) | 8 (20%) |
| **Smoking History** | | | | | |
| Smoker | 2 (20%) | 8 (80%) | 6 (60%) | 6 (60%) | 22 (55%) |
| Non-smoker | 8 (80%) | 2 (20%) | 4 (40%) | 4 (40%) | 18 (45%) |
| **Alcoholism** | | | | | |
| Yes | 6 (60%) | 6 (60%) | 6 (60%) | 4 (40%) | 22 (55%) |
| No | 4 (40%) | 4 (40%) | 4 (40%) | 6 (60%) | 18 (45%) |

**Demographic details of Study population:**

**6.1.1) Sex:**

Patients of both sex were included 6 males and 4 females in control group, 8 males and 2 female in newly diagnosed TB patients, 6 males and 4 females in Three months treated TB patients, 8 males and 2 female in Six months treated TB patients. Out of 40 patients 28 (70%) were males and 12 (30%) were females. It is given in Fig.1

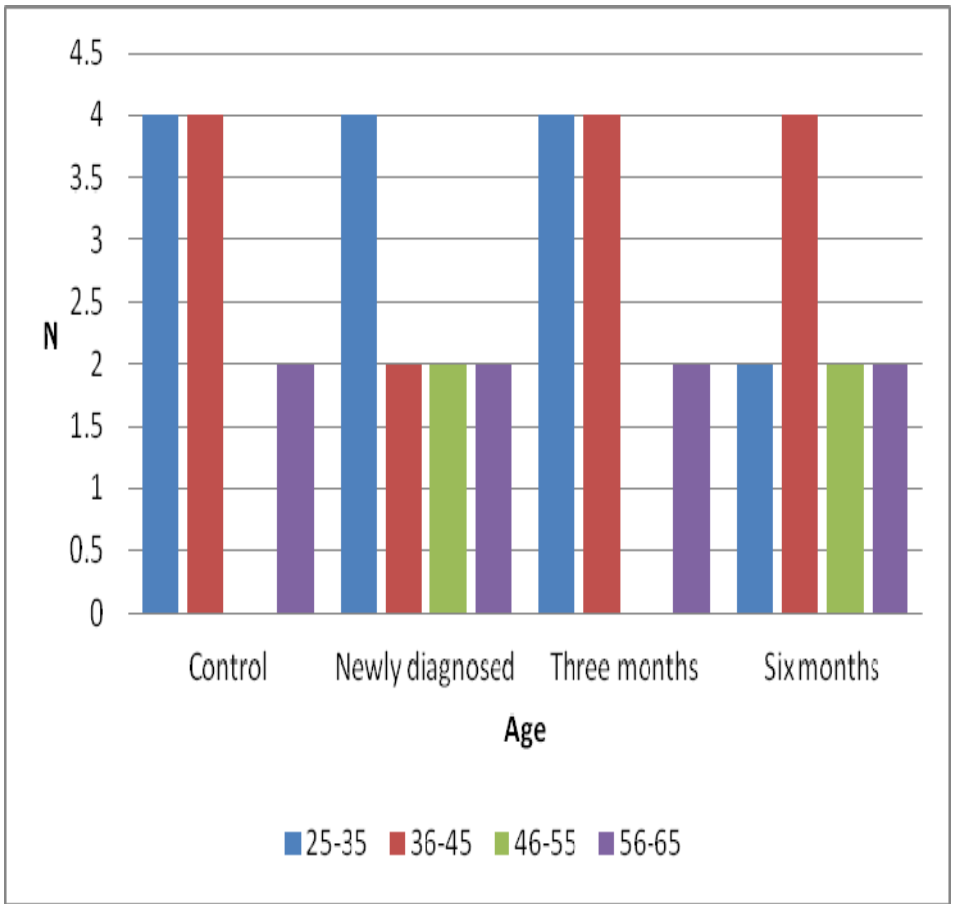
**Fig 1: Gender distribution for different groups of study population**



### 6.1.2) Age:

Respondents with 25-65 years of age were included in the study. Out of 40 Subjects in this study, 14 (35%) were in age group between 25-35 yrs, 14 (35%) were in age group between 36-45 yrs, 4 (10%) were in age group between 46-55yrs and 8 (20%) were in age group between 56-65 yrs were included. (Fig.2).

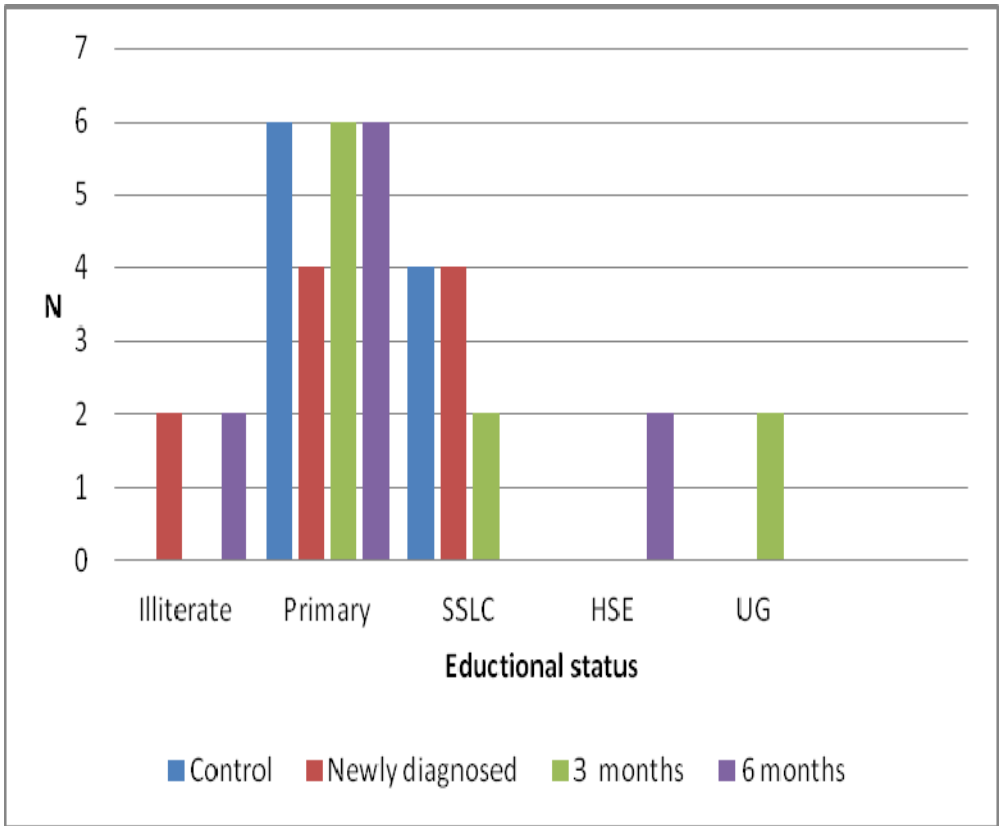
**Fig 2: Age distribution for different groups of study population**



### 6.1.3) Education status:

The study population were categorised in to five groups according to their educational status: 4 (10%) were Illiterate, 22 (55%) had primary education, 10 (25%) completed SSLC education, 2 (5%) in Higher Secondary education and 2 (5%) were graduates. (Fig.3)

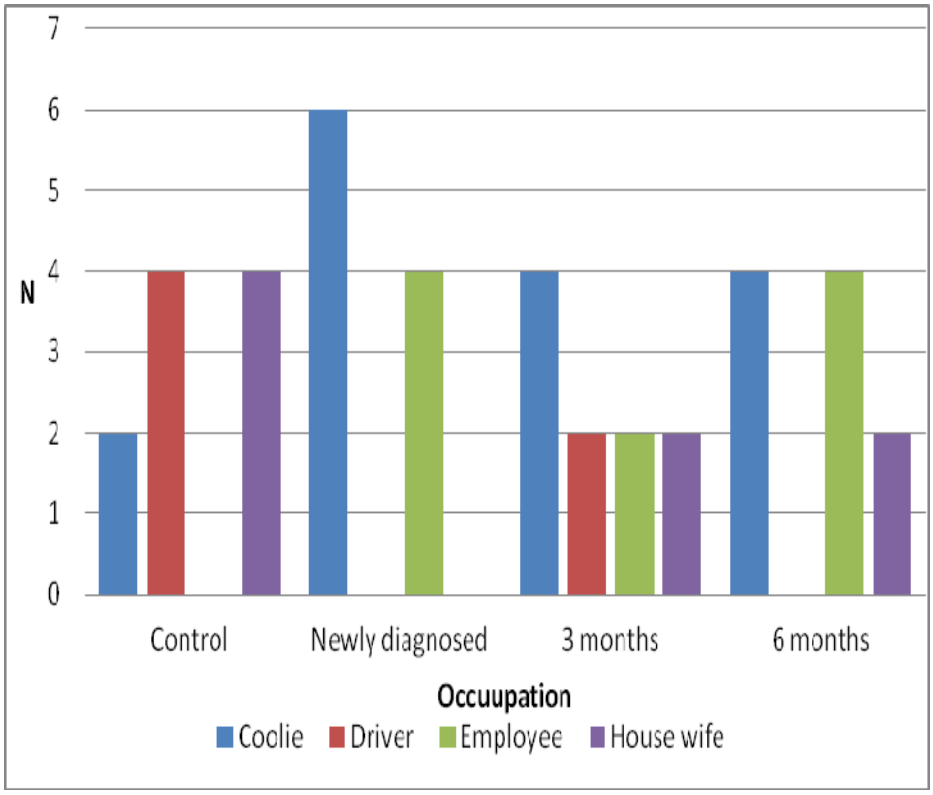
**Fig 3: Educational status for different groups of study population**



### 6.1.4) Occupation:

Out of 40 subjects, 16 (40%) were coolie workers, 6 (15%) were drivers, 10 (25%) were employees, 8 (20%) were house wives.(Fig 4).

**Fig 4: Occupation for different groups of study population**



### 6.1.5) Social History:

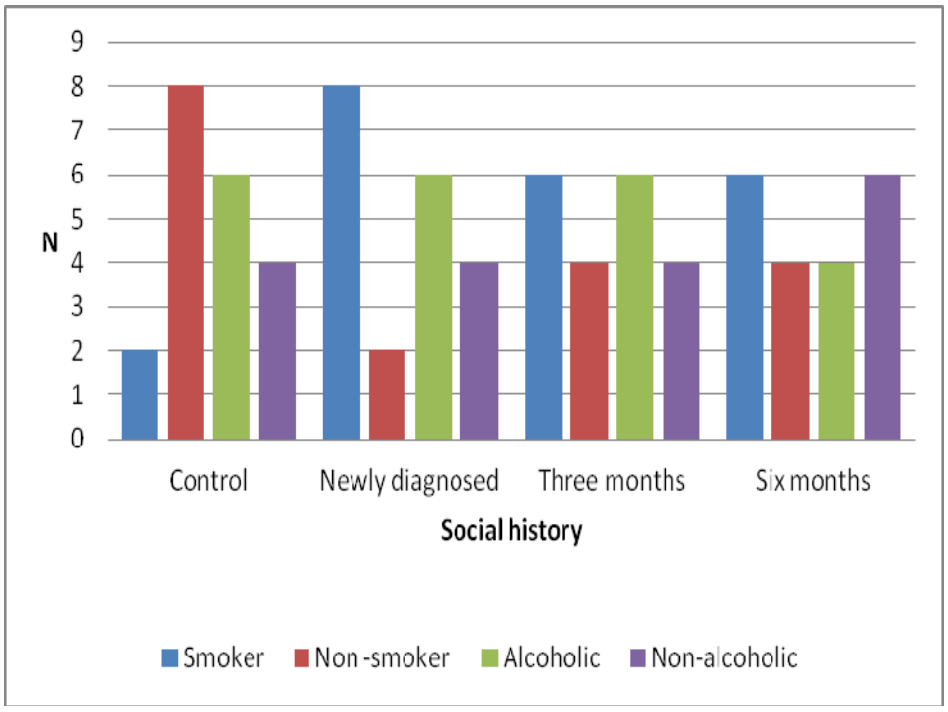
**Smoking history:**

Out of 40 Subjects, 22 (55%) are smoker and 18 (45%) are non-smoker. Out of 30 TB patients, 20 (66.67%) patients are smokers and 10 (33.33%) patients are non-smokers.

**Alcoholism:**

Out of 40 Subjects, 22 (55) are alcoholic and 18 (45%) patients are non-alcoholic. Among 30 TB patients, 16 (53.33%) patients are Alcoholic and 14 (46.67%) are Non-Alcoholic ( Fig 5).

**Fig 5: Social History for different groups of study population**



### 6.2) Quantitative analysis of cellular damage:

The single cell gel electrophoresis technique or comet assay is widely regarded as a quick and reliable method of analysing DNA damage in individual cells. The whole-cell comet assay results obtained in this current study are summarised in table 2. Result of the different comet parameters are given below.

**Table 2: Impact of Tuberculosis on Comet assay indices**

| Parameters | G0 | G1 | G2 | G3 | P value |
|---|---|---|---|---|---|
| **Comet length (µm)** | 80.476 ± 3.624 | 117.61 ± 13.68 | 175.42 ± 61.275** | 193.37± 10.027*** | 0.0005*** |
| **Comet height(µm)** | 122.676 ± 65.623 | 145.19 ± 9.74 | 192.586 ± 47.432 | 163.848 ± 14.617 | 0.2916 |
| **Comet Area(µm²)** | 1380.292 ± 119.51 | 3040.782 ± 1726.7 | 3490.728 ± 900.40 | 4333.006 ± 1048.4 | 0.3221 |
| **Comet Intensity** | 83.939 ± 7.760 | 86.148 ± 1.100 | 137.537 ± 1.958*** | 84.331 ± 1.036 | <0.0001*** |
| **Comet mean Intensity** | 58.942 ± 4.748 | 116.074 ± 9.065*** | 137.704 ± 10.356*** | 102.998 ± 1.639 | <0.0001*** |
| **Head diameter(µm)** | 77.672 ± 9.165 | 88.796 ± 12.520 | 80.226 ± 9.958 | 94.646 ± 8.537 | 0.6287 |
| **Head Area(µm²)** | 6945.414 ± 422.72 | 7759.674 ± 114.20 | 2284.67 ± 542.26*** | 7188.508 ± 202.37 | <0.0001*** |
| **Head intensity** | 5482.84 ± 352.78 | 5693.214 ± 647.64 | 5965.006 ± 1064.1 | 5653.478 ± 227.20 | 0.9632 |
| **Head Mean intensity** | 46.676 ± 5.973 | 80.736 ± 6.196 | 72.238 ± 11.842 | 82.116 ± 9.190* | 0.0363* |
| **%DNA in head** | 92.186 ± 1.406 | 91.734 ± 1.188 | 85.558 ± 7.036 | 83.908 ± 4.378 | 0.421 |
| **Tail length(µm)** | 5.81 ± 1.120 | 7.712 ± 0.613 | 7.486 ± 0.770 | 5.494 ± 0.675 | 0.1462 |
| **Tail Area(µm²)** | 30.52 ± 4.998 | 43.122 ± 6.461 | 66.746 ± 6.896* | 61.624 ± 9.071* | 0.0075 |

| | | | | | |
|---|---|---|---|---|---|
| **Tail intensity** | 565.47 ± 138.60 | 274.656 ± 65.111 | 312.856 ± 29.384 | 434.682 ± 30.022 | 0.0774 |
| **Tail mean intensity** | 361.798 ± 61.444 | 663.144 ± 157.72 | 1001.86 ± 92.912** | 587.856 ± 86.700 | 0.0051** |
| **%DNA in tail** | 2.454 ± 0.5919 | 5.328 ± 0.6341** | 5.09 ± 0.4283* | 3.812 ± 0.4131 | 0.0049** |
| **Tail Moment** | 0.072 ± 0.0143 | 0.059 ± 0.009 | 0.0686 ± 0.0085 | 0.0582 ± 0.008 | 0.8360 |
| **OTM** | 0.098 ± 0.0124 | 0.162 ± 0.0174 | 0.234 ± 0.0457 | 0.242 ± 0.0538 | 0.0449* |

All the data was shown as mean ± S.E.M values. *, **, ***, p< 0.0001: Statistically significant difference from controls; (One way ANOVA with post test)

* considered as significant

** considered as very significant

*** considered as extremely significant.

ns considered as not significant.

Comet assay of white blood cells of study subjects shows extremely significant increase in comet length in TB patients, when compared to control group. (G3=193.37; G2=175.42; G1=117.61; G0=80.47; P=0.0005) Similarly a significant increase in Comet intensity, Comet mean intensity, Head Area, Head mean intensity, Tail Area, tail mean intensity, %DNA in tail and Olive tail moment were found in TB patients when compared to control.

When a comparison is made among the TB patients ( G1, G2, G3), Six months Anti-tubercular drug treatment exhibited significantly extensive damage in DNA than 3 months treated patients and newly diagnosed patients.

Some comet parameters like Comet height, Comet Area, head diameter, head intensity, %DNA in head, Tail length, Tail intensity, Tail moment showed increase but not statistically significant.

**Comet area:** The number of pixels in the binary comet image (including pixels that belong to fragments) is counted. The area is returned in calibrated units.

**Comet intensity**: Sums all pixel intensities from the grayscale image when there is a corresponding pixel in the binary mask image. The resulting value is normalized by the maximum intensity value that can be represented in the data buffer.

**Tail moment:** The product of distance and normalized intensity integrated over the tail length. A damage measure combining the amount of DNA in the tail with the distance of migration.

**Tail area:** The number of pixels in the binary tail region of image (including pixels that belong to fragments) is counted. The area is returned in calibrated units.

**Tail length:** Tail length is the distance of DNA migration from the body of the nuclear core and it is used to evaluate the extent of DNA damage.

**%DNA in Tail:** The integrated tail intensity x 100 divided by the total integrated cell intensity for a normalized measure of the percent of total cell DNA found in the tail.

**Olive Tail moment:** Tail moment is defined as the product of the tail length and the fraction of total DNA in the tail. Tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed / broken pieces (represented by the intensity of DNA in the tail).

**COMET ASSAY IMAGE:**

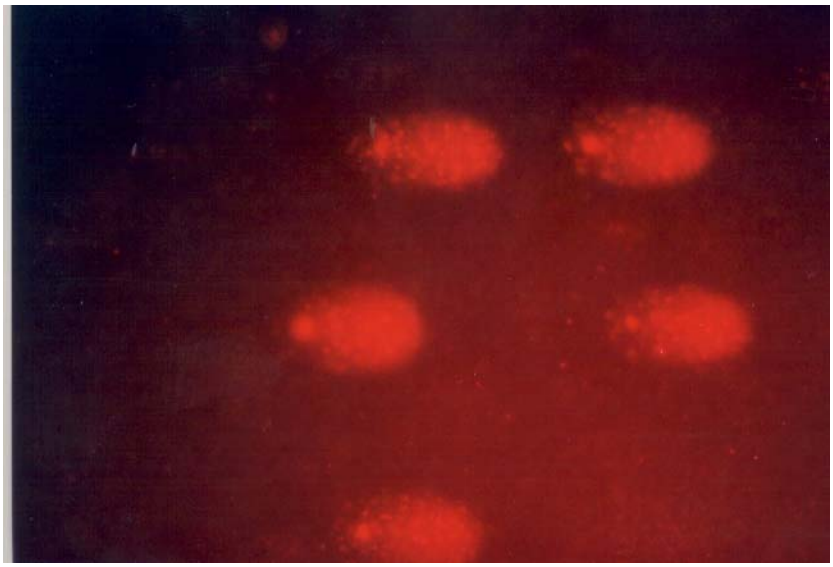


Fig 6 : Comet assay image.Ethidium bromide stained slide when viewed under fluorescent microscope at 40x.

**Table 3: Tukey-Kramer Multiple Comparison test for Comet parameters among study population**

| Parameters | Groups | Mean Difference | q Value | P value |
|---|---|---|---|---|
| Comet Length (μm) | G0 vs G1 | -37.134 | 2.279 | ns>0.05 |
| | G0 vs G2 | -94.950 | 5.827 | <0.01** |
| | G0 vs G3 | -112.90 | 6.928 | <0.001*** |
| | G1 vs G2 | -57.816 | 3.548 | ns>0.05 |
| | G1 vs G3 | -75.764 | 4.650 | <0.05* |
| | G2 vs G3 | -17.948 | 1.101 | ns>0.05 |
| Comet intensity | G0 vs G1 | -2.209 | 0.5425 | ns>0.05 |
| | G0 vs G2 | -53.593 | 13.162 | <0.001*** |
| | G0 vs G3 | -0.3918 | 0.09622 | ns>0.05 |
| | G1 vs G2 | -51.389 | 12.620 | <0.001*** |
| | G1 vs G3 | 1.817 | 0.4463 | ns>0.05 |
| | G2 vs G3 | 53.206 | 13.066 | <0.001*** |
| Comet mean intensity | G0 vs G1 | -57.132 | 7.799 | <0.001*** |
| | G0 vs G2 | -78.762 | 10.752 | <0.001*** |
| | G0 vs G3 | -44.056 | 6.014 | <0.01** |
| | G1 vs G2 | -21.630 | 2.953 | ns >0.05 |
| | G1 vs G3 | 13.076 | 1.785 | ns>0.05 |
| | G2 vs G3 | 34.706 | 4.738 | <0.05* |
| Head Area | G0 vs G1 | -814.26 | 2.244 | ns>0.05 |
| | G0 vs G2 | 4660.7 | 12.843 | <0.001*** |
| | G0 vs G3 | -243.09 | 0.6699 | ns>0.05 |

| | | | | |
|---|---|---|---|---|
| | G1 vs G2 | 547.9 | 15.087 | <0.001*** |
| | G1 vs G3 | 571.17 | 1.574 | ns>0.05 |
| | G2vs G3 | 4903.7 | 13.513 | <0.001*** |
| Head mean intensity | G0 vs G1 | -34.060 | 3.941 | ns>0.05 |
| | G0 vs G2 | -25.562 | 2.958 | ns>0.05 |
| | G0 vs G3 | -35.440 | 4.101 | <0.05* |
| | G1 vs G2 | 8.498 | 0.9833 | ns>0.05 |
| | G1 vs G3 | -1.380 | 0.1597 | ns>0.05 |
| | G2 vs G3 | -9.878 | 1.143 | ns>0.05 |
| Tail area ($\mu m^2$) | G0 vs G1 | -12.602 | 1.798 | ns>0.05 |
| | G0 vs G2 | -36.226 | 5.168 | <0.05* |
| | G0 vs G3 | -31.104 | 4.437 | <0.05* |
| | G1 vs G2 | -23.624 | 3.370 | ns>0.05 |
| | G1 vs G3 | -18.502 | 2.639 | ns>0.05 |
| | G2 vs G3 | 5.122 | 0.7307 | ns>0.05 |
| Tail mean intensity | G0 vs G1 | -301.35 | 2.847 | ns>0.05 |
| | G0 vs G2 | -640.06 | 6.048 | <0.01** |
| | G0 vs G3 | -226.06 | 2.136 | ns>0.05 |
| | G1 vs G2 | -338.72 | 3.201 | ns>0.05 |
| | G1 vs G3 | 75.288 | 0.7114 | ns>0.05 |
| | G2 vs G3 | 3.912 | 3.912 | ns>0.05 |

| | | | | |
|---|---|---|---|---|
| %DNA in tail | G0 vs G1 | -2.874 | 5.464 | <0.01** |
| | G0 vs G2 | -2.636 | 5.012 | <0.05* |
| | G0 vs G3 | -1.358 | 2.582 | ns>0.05 |
| | G1 vs G2 | 0.2380 | 0.4525 | ns>0.05 |
| | G1 vs G3 | 1.516 | 2.882 | ns>0.05 |
| | G2 vs G3 | 1.278 | 2.430 | ns>0.05 |
| Olive Tail Moment | G0 vs G1 | -0.06400 | 1.734 | ns>0.05 |
| | G0 vs G2 | -0.1360 | 3.685 | ns>0.05 |
| | G0 vs G3 | -0.1440 | 3.902 | ns>0.05 |
| | G1 vs G2 | -0.0720 | 1.951 | ns>0.05 |
| | G1 vs G3 | 0.0800 | 2.168 | ns>0.05 |
| | G2 vs G3 | 0.0080 | 0.2168 | ns>0.05 |

The result shown in (Table 2) was analyzes by Tukey-Kramer multiple Comparison Test in order to verify the difference between the groups.

* considered significant

** considered very significant

*** considered extremely significant

ns considered not significant.

TB patients showed significant increase in Comet length when compared to control (P value is 0.0005), when further analyzed with Tukey- Kramer Multiple Comparison Test it showed no significant difference in G1 compared to G0. Significant difference in newly diagnosed TB patients compared to control.

Significant difference is observed in TB drug treatment patients ( ie), G2 & G3, when compared to G0.

TB patients showed significant increase in comet intensity when compared to control (P value is <0.0001), when further analyzed with Tukey- Kramer multiple Comparison test, it showed a significant difference in three months TB drug treatment patients compared to control and G1. When three months & six months treated Tb patients are compared, six months treated TB patients shows extreme significant difference.

TB patients showed significant increase in Comet mean intensity when compared to control group (P value is <0.0001), when further analysed with Tukey- Kramer multiple Comparison Test, an extremely significant difference in G1 & G2 when compared with G0. When compared with six months and three months treated patients, three months treated TB patients showed significant difference.

TB patients showed significant increase in Head Area when compared to control group (P value is <0.0001), when further analyzed with Tukey- Kramer Multiple Comparison, three months TB drug treatment patients shows extremely significant difference when compared with control group. TB drug treatment patients showed extremely significant difference when compared to newly diagnosed patients.

TB patients showed significant increase in Head mean intensity when compared to control group (P value is 0.0363), when further analyzed with Tukey- Kramer multiple Comparison test, it shows a significant difference in six months treated TB patients when compared with control group (G0).

TB patients showed significant increase in Tail area when compared to control group (P value is 0.0075), when further

analyzed with Tukey-Kramer Multiple Comparison Test it showed, no significant difference in G1 compared to G0. Very significant difference is observed in three months TB drug treated and six months treated TB patients compared to G0.

TB patients showed significant increase in Tail mean intensity when compared to control group (P value is 0.0051), when further analyzed with Tukey- Kramer Multiple Comparison test it showed, very significant difference was observed in G2 group compared with G0. And there is no significant difference is observed in any other groups.

TB patients showed significant increase in %DNA in tail when compared to control group (P value is 0.0049), when further analyzed with Tukey- Kramer Multiple Comparison test it showed, there is very significant difference in newly diagnosed patients and three months treated TB patients. No significant difference is observed in six months treated TB patients.

TB patients showed significant increase in Olive tail moment when compared to control group (P value is 0.0449), when further analyzed with Tukey- Kramer Multiple Comparison Test it showed, there is no significant difference is observed in all cases.

**Table 4: Comparison of the Comet parameters between cases and control**

| Parameters | G0 | G1 | G2 | G3 | P value |
|---|---|---|---|---|---|
| **Comet Length (µm)** | 80.47 ± 3.62 | 117.61 ± 13.68 | 175.426 ± 27.587** | 193.374± 10.027*** | 0.0005*** |
| **Tail Length (µm)** | 5.81 ± 1.12 | 7.712 ± 0.613 | 7.486 ± 0.770 | 5.494 ± 0.675 | 0.1462 |
| **Head Diameter (µm)** | 77.67 ± 9.16 | 88.79 ± 12.52 | 80.226 ± 9.958 | 94.646 ± 8.537 | 0.6287 |

The values are expressed as mean ± S.E.M; (One way ANOVA with post test).

** considered very significant

*** considered as extremely significant

ns considered not significant

During Electrophoresis, the negatively charged DNA fragments migrate from the nucleus of each cell towards the anode producing the images which appear like comets. A comet thus formed with the tail of the comet being directly proportional to the extent of DNA damage in a single cell.

Comet length showed statistically significant increase in patients than that of control groups. (117.61 ± 13.68; 80.476 ± 3.624), two fold increase in comet length was found in three months treated TB patients and more than two fold increase in six months treated TB patients group. P value is 0.0005.

**Comparison of the Comet parameters between cases and control**

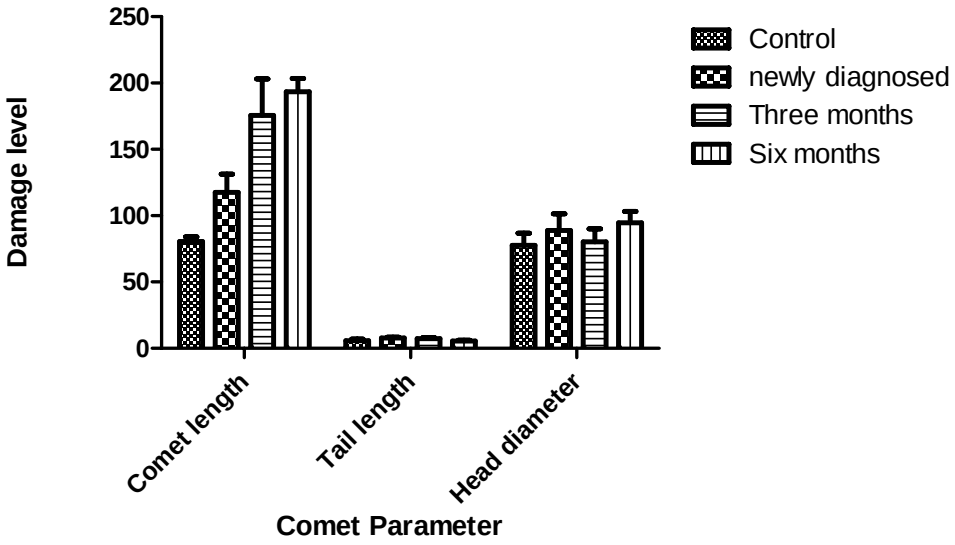



Fig 7: Comparison of comet parameters between cases and control (ie), Comet length, Tail length, Head diameter.

**Table 5: Comparison of some commonly used metrics in leucocytes in tuberculosis subjects with control**

| Parameters | G0 | G1 | G2 | G3 | P value |
|---|---|---|---|---|---|
| **Tail length (µm)** | 5.81 ± 1.12 | 7.832 ± 0.613 | 7.486 ± 0.77 | 5.494 ± 0.675 | (0.1462) |
| **%DNA in Tail** | 2.454 ± 0.5919 | 5.328 ± 0.6341** | 5.09 ± 0.43* | 3.812 ± 0.41 | (0.0049**) |
| **OTM** | 0.098 ± 0.0124 | 0.162 ± 0.01744 | 0.234 ± 0.045 | 0.242 ± 0.054 | (0.0449)* |

The values are expressed as mean ± S.E.M; (One way ANOVA with post test) ** considered very significant, * considered significant, ns considered not significant.

Results of three different parameters namely, tail length, %DNA in tail and Olive tail moment have been presented in Table 5.

**Tail length:** Tail length is the distance of DNA migration from the body of the nuclear core, which is related directly to the fragment size and it is expected to be proportional to the extent of DNA damage. Newly diagnosed TB patients demonstrated non significantly increased tail length values (7.832 ± 0.613) when compared to control subjects and drug treated patients (5.81 ± 1.12; 7.486 ± 0.77; 5.494 ± 0.675).

**%DNA in tail:** It gives an idea regarding the damaged DNA content in individual cells, measured as the total intensity of ethidium bromide in each comet tail, verified by DNA leached out of the cell when exposed to alkaline electrophoretic conditions. It is defined as the 'ratio of tail optical intensity to the sum of tail and head optical intensity', multiplied by 100. Very significant increase in %DNA in tail in newly diagnosed TB patients were observed (5.328 ± 0.634) when compared to control group. Three months treated TB patients showed significant difference (5.09 ± 0.4283) when compared with control group. P value is 0.0049.

**Olive tail Moment (OTM):** It is defined as the fraction of tail DNA multiplied by the distance between the profile centres of gravity for DNA in head and tail. OTM incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed/ broken pieces (represented by the intensity of DNA in the tail). OTM was observed to be least in controls and highest in Six months treated group, and the difference was significant (0.098 ± 0.0124; 0.162 ± 0.0174; 0.234 ± 0.0457; 0.242 ± 0.0538). P value is o.0449.

**Comparison of some commonly used metrics in leucocytes in tuberculosis subjects with control**

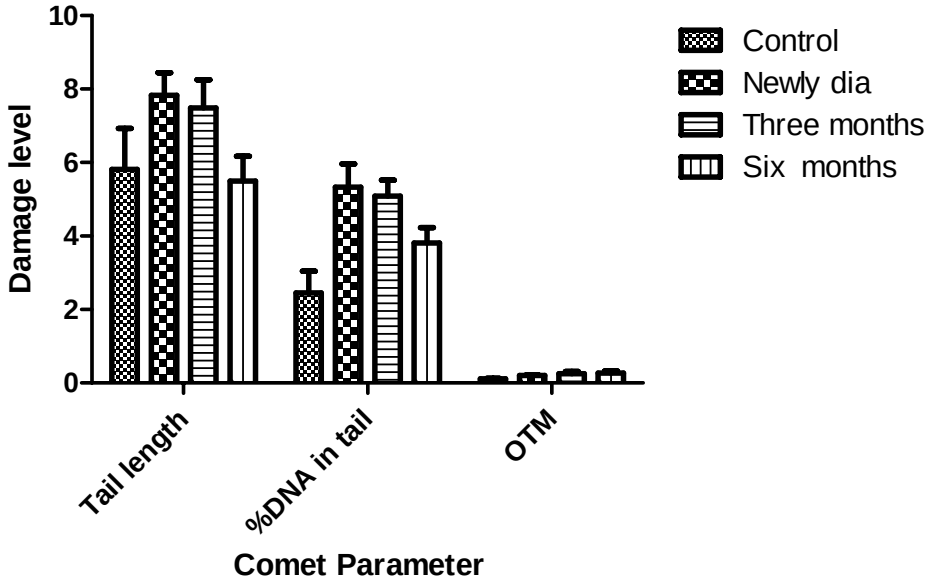



Fig 8: Comparison of some commonly used comet parameters are Tail length, %DNA in tail, OTM-Olive tail moment.

**Table 6: Qualitative evaluation of cell damage in tuberculosis patients**

The number of cells >C1 were found increased in G2 and G3 group patients. Although the G1 group patients did not show any significant changes when compared with Control (G0) group at p value <0.0001.

| | G0 | G1 | G2 | G3 | P value |
|---|---|---|---|---|---|
| **C0** | 52.09 ± 4.72 | 34.36 ± 3.510** | 13.216 ± 2.46*** | 11.2 ± 1.49*** | < 0.0001 |
| **C1** | 26.38 ± 2.21 | 47.6 ± 4.659** | 64.84 ± 3.83*** | 75.29 ± 3.36*** | < 0.0001 |
| **>C1** | 1.02 ± 0.25 | 1.73 ± 0.33 | 12.88 ± 1.63*** | 15.31 ± 1.74*** | < 0.0001 |

The values are expressed as mean ± S.E.M ;( One way ANOVA with post test).

Co=undamaged cells, C1=Mild and moderate cells, >C1= High Damage cells

** considered very significant

*** considered extremely significant

The result was shown in ( Table 6 and fig 8) p value was found to be extremely significant for C0, C1, and >C1 in all the four groups (ie), Control, Newly diagnosed TB patients, Three months treated TB patients and Six months treated Tb patients.

White blood cells of study subjects were used for comet assay. When the cells were viewed through Fluorescent microscope, the field showed both damaged and undamaged cells. The numbers of undamaged cells are significantly more in healthy volunteers when compared to newly diagnosed Tuberculosis patients. (52.09 ± 4.720; 34.36 ± 3.510; p<0.0001). Undamaged cells were very much decreased in six months drug treated group than three

months drug treated and newly diagnosed patients. (11.2 ± 1.495; 13.21 ± 2.467; 34.3 ± 3.510; $p<0.0001$).

Mild to moderately damaged cells were less in number in healthy subjects and showed significant increase in diseased patients, and showed greater increase in TB drug treated patients (Table 6).

Similarly, highly damaged cells also were very high when compared to control subjects and TB patients before the initiation of treatment. The study results indicate the influence of Mycobacterium tuberculi bacilli infection and the impact of anti-tubercular drugs on cell damage.

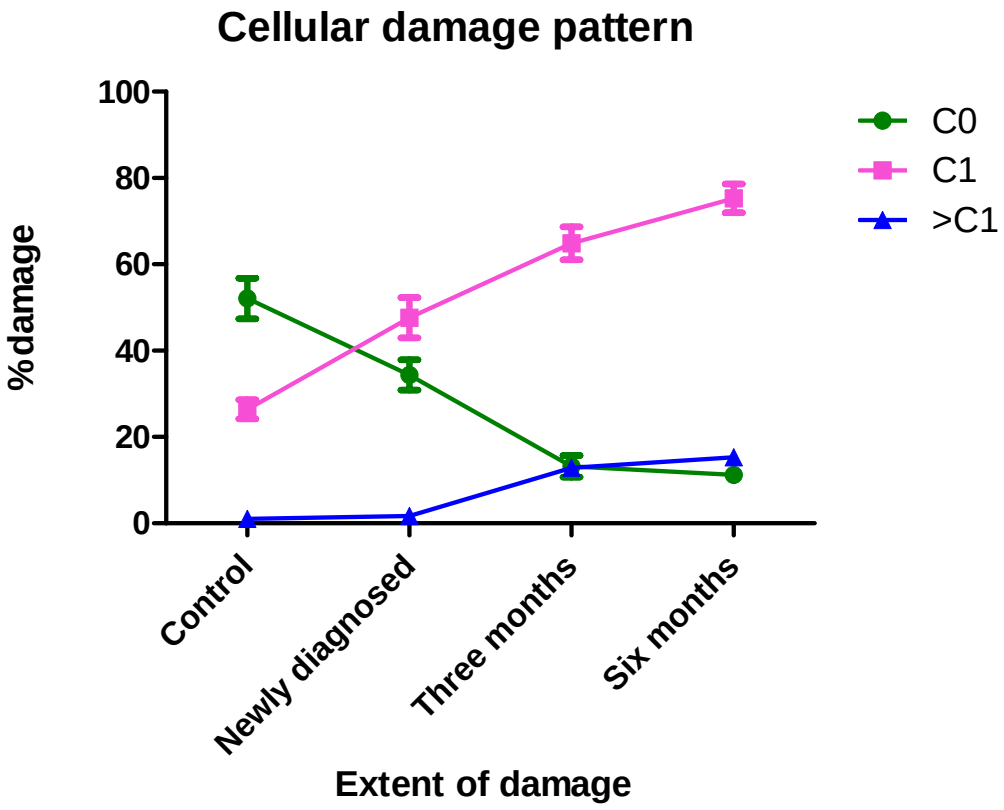



Fig 9: Cellular damage pattern (all the data are shown in mean ± S.E.M values. C0= undamaged cells, C1= mild and moderate cells, >C1= highly damaged cells.

**Table 7: Comparison of sex for Comet length and its significance**

The comparison of both male and female sex groups, the comet length shows extremely significant difference in G2 group, when compared with the control group. And in G3 group shows very significant difference when compared with the Control group in male patients. P value is <0.0003. Female patients didn't show any significant difference in all groups, when compared with control.

| Sex | G0 | G1 | G2 | G3 | P value |
|---|---|---|---|---|---|
| **Male** | 85.09 ± 2.63 | 120.82 ± 17.17 | 203.53 ± 12.56*** | 189.17 ± 11.75** | 0.0003*** |
| **Female** | 73.55 ± 5.53 | 104.74 ± 0.00 | 133.26 ± 64.60 | 210.16 ± 0.00 | ns |

*** considered extremely significant
**considered very significant
ns considered not significant

**Fig 10: Comparison of sex for Comet length and its significance**

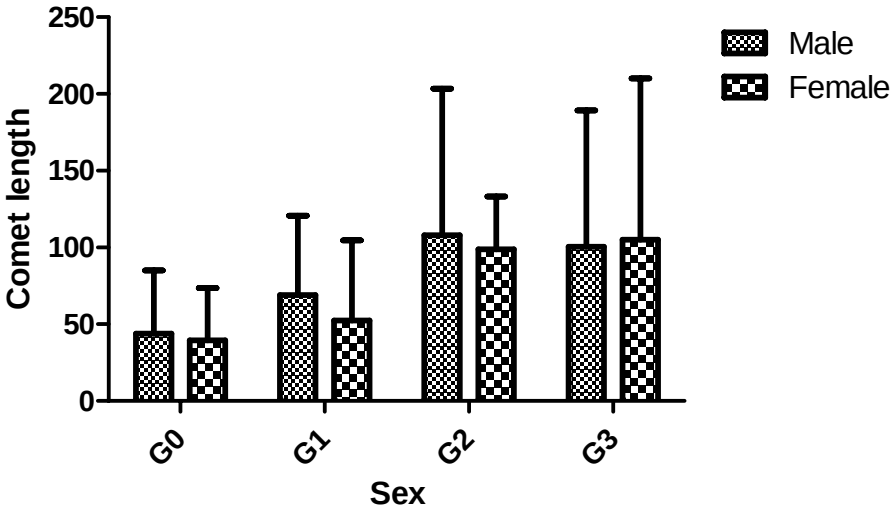


**Table 8: Comparison of Alcoholism for comet length and its significance**

Comparison of both the Alcoholic and non-alcoholic group, comet length shows very significant difference in both three months treated TB patients and in six months treated TB patients when compared with control group in Alcoholism. P value is <0.0010. And in Non-alcoholic group didn't shows any significant differences in all groups when compared with G0 group.

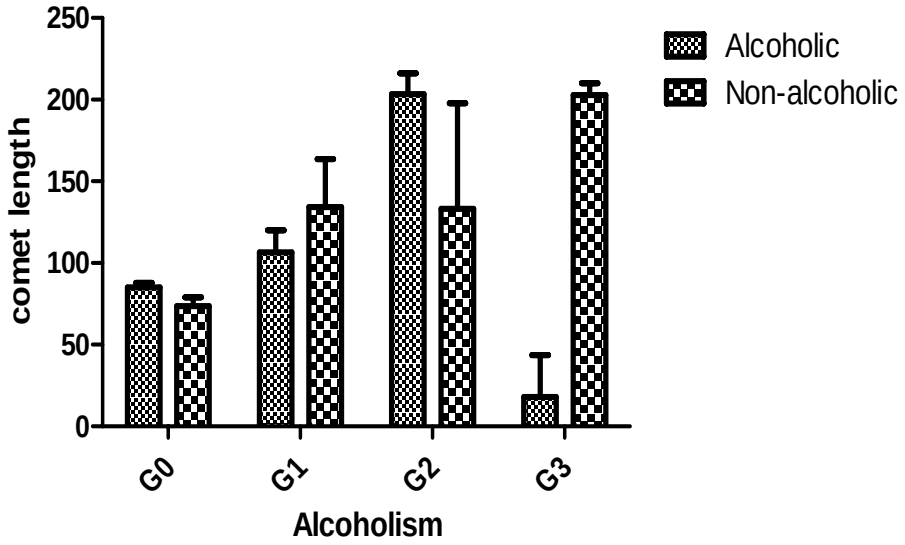
| **Parameters** | **G0** | **G1** | **G2** | **G3** | **P value** |
|---|---|---|---|---|---|
| **Alcoholic** | 85.09 ± 2.63 | 106.54 ± 13.49 | 203.53 ± 12.56** | 179.99 ± 25.56** | 0.0010*** |
| **Non-alcoholic** | 73.55 ± 5.53 | 134.20 ± 29.46 | 133.26 ± 64.60 | 202.91 ± 7.24 | ns |

*** considered extremely significant

** considered very significant

ns considered not significant

**Fig 11: Comparison of Alcoholism for comet length and its significane**



# 7. DISCUSSION

The importance of studying DNA damage has been recognized by many scientists. Subjects with Healthy volunteers (G0), newly diagnosed TB patients (G1), Three months treated TB patients (G2), Six months treated TB patients(G3) were included in the present study. Age and long duration of disease are two such factors which contribute to the development of DNA damage and thereby cell damage (Fournie GJ et al.1993)[54]. Thus, the present study may help us to identify the extent of cellular damage in individuals suffering from the Bacterial infectious disease. It can also help to identify the effect of the medication on reactive oxygen species generation and thereby DNA damage

The cellular damage in TB patients may also occur due to the environmental conditions, Food intake, Social history, allergic substances, also some chemical substances and radiations. Humans are continuously exposed to genotoxic chemicals which can damage the DNA. Today it is well known that DNA damaging effects of chemical agents are associated with mutagenic and carcinogenic events, which could be the starting point for the development of Cancer (Pitot and Dragan et al.1994) [55]. Comet assay are useful combination when testing for the potential DNA damaging effects of chemicals (Maria Andersson et al. 2006)[56].

Ionizing radiation produces DNA damage by direct hits. Free radical production is an important aspect of ionizing radiation. In addition to the other effects, free radicals also cause DNA damage. (Anderson et al.2000)[57]. The mechanistic studies (Curtis et al .1988) have shown that micronucleus formation may be due to free radical generation from an agent leading to lipid peroxidation of membrane causing the breakage of the DNA and covalently binding between the product of lipid peroxidation and DNA. The free radicals play an important role in a number of biological processes, some of which are necessary for life, such as intercellular killing of bacteria by neutropil granulocytes and also in certain cell signalling process[58].

The earlier study suggests that the Rifampacin has damaging effect on the DNA and this damage may be induced by free radicals generated by this drug (O. Awodele et al. 2010) [59].

In the present study, it is attempted to define the most reliable comet measurements that would truly reflect the extent of DNA damage induced by the bacterial infectious disease. And was assessed by performing the alkaline comet assay on peripheral blood samples of the patients. Measure of number of highly damaged cells (>C1), Mild and moderate damaged cells (C1) and undamaged cells (C0) formed the qualitative evaluation. In this study, it was found that percentage of highly damage cells (>C1) was significantly high in numbers in Three months treated TB patients and in Six months treated TB patients. These results show that Mycobacterium tuberculi infection may contribute towards early cell damage of these patients.

The length of DNA migration commonly referred as the Tail length (it is measured from the centre of the head to the end of the tail) is the first comet measurement outcome used to quantify DNA damage. The length of DNA migration is directly related to the loops of released DNA or the size of DNA fragments and is proportional to the amount of strand breaks and alkali labile sites (Kumaravel TS et al. 2006)[60]. The difference observed in the increase tail length in the Tuberculosis patients when compared to control was insignificant. The percentage DNA in the tail is the second primary comet measurement on which other derived units are based. The percentage DNA in tail is directly proportional to the amount of damaged DNA (Collins AR et al 1997) [35].

Olive tail moment (OTM) is the tail moment the product of the tail length and the fraction of total DNA in the tail. Tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed / broken pieces (represented by the intensity of DNA in the tail). A significant difference of (P value is 0.0449), control group was compared with newly diagnosed, three months, and six months. Any change in the level of DNA damage will be reflected most

accurately by Olive tail moment measurements. Increase in Olive tail moment in our study confirms the role of Mycobacterial infection on DNA damage.

(Patricia C et al. 2011) have analyzed the expression following DNA damage in M.tuberculosis of a number of other genes which are DNA damage inducible in Escherichia coli. While many of these genes were also induced by DNA damage in M.tuberculosis, some were not. In addition, one gene (ruvC) which is not induced by DNA damage in E.coli was induced in M.tuberculosis, a result likely linked to its different transcriptional arrangement in M.tuberculosis[2]. The repair of DNA damage is expected to be particularly important to intracellular pathogens such as Mycobacterium tuberculosis, and so it is of interest to examine the response of M. tuberculosis to DNA damage. The expression of recA, a key component in DNA repair and recombination, is induced by DNA damage in M. Tuberculosis (Brooks PC et al. 2011) [2].

The mean± S.E.M value of comet length in control group of patient was 80.476 ± 3.624, for newly diagnosed TB patients it was117.61 ± 13.689, for Three months treated patients it was 175.426 ± 27.587 and for Six months treated patients193.374± 10.027 which was found to be extremely significant (P value 0.0005). Comet length showed extremely significant difference among the cases when compared with control subjects indicating increased DNA damage among cases and this is inconsistent with other studies.

The comet length shows significant difference in Alcohol consumption and smokers, it is greater in males taking anti-tubercular drug treatment when compared with females, which clearly indicates the influence of alcohol induced oxidative stress on cell damage. Oxidative stress and DNA damage are increased in pulmonary tuberculosis patients. Increased oxidative stress associated DNA damage may be one of the pathogenetic mechanisms involved in the disorders suggested to be associated with pulmonary tuberculosis. (Sahbettin Selek et al.2011)[5].

Certain drugs are known to induce DNA damage in healthy cells and potentiate the oxidative stress generated during cellular events (Gonzalez c et al 2002)[36.] To the best of our knowledge, no study has been performed to

understand the effect of anti-tubercular drugs on cell damage with the help of comet assay. It was observed that Six months treated Tuberculosis patients taking anti-tubercular drug treatment of Rifampacin and Isoniazid showed an extremely significant cellular damage. And in three months treated Tb patients taking anti-tubercular drugs like Rifampacin, Isoniazid, Pyrazinamide, and Ethambutol also showed a significant difference in cellular damage. Major anti-TB drugs include isoniazid (INH), Rifampacin (RMP), and Ethambutol (EMB). The co-administration of Rifampacin and antioxidants (Vit.C & Vit. E) has protective effect on the damaging potentials of Rifampacin on the DNA. It may then be recommended that the clinician may incorporate antioxidants in the regimen of patients with tuberculosis so as to reduce the possible adverse effect on the DNA (O.Awodele et al 2010)[59].

Thus, in the light of our observation, it is suggested that Tuberculosis patients showed increased DNA damage as significant differences were detected between Control, newly diagnosed TB patients, Three months treated TB patients and Six months treated TB patients in terms of frequencies of damaged cells. The result of the present study reveal that patients undergoing therapy had significantly greater DNA damage as compared with untreated patients, indicating that bacterial infection and drug therapy are causal factors.

# 8. SUMMARY & CONCLUSION

- In conclusion, the present study suggest the presence of significant cellular damage in Six months treated tuberculosis patients and in three months treated tuberculosis patients compared with normal healthy individuals, which is suggestive of detrimental effects of tuberculosis.

- Further, the DNA damaging effects of anti-tubercular drugs like Rifampacin and Isoniazid showed greater possibility of cellular damage.

- Extent of cell damage is high in males when compared with females, due to adduction of Alcohol and smoking.

- The high level of concordance of the result obtained in the comet assay showed that the comet assay is not only sensitive enough to detect low levels of DNA damage in human lymphocytes, but it also highly specific and give an idea about how the anti-tubercular drugs causes DNA damage to the human.

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## DATA ENTRY FORM

**PATIENTS DETAILS**

**NAME:**

**SEX:** **AGE:**

**PAST MEDICATION HISTORY**

**FAMILY HISTORY:**
**SOCIAL HISTORY:**
**SMOKER:** **ALCOHOLIC:**

**DURATION OF TUBERCULOSIS (TB):**

**PHASE (INTENSIVE / CONTIUATION):**

# CONSENT FORM

I Mr/Mrs/Miss..... patient of Dr.....Tiruchengodu Govt. Hospital, hereby declare that I have been told in detail by Mrs. Ushananthini A.S. [II M.Pharm (Pharmacy Practice), Swamy Vivekanandha College of Pharmacy,Elayampalayam]about the study titled, **"ASSESSMENT OF DNA DAMAGE IN PULMONARY TUBERCULOSIS PATIENTS BY SINGLE CELL GEL ELECTOPHORESIS/ COMET ASSAY".** I have understood completely and I agree to take part in this research and to donate venous blood samples which will help in acquiring knowledge for the benefit of the mankind.