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Assessment of DNA damage in pulmonary tuberculosis patients by single cell gel electrophoresis / comet assay

M.P. Narmadha[1], A. S. Ushananthini[1], A. Murugan[2], R. Saravanan[3], D. Sridhar[4]

[1]Department of Pharmacy Practice, Swamy Vivekanandha College of Pharmacy, Tiruchengodu-637205, Tamilnadu, India. [2]UGC-NRCBS Program,School of Biological Sciences, Madurai Kamaraj Unversity, Madurai-625021, Tamilnadu ,India. [3]Department of Animal Genetics and Breeding, Veterinary College and Research Institute, Namakkal-637002, Tamilnadu, India. [4]Treatment Laboratory Unit, Tuberculosis Unit, Tiruchengodu-637211 Tamilnadu, India.

Abstract

Background: The role of antitubercular drugs as anti bacterial agents on mycobacterium tuberculi is established. However their impact on human cell is not much explored. **Objective**: The present study was aimed to determine the extent of cell damage in Pulmonary Tuberculosis patients with the help of comet assay. This is a prospective study carried out in Tiruchengodu Govt. Hospital from September 2011 to April 2012. Blood samples were collected from 100 subjects [Healthy volunteers (Go), newly diagnosed TB patients (G1), Three months treated TB patients (G2), Six months treated TB patients (G3). n=25 subjects in each group]. **Methodology:** Blood samples from anticubital vein were collected, embedded in agarose 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. **Results:** Subjects of newly diagnosed TB (G1) patients did not show any remarkable cell damage as compared to TB patients under treatment. Extent of cell damage in six months treated TB patients (G3) was greater than three months treated TB patients (G2) (P<0.0001). Among quantitative comet metrics, comet length was significantly greater in males compared with females (P= <0.0003). Extent of cell damage was observed significantly high in anti-tubercular drug treatment patients both three months treated and six months treated TB patients. **Conclusion**: DNA damage was found to be significantly increased in smoking & alcoholic tuberculosis patients. Anti-tubercular drug and social habits seem to be the major contributor of the cell damage.

Keywords: *Tuberculosis, Anti-tubercular drugs, Comet assay, Comet length, Tail length*

1. Introduction

Tuberculosis (TB) is a highly contagious infection caused by the bacterium called Mycobacterium tuberculosis. 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[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].

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[3]. 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[4].

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[5]. To the best of our knowledge, no study has

**Corresponding author*
Full Address :
[1]Department of Pharmacy Practice, Swamy Vivekanandha College of Pharmacy, Tiruchengodu-637205
Phone no. +91– 9578583490
E-mail: narmadhasam@yahoo.co.in

Table III: Comparison of some commonly used comet metric in leucocytes of tuberculosis patients with control

Parameters	G0	G1	G2	G3	Pvalue
Taillength (µm)	5.81±1.12	7.832±0.613	7.486±0.77	5.494±0.675	(0.1462)
%DNA inTail	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)*

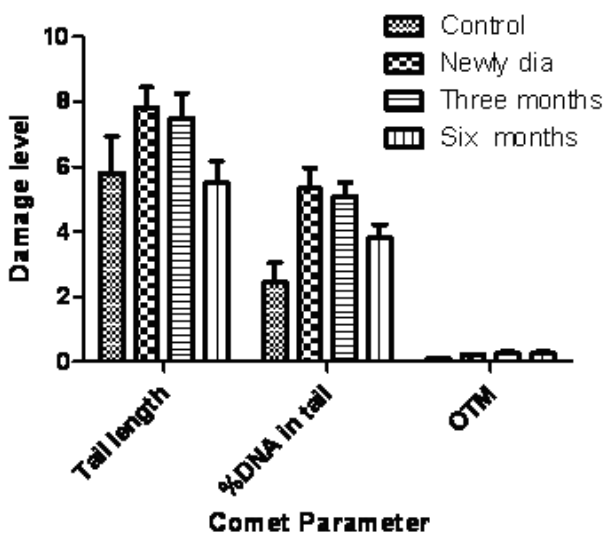

Fig II: Comparison of some commonly used comet parameters are Tail length, %DNA in tail, OTM-Olive tail moment.

Fig III: Impact of Alcoholism on comet length and significance

show any significant difference in all groups when compared with G0 group (Table IV) (Figure III).

4. Discussion

The importance of studying DNA damage has been recognized by many scientists. Age and long duration of disease are two such factors which contribute to the development of DNA damage and thereby cell damage[6]. 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[7]. Comet assay are useful combination when testing for the potential DNA damaging effects of chemicals[8].

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[9]. 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 venous 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 and anti-tubercular drug 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[10]. The difference observed in the increase of 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

Table IV: Impact of Alcoholism on comet length and its significance

Parameters	G0	G1	G2	G3	P value
Alcoholic	85.09 ± 2.63	106.54 ± 13.49	203.53 ± 2.56**	179.99 ± 5.56**	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

been performed to understand the effect of anti-tubercular drugs on cell damage with the help of comet assay.

The aim of this work is to study qualitatively the development of comet images evaluating extent of cellular damage and quantify different comet metrics and to evaluate the effect of drug treatment on cell damage.

Material and Methods

2. Chemicals

Low melting agarose, Normal melting agarose, Triton X-100 and Phosphate buffer saline (PBS; Ca^{++}, Mg^{++} free) were purchased from HiMedia pvt. Laboratories (Mumbai). All other chemicals were of the highest purity available.

2.2 Subject selection and collection of blood samples

DNA damage is an indication of cellular damage. Comet assay is widely regarded as a quick and reliable method for analyzing DNA damage in individual cells. 2ml of venous blood samples were collected in ETDA tube from subjects of Control group (G0), newly diagnosed TB patients (G1), three months treated TB patients (G2) and six months treated TB patients (G3).

Institutional ethical committee of Swamy Vivekanandha College of pharmacy, Tiruchengodu, approved the protocol for the present study. All the patients were given verbal and written information about the study prior to withdrawal of blood sample. Three months treated TB patients were receiving Rifampacin, Isoniazid, Pyrazinamide and Ethambutol. Six months treated TB patients were receiving Rifampacin and Isoniazid. Newly diagnosed TB patients were recruited into the present study (n=25 for each group). All the study groups were compared with the 25 volunteers who constituted control group.

2.3 Single gel electrophoresis

Supernatant liquid is discarded after centrifugation of blood. A small amount of remaining cells are placed in the glass slide with the help of micropipette. Half frosted slides were dipped into a chromic acid solution and then 100 % methanol is used to remove particulate matters. Half-frosted slides were dipped into 1% normal melting agarose (NMA), underside of the slide was wiped and slide was laid on flat surface to dry (First layer). To the coated slide, 75 µL 0.5% low-melting point agarose (LMPA) (prepared in PBS; Ca^{++}, Mg^{++} free) was added to prepare second layer. Third agarose layer with 80 µL of 0.5% LMPA then followed (Third layer). Slides were kept in the lysing solution (2.5M NaCl, 100mM EDTA and 10 mM Trizma Base, 1 % Triton X-100 and 10 % DMSO were added freshly) at 40 C overnight.

After lysis, slides were kept in electrophoresis chamber containing electrophoresis buffer (30 ml 10N NaOH, 5 ml 200mM EDTA q.s. 1000 ml, pH>13). Slides were allowed to sit in alkaline buffer for 20 mins to allow unwinding of DNA and the expression of alkali labile damage, and then electrophoresed for 30 mins (24 volts, 300 milliamperes). Slides were then coated with the neutralization buffer (0.4 M Tris in dH_2O, pH 7.5).

Each cell had the appearance of a comet, with a brightly fluorescent head and a tail to one side formed by the DNA containing strand breaks that were drawn away during electrophoresis. Numbers of comet parameters were calculated with TriTek CometScore TM Freeware version 1.5. Samples were run in duplicate, and 50 cells were randomly analyzed per slide for a total of 100 cells per sample. For quantitative evaluation, undamaged cells (C0), mild and moderate damaged cells (C1), highly damaged cells (>C1) cells were taken into account. It has been documented that any change in the level of DNA damage reflect most accurately in these three parameters (i.e) tail length, %DNA in tail and Olive tail moment[10].

2.4 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.

3. Results

3.1 Qualitative analysis of cellular damage

The number of cells >C1 were found increased in Three months treated TB patients (G2) and Six months treated TB patients (G3). Newly diagnosed patients (G1) did not show any significant change in >C1 when compared with Control (G0) group at $p<0.0001$ (Table I). There was a linear increase in mild to moderately damaged cells (C1) with respect to increase in the time duration of treatment (Figure I).

3.2 Quantitative analysis of cellular damage

Comet assay of white blood cells of study subjects show 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 (Table II). 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

3.3 Comparison of some commonly used comet metrics:

Results of three different parameters namely, tail length, %DNA in tail and Olive tail moment have been presented in (Table III) (Figure II).

3.3.1 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).

Table I: Qualitative evaluation of cell damage in tuberculosis patients

	G0	G1	G2	G3	P Value
C0	52.09±4.72	34.36± 3.51**	13.216± 2.46***	11.2±1.49***	<0.0001
C1	26.38± 2.21	47.6± 4.69**	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

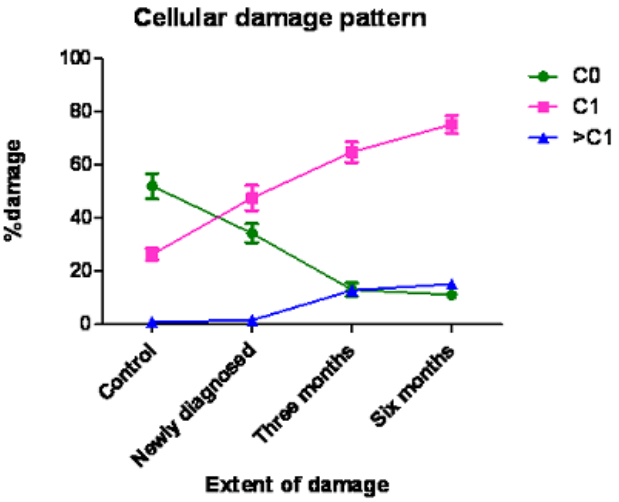

Fig I: Cellular damage pattern (all the data are shown in mean ± S.E.M values. C0= undamaged cells, C1= mild and moderate cells, >C1= highly damaged cells

3.3.2 %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 =0.0049).

3.3.3 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 =0.0449).

3.4 Impact of Alcoholism on 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 (P<0.0010). Non-alcoholic group didn't

Table II: Impact of Tuberculosis on Comet assay indices

Parameters	G0	G1	G2	G3	Pvalue
Cometlength(µm)	80.476±3.624	117.61±13.68	175.42±61.275**	193.37±10.027***	0.0005***
Cometheight(µm)	122.676±65.623	145.19±9.74	192.586±47.432	163.848±14.617	0.2916
CometArea(µm²)	1380.292±119.51	3040.782±1726.7	3490.728±900.40	4333.006±048.4	0.3221
CometIntensity	83.939±7.760	86.148±1.100	137.537±1.958***	84.331±1.036	<0.0001***
CometmeanIntensity	58.942±4.748	116.074±9.065***	137.704±10.35***	102.998±1.639	<0.0001***
Headdiameter(µm)	77.672±9.165	88.796±12.52	80.226±9.958	94.646±8.537	0.6287
HeadArea(µm²)	6945.414±422.72	7759.674±114.20	2284.67±542.26***	7188.508±202.37	<0.0001***
Headintensity	5482.84±352.78	5693.21±647.64	5965.006±1064.1	5653.478±227.20	0.9632
HeadMeanintensity	46.676±5.973	80.736±6.196	72.238±11.842	82.116±9.190*	0.0363*
%DNAinhead	92.186±1.406	91.734±1.188	85.558±7.036	83.908±4.378	0.421
Taillength(µm)	5.81±1.120	7.712±0.613	7.486±0.770	5.494±0.675	0.1462
TailArea(µm²)	30.52±4.998	43.122±6.461	66.746±6.896*	61.624±9.071*	0.0075
Tailintensity	565.47±138.60	274.656±65.111	312.856±29.384	434.682±30.022	0.0774
Tailmeanintensity	361.798±61.44	663.144±157.72	1001.86±92.912**	587.856±86.700	0.0051**
%DNAintail	2.454±0.5919	5.328±0.6341**	5.09±0.4283*	3.812±0.4131	0.0049**
TailMoment	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)

derived units are based. The percentage DNA in tail is directly proportional to the amount of damaged DNA[11].

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 in (P=0.0449), control group when compared with newly diagnosed, three months, and six months was observed. 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 and anti-TB drugs on DNA damage.

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 was 117.61 ± 13.689 for Three months treated patients it was 175.426 ± 27.587 and for Six months treated patients 193.374± 10.027 which was found to be extremely significant (P= 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 in consistent with other studies[12].

The comet length also showed significant increase in Alcoholics 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[3].

Certain drugs are known to induce DNA damage in healthy cells and potentiate the oxidative stress generated during cellular events[13]. It was observed that six months treated Tuberculosis patients taking anti-tubercular drug treatment of Rifampacin and Isoniazid showed an extremely significant increase in cellular damage. Three months treated TB patients taking anti-tubercular drugs like Rifampacin, Isoniazid, Pyrazinamide, and Ethambutol also showed a significant difference in cellular damage. 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[9].

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.

5. *Conclusion*

Study results strongly indicate that antitubercular drugs cause cellular damage in TB patients and the extent of damage is more pronounced in alcoholics and smokers. 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 also highly specific to detect DNA damage.

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