Changes in the levels of comet parameters before and after fluoxetine therapy in major depression patients

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Abstract: Major depression belongs to mood disorders and characterized by worthlessness, no interest or happiness in any activity; lasting for atleast two weeks. Etio-pathological changes of major depression include oxidative stress leading to free radical synthesis which causes damage to carbohydrates, proteins, lipids and nucleic acids. Nucleic acid damage can be identified by either single or double strand breaks and for quantitative estimation of the same, neutral or alkaline comet assay is performed. Fluoxetine is the drug of choice for treatment of major depression having antioxidant function. In the current study eighty drug naïve major depression patients were recruited and comet parameters namely total comet length, head diameter and tail length were measured before starting the treatment and after completion of eight week fluoxetine therapy. The levels of comet parameters were higher in females than males suggesting higher prevalence of major depression among females. On categorizing into three age groups, the numbers of major depression patients belonging to 18–30 year age group were higher than 31–40 and 41–50 year age groups. All the parameters of deoxyribonucleic acid damage were reduced after eight week of fluoxetine therapy indicating that fluoxetine has anti-oxidant action along with its antidepressant properties, which cause reversal of oxidative stress induced damage occurring during major depression.

Key words: Major depression, Oxidative stress, Deoxyribonucleic acid Damage, Comet assay, Fluoxetine

Introduction

Major depression is one sub-type of mood disorders and can be identified by presence of low esteem, feeling of no relevance or no happiness in previously interesting and pleasurable activities; lasting continuously for atleast two weeks [1, 2]. There can be extrinsic causative factors such as genetic factors, family history and immune-inflammatory response changes or the intrinsic factors such as either infectious like herpes virus, Human immunodeficiency virus or non-infectious such as Alzheimer's disease, autoimmune diseases etc. The etiopathogenesis mainly involves the contribution of oxidative stress and immune-inflammatory modification resulting in synthesis of free radicals such as reactive oxygen species and reactive nitrogen species. The free radicals have highly reactive oxidizing properties resulting in damage to carbohydrates, nucleic acids, proteins, and lipids [3-8]. The free radicle-mediated, oxidative stress-induced DNA damage cause numerous structural changes in DNA [9]. Structural changes in DNA can be due to reaction of heme iron with hydroxyl radical in nuclear and mitochondrial DNA [10].

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Elevated levels of breakage in DNA strands, alkali-labile sites and oxidative DNA damage in the recurrent depressive disorder patients were found by Czarny [11], which were estimated with the help of comet assay method. The principle of comet assay utilizes single cell gel electrophoresis (SCGE) technique for estimation of the migration of the damaged DNA [12]. The degree of DNA strand breakage can be identified by analyzing the percentage of DNA present in the tail [13]. The comet assay protocol was proposed by Singh et al. [14] and later simplified by Collins et al. [15].

Fluoxetine is the drug of choice for major depression causing decrease in oxidative stress-induced DNA damage and subsequent restoration of anti-oxidant function [16]. Fluoxetine inhibits serotonin re-uptake and causes continuous activation of post-synaptic receptors by serotonin and it has enhanced antioxidant capacity which partially contributed to its therapeutic efficacy [17, 18].

Thus study of DNA damage parameters can be valuable for investigating the grade of depression and for monitoring the treatment efficacy. Only few studies have been done in the past for the estimation of DNA damage in patients diagnosed with major depression. The current study has been aimed to estimate the DNA damage in newly diagnosed drug-naive cases of major depression and also after fluoxetine course for eight-weeks in the same patients.

Materials and Methods

The study was performed in the Department of Anatomy, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Pondicherry (India) from October 2016 to March 2018. This was an interdepartmental project between the departments of Anatomy and department of Psychiatry. It was a prospective clinical study for which prior clearance was obtained from the Postgraduate Research Monitoring Committee and Institute Ethics Committee (Human Studies), respectively (approval No. JIP/IEC/2016/28/941 dated 19.10.2016).

The study recruited single group of forty drug-naïve major depression patients of 18 to 50 year age, newly diagnosed as per Diagnostic and Statistical Manual of Mental Disorders, 5th Edition criteria, attending the psychiatry out-patient department. Pregnant women, diabetics, hypertensive, cancer patients and patients under antidepressant treatment, were excluded. After explaining the procedure to the patients and legally acceptable representative and obtaining written consent, blood samples were collected before starting the treatment and after eight week fluoxetine therapy.

Alkaline comet assay

DNA damage was analyzed by using comet assay method in which SCGE technique was employed. The alkaline comet assay method standardized by Nandhakumar et al. [19] was used for the assessment of DNA damage.

Principle

The alkaline comet assay involved the movement of negatively charged damaged DNA fragments towards the anode during electrophoresis resulting in comet-like tail like appearance under the microscope.

Procedure

The comet assay is a three-day procedure consisting of blood collection to cell lysis on day one, electrophoresis and cell fixation on day two, and staining and comet scoring on day three.

Day one – Blood collection, lymphocyte isolation, slide preparation and cell lysis

A 2 ml venous blood was taken in heparinized syringes, added to a centrifuge tube containing 2 ml of histopaque (lymphocyte separation media) without allowing mixing of the two and subjected to centrifugation. On completion of centrifugation at 1,500 rpm for thirty minutes, lymphocytes occupied the middle layer; plasma in the topmost layer, histopaque in lower layer and red blood corpuscles occupied the bottom. The first layer formed by coating the slide with normal melting agar (NMA), second layer consisted of mixture of lymphocytes and low melting agar (LMA) over the NMA-coated slides and the third layer formed by coating with LMA. Afterwards, slides were immersed in lysis solution leading to lysis of the cell membrane of the lymphocytes to expose the nucleoids.

Day two – Electrophoresis, neutralization and cell fixation

The slides were subjected to electrophoresis in a solution containing Sodium Hydroxide and disodium (ethylene diamine tetra acetate), without current for thirty minutes resulting in unwinding of DNA, followed by passage of current for thirty minutes leading to migration of negatively charged DNA fragments towards the anode. The slides were neutral-

ized with trisaminomethane buffer, fixed in fixative solution and dried at room temperature overnight or 1–2 hours in the incubator at 37.9°C temperature.

Day three – Staining, photomicrography and comet scoring

A 40–50 randomly selected cells per stained slide were observed under the bright field microscope (Olympus BX53; lympus, Tokyo, Japan) and the captured images were utilized for comet parameter scoring using comet software (TriTek Comet Score Freeware v1.5; TriTek Co., Sumerduck, VA,
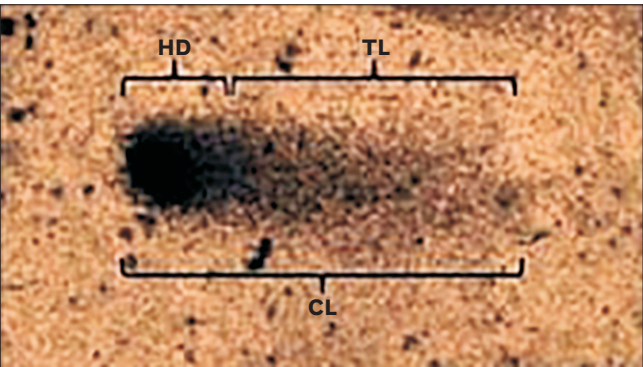

Fig. 1. Parts of comet. CL, comet length; HD, head diameter; TL, tail length.

USA). The analyzed parameters were generated as notepad file and they included Total comet length (CL) i.e. the total amount of DNA present in single lymphocyte; head diameter (HD) i.e. the amount of undamaged DNA present in the head which is not dispersed and tail length (TL) i.e. the damaged DNA which moves towards anode because of more negatively charged molecules (Figs. 1, 2).

Sample size calculation and statistical analysis

The sample size was computed using the Open Epi programme 9 open sources Epidemiology statistics for Public Health version 3.01 formula (Emory University, Atlanta, GA, USA) [20]. On assuming alpha error of 0.05, power 90% and dropout rate of 10%; the eighty patients were recruited. The comparison of categorical data before starting and after completion of the treatment, was completed by utilizing Student's paired t-test and Wiloxons signed ranksum test. The continuous parameters, such as age and comet parameters were expressed as mean±standard deviation (SD) or median with interquartile range (IQR) and the comparision of the comet parameters between the groups were carried by utilizing Student's paired t-test/Wiloxons signed ranksum test based on distribution of data (Normal/Non-normal). Considering 5% level of significance, P-value of <0.05 was considered as significant. PASW Statistics for Windows, Version
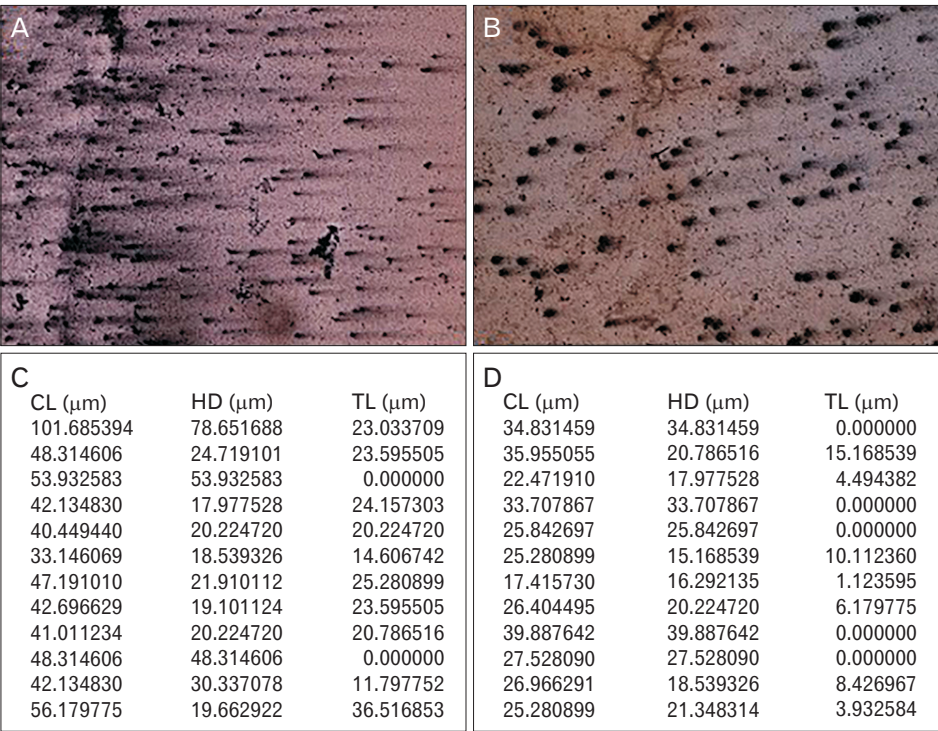

CL (µm)	HD (µm)	TL (µm)	CL (µm)	HD (µm)	TL (µm)
101.685394	78.651688	23.033709	34.831459	34.831459	0.000000
48.314606	24.719101	23.595505	35.955055	20.786516	15.168539
53.932583	53.932583	0.000000	22.471910	17.977528	4.494382
42.134830	17.977528	24.157303	33.707867	33.707867	0.000000
40.449440	20.224720	20.224720	25.842697	25.842697	0.000000
33.146069	18.539326	14.606742	25.280899	15.168539	10.112360
47.191010	21.910112	25.280899	17.415730	16.292135	1.123595
42.696629	19.101124	23.595505	26.404495	20.224720	6.179775
41.011234	20.224720	20.786516	39.887642	39.887642	0.000000
48.314606	48.314606	0.000000	27.528090	27.528090	0.000000
42.134830	30.337078	11.797752	26.966291	18.539326	8.426967
56.179775	19.662922	36.516853	25.280899	21.348314	3.932584

Fig. 2. Comet images and comet parameter. (A, C) Before treatment (B, D), after treatment. CL, comet length; HD, head diameter; TL, tail length.

18.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis and the charts were plotted utilizing Microsoft Excel 2007 (Microsoft, Redmond, WA, USA).

Results

Patients were categorized into three age-groups namely 18–30, 31–40, and 41–50 years respectively, among which highest number of patients comprising of 38.8% of total patients belonged to 18–30 years age group followed by 37.5% belonging to 31–40 years age-group and 23.8% belonging to 41–50 years age-group, respectively (Table 1). The percentage of female patients (58.8%) was significantly higher than the male patients (41.3%) (Table 2). Among all the comet parameters, only HD followed normal distribution, remaining comet parameters i.e. CL and TL followed non-normal distribution.

Levels of comet parameters before starting and after eight-week fluoxetine therapy

The mean value of HD was 27.165 with SD of ±6.930 while the median values of CL and TL along with IQR were 33.919 (14.567) and 6.843 (8.579), respectively. After eight-week fluoxetine therapy, the mean value of HD was 20.324 with SD of ±6.451 while the median values of CL and TL along with IQR were 23.004 (5.188) and 2.000 (2.543), respectively.

The mean value of HD and the median values of CL and TL were reduced after eight-week fluoxetine therapy. However, the reduction in the levels of CL and TL was statistically significant ($P<0.001$) and reduction in the levels of HD was statistically not significant ($P=0.002$) (Fig. 3).

Table 1. Age distribution among major depression patients

Variable	Value (n=80)
Age (yr)	
18–30	31 (38.8)
31–40	30 (37.5)
41–50	19 (23.8)

Values are presented as number (%).

Table 2. Sex distribution in major depression patients

Variable	Value (n=80)
Sex	
Male	33 (41.3)
Female	47 (58.8)

Values are presented as number (%).

Fig. 3. Mean scores of comet parameters in major depression patients BT and AT with fluoxetine for eight weeks. AT, after treatment; BT, before treatment; CL, comet length; HD, head diameter; IQR, inter quartile range; SD, standard deviation; TL, tail length.

Table 3. Age-wise distribution of comet parameters among major depression patients before starting and after completion of the eight-week therapy with fluoxetine

Comet parameter	18–30 yr		31–40 yr		41–50 yr	
	Before	After	Before	After	Before	After
CL (µm)	37.68±11.43	23.77±4.16	34.97±11.13	22.79±5.76	32.51±8.18	20.93±3.90
HD (µm)	28.44±7.29	21.49±4.90	26.32±6.81	19.64±5.94	26.40±6.53	19.48±3.95
TL (µm)	8.22 (28.78)	2.65±1.782	6.54 (32.60)	2.45 (10.61)	6.21±4.43	1.24 (7.19)

Values are presented as mean±SD or median (interquartile range). CL, comet length; HD, head diameter; TL, tail length.

Table 4. Comparison of comet parameters among female and male major depression patients before starting and after completion of the eight-week therapy with fluoxetine
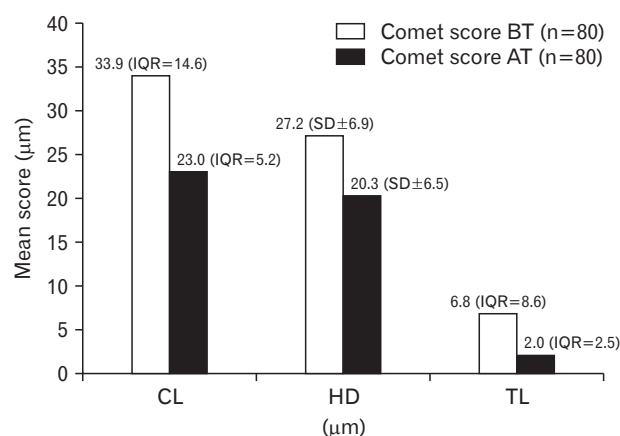
Comet parameter	Female		Male	
	Before	After	Before	After
Total comet length	33.579 (15.603)	23.254±4.903	35.706±9.398	22.024±4.739
Head diameter	27.162±7.460	20.489±5.366	27.171±6.252	20.103±4.917
Tail length	8.233±6.835	2.305 (3.036)	8.604±7.025	1.568 (2.263)

Values are presented as mean±SD or median (interquartile range).

Comparison of levels of comet parameters among different age groups and different sex

Levels of all the comet parameters namely CL, HD, and TL were reduced after eight weeks treatment with fluoxetine. In all the age groups, reduction in the levels of all the parameters was statistically significant except in the age group 31–40 and 41–50 years age groups where reduction in the levels of TL were not significant. Reductions in the levels of CL, HD, and TL were statistically significant among both male and female groups (Tables 3 and 4).

Discussion

Major depression consists of isolated spells of changes in concern, intellect, psychosomatic functions manifesting over a period of atleast 2 weeks combined with intermediate symptomless periods [2]. Few hypothesis suggested oxidative stress leading to synthesis of free radicals resulting in damage to nucleic acids, lipids, carbohydrates and proteins as one of the cause of major depression [8]. As a result of oxidative stress induced damage, DNA structure is characterized by apurinic/apyrimidinic or baseless sites, cyclobutane-type pyrimidine dimer, chemical alteration in small bases, distortion in helix structure or breaks in single strands and double strands etc. [9]. Excessive heme iron released as a result of oxidative-stress induced-hemolysis reacts with hydroxyl radical leading to single or double-strand breaks in nuclear and mitochondrial DNA [10]. Czarny [11] estimated elevated levels of breakage in DNA strands, alkali-labile sites and oxidative DNA damage in the recurrent depressive disorder patients the help of comet assay method. SCGE technique is used during comet assay for estimation of the migration of the damaged DNA [12]. On the passage of current, there is migration of relaxed DNA loops from nucleus towards the anode, giving an appearance of the comet tail. Collins et al. [15] suggested a single layer of agarose gel for cell-embedding on a plain slide pre-coated with agarose and dried. The breaks in single strand or double-strand DNA can be assessed quantitatively by comet assay; either neutral or alkaline [14].

The lifetime risk of major depressive disorders in United States was highest in the age-group 45–64 years (15.91% with standard error i.e. SE±0.50), followed by age groups 30–44 years (14.03% with SE±0.46) and 18–29 years (12.02% with SE±0.49) [21]. Reddy and Chandrashekar [22] suggested highest prevalence of mental disorder in 35–44 year

age group, depression being highest among the all mental disorders (41.7%) and higher prevalence of depression among indian females than the male counterparts (female: 49.9%, male: 40.5%). Judd et al. [23] reported higher occurrence of major depression in females as compared to males (female: 60.6%, male: 39.4%). Present study showed highest prevalence of the major depression in 18–30 year age-group (38.8%), closely followed by 31–40 year age-group (37.5%) and higher prevalence of major depression in females than the males.

In the current study, comet images had good comet tails and higher CL, HD, and TL in the cases before the treatment than after concluding the treatment. The breaks in the DNA strands resulted in liberation of supercoils leading to migration of the fragmented DNA towards the anode. Hence, TL is believed to be the best indicator of DNA damage [24, 25]. Different parameters such as TL, tail movement (TM), percentage of DNA in tail (%T), olive tail movement (OTM) have been scrutinized in the past as indicators of DNA damage in the cases before and after the interventions [26-29]. The therapeutic agent (fluoxetine) had antioxidant effect which resulted in decrease in TL after completion of the treatment.

The available literature about assessment of the levels of parameters of oxidative stress in the cases before and after treatment with fluoxetine in major depression patients was limited. Galecki et al. explained the role of fluoxetine in influencing the antioxidant defences by estimating superoxide dismutase and catalase, malondialdehyde and Hamilton depression rating scale (HDRS) scores and it showed significant reduction in the HDRS scores on completion of fluoxetine therapy [30].

Fluoxetine is preferred drug for major depression because of its action post-synaptic receptors by prolonging activation of the receptors and delaying the re-uptake of serotonin [31]. Novío et al. [16] demonstrated a decrease in oxidative stress-induced damage and restoration of anti-oxidant function caused by fluoxetine. Zafir and Banu [18] suggested antioxidant properties of fluoxetine. Perić et al. [17] suggested the role of fluoxetine in GSH-dependent defense systems along with decrease in levels of pro-inflammatory cytokines in socially isolated mice.

Czarny [11] assessed the degree of endogenous damage to the DNA in individuals having recurrent depressive disorder (rDD) and healthy subjects, DNA damage due to hydrogen peroxide and changes occurring in DNA damage parameters

after repair incubation and reported considerably greater endogenous DNA damage in the rDD patients than the healthy subjects ($P<0.001$) which was indicated by the increased %T. DNA damage after exposure to H_2O_2 was substantially more in the patients than the controls ($P<0.001$). Post-incubation analysis showed complete repair of H_2O_2–induced DNA damage in the controls but in the patients, the damage to the DNA was still more than the endogenous DNA damage levels ($P<0.001$).11 The current study showed fluoxetine as antioxidant agent in addition to its antidepressant action; as there was notable reduction in TL in the cases along with symptomatic relief.

For assessing the oxidative stress induced cellular damage by alkaline comet assay among the psychiatric diseases, other than depression, one study was done among schizophrenia patients [32]. The levels of parameters of DNA damage were analyzed among cases and controls and again these values were compared after incubating the cells in the nutrient medium among the cases. The DNA damage parameters namely CL, HD and TL were reduced after the repair incubation in the cases.

The stratification of all the comet parameters was done based on gender and different age groups and the widest disparity between pre and post-treatment levels of CL, HD, and TL was found in the 18–30 year age-group. In both the genders, the changes in the levels of all the comet parameters before starting and after completing eight-week fluoxetine therapy were statistically significant.

The current study established relationship between age and oxidative stress because varying levels of different comet parameters were reported among different age groups and there were differences in efficiency of mechanisms for repairing the oxidative stress-induced DNA damage in different age groups.

In conclusion, adult patients who were suffering from major depression, had increased levels of DNA damage parameters before starting the treatment which were reduced after finishing the eight-week antidepressant therapy with fluoxetine. The levels of the parameter indicated damage to the DNA were more in males than in the females.

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Conceptualization: RP, MS. Data acquisition: RP, VM, BV. Data analysis or interpretation: RP, MS, VM, BV. Drafting of the manuscript: RP, MS. Critical revision of the manuscript: RP, MS, VM, BV. Approval of the final version of the manuscript: all authors.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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