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ANALYSIS OF DNA DAMAGE USING THE COMET ASSAY IN FEMALE PATIENTS TREATED WITH FLUOXETINE AGAINST DEPRESSION

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ABSTRACT: Depression during pregnancy is not uncommon. One of the main concern with psychotropic drugs during pregnancy is teratogenicity. Although fluoxetine is one of the most frequently prescribed antidepressant, its safety in women considering possible toxic effecst has not been established well. To investigate the potential toxic effect of long term fluoxetine therapy on the DNA in women, comet assay was performed in peripheral lymphocytes of 25 depressive female patients who has been receiving fluoxetine for at least six months. 25 healthy, drug free female volunteers were selected for the control group. The frequencies of comet scores in the patient group were significantly higher than that of the control group (p=0.01) indicating that DNA damaging effect of fluoxetine in human lymphocytes. Our preliminary data were based on a few subjects and etiological and pathological mechanism remain obscure, further large-scale experimental and clinical studies are needed.
[Keywords: Flyoxetine, Teratogenicity, Depression]

INTRODUCTION

An estimated 8 to 20 percent of woman have depression at some time in their lives, most commonly during childbearing years and often requiring drug therapy (1, 2). The decision to continue or initiate pharmacotherapy for depression during pregnancy is complicated by the need to balance maternal well-being with fetal safety. Although the first trimester of pregnancy, in particular week 2 to 8 after conception, is the most critical period for drug-induced malformations, the brain develops throughout pregnancy and some defects may occur after the first trimester (3).

There is ample evidence that discontinuation of antidepressant-drug therapy in patients with medication-responsive illness may be detrimental, with high relapse rates (4). The main drugs currently used for treating major depression are agents that selectively inhibit the reuptake of serotonin (SSRI). They have fewer anticholinergic and cardioarrhythmic effects (5), but they cause anxicty, nausea, and insomnia in a substantial proportion of patients (6-8). They are used primarily for the treatment of depression, anxiety, obsessive compulsive disorder and impulse control disorders, but they are also useful in the treatment of other psychiatric disorders. Although fluoxetine is one of the most frequently prescribed SSRI, its toxicity on DNA has not been enough clear yet.

The comet assay, also called single-cell gel electrophoresis, is a simple and, powerful tool for demonstrating the damaging effects of different compounds or physical treatments on DNA at the individual cell level. In the last 20 years, advances in DNA damage detection have allowed scientists to determine the impact of certain environmental factors or medical treatments on DNA integrity (9-11). Although other methodologies exist for detecting damaged DNA, as the comet assay have some advantages (12-14), it is rapidly gaining in popularity.

The purpose of this study was to determine the potential toxic effects of long term fluoxetine therapy using comet assay of peripheral blood lymphocytes of female patients with depression.

MATERIALS AND METHODS

Subjects

Twenty-five female patients between the age of 21 to 37 years who have receiving fluoxetine for six months to 1.5 years were chosen for the study. Patients were accepted if they are not smoker, they did not use any other drug for at least a year and if they have no problem with irregular menstruation. Fluoxetine was prescribed for all subjects diagnosed by DSM-IV (major depression). The control group was selected from the healthy non-smoker females. Their ages matched to the patient group. They have normal menstruation and no long-term drug usage story. According to our inspection, neither the patients received fluoxetine nor the control group were exposed to any other mutagenic agents (e.g. , radiation , chemicals, lifestyle, smoking, drugs , or viruses) during the at least one year before the study. Neither did any of them presented any chronic or neoplastic diseases. The study was approved by the hospital's research ethics board and informed consent was form provided to all subjects. The blood samples were taken from the patient and control groups within 20^{th} and 27^{th} days after their menstruation. All subjects were healthy at the time of sampling.

The alkaline comet assay

Chemicals

All chemicals were purchased from the Sigma Chemical unless otherwise stated. Lymphocyte Separation Medium was from ICN Flow and TC-199 from Gibco. Superfrost 1.0 - 1.2 mm thick microscope slides from Merck were used. Normal and low melting point agarose were obtained from Gibco. Dulbecco's phosphate-buffered salts (PBS), without Mg and Ca, was from ICN Flow.

Peripheral blood lymphocyte preparation

Five ml of blood was carefully layered over 8 ml Lymphocyte Separation Medium and centrifuged at 2000 x g for 15 min. After the plasma layer was removed and saved, the buffy coat was carefully removed and the cells were washed with TC-199 medium and then collected by 10 min centrifugation at 1000 x g. Lymphocytes were resuspended at approximately 10^7 / ml in TC-199 medium with 20% v/v plasma and 10% v/v plasma and v/v DMSO. Lymphocytes were transferred to microfuge tubes and stored at -20°C.

The application alkaline comet assay (single cell gel electrophoresis)

The comet assay, as described by Singh et al (9), was used with some modifications. The comet assay protocol was carried out under dim light to prevent any additional DNA damage. Darkin fully frosted microscope slides were each covered with 100 µl of 0.5% normal melting point agarose in Ca^{+2} and Mg^{+2} - free PBS at 45°C. They were immediately covered with a large no. 1 cover slip and then kept at 4°C until the agarose had solidified. Seventy-five µl of 0.5% low melting point agarose (LMA) at 37°C was added to the lymphocytes (1500-100000 cells) suspended in 10 µl of PBS. After gently removing the cover slip, the cell suspension was rapidly pipetted on to the first agarose layer, spread using a cover slip, and allowed to solidify at 4°C. A final layer of 75 ml of 0.5% LMA was applied in the same way. The slides were immersed in freshly prepared, cold lysing solution (2.5 M NaCl2, 100mM Na2 EDTA, 10 mM Tris, pH 10, 1% sodium sancosinate with 1% Triton X-100 and 10% DMSO added just before use) for 1 hr at 4°C. Slides were removed from the lysing solution, drained and placed in a horizontal gel electrophoresis tank side by side with the agarose end facing the anode. The tank was filled with fresh electrophoresis buffer (300 mM NaOH and 1 mM Na2 EDTA) at 12-15°C to a level approximately 0.25 cm above the slides. The slides were left in the alkaline buffer (pH 13) for 20 min to allow unwinding

of the DNA to occur before electrophoresis. Electrophoresis was conducted for 20 min at 25 V adjusted to 300 mA by raising or lowering the buffer level in the tank. Slides were than drained, placed on a tray and flooded slowly with 3 changes of neutralization buffer (0.4 M Tris, pH 7.5) for 5 min each, to remove alkali and detergents. The slides were again drained before being stained with 50 µl of 20 µg/ml ethidium bromide and a cover slip was placed on top. Slides were stored in a closed container at 4°C and analyzed within 24 h, gel dehydration over longer storage times led to detoriation in slide quality.

COMET CAPTURE AND ANALYSIS

Analysis was performed immediately after staining, using a 200 X objective with a Zeiss optiphot equipped with an excitation filter of 515-560 nm from a 100-W mercury lamp and a barrier filter of 590 nm. Comets from as broken ends of the negatively charged DNA molecule becomes free to migrate in the electric field towards the anode. The assay provides direct determination of the extend of DNA damage in individual cells and the extend of DNA damage can be assessed from the length of DNA migration which is derived by substracting the diameter of the nucleus from the total length of the image. Several software systems are now available commercially, and can be configured to estimate total DNA content and comet image length. The tail length can also be measured from the trailing edge of the nucleus to the leading edge of tail, using a calibrated scale in the ocular of the microscope. We determined the degree of damage by grading the cells as; normal (undamaged - no migration), limited migration (at low damage levels, stretching of attached strands of DNA, rather than migration of individual pieces is likely to occur), and extensive migration (with increasing numbers of breaks, DNA pieces migrate freely into the tail forming comet images).

A minimum of 100 cells were analyzed for each sample population. Slides were scored by the independent investigators and cells were graded as; normal (undamaged), limited migration and extensive migration.

Statistics

Statistical comparisons between the grade of DNA damages in control/patient groups were analyzed by using Student-t test which assumes Gaussian populations with equal standart deviations. Two sided p values were used.

RESULTS:

The ages of the patient group ranged from 21 to 37 years (mean 28,88). The ages of the controls ranged from 22 to 37 years (mean 28.92 years). The statistical comparison of the ages in two groups showed no significant difference ($p > 0,05$). The comet scores and clinical data of the patient and control groups are listed on Table-I and Table-II respectively. The statistical comparison of the comet scores of two groups demonstrated a significant difference in number of damaged cells. Damaged (limited and extensive migrated) cells in the depressive women who were taking fluoxetine were higher than those of the controls ($p < 0.01$) (Table III).

DISCUSSION

The single cell gel electrophoresis (SCGE) assay also known as comet assay is a rapid simple, visual and sensitive technique for measurement and analyzing DNA breakage in mammalian cells. One of the advantages of SCGE assay is that it can be used to measure DNA breaks in virtually any cell type. DNA damage is known as responsible from teratogenity and cancerogenesis (9-14). The aim of this study was to evaluate the association of exposure to fluoxetine with DNA damage (teratogenity and cancerogenesis).

Table I. Individual data (age, duration of treatment, grade of DNA damage by comet assay) from patients treated with fluoxetine

Subject Number	Age (years)	Duration of treatment (months)	Grade of damage in 100 cells		
			Undamaged (no migration)	Limited Migration	Extensive migration
1	21,00	7,00	91,00	7,00	2,00
2	26,00	9,00	90,00	8,00	2,00
3	23,00	11,00	95,00	5,00	0,00
4	37,00	14,00	90,00	7,00	3,00
5	32,00	18,00	88,00	6,00	6,00
6	34,00	10,00	89,00	6,00	5,00
7	28,00	9,00	87,00	7,00	6,00
8	27,00	8,00	92,00	4,00	4,00
9	31,00	10,00	96,00	2,00	2,00
10	33,00	7,00	90,00	7,00	3,00
11	26,00	9,00	88,00	7,00	5,00
12	21,00	8,00	87,00	7,00	6,00
13	22,00	8,00	90,00	6,00	4,00
14	29,00	7,00	92,00	5,00	3,00
15	30,00	5,00	91,00	5,00	4,00
16	30,00	10,00	88,00	6,00	6,00
17	30,00	9,00	87,00	8,00	5,00
18	32,00	9,00	85,00	8,00	7,00
19	27,00	9,00	88,00	6,00	6,00
20	35,00	8,00	91,00	6,00	3,00
21	34,00	9,00	90,00	7,00	3,00
22	30,00	9,00	90,00	7,00	3,00
23	28,00	8,00	88,00	6,00	6,00
24	31,00	6,00	85,00	7,00	8,00
25	25,00	8,00	88,00	5,00	7,00
Mean	28,88	9,00	89,44	6,20	4,36
SD	4,34	2,55	2,63	1,35	1,96
SEM	0,87	0,51	0,53	0,27	0,39

In order to investigate the association of antidepressants with cancers, it has been searched the medline for relevant articles. Four human studies and nine experimental models have been found. Human studies showed transiently statistically positive association between amitriptyline and liver cancer and a negative association with pancreatic cancer; and the antidepressants amitriptyline, nortriptyline, desipramine, and phenelzine may increase breast cancer. Amitriptyline was found to promote tumour growth, fluoxetine and clomipramine were reported to be both tumour promoters and antineoplastic agents, and imipramine and citalopram both demonstrated antineoplastic properties (15).

It has been reviewed the literature on the use of psychotropic drugs in pregnancy and lactation and stated as it appeared that most, but not all, current psychotropic drugs fairly safe for use in pregnancy (16). It has been investigated the site specific malformations in the mouse embryo following exposure to serotonin receptor uptake inhibitors and it has

been concluded that inhibition of serotonin uptake into craniofacial epithelia may produce developmental defects by interference with serotonergic regulation of epithelial-mesenchymal interactions important for normal craniofacial morphogenesis (17). In a study, it has been identified 254 women taking fluoxetine. It has been concluded that women who take fluoxetine during pregnancy do not have increased risk of spontaneous pregnancy loss or major fetal anomalies, but women who take fluoxetine in the third trimester are at increased risk of perinatal complications (18).

Table II. Individual data (age, grade of DNA damage by comet scores) of control group.

Subject Number	Age (years)	Grade of damage in 100 cells		
		Undamaged (no migration)	Limited Migration	Extensive migration
1	23,00	91,00	5,00	4,00
2	27,00	92,00	5,00	3,00
3	33,00	95,00	4,00	1,00
4	29,00	93,00	4,00	3,00
5	35,00	93,00	3,00	4,00
6	37,00	92,00	4,00	4,00
7	30,00	92,00	4,00	4,00
8	29,00	95,00	4,00	1,00
9	26,00	93,00	4,00	3,00
10	19,00	95,00	4,00	1,00
11	24,00	94,00	4,00	2,00
12	34,00	94,00	5,00	1,00
13	31,00	92,00	4,00	4,00
14	29,00	95,00	4,00	1,00
15	22,00	94,00	4,00	2,00
16	26,00	95,00	4,00	1,00
17	29,00	94,00	4,00	2,00
18	32,00	95,00	4,00	1,00
19	27,00	94,00	4,00	2,00
20	30,00	95,00	4,00	1,00
21	31,00	95,00	4,00	1,00
22	30,00	92,00	6,00	2,00
23	39,00	94,00	4,00	2,00
24	26,00	96,00	3,00	1,00
25	25,00	94,00	4,00	2,00
Mean	28,92	93,76	4,12	2,12
SD	4,66	1,33	0,60	1,17
SEM	0,93	0,27	0,12	0,23

On the other hand, it has been reported that the maternal fluoxetine use during the third trimester results in significant postnatal complications is unlikely (19). In an investigation, it has been found that the rates of major malformations in children exposed in utero fluoxetine, tricyclic antidepressants and non-teratogenic drugs did not differ from the

rates in general population (20). In some studies, it has been reported that SSRI's use during pregnancy do not increase teratogenic risk when used in recommended dosages (21,22). Addis and Koren examined all published and unpublished reports and made meta analysis. As a result, they reported that the use of fluoxetine during the first trimester of pregnancy is not associated with measurable teratogenic effects in human (23) .

Table III. Statistical results

	N	Mean	Std. Deviation	Std. Error Mean	P
Age Patient Control	25 25	28.88 28.92	4.34 4.66	0.87 0.93	0,3651 p>0.05
No Migration Patient Control	25 25	89.44 93.76	2.63 1.33	0.53 0.27	0.0007 p<0,05
Limited Migration Patient Control	25 25	6.20 4.12	1.35 0.60	0.27 0.12	0.0001 p<0.05
Excessive migration Patient Control	25 25	4.36 2.12	1.96 1.17	0.39 0.23	0.0072 p<0.005

Although there is not enough data in the literature that the fluoxetine is associated with teratogenity and cancerogenesis, the mean limited damage and excessive damage of the patient group in this study was found as significantly higher than the control group. These results support the idea that the exposure to fluoxetine is associated with DNA damage which may be associated with teratogenity and cancers.

Based on these data, more controlled prospective studies with the association of exposure to fluoxetine and DNA damage are needed.

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