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Sister chromatid exchanges and sperm abnormalities produced by antidepressant drug fluoxetine in mouse treated *in vivo*

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Abstract. – OBJECTIVES: The aim of this investigation was to determine the capacity of serotonin reuptake inhibitor (SSRI) antidepressant drug fluoxetine (FLX) to induce genotoxic damage in somatic and germ cells.

METHODS: For this study, sister-chromatid exchanges (SCE's) in bone marrow cells and sperm abnormalities assays in male mice were used. The animals were organized in four groups constituted by five mice. They were orally administered with the test substance as follows: a negative control group; three groups treated with FLX (2.6, 7.8 and 13.0 mg/kg b.wt.) for 5 consecutive days. Animals were sacrificed 24h after the last treatment for analysis SCE's and left for 35 days from the first treatment for analysis sperm-shape abnormalities.

RESULTS: The results showed that the drug was SCE and sperm abnormalities inducer. The response of this compound was dose-dependent, and showed that the highest tested dose increased about two times SCE and four times the sperm abnormalities control level. The cellular proliferation kinetics was not affected by the chemical, and the mitotic indexes were slightly diminished with the highest dose. The percentage of sperm count and sperm motility decreased ($p < 0.01$) with increased the dose of treatment.

CONCLUSIONS: These results indicate an *in vivo* genotoxic potential for the antidepressant drug FLX.

Key Words:
Fluoxetine, Sister chromatid exchanges, Sperm head abnormalities, Mice.

Introduction

Fluoxetine (FLX), known also as Prozac, is a clinically used potent antidepressant compound[1]. FLX is a selective serotonin reuptake inhibitor (SSRI) with a high selectivity for the 5-hydroxytryptamine (5-HT) transporter and, thus, in the brain, modulates synaptic serotonin concentration[2]. However, FLX produces undesired side effects including anxiety, sleep disturbances, sexual dysfunction and gastrointestinal disturbances[3]. Besides the well-known actions, FLX exerts other effects, such as blockade of muscular and neuronal nicotinic receptors[4] and inhibition of monoamine oxidase A and B[5]. FLX has also been reported to inhibit the activity of the voltage-dependent Na^+ and K^+ and Ca^{2+} channels[6,7]. In addition, FLX inhibits the multi-drug resistance extrusion pump and thus enhances the response to chemotherapy. Indeed, FLX enhances doxorubicin accumulation within tumors[8].

Several studies have linked FLX with cell proliferation and an increased risk of developing cancer[9-11]. FLX has been shown to enhance cell proliferation and to prevent apoptosis in dentate gyrus[10], to stimulate DNA synthesis[9] and inhibit UV-induced DNA fragmentation in U937 cells[12]. Contradicting results showing enhancement of programmed cell death in various cell lines have also been reported[13]. FLX was found to trigger rapid and extensive apoptosis in Burkitt lymphoma cells that is prevented by over-expression of the anti-apoptotic Bcl-2[13].

In male rats, acute administration of FLX results in an inhibition of sexual behavior evidenced by prolonged ejaculation latency and/or by increased number of mounts and/or intromissions exhibited prior to ejaculation[14-17]. Additionally, genital grooming was increased in FLX treated rats[17]. The antidepressant effects of FLX are typically realized after 2-4 weeks of treatment. Thus, the effect of chronic administration of the drug is of interest from both theoretical and practical perspectives. Taylor et al[18] investigated the effects of chronic administration of

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FLX (0.75 mg/kg; 28 days) on social behavior and copulation. Vega Matuszcyk et al[19] reported that subchronic administration of FLX (10 mg/kg; 13 and 28 days) inhibited the copulatory pattern by increasing latency to ejaculation and increasing the frequency of mounts. Further, they reported that FLX reduced the apparent motivation of the male rat to pursue an estrous female based on the amount of time the male spent near the female rat.

Although many clinical and basic research studies have examined the safety and efficacy of SSRI's in the adult population[20,21], little research exists regarding their use during development. Still, the United States Food and Drug Administration (USFDA) recently approved, FLX for use in children age 7-17 years old[22], despite serious reservations regarding the drug's efficacy in children[23,24]. A subsequent report from the U.S. Department of Health and Human Services' Panel on Developmental Toxicity of Fluoxetine determined that "Sufficient evidence exists for the Panel to conclude that FLX exhibits developmental toxicity…"[25]. Although a meta-analysis conducted by Whittington et al[26], who examined both published and unpublished clinical trials of adolescent FLX use, reported that FLX seems to have a favorable risk-benefit profile in adolescents. Consequently, based on this lack of adolescent FLX exposure studies, along with the adverse effects reported to occur with other SSRI's[27,28], caution needs to be heeded before the use of SSRI treatments in the adolescent population is fully accepted.

In spite of the extensive use of such medication there is no clear definition in regard to their genotoxic capacity; the literature shows heterogeneous data and almost a lack of *in vivo* studies. Therefore, the genotoxic evaluation of FLX using various *in vivo* endpoints was undertaken.

Materials and Methods

Animals

Male white Swiss mice aged 9-12 weeks were used in all experiments. The animals were obtained from a closed random-bred colony at the College of Pharmacy, University of King Saud in Rhiyad. The mice used for any one experiment were selected from mice of similar age (±1 week) and weight (±2 g). Animals were housed in polycarbonate boxes with steel-wire tops (not more than five animals per cage) and bedded with wood shavings. Ambient temperature was controlled at 22±3°C with a relative humidity of 50±15% and a 12-h light/dark photoperiod. Food and water were provided *ad libitum*. Animals were sacrificed after treatment by cervical dislocation.

Chemicals

Fluoxetine was purchased from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals used were of analytical grade.

Doses

The human therapeutic doses of the tested drug was converted to mice therapeutic equivalent doses using the dose-conversion table of Paget and Barnes[29]. Animals were divided into 4 groups of 5 animals each. Group I were used as negative control. Groups II, III and IV were treated orally with 2.6, 7.8 and 13.0 mg FLX/kg b.wt. for 5 consecutive days, respectively. Animals were sacrificed 24h after the last treatments for analysis SCE's and left for 35 days from the first treatment for analysis sperm-shape abnormalities.

Sister Chromatid Exchanges (SCE's)

The method described by Allen[30], for conducting *in vivo* SCE's induction analysis in mice was applied with some modifications. Approximately 55 mg 5'-bromodeoxyuridine (BrdU, Fluka AG, Buchs SG, Riedstr, Steinheim, Switzerland) tablets were inserted in mice subcutaneously (s.c.) 21-23h before sacrifice. Mice were injected intraperitonealy with colchicine at a final concentration of 3 mg/kg body wt. 2hrs before sacrifice. Bone-marrow cells from both femurs were collected. The fluorescence-photolysis Giemsa technique was used[31] (Litz light microscope, Wetzlar, Germany). The microscopic analysis per mouse was carried out as follows: 40 second-division metaphases to determine the frequency of SCE's, 1000 cells to determine mitotic indexes (MI) which was equal to 1/(M1+2M2+3M3)100 and 100 cells to establish the cellular proliferations kinetics (CPK). Based on the CPK values, we obtained the average generation time (AGT) which was equal to 24/ (M1+2M2+3M3)100. M1, M2 and M3 corresponded to the number of cells in first, second and third cellular division, respectively.

Epididymal Sperm Count, Motility and Abnormal Sperm

The mice were sacrificed by cervical dislocation. The epididymes were excised and placed in a prewarmed Petri dish containing 1 ml phosphate buffered saline (PBS, pH 7.4) at 37°C and placed in a 37°C incubator for 15 min, prior to determining sperm motility. The suspension was stirred, one drop was placed on a warmed microscope slide, and a 22 × 22 mm cover slip was added. Microscopic fields were observed at 400 × magnification using a standard light microscope, and the percentage of motile sperm was determined. Five micro liters of the sperm suspension was transferred into an Eppendorf tube and diluted with 95 µl of PBS. After mixing, the sperm suspensions were counted. Sperm counts were made using a Thoma counting chamber and expressed as $X10^6$/ml. A drop of sperm suspension was smeared onto a slide and stained with Eosin Y stain[32]. 1000 sperm per animal (5 animals/group) were assessed for morphological abnormalities of the sperm shape.

Statistical Analysis

The significance of the results from the control data was calculated using Student's *t*-test. A difference in the mean value of $p < 0.05$ was considered to be statistically significant.

Results

Sister Chromatid Exchanges

The frequency of SCE's induced by FLX is shown in Table I. The low dose administered (2.6 mg/kg b.wt) did not increase the number of SCE's with respect to the value of the negative control group. However, the two high doses produced a genotoxic effect. With 13 mg/kg b.wt. the increase over the control level was 3.31 SCE's. The MI and the AGT produced by the compound are also shown in Table I. With respect to the first parameter, the chemical produced a cytotoxic effect with only the highest dose tested, which inhibited the MI 28% with respect to the control mean. The CPK was characterized by the number of mitosis in M1, in M2, and in M3, which was very close to the rate observed in the control mice; these results produced a homogeneous AGT value in the experiment (between 12.41 and 12.44 h).

Epididymal Sperm Count, Motility and Abnormal Sperm

Administration of FLX with the three different doses once daily for 5 consecutive days significantly ($p < 0.01$) reduced sperm count in all tested doses compared to the control group (Table II). Furthermore, the drug caused a significant decrease in the sperm motility. The mean value of Johnsen's score in control group was 88.64±4.54; the treatment reduced significantly ($p < 0.01$) the mean of the score to 62.22±4.82 after treatment with the high tested dose of FLX. The mean percentage of sperm shape abnormalities for animals treated with FLX was increased with dose response (Table II). The percentage of sperm abnormalities was statistically significant ($p < 0.01$) with all the tested doses. The maximum percentage reached 6.16±0.52 ($p < 0.01$) compared to the control group 1.32±0.40. Table II also represents the number and means percentage of sperm shape abnormalities and the main types demonstrated with head abnormalities.

Table I. Frequency of sister chromatid exchanges (SCE's) in mouse bone-marrow cells treated with FLX.

Dose (mg/kg b. wt.)	No. of different types of SCEs/chromosome			Total No. of SCE's[a]	SCE's/Cell[b] mean ± SE	MI $\bar{X}$ ± SE	AGT $\bar{X}$ ± SE
	Single	Double	Triple				
I. Control	433	59	3	560	2.80 ± 0.35	5.30 ± 0.35	12.41 ± 0.28
II. FLX							
2.6	636	60	4	768	3.84 ± 0.40	5.35 ± 0.42	12.42 ± 0.34
7.8	798	75	8	972	4.86 ± 0.31*	5.18 ± 0.29	12.44 ± 0.22
13.0	976	99	16	1222	6.11 ± 0.50**	5.02 ± 0.31*	12.37 ± 0.24

[a]The total number of chromosomes is 8000; [b]The total number of scored cells is 200 (5 animals/group); *Significant $p < 0.05$ level; **Significant $p < 0.01$ level (*t*-test).

Table II. Percentage of sperm abnormalities induced in male mice after oral treatment with different doses of FLX.

Dose (mg/kg b. wt.)	Abnormal sperm		Number of sperm head abnormalities					Coiled tail	Sperm motility mean % ± SE	Sperm count (x 10^6/ml) mean ± SE
	No	Mean % ± S.E.	Amorphous	Without hook	Triangle	Banana	Small			
I. Control	66	1.32 ± 0.40	24	12	20	–	2	8	88.64 ± 4.54	25.43 ± 1.90
II. FLX										
2.6	170	3.40 ± 0.46**	45	21	65	3	2	34	72.18 ± 4.82*	14.14 ± 4.92*
7.8	227	4.54 ± 0.28**	52	45	80	8	4	38	66.63 ± 6.32**	10.10 ± 5.52**
13.0	308	6.16 ± 0.52**	82	55	101	14	5	51	62.22 ± 4.82**	8.54 ± 5.36**

Total No of sperm count = 5000; *Significant $p < 0.05$ level; **Significant $p < 0.01$ level (*t*-test).

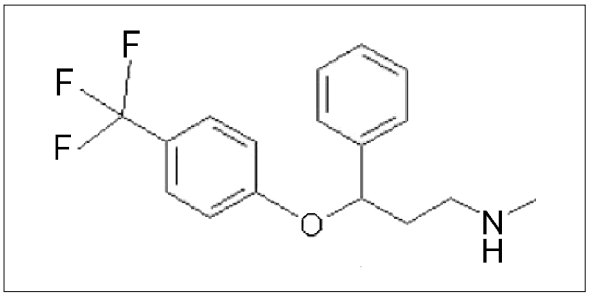

Figure 1. Fluoxetine.

Discussion

Medication in most cases may produce secondary health effects of variable degree, which in some cases may be a serious human health hazard. This potential damage is of particular concern with respect to compounds used for long periods and/or during pregnancy. The antidepressants studied are medicaments that may be continuously consumed for 6 months or longer, with a possible repetition of the treatment[33]. Also, there have been reports showing collateral health effects, mainly on the cardiovascular system[34,35], although other types of alterations have been described as well: for example, myoclonus, sexual dysfunction, and hyponatremia[36-38]. Besides, the development of mammary cancer and pheochromocytoma has been described[39,40], as well as a few cases of neonatal adaptation impairment and withdrawal syndrome when administered in the third trimester of pregnancy[41,42]. On the other hand, it is known that therapeutic drugs may produce genotoxic damage by a direct interaction with DNA or after their metabolic transformation, and that by establishing their genotoxic level it is possible to propose preventive measures[43].

There is an almost lack of *in vivo* mammalian studies to evaluate the effect of FLX on the genetic material. Our findings confirm the usefulness of the SCE evaluation to detect genotoxicity; it show a dose-dependent effect produced by

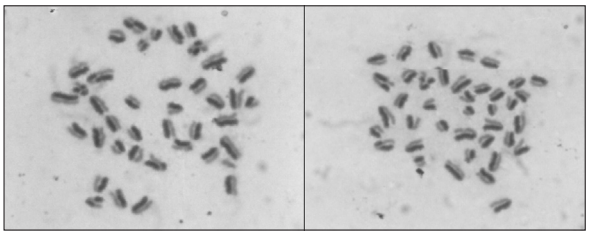

Figure 2. Metaphases from mice treated with FLX showing sister chromatid exchanges from mouse bone marrow cells.

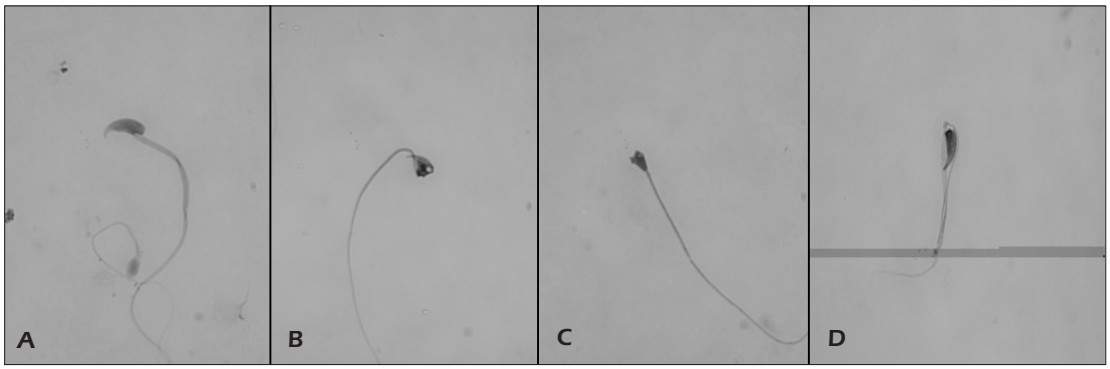

Figure 3. Sperm shape abnormalities induced in male mice treated with FLX showing ***(A)*** normal, ***(B)*** amorphous, ***(C)*** triangular and ***(D)*** coiled tail.

FLX, and a clear SCE increase with the median and highest doses treatment for five consecutive days. The results agree with Saxena and Ahuja[44] who observed that desipramine an antidepressant drug increases the frequency of SCE's and chromosomal aberrations in human lymphocyte cultures. Paniagua-Perez et al[45] reported that imipramine and desipramine have the ability to induced SCE's in mice bone marrow cells *in vivo*. Also, desipramine induced the genotoxic damage using the wing somatic and recombination test in *Drosophila*[46]. Kusakawa et al[47] revealed that FLX exhibits strong embryonic toxicity by assessment with IC50 and ID50 values, using a mouse ES cell differentiation system.

Sperms are important target cells in reproductive toxicology for assessment of spermatogenic damage, fertility and heritable genetic mutations[48,49]. Although not widely used in mutagenicity testing, the sperm morphology test proves to be a sensitive one. Sperm tests have also been used to study chemically induced sperm-mutagenic dysfunction in other mammalian species, including humans[49].

There are few data concerning the effects of antidepressant drug on the reproductive system of male mice. The results of the present study indicated that FLX administration at the doses of 2.6, 7.8 and 13 mg/kg b.wt resulted in a significant decrease in both sperm motility and count ($p < 0.01$) of the mice. Moreover, it induced increase in abnormal spermatozoa ($p < 0.01$).

The present results indicate that the administration of FLX causes a strong toxic effect on mouse seminiferous epithelium. The significant increase in sperm shape abnormalities may reflect chromosome abnormalities in primary spermatocytes and spermatids, and has always been associated with infertility[32,50]. Sperm-shaping is polygenetically controlled by numerous autosomal and sex-linked genes[51]; while sperm head abnormality was found to be correlated to germ cell mutational activity[51]. Induced sperm abnormalities indicate point mutations in germ cells[52], which should have triggered structural changes in cell organelles involved in head and tail formation, leading to sperm abnormalities.

Sexual dysfunction in both men and women has been reported frequently, but it is not always possible to differentiate drug-induced adverse effects from those induced by the underlying disease. Studies in men have suggested that SSRIs may damage normal sperm DNA integrity and thereby adversely affect fertility[53,54]. Safarinejad[53] reported that patients receiving SSRIs have sperm count and sperm motility ($p < 0.01$) lower than the normal control. Furthermore, SSRIs induced sperm abnormality ($p < 0.01$) higher compared to the normal patients. In men with normal semen parameters, paroxetine induced abnormal sperm DNA fragmentation in a significant proportion of subjects. The fertility potential of a substantial number of men on paroxetine may be adversely affected by these changes in sperm DNA integrity[54]. Also, lower serum gonadotropin and testosterone levels have been reported in depressed men treated with SSRIs compared to healthy men[55]. However, it is not known if these changes are related to depression or the medication.

Lister et al[56] reported that exposure of zebrafish (*Danio rerio*) to FLX for 7 d at environmentally relevant concentrations (0.32-32 μg L^{-1}) can significantly decrease egg production. Japanese medaka (*Oryzias latipes*) exposed to FLX at concentrations as low as 0.1 μg L^{-1} for 4 weeks showed significantly elevated plasma estradiol

and developmental deformities among offspring[57]. In goldfish (*Carassius auratus*), FLX (five injections of 5 μg g^{-1}over 14 d) decreased transcript levels in the brain of isotocin, the fish homolog of the mammalian neuropeptide oxytocin, indicating a mechanistic link between FLX exposure and reproductive dysfunction[58].

Reduced sperm concentration in the present study can be explained by a toxic effect of the doses of antidepressant on spermatozoa as well as spermatogonia and additionally a secondary effect related with negative feedback on steroid hormone negative feedback via the testes on the hypothalamus[59]. Friedman et al[59], moreover, reported that a higher cycle cancellation rate was observed secondary to poor ovarian response in women using SSRIs compared to nonusers. The Authors speculated that SSRI drugs may interfere with the hypothalamic-gonadal axis in subtle ways.

The chemical structure of the antidepressants includes two potentially dangerous components related with mutagenic and carcinogenic events, and particularly with the formation of SCE's: one of these components is the aromatic ring, and the other the nitro group[60,61]. The latter may be transformed into nitroso compounds causes genotoxic damage[62]. So much more care should be taken when we used antidepressant drugs.

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