DOI: 10.15386/cjmed-474

ESTROGENIC/ANTIESTROGENIC ACTIVITY OF SELECTED SELECTIVE SEROTONIN REUPTAKE INHIBITORS

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Abstract

Background and aims. *Selective serotonin reuptake inhibitors (SSRIs) are one of the most prescribed classes of psychotropics. Even though the SSRI class consists of 6 molecules (citalopram, escitalopram, fluoxetine, fluvoxamine, paroxetine and sertraline), only fluoxetine was intensively studied for endocrine disruptive effects, while the other SSRIs received less attention. This study was designed to evaluate the estrogenic/antiestrogenic effect of fluoxetine, sertraline and paroxetine.*

Methods. *The in vitro (anti)estrogenic activity was assessed using a firefly luciferase reporter construct in the T47D-KBluc breast cancer cell line. These cells express nuclear estrogen receptors that can activate the transcription of the luciferase reporter gene upon binding of estrogen receptor agonists.*

Results. *All three compounds were found to interact with the estrogen receptor. Fluoxetine had dual properties, weak estrogenic at lower concentrations and antiestrogenic effect at higher concentrations. Sertraline shared the same properties with fluoxetine, but also increased the estradiol-mediated transcriptional activity. Paroxetine presented only one type of effect, the ability to increase the estradiol-mediated transcriptional activity.*

Conclusions. *Overall, our results indicate a possible interaction of SSRIs with the estrogen receptor. As SSRIs are being used by all categories of population, including pregnant women or children, establishing whether they can affect the endocrine mediated mechanisms should be a priority.*

Keywords: fluoxetine, sertraline, paroxetine, luciferase assay

Background and aims

Selective serotonin reuptake inhibitors (SSRIs) are one of the most prescribed classes of psychotropics [1]. Fluoxetine (FLX), one of the most prescribed SSRIs, was quantified in the aquatic systems via sewage-treatment effluents [2]. Concerns were raised after a number of studies demonstrated that FLX can have hormonal effects. Studies that evaluated FLX in aquatic systems showed that it can affect the fecundity, circulating hormone levels and reproductive success of fish [2,3,4,5]. In addition, rodent studies confirmed that FLX exposure could lead to adverse endocrine and reproductive effects [3,6]. Erdemir et al showed that paroxetine (PRX) could affect spermatogenesis, PRX and FLX decreased the follicle-stimulating hormone (FSH) levels, while sertraline (SRT) lowered the levels of testosterone [7]. FLX, SRT, PRX were found to negatively affect sperm parameters [8,9].

SSRI therapy during pregnancy was linked to miscarriages, fetal defects and preterm births, but the results were mixed and inconclusive [10,11]. Croen et al presented a possible link between the fetal exposure to SSRIs, especially during the first trimester, and a modestly increased risk of developing autism spectrum disorders [12]. Withdrawal symptoms in the newborn were observed if the exposure to SSRIs was during the third semester [13].

Manuscript received: 18.05.2015
Received in revised form: 17.06.2015
Accepted: 19.06.2015
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Long-term developmental effects of prenatal and neonatal exposure to SSRIs is still under investigation.

SSRIs have been shown to cause sexual dysfunctions in patients, with an incidence of over 70% [14]. The affective dimensions of romantic love and attachment could also be influenced by AD [15].

Due to their interfering with normal sexual function, SSRIs may be used as anti-libidinal medication in sex offenders [16].

All these demonstrated effects may suggest an endocrine disruptive effect of SSRIs on the hypothalamic–pituitary–gonadal axis (HPT), on sexual steroid metabolism or on estrogenic or androgenic receptors, as SSRI use can be associated with low free testosterone serum levels [17].

Even though the SSRIs consist of 6 molecules (citalopram, escitalopram, fluoxetine, fluvoxamine, paroxetine and sertraline), only FLX was intensively studied for endocrine disruptive effects, while the other SSRIs received less attention [1].

This study was designed to evaluate the estrogenic/antiestrogenic effect of FLX, SRT and PRX using the T47D-KBluc breast cancer cell line. All compounds were tested individually and in mixtures with 17beta-estradiol (E_2) to detect potential additive, synergistic or antagonistic effects. FLX was chosen in this study in order to compare, if the case, its' potency with the other two molecules from SSRIs class.

Materials ans methods

Chemicals and reagents

Fluoxetine, sertraline and paroxetine were purchased from LGC Standards (Germany), 17-beta estradiol, resazurin, tricine, EDTA, dithiothreitol (DTT), and ATP were purchased from Sigma Aldrich (Steinheim, Germany). (MgCO3)4Mg(OH)2.5H2O was obtained from Acros Organics (Geel, Belgium), 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA) and Tris were from Fluka (Buchs, Switzerland) and luciferin was from Roth (Karlsruhe, Germany). All solvents and reagents were of analytical grade. RPMI 1640+GlutaMax medium, trypsin, Dulbecco's Phosphate Buffered Saline (PBS) and charcoal stripped FBS were purchased from Gibco (Paisley, UK). Fetal Bovine Serum (FBS) and Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham were obtained from Sigma (Steinheim, Germany).

Cell line

T47D-KBluc human breast cancer cells (estrogen receptor positive) were obtained from American Type Culture Collection (ATCC, USA). The cells were grown in RPMI 1960+ GlutaMax medium supplemented with 10% fetal bovine serum at 37°C with 5% CO_2 under saturating humidity and passaged every 2 to 3 days. Dulbecco's Phosphate Buffered Saline was used to rinse the cells and trypsin was used to detach cells from plastics. During the experiments, the cells were cultured in phenol red-free Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham, containing 10% charcoal stripped FBS.

Preparation of stock solutions

All compounds were prepared as 1000µL stock solutions in dimethyl sulfoxide solutions (DMSO, ≥99.5%, Riedel-de Haën, Seelze, Germany) at a concentration of 30 mM. Solutions of 0.005, 0.015, 0.05, 0.15, 0.5, 1.5, 5, 7.5, 10 and 15 mM for all three chemicals were obtained from stock solutions that were subsequently diluted with DMSO. These serial dilutions were then used to obtain the desired test concentration range for individual compounds.

Viability assay

For viability assay, cells were left to attach for 24 h, rinsed with 200µL phosphate buffered saline (PBS) and then exposed to individual test compounds at increasing concentrations and mixtures compound-estradiol (30 pM E_2) for 24 h. Cell viability was evaluated by measuring the capacity of the cells to reduce resazurin (final concentration 100 µM), a non-fluorescent reagent, to resorufin, a fluorescent product. The fluorescence was measured at $\lambda_{excitation}$=530/25; $\lambda_{emission}$=590/35, using a plate-reader (Synergy 2 Multi-Mode Microplate Reader, BioTek).

Luciferase assay

For luciferase induction, cells were seeded in 96 wells/plates and incubated for 24 h at 37°C. After this period, the medium was removed, the cells were rinsed with PBS then 100 uL medium was added together with another 100 uL medium that contained the tested compounds at increasing concentrations and mixtures compound-E_2. E_2 at a concentration of 30 pM served as a positive control and was used in combination with test chemicals to screen for anti-estrogenic effect. Each chemical was tested in three independent experiments in the presence or absence of E_2. The assay medium contained DMSO at a concentration of 0.2%. After exposure to test chemicals, the cells were lyzed using a low salt buffer containing 10 mM Tris, 1.99 mM DTT and 2 mM CDTA. Following a 15-minute incubation at -20°C, the plates were frozen at -80°C for a minimum of 30 minutes. Plates were thawed on ice and shaken for 30 mins at room temperature. Luciferase activity was measured using a luminometer (Synergy 2 Multi-Mode Microplate Reader, BioTek) with automatic injection of 100µL luciferin FlashMix [0.47 mM luciferin, 20 mM tricine, 1.07 mM (MgCO3)4Mg(OH)2.5H2O, 0.1 mM EDTA, 2 mM DTT and 5 mM ATP, pH 7.8] in each well. Light emission was extinguished with 50 µL NaOH 0.2 M to stop the reaction.

Data analysis and statistics

The results were expressed as mean ± standard deviation (S.D.). For each experiment, relative light units (RLUs) in every well were corrected by substracting the mean response of control wells. To compare data, the mean induction of luciferase, obtained at 30 pM E_2, was set at 100%. Data were expressed as percentages of the signal observed at 30 pM E_2. Statistical Analysis Origin software (OriginLab, Northampton, USA) was used

for graphical analyses. Data were analyzed by one-way analysis of variance (ANOVA). Differences in p values of <0.05 were considered statistically significant. To test for agonist properties, the signal from the cells treated with compounds was compared to the signal of the cells exposed to medium and DMSO (negative control). For estrogen antagonist properties, the comparison was made with the cells exposed to E_2.

Results

Viability assay

To assess cell viability for the estrogenic assay, the T47D-KBluc cell line was exposed to FLX, SRT and PRX in increasing concentrations (0.01, 0.03, 0.1, 0.3, 1, 3, 10, 15, 20 μM) for 24 hours. PRX was the most toxic compound, with 3μM as the highest concentration that did not have a significant decrease in the cell response to resazurin when compared to control. For SRT, concentrations lower than 15 μM did not decrease cell viability in a significant manner, while for FLX it was possible to test for estrogenic activity concentrations up to 15 μM.

For the antiestrogenic assay, cytotoxicity was assessed by incubating the cells for 24 hours to binary mixtures of the studied compounds (increasing concentrations) and 30 pM E_2. The concentration for estradiol was chosen as the minimal concentration where the maximum signal was obtained. The range of tested compound concentrations in the binary mixtures were as follows: FLX 0.01-15 μM, SRT 0.01-10 μM and PRX 0.01-3 μM.

(Anti)estrogenic assay

Fluoxetine and sertraline presented weak estrogenic effects. The maximal induction for FLX represented 7% from the activity of E_2 and it was observed at 1μM, while for SRT the maximal induction represented 8% from estradiol's activity and was observed at a lower concentration (at 0.1 μM) than in case of FLX. None of the tested concentrations of PRX was able to induce a significant luciferase expression, when compared to control.

When tested in the presence of E_2, FLX and SRT presented an antiestrogenic effect by decreasing in a significant manner the estradiol induced signal at 15 and 10μM for FLX+E_2 and at 10 μM in case of SRT+E_2 exposure (Figure 1a, b), but the decrease did not allow us to calculate the IC_{50}. Since at individual testing, in case of SRT at 0.01 μM and of PRX at 0.01 and 0.03 μM, no estrogenic effect was observed, the increase in the cellular response obtained during the mixture assay (compound+E_2), at 0.01 μM SRT (112%) (Figure 1b) and at 0.03 and 0.01 μM PRX (112% and 114%) (Figure 1c) indicate a potential synergistic effect between SSRIs and E_2.

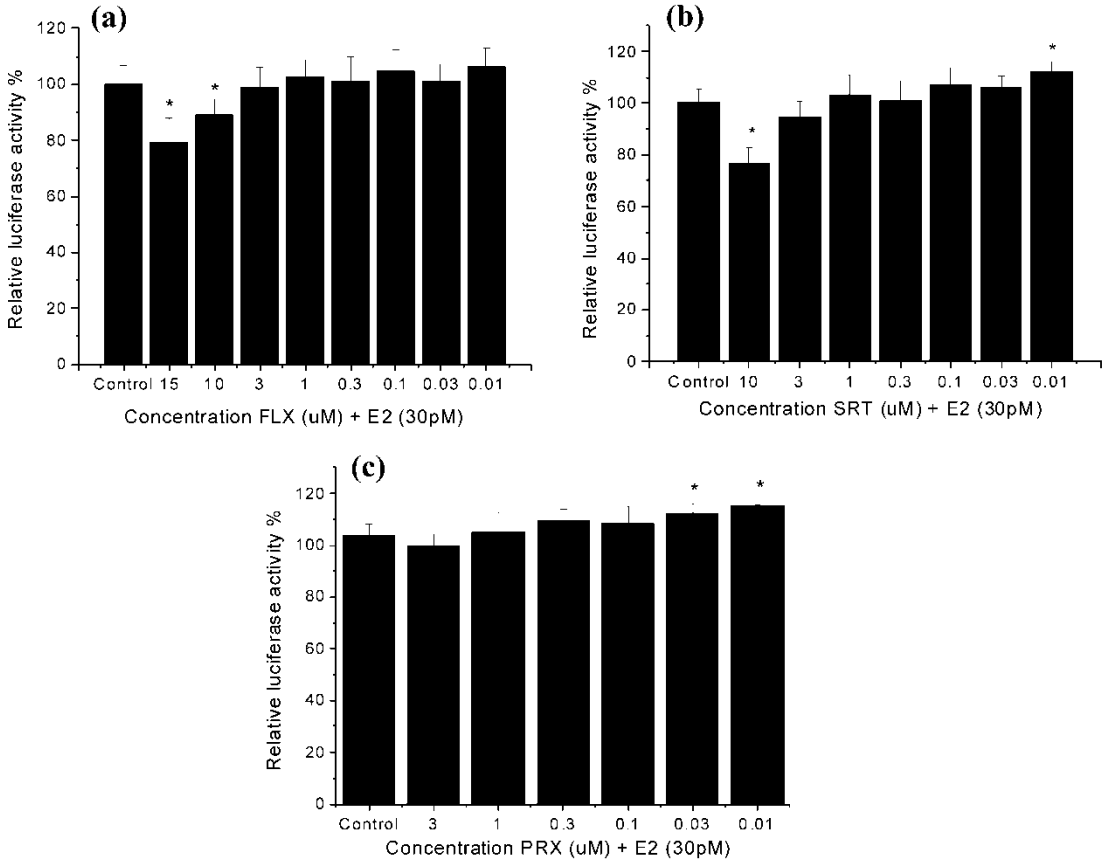

Figure 1. Effects of mixtures compound (increasing concentrations)-E_2 (30 pM) on luciferase activity in T47D-KBluc breast cancer cells: (a) FLX, (b) SRT (c) PRX. (*) statistical significance compared to positive control (E_2) ($p<0.05$).

Discussion

The use of antidepressants (AD) has increased considerably in the last decade, SSRIs being the most widely used class of AD [18]. Fluvoxamine was the first SSRI introduced in England in 1983, followed by FLX in 1989 [19], SRT in 1991 [20] and PRX in 1992 [21] in the United States. The blood concentrations of SSRIs are highly variable between individuals, but they are in the nanomolar to low micromolar range [22].

Even though the medical practice does not encourage the use of drugs during pregnancy or breastfeeding, the SSRIs representatives are among the drugs accepted, only after a risk-benefit analysis. SSRIs can pass through the placental barrier and maternal milk affecting the development of the fetus and the neonate, therefore it is of great importance the evaluation of the endocrine disruptive effect of the SSRIs representatives [23,24,25]. The results of the existing studies are mixed and inconclusive [11].

FLX is the most studied molecule from the SSRI group [26]. Studies were performed both *in vitro* and *in vivo* (in different types of fish, rodents). Studies on fish show that FLX can reduce the expression of estrogen receptors (ER) in the hypothalamus. Also, FLX can interfere with the neuroendocrine regulation of steroidogenesis and gametogenesis [2].

Rodent studied demonstrated that FLX can affect sexual brain differentiation, sexual behavior, testicular development and sperm production in rat offspring exposed *in utero* and/or through lactation [27,28,29,30]. Mueller et al showed that FLX can act as an estrogen receptor agonist both *in vitro* and *in vivo* [3].

Our results support the data from Muller et al., as FLX presented estrogenic activity, but at a lower concentration than that reported on MCF-7-ERE (1 μM vs 17 μM) [3]. Also, the relative response was smaller (20% vs 7%). In our case, due to cytotoxicity, the maximum concentration tested for FLX was 15 μM [3]. Unlike for Muller et al, the cells exposed to FLX and E_2 revealed an antiestrogenic activity at 15 and 10 μM. No increase in the cellular response was observed when compared to the E_2 signal. Therefore, FLX presented itself as a compound with dual properties: at lower concentrations it acts as a weak estrogen, while at higher concentrations, that could have biological relevance [31], it acts as a weak antiestrogen.

Besides FLX, an estrogenic effect was also detectable in case of exposure to SRT, but at a concentration 10 times lower than the estrogenic concentration for FLX. These *in vitro* results revealed that FLX and SRT present estrogenic activity without any metabolic activation. In this case, our results do not support the *in vivo* results of Montagnini et al where SRT was found not to have any estrogenic properties [1]. This finding could be explained by: a different mechanism, other than the interaction of SRT with the estrogenic receptor, when using our *in vitro* system, or the differences in bioavailability of SRT *in vivo* vs *in vitro*, or by the possibility that, *in vivo*, SRT could activate more than just one pathway. Also, depending on the moment of exposure (age) and the duration of exposure, the body could, by its mechanisms of protection, compensate this endocrine disruptive effect.

In case of co-exposure to SRT+E_2, at the highest concentration tested a decrease in the relative luminescence was noticed, SRT being able to antagonize a part of E_2 activity. Interestingly, at the lowest concentration tested (0.01 μM) an increase in the estradiol-mediated transcriptional activity was observed, this increase being statistically significant when compared to the positive control. The concentration where this effect appeared was 10 times lower than the concentration where the estrogenic effect was observed. SRT presented thus multiple proprieties, weak estrogen, weak antiestrogen and also modulator of the estradiol-mediated transcription.

Since the estrogenic effect and also the synergistic effect of SRT+E_2 were observed at submicromolar concentrations, this might suggest that this compound could interfere with estrogenic signaling at therapeutic plasma concentrations and this could be a reason for concern.

An increase in the E_2-mediated transcriptional activity was demonstrated also in case of the cells exposed to PRX, 0.03 and 0.01 μM being the concentrations where this type of activity was observed. This was the only effect observed when testing PRX on T47D-KBluc.

To our knowledge, this is the first time when the selected SSRIs are analyzed in the same sets of experiments, using the same end-points in order to compare their potency.

The limitation of our study would be the lack of testing the compounds in the presence of ERs antagonists, or confirming the ER interaction by using binding affinity studies.

Conclusions

Overall, our results indicate a possible interaction of SSRIs with the estrogen receptor. Taking into consideration that the estrogenic effects and the increase in the estradiol-induced activity appear at lower concentrations that can be therapeutically relevant, it would be of help to elucidate the specific mechanism(s) of action in case of exposure to SSRIs in order to estimate to which extent they can affect the endocrine-mediated activity.

Acknowledgement

This paper was published under the frame of European Social Found, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/136893.

References

1. Montagnini BG, Bortolan S, dos Santos BD, Moreno AP, Camin Nde A, Gerardin DC, et al. Evaluation of escitalopram, sertraline, and methylphenidate in the immature rat uterotrophic

assay. Int J Toxicol. 2013;32(6):426-430.
2. Mennigen JA, Lado WE, Zamora JM, Duarte-Guterman P, Langlois VS, Metcalfe CD, et al. Waterborne fluoxetine disrupts the reproductive axis in sexually mature male goldfish, Carassius auratus. Aquat Toxicol. 2010;100(4):354-364.
3. Müller JC, Imazaki PH, Boareto AC, Lourenço EL, Golin M, Vechi MF, et al. In vivo and in vitro estrogenic activity of the antidepressant fluoxetine. Reprod Toxicol. 2012;34:80–85.
4. Lister A, Regan C, Van Zwol J, Van Der Kraak G. Inhibition of egg production in zebrafish by fluoxetine and municipal effluents: a mechanistic evaluation. Aquat Toxicol. 2009;95(4):320-329.
5. Weinberger J 2nd, Klaper R. Environmental concentrations of the selective serotonin reuptake inhibitor fluoxetine impact specific behaviors involved in reproduction, feeding and predator avoidance in the fish Pimephales promelas (fathead minnow). Aquat Toxicol. 2014;151:77-83.
6. Uphouse L, Hensler JG, Sarkar J, Grossie B. Fluoxetine disrupts food intake and estrous cyclicity in Fischer female rats. Brain Res. 2006;1072(1):79-90.
7. Erdemir F, Atilgan D, Firat F, Markoc F, Parlaktas BS, Sogut E. The effect of sertraline, paroxetine, fluoxetine and escitalopram on testicular tissue and oxidative stress parameters in rats. Int Braz J Urol. 2014;40(1):100-108.
8. Tanrikut C, Feldman AS, Altemus M, Paduch DA, Schlegel PN. Adverse effect of paroxetine on sperm. Fertil Steril. 2010;94(3):1021-1026.
9. Safarinejad MR. Sperm DNA damage and semen quality impairment after treatment with selective serotonin reuptake inhibitors detected using semen analysis and sperm chromatin structure assay. J Urol. 2008;180(5):2124-2128.
10. Domar AD, Moragianni VA, Ryley DA, Urato AC. The risks of selective serotonin reuptake inhibitor use in infertile women: a review of the impact on fertility, pregnancy, neonatal health and beyond. Hum Reprod. 2013;28(1):160-171.
11. Ellfolk M, Malm H. Risks associated with in utero and lactation exposure to selective serotonin reuptake inhibitors (SSRIs). Reprod Toxicol. 2010;30(2):249-260.
12. Croen LA, Grether JK, Yoshida CK, Odouli R, Hendrick V. Antidepressant use during pregnancy and childhood autism spectrum disorders. Arch Gen Psychiatry. 2011;68(11):1104-1012.
13. Rudy E. SSRI antidepressant withdrawal syndrome in newborns. Drug Therapy Topics 2002;31(2):7-11
14. Clark MS, Jansen K, Bresnahan M. Clinical inquiry: How do antidepressants affect sexual function?. J Fam Pract. 2013;62(11):660-661.
15. Andrews PW, Thomson JA Jr, Amstadter A, Neale MC. Primum non nocere: an evolutionary analysis of whether antidepressants do more harm than good. Front Psychol. 2012;3:117.
16. Bradley DB. How to select pharmacologic treatments to manage recidivism risk in sex offenders. Current Psychiatry. 2009;8(10):60-72.
17. Cohen AJ. Antidepressant-induced sexual dysfunction associated with low serum free testosterone. 1999. Available from http://www.mental-health-today.com/rx/testos.htm
18. Health at a Glance 2013: OECD Indicators. Organisation for Economic Co-operation and Development. 2013. Available from: http://www.oecd-ilibrary.org/docserver/download/8113161e.pdf?expires=1428189161&id=id&accname=guest&checksum=5EE841ABAF519EF76895C3FA6DD62E40.
19. DeVane CL. Metabolism and pharmacokinetics of selective serotonin reuptake inhibitors. Cell Mol Neurobiol. 1999;19(4):443-466.
20. LiverTox. Clinical and research information on drug-induced liver injury. 2013. Available from: http://livertox.nih.gov/Sertraline.htm
21. LiverTox. Clinical and research information on drug-induced liver injury. 2014. Available from: http://livertox.nih.gov/Paroxetine.htm
22. Schulz M, Iwersen-Bergmann S, Andresen H, Schmoldt A. Therapeutic and toxic blood concentrations of nearly 1,000 drugs and other xenobiotics. Crit Care. 2012;16(4):R136.
23. Cooper WO, Willy ME, Pont SJ, Ray WA. Increasing use of antidepressants in pregnancy. Am J Obstet Gynecol. 2007;196(6):544.e1-e5.
24. Gentile S. The safety of newer antidepressants in pregnancy and breastfeeding. Drug Saf. 2005;28(2):137-152.
25. Rampono J, Simmer K, Ilett KF, Hackett LP, Doherty DA, Elliot R, et al. Placental transfer of SSRI and SNRI antidepressants and effects on the neonate. Pharmacopsychiatry. 2009;42(3):95-100.
26. Sumpter JP, Donnachie RL, Johnson AC. The apparently very variable potency of the anti-depressant fluoxetine. Aquat Toxicol. 2014;151:57-60.
27. Rayen I, Steinbusch HWM, Charlier TD, Pawluski JL. Developmental fluoxetine exposure and prenatal stress alter sexual differentiation of the brain and reproductive behavior in male rat offspring. Psychoneuroendocrinology. 2013;38:1618—1629.
28 Rayen I, Steinbusch HWM, Charlier TD, Pawluski JL. Developmental fluoxetine exposure facilitates sexual behavior in female offspring. Psychopharmacology (Berl). 2014;231:123–133.
29. de Oliveira WM, de Sá IR, de Torres SM, de Morais RN, Andrade AM, Maia FC, et al. Perinatal exposure to fluoxetine via placenta and lactation inhibits the testicular development in male rat offspring. Syst Biol Reprod Med. 2013;59:244–250.
30. Monteiro Filho WO, De Torres SM, Amorim MJ, Andrade AJ, de Morais RN, Tenorio BM, da Silva Junior VA. Fluoxetine induces changes in the testicle and testosterone in adult male rats exposed via placenta and lactation. Syst Biol Reprod Med. 2014;60(5): 274–281.
31. Bolo NR, Hodé Y, Nédélec JF, Lainé E, Wagner G, Macher JP. Brain pharmacokinetics and tissue distribution in vivo of fluvoxamine and fluoxetine by fluorine magnetic resonance spectroscopy. Neuropsychopharmacology. 2000;23(4):428-438.