JULIANE CENTENO MÜLLER

POSSÍVEIS ATIVIDADES (ANTI)ESTROGÊNICA E (ANTI)ANDROGÊNICA E EFEITOS REPRODUTIVOS E COMPORTAMENTAIS EM RATOS WISTAR EXPOSTOS À FLUOXETINA EM PERÍODOS CRÍTICOS DE DESENVOLVIMENTO

Tese apresentada como requisito parcial à obtenção do grau de Doutor em Farmacologia, Curso de Pós-Graduação em Farmacologia, Setor de Ciências Biológicas da Universidade Federal do Paraná.

Orientador: Prof. Dr. Paulo Roberto Dalsenter.
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PARECER

A Comissão Examinadora da Tese de Doutorado "POSSÍVEIS ATIVIDADES (ANTI)ESTROGÊNICA E (ANTI)ANDROGÊNICA E EFEITOS REPRODUTIVOS E COMPORTAMENTAIS DE RATOS WISTAR EXPOSTOS À FLUOXETINA EM PERÍODOS CRÍTICOS DE DESENVOLVIMENTO.", de autoria da pós-graduanda **JULIANE CENTENO MÜLLER,** sob orientação do Prof. Dr. Paulo Roberto Dalsenter e banca composta pelos professores: Prof. Dr. Paulo Roberto Dalsenter (Presidente - Farmacologia - UFPR); Profª. Drª. Estefânia Gastaldello Moreira (Ciências Fisiológicas-UEL); Prof. Dr. Roberto Andreatini (Farmacologia-UFPR); Prof.ª Dr.ª Rosana Nogueira de Morais (Fisiologia-UFPR); Prof. Dr. Sérgio Noboru Kuriyama (FIOCRUZ/RJ) reuniu-se e de acordo com o Regimento Interno do Programa de Pós-Graduação em Farmacologia, a pós-graduanda foi APROVADA. Para a devida publicação o trabalho deverá sofrer as modificações sugeridas, que serão conferidas pelo seu orientador. Em Curitiba, 15 de junho de 2012.

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Para onde quer que vá, vai todo, leva junto teu coração.

Confúcio

SUMÁRIO

APRESENTAÇÃO

Esta tese está apresentada na forma de três artigos, que correspondem aos itens 6, 7 e 8. Os itens materiais e métodos, resultados, discussão e referências encontram-se em cada artigo e representam a íntegra deste trabalho. O item 9, discussão geral, apresenta comentários gerais a respeito dos resultados obtidos nos três artigos. No item 10, conclusões, são apresentadas as conclusões gerais da tese. As referências referem-se às citações que aparecem na introdução, na revisão de literatura e na discussão da tese.

LISTA DE FIGURAS

LISTA DE SIGLAS

FDA *Food and Drug Administration:* Administração Federal de Alimentos e Medicamentos dos Estados Unidos da América.

OECD *Organization for Economic Cooperation and Development:* Organização para a Cooperação e o Desenvolvimento Econômico.

LISTA DE SÍMBOLOS E ABREVIATURAS

%	- Por cento
®	- Marca registrada
°C	- Grau Celsius
±	- Mais ou menos
α	- Alfa
β	- Beta
µL	- Microlitro
µg	- Micrograma
5-HT	- 5-hidroxitriptamina ou serotonina
5-HIAA	- Ácido 5-hidroxiindolacético
CYP	- *Cytochrome P450 superfamily*: superfamília do citocromo P450
DA	- Dopamina
DES	- *Endocrine-Disrupting Substances:* substâncias desreguladoras endócrinas
DNA	- *Deoxyribonucleic Acid*: ácido desoxirribonucléico
DOPAC	- Ácido 3,4-dihidroxifenilacético
DPP	- Depressão Pós-Parto
DSM-IV	- *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition:* manual diagnóstico e estatístico de transtornos mentais, quarta edição
h	- Hora
HPA	- Hipotálamo-Hipófise-Adrenais
HPG	- Hipotálamo-Hipófise-Gônadas
ISRS	- Inibidor Seletivo da Recaptação de Serotonina
kg	- Kilograma
L	- Litro
mL	- Mililitro
mg	- Miligrama
NA	- Noradrenalina
ng	- Nanograma
n°	- Número

RNA - *Ribonucleic Acid:* ácido ribonucleico

TDM - Transtorno Depressivo Maior

TDPM - Transtorno Disfórico Pré-Menstrual

TOC - Transtorno Obsessivo Compulsivo

TP - Transtorno do Pânico

™ - Trademark: marca registrada

RESUMO

O tratamento antidepressivo durante a gestação e lactação é desejável ou necessário quando os riscos da depressão não tratada para a mãe e o bebê são maiores do que aqueles associados com o uso de antidepressivos. Nas últimas décadas houve um aumento considerável no uso de antidepressivos durante a gestação e lactação, e a fluoxetina foi reportada como a droga mais utilizada durante estes períodos. A fluoxetina atravessa facilmente a barreira placentária, alcançando níveis séricos fetais semelhantes aos maternos, e é excretada no leite materno no momento da amamentação. Assim, um grande número de crianças em desenvolvimento é exposta a este antidepressivo *in utero* e durante a lactação. Estudos clínicos e pré-clínicos mostraram alterações reprodutivas em diferentes espécies após a exposição à fluoxetina. A partir desses estudos nossa primeira hipótese foi que a fluoxetina poderia estar mediando tais efeitos através de uma possível atividade estrogênica e/ou anti-androgênica. Esta hipótese foi testada pela investigação de possível atividade da fluoxetina (0,4, 1,7 e 17 mg/kg/dia) sobre receptores estrogênicos e/ou androgênicos pelos testes de Hershberger, uterotrófico e do gene repórter. Os resultados indicaram ação estrogênica da fluoxetina tanto *in vivo* (1,7 e 17 mg/kg) como *in vitro* (17 µM). Substâncias que atuam no sistema endócrino são passíveis de interferir com a regulação hormonal através de diferentes mecanismos e dessa maneira causar vários efeitos adversos na reprodução. Assim, a segunda parte deste estudo teve por objetivo testar a hipótese de que se a fluoxetina é capaz de interferir com a ação de hormônios gonadais, tais como o estradiol, a exposição à fluoxetina durante períodos críticos de desenvolvimento poderia produzir efeitos reprodutivos a curto e a longo-prazo. Esta hipótese foi testada através da avaliação dos parâmetros gestacionais e do desenvolvimento sexual dos descendentes no teste de exposição *in utero* e lactação em ratos Wistar. Os resultados da exposição *in utero* e lactação revelaram que a fluoxetina causou redução no ganho de massa corporal durante a prenhez, redução na massa corporal no 21° dia de prenhez (corrigido pela soma do peso dos filhotes no 1° dia pós-natal), menor peso ao nascimento, redução no índice de viabilidade e desmame, além de alterações hormonais e no peso de órgãos nas progenitoras tratadas com 17 mg/kg de fluoxetina durante a gestação e a lactação. Não foram observadas alterações no desenvolvimento sexual dos filhotes expostos à fluoxetina *in utero* e lactação. Outro aspecto a ser considerado é sobre os possíveis efeitos da exposição à fluoxetina em períodos de neurodesenvolvimento, já que a serotonina desempenha um papel importante na neuroplasticidade durante críticos estágios embrionários. Com isso, a terceira parte deste estudo teve por objetivo investigar se a exposição à fluoxetina *in utero* e lactação a ratos Wistar poderia causar alterações comportamentais e neuroquímicas a longo prazo através de dosagens de neurotransmissores e da avaliação nos testes de campo aberto, labirinto em cruz elevado, natação forçada modificado e preferência à sacarose. Os resultados do terceiro estudo mostraram que a exposição à fluoxetina produziu redução nos níveis de noradrenalina, do ácido 5-hidroxindolacético e do ácido 3,4-dihidroxifenilacético no córtex pré-frontal e hipocampo dos animais na vida adulta. Porém, não foram observadas alterações relevantes em comportamentos que expressam ansiedade e depressão nestes animais. As consequências funcionais e implicações clínicas

destas alterações neuroquímicas nas estruturas cerebrais permanecem a ser elucidadas. Concluímos que a fluoxetina demonstrou atividade estrogênica, causou toxicidade fetal e materna, além de induzir alterações hormonais em progenitoras tratadas com esta droga durante a gestação e lactação. A maioria destas alterações reprodutivas foi observada apenas com a maior dose de fluoxetina investigada (17 mg/kg). Além disso, a exposição à fluoxetina *in utero* e lactação produziu alterações a longo prazo nos níveis de neurotransmissores dos descendentes, e estas alterações variaram de acordo com o sexo e as doses de fluoxetina.

Palavras-chave: Toxicologia reprodutiva; fluoxetina, atividade estrogênica, *in utero* e lactação; toxicidade materna, toxicidade fetal, alterações hormonais, desenvolvimento sexual.

ABSTRACT

The use of antidepressant medications is currently acceptable for pregnant and lactating women in situations where the risks to the mother and child of not treating the disease are greater than the risks associated with exposure to the antidepressant drug. In recent decades has seen a large increase in the use of antidepressants during pregnancy and lactation, and fluoxetine was reported as the most widely used antidepressant during these periods. Fluoxetine readily cross the placenta reaching similar levels in maternal and fetal serum, and is also secreted into breast milk during lactation. Thus, a large number of developing children is exposed to fluoxetine *in utero* and during lactation. Clinical and preclinical studies showed reproductive changes in different species after exposure to fluoxetine. From these studies we hypothesized that fluoxetine could be mediating these effects through a possible estrogenic and/or anti-androgenic activity. This hypothesis was tested by investigating possible activities of fluoxetine (0.4, 1.7 and 17 mg/kg) on estrogenic and/or androgenic receptors in the Hershberger, uterotrophic, and reporter gene assays. The results indicated estrogenic activity of fluoxetine in both, *in vivo* (1.7 and 17 mg/kg) and *in vitro* (17 µM), assays. Substances that act on the endocrine system are likely to interfere with the hormonal regulation through different mechanisms and to produce several adverse effects on reproduction. Thus, the second part of this study was to test the hypothesis that if fluoxetine is able to interfere with the gonadal hormones action, such as estradiol, fluoxetine exposure during critical periods of development could produce reproductive effects in the short and long-term. This hypothesis was tested by evaluating pregnancy outcomes and sexual development of offspring in the *in utero* and lactation assay using Wistar rats. The results revealed that fluoxetine caused a reduction in body weight gain during pregnancy, body weight reduction on gestational day 21(corrected by subtracting the sum of the weights of the pups on postnatal day 1), low birth weight, reduction in viability and weaning indices, as well as hormonal and organ weights changes in dams treated with 17 mg/kg of fluoxetine during pregnancy and lactation. There were no changes in sexual development of offspring exposed to fluoxetine *in utero* and lactation. Another aspect to be considered is the possible effects of fluoxetine exposure in neurodevelopment periods. It is known that serotonin plays an important role in neuroplasticity in critical embryonic stages. Thus, the third part of this study was to investigate whether exposure to fluoxetine *in utero* and lactation could cause long-term neurochemical and behavioral changes in rats by measurement of neurotransmitters and assessment in the open field, elevated plus maze, forced swimming and sucrose preference tests. The results of the third study showed that exposure to fluoxetine produced a reduction in the levels of noradrenaline, 5-hydroxyindoleacetic acid and 3,4-dihydroxyphenylacetic acid in the prefrontal cortex and hippocampus in adulthood. However, there were no significant changes in behaviors that express anxiety and depression in these animals. The functional consequences and clinical implications of these neurochemical

changes in brain structures remain to be elucidated. We concluded that fluoxetine showed estrogenic activity, caused maternal and fetal toxicity, and induced hormonal changes in dams treated with this drug during pregnancy and lactation. Most of these reproductive changes was observed only with the highest dose of fluoxetine (17 mg/kg). Furthermore, exposure to fluoxetine *in utero* and lactation produced long-term changes in neurotransmitter levels of the offspring, and these changes varied according to the sex and the doses of fluoxetine.

Key-words: Reproductive Toxicology, fluoxetine, estrogenic activity, *in utero* and lactation, maternal toxicity, fetal toxicity, hormonal changes, sexual development.

1. INTRODUÇÃO

O transtorno depressivo é um grave problema de saúde pública com estimativas altas de prevalência ao longo da vida, chegando até 21% da população geral em alguns países desenvolvidos (Cryan et al., 2002). A Associação Psiquiátrica Americana define depressão como um transtorno psiquiátrico heterogêneo, freqüentemente manifestado a nível psicológico, comportamental e fisiológico (American Psychiatric Association, 1994).

A depressão está entre os distúrbios mais comuns na saúde das mulheres e estima-se que uma em quatro mulheres irá manifestar depressão em algum momento da sua vida (Kessler et al., 1999). Além disso, episódios depressivos estão frequentemente mais presentes durante a idade reprodutiva, e estima-se que aproximadamente 10% das mulheres irão apresentar depressão durante a gestação (Marcus et al., 2001; Marcus et al., 2003; Bennett et al., 2004) e 20% após o parto (Moraes et al., 2006). A própria gestação é acompanhada por mudanças hormonais marcantes, já que os níveis de hormônios produzidos pelos ovários e placenta durante a gestação são aproximadamente cem vezes maiores que em mulheres não grávidas (Bloch et al., 2003). Esse fator pode estar envolvido nas alterações de humor que ocorrem nesta fase, aumentando a predisposição do desenvolvimento ou recorrência de episódios de transtornos psiquiátricos em mulheres mais sensíveis a variações hormonais (Joffe e Cohen, 1998; Bloch et al., 2005; Camacho et al., 2006). De maneira semelhante, a queda brusca destes hormônios após o parto pode estar envolvida na etiologia da depressão puerperal (Bloch et al., 2003).

O tratamento farmacológico do Transtorno Depressivo Maior (TDM) em mulheres grávidas e lactantes ainda representa um desafio clínico. O uso de antidepressivos tem sido associado a complicações neonatais, incluindo baixa idade gestacional, parto prematuro, baixo peso ao nascimento, e pobre adaptação neonatal (Hackley, 2010; Roca et al., 2012). Em contrapartida, estudos clínicos têm mostrado que transtornos psiquiátricos, tais como a ansiedade e a depressão durante a gestação e puerpério, também estão relacionados ao estresse materno e a graves complicações materno-fetais, incluindo parto prematuro, restrição do crescimento fetal, prejuízo no

desenvolvimento mental, entre outros (Laplante et al., 2004; Jablensky et al., 2005; Austin et al., 2005; Dayan et al., 2006, Field et al., 2006, Marcus and Heringhausen, 2009; Hackley, 2010). Além disso, resultados dramáticos como infanticídio ou suicídio maternal podem ser consequências desastrosas de depressão não tratada durante a gestação e pós-parto (Camacho et al., 2006). Neste contexto, é considerado o uso de medicamentos antidepressivos para gestantes e lactantes em situações em que os riscos da doença não tratada, como o estresse e a morbidade substancial, possam ameaçar o desenvolvimento pré e pós-natal (Altshuler, 1996; American Psychiatric Association, 2010). O histórico clínico da paciente também deve ser avaliado, pois o TDM é hoje reconhecido como um transtorno recorrente, com risco aumentado de desenvolvimento e recaída para aqueles com histórico de episódios anteriores e descontinuação do tratamento com antidepressivos (Yonkers et al., 2009; American Psychiatric Association, 2010).

De acordo com dados de estudos retrospectivos, houve um aumento no uso de drogas antidepressivas durante a gestação, sobretudo da classe dos inibidores seletivos da recaptação de serotonina (ISRSs). Dentre estas, a fluoxetina foi reportada como o antidepressivo mais utilizado durante a gestação (Mc Connell et al., 1998; Cooper et al., 2007; Andrade et al., 2008). É importante ressaltar que a fluoxetina atravessa a barreira placentária, alcançando níveis séricos fetais semelhantes aos maternos (Heikkine et al., 2002), assim como é secretada no leite materno durante a amamentação (Hendrick et al., 2001, Suri et al., 2002). Dessa maneira devem ser considerados os riscos do uso deste medicamento sobre o feto e/ou neonato.

A diferenciação sexual gonadal e cerebral ocorrem predominantemente durante o período fetal (humanos e ratos) e perinatal (ratos) (Wilson e Davies, 2007). Assim, o feto e o recém-nascido são mais suscetíveis a efeitos adversos reprodutivos. Durante estes períodos sensíveis de desenvolvimento, substâncias capazes de interferir com a organização celular, podem provocar efeitos irreversíveis, que muitas vezes serão percebidos apenas na idade adulta, após o completo amadurecimento do sistema reprodutivo (Louis et al., 2008). As investigações a respeito dos possíveis efeitos adversos reprodutivos dos antidepressivos ISRSs usados durante a gestação e lactação são promissoras, porém ainda carecem de muitas informações sobre o modo de

ação destas substâncias nas diferentes etapas do desenvolvimento dos animais e seres humanos.

Vários estudos que avaliaram os efeitos reprodutivos da exposição à fluoxetina mostraram que esta droga pode interferir com o sistema endócrino de animais aquáticos (Fong, Huminski e D'Urso, 1998; Fong, 1998; Flaherty, Kashian e Dodson, 2001; Brooks et al., 2003ab; Foran et al., 2004; Henry e Black, 2008; Gust et al., 2009; Lister et al., 2009; Sánchez-Argüello, Fernández e Tarazona, 2009; Mennigen et al., 2010), roedores (Hoyt et al., 1989; Matuszczyk, Larsson e Eriksson, 1998; Tabacova, 2001; Taylor et al., 2004; Uphouse et al., 2006; Maswood, Sarkar e Uphouse, 2008; Silva et al., 2008; Bauer et al., 2010), e também de humanos (Iancu et al., 1992; Menkes et al., 1993; Strain, 1994; Warnock et al., 1995; Arya e Taylor, 1995; Egberts et al., 1997; Steiner et al., 1997; Simon, Cunningham e Davis, 2002; National Toxicology Program, Center for the Evaluation of Risks to Human Reproduction, 2003; Safarinejad, 2008ab). Estes resultados indicam uma possível ação estrogênica ou antiandrogênica da fluoxetina. Com isso, nossa hipótese para o primeiro estudo foi que a fluoxetina poderia estar mediando tais efeitos através de uma possível atividade estrogênica e/ou antiandrogênica. Esta hipótese foi testada usando ensaios *in vivo* e *in vitro* validados pela Organização para a Cooperação e o Desenvolvimento Econômico (OECD - Organisation for Economic Co-operation and Development) para triagem (identificação) de substâncias desreguladoras endócrinas (Organisation for Economic Co-operation and Development, 2006, 2007).

Substâncias com ação desreguladora endócrina são passíveis de interferir com a regulação hormonal através de diferentes mecanismos e causar vários efeitos adversos, como afetar o desenvolvimento, a diferenciação, a reprodução e o comportamento. Além disso, estudos pré-clínicos indicam que a exposição pré-natal ou perinatal à substâncias com potencial ação desreguladora endócrina podem resultar em efeitos reprodutivos irreversíveis que podem ter impacto ao longo da vida e até mesmo entre as gerações (Woodroof et al., 2008). Assim, a segunda parte deste estudo teve por objetivo testar a hipótese de que se a fluoxetina é capaz de interferir com a ação de hormônios gonadais, tais como o estradiol e/ou a testosterona, a exposição à fluoxetina durante períodos críticos de desenvolvimento poderia produzir

efeitos reprodutivos a curto e a longo-prazo. Esta hipótese foi testada através da avaliação de parâmetros gestacionais e lactacionais e de marcos do desenvolvimento sexual dos descendentes através do teste de exposição *in utero* e lactação em ratos Wistar (Environmental Protection Agency, 2005).

Outro aspecto a ser considerado é sobre os possíveis efeitos da exposição à fluoxetina em períodos de neurodesenvolvimento. Sabe-se que a serotonina desempenha um papel importante na neuroplasticidade durante críticos estágios embrionários (Gaspar, Cases e Maroteaux, 2003; Homberg, Schubert e Gaspar, 2010). Dessa maneira, também são necessários estudos mais aprofundados sobre as possíveis alterações comportamentais e neuroquímicas a longo-prazo que poderiam ser causadas pelo aumento dos níveis de serotonina durante o desenvolvimento encefálico do feto e do neonato pelo uso da fluoxetina durante a gestação e lactação. Com isso, a terceira parte deste estudo teve por objetivo investigar se a exposição à fluoxetina *in utero* e lactação a ratos Wistar poderia causar alterações comportamentais e neuroquímicas irreversíveis através de dosagens de neurotransmissores e testes comportamentais que avaliam comportamentos de locomoção, ansiedade e depressão.

2. REVISÃO DE LITERATURA

2.1. FLUOXETINA

2.1.1. Nomenclatura e propriedades físicas e químicas

A fluoxetina (CAS RN 54910-89-3) é o N-metil-gama-(4-(trifluorometil)-fenoxi) -, (+ -)-benzenopropanamina.

Outros nomes identificados são:

- (+-)-N-metil-3-fenil-3-((alfa, alfa, alfa-trifluoro-p-tolil) oxi) propilamina;
- (+-)-N-metil-gama-(4 - (trifluorometil) fenoxi) benzenopropanamina;
- (+-)-N-metil-3-fenil-3-((alfa, alfa, alfa-trifluoro-p-tolil) oxi) propilamina;
- (+-)-N-metil-gama-(4 - (trifluorometil) fenoxi) benzenopropanamina;
- N-metil-gama-(4 - (trifluorometil)-fenoxi) -, (+ -)-benzenopropamina;
- N-metil-3-(p-trifluorometilfenoxi)-3-fenilpropilamina;
- dl-3-(p-trifluorometilfenoxi)-N-metil-3-fenilpropilamina;

O cloridrato de fluoxetina (CAS RN 59333-67-4) é comercializado sob o nome Prozac® e Sarafem™ por Eli Lilly & Co, Indianápolis IN - EUA. Os dois nomes comerciais representam formulações químicas idênticas.

A fluoxetina é uma mistura racêmica 50/50 de R e S-enantiômeros. O cloridrato de fluoxetina é um sólido branco cristalino a esbranquiçado com um ponto de fusão de 158,4-158,9 °C e uma solubilidade de 14 mg/mL em água. A (S)-fluoxetina é dextrógira (1,60) em metanol, mas é levógira (-10,85) em água (National Toxicology Program, Center for the Evaluation of Risks to Human Reproduction, 2003).

A norfluoxetina, metabólito ativo da fluoxetina, também é uma mistura racêmica de R e S-enantiômeros. O enantiômero S é mais potente do que o enantiômero R. Nenhuma outra informação está disponível sobre as propriedades químicas e físicas de norfluoxetina (National Toxicology Program, Center for the Evaluation of Risks to Human Reproduction, 2003).

2.1.2. Estrutura, fórmula e massa molecular

A fórmula química da fluoxetina é $C_{17}H_{18}F_3NO$, e sua massa molecular é de 309,33, enquanto o cloridrato de fluoxetina tem uma massa molecular de

345,79. A fluoxetina é metabolizada em norfluoxetina, que possui a fórmula química $C_{16}H_{16}F_3NO$ (National Toxicology Program, Center for the Evaluation of Risks to Human Reproduction, 2003). A estrutura química da fluoxetina e da norfluoxetina estão ilustradas na Figura 1.

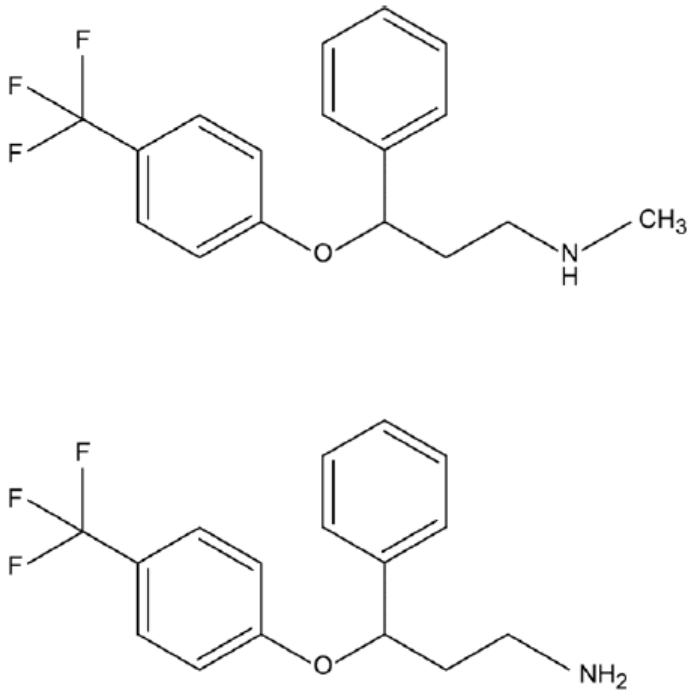

Figura 1: Estrutura química da fluoxetina (acima) e do seu metabólito norfluoxetina (abaixo).

Fonte: National Toxicology Program, Center for the Evaluation of Risks to Human Reproduction (2003).

2.1.3. Uso

A fluoxetina é um Inibidor Selevivo da Recaptação de Serotonina (ISRS), indicado pela *Food and Drug Administration* (FDA) para o tratamento do TDM, Transtorno Obsessivo-Compulsivo (TOC), bulimia nervosa, Transtorno do Pânico (TP) e Transtorno Disfórico Pré-Menstrual (TDPM) (Stokes e Holtz, 1997). Apesar dos ISRSs serem comercializados para várias indicações, a melhor atividade conhecida destes agentes é no tratamento da depressão. A fluoxetina foi reportada ser eficaz para o tratamento de todos os graus de depressão, que vão desde leve a grave (Stokes e Holtz, 1997). Além disso, alguns estudos mostraram que a fluoxetina foi tão eficaz quanto os

antidepressivos tricíclicos no tratamento da depressão grave (Wong, Bymaster e Engleman, 1995; Stokes e Holtz, 1997). Em 2003 a FDA aprovou o uso de fluoxetina para o tratamento do TDM e TOC em crianças e adolescentes (7-17 anos de idade) (Food and Drug Administration, 2003). Apesar da bula deste medicamento relatar que a fluoxetina esta bem estabelecida para o tratamento do TOC e do TDM, a sua segurança e eficácia em crianças menores de 7 anos com TOC e menores de 8 anos com TDM não foram estabelecidas (Eli Lilly, 2003).

2.1.4. Dose

De acordo com a bula do medicamento Prozac®, a dose inicial de fluoxetina indicada para o TDM em adultos é de 20 mg cada manhã, com um aumento da dose após várias semanas, se necessário, até um máximo de 80 mg por dia. Para a terapia semanal em adultos, a dose é de 90 mg uma vez por semana (Eli Lilly, 2003). A dose de 20 mg/dia é a mais amplamente prescrita e responde por cerca de 70% de todas as receitas aviadas (National Toxicology Program, Center for the Evaluation of Risks to Human Reproduction, 2003). As alterações fisiológicas que ocorrem durante a gravidez podem exigir que a dosagem de fluoxetina seja aumentada para manter a eficácia clínica (Hostetter et al., 2000).

Em 2002, cerca de 26,7 milhões de prescrições foram dispensadas para a fluoxetina nos Estados Unidos, com 1,2 milhões de receitas dispensadas aos pacientes pediátricos e adolescentes (1-18 anos) e 8,4 milhões para as mulheres em idade fértil (19-44 anos) (National Toxicology Program, Center for the Evaluation of Risks to Human Reproduction, 2003). O número de pessoas para as quais essas prescrições foram prescritas é desconhecido.

2.1.5. Exposição intrauterina e no leite materno

A exposição fetal pelo uso da fluoxetina por mulheres grávidas tem sido estimada através da dosagem de fluoxetina no sangue de cordão umbilical logo após o nascimento. As concentrações detectadas variaram entre 26 e 112 ng/mL para fluoxetina (Spencer, 1993; Mhanna e Bennet, 1997; Mohan e Moore, 2000; Heikkine et al., 2003), e 54 e 209 ng/mL para norfluoxetina (Spencer, 1993; Heikkine et al., 2003). Outros estudos também estimaram a

exposição fetal/neonatal pela dosagem de fluoxetina somada a norfluoxetina no sangue de cordão umbilical, e os valores encontrados variaram entre 65 e 114 ng/mL (Laine et al., 2003).

Vários trabalhos quantificaram as concentrações de fluoxetina no sangue e no leite de lactantes e lactentes (Taddio, Ito e Koren, 1996; Yoshida et al., 1998; Kristensen et al., 1999; Hendrick et al., 2001; Suri et al., 2002; Heikkine et al., 2003). Os intervalos de concentrações encontrados para a fluoxetina e norfluoxetina no leite, foram respectivamente <2-384 ng/mL e <2-321 ng/mL. As concentrações sanguíneas de fluoxetina e norfluoxetina em infantes variaram de indetectável a 340 ng/mL e 265 ng/mL, respectivamente. As concentrações no sangue materno foram medidas em 21-506 ng/mL para fluoxetina, e 43-674 ng/mL para norfluoxetina. A fluoxetina parece estar mais concentrada no leite mais rico em gordura (posterior ao colostro) do que no colostro. A razão leite:plasma varia de 0,05 a 6,09 para a fluoxetina e de 0,085 a 2,08 para norfluoxetina. Na maioria das vezes a razão foi menor do que 1. A exposição dos lactentes, estimado pela concentração sérica de norfluoxetina, está fortemente relacionada com a dose materna da fluoxetina, e com as concentrações séricas maternas da fluoxetina e norfluoxetina.

2.1.6. Farmacocinética

A fluoxetina é absorvida após ingestão oral em seres humanos, e uma dose oral única de 40 mg produz concentrações plasmáticas máximas de fluoxetina de 15-55 ng/mL após 6-8 horas (Eli Lilly, 2003). A média (± desvio padrão), dos níveis plasmáticos máximos após a dosagem de 20 mg de fluoxetina em seres humanos sob a forma de comprimidos ou cápsulas foram relatados em 8,88 ± 3,42, e 8,99 ± 2,95 ng/mL, respectivamente (National Toxicology Program, Center for the Evaluation of Risks to Human Reproduction, 2003). O alimento parece não afetar a biodisponibilidade da fluoxetina, embora possa retardar a sua absorção por uma a duas horas (Altamura, Moro e Percudani, 1994; Eli Lilly, 2003).

No plasma humano, 94,5% da fluoxetina liga-se a proteínas de ligação, principalmente a albumina e a α_1-glicoproteína (Eli Lilly, 2003). O volume de distribuição em humanos tem sido relatado como 20-42 L/kg (revisto por Altamura, Moro e Percudani, 1994). As concentrações reportadas no plasma

humano após 30 dias de administração de 40 mg/dia foram de 91 a 302 ng/mL para a fluoxetina e de 72 a 258 ng/mL para a norfluoxetina (Eli Lilly, 2003).

A fluoxetina é desmetilada para norfluoxetina pelo citocromo P450 (CYP) (revisado por Caccia et al., 1998). Preparações *in vitro* de enzimas humanas microssomais mostraram que estas enzimas são ativadas no processo de N-desmetilação. Para (R)-, (S) -, e fluoxetina racêmica, CYP2D6 produziu os maiores valores de depuração (calculado a partir de um modelo farmacocinético), seguido em ordem pelo CYP2C9, CYP3A4 e CYP2C19 para (R)-fluoxetina, e pelo CYP3A4, CYP2C9, e CYP2C19 para (S)-fluoxetina (Margolis et al., 2000). Quando os valores *in vitro* foram corrigidos pela quantidade prevalente das isoformas CYP no fígado humano, CYP2C9, CYP3A4, e CYP2D6 foram estimados serem responsáveis por 43%, 32%, e 20% da depuração da fluoxetina *in vivo*. Tanto a fluoxetina como a norfluoxetina são glicuronizadas no fígado. Outro metabólito nos seres humanos é o ácido hipúrico, um conjugado de glicina de ácido benzóico (Altamura, Moro e Percudani, 1994). Outras destinos metabólicos não foram bem caracterizados.

Nos seres humanos, cerca de 80% de fluoxetina é excretada na urina e de 15% nas fezes. Os produtos de excreção de urina consistem em fluoxetina, fluoxetina glicuronídeo, norfluoxetina, norfluoxetina glicuronídeo, e ácido hipúrico. A meia-vida plasmática da fluoxetina é de 1-4 dias e a meia vida da norfluoxetina é 7-10 dias. A insuficiência renal não influencia as meias-vidas plasmáticas, mas a insuficiência hepática aumenta as meias-vidas (Altamura, Moro e Percudani, 1994).

2.1.7. Farmacodinâmica

A ação farmacológica da fluoxetina e de outros ISRSs foi revisada por Wong et al. (1995) e por Stokes e Holtz (1997). A serotonina é a 5-hidroxitriptamina (5-HT) (Figura 2), um neurotransmissor que também possui funções fisiológicas em plaquetas, no trato gastrointestinal, e em outras partes do corpo. No encéfalo, neurônios contendo 5-HT têm seus corpos celulares principalmente na linha média do tronco cerebral, mas as projeções axonais desses neurônios se projetam por todo o cérebro. Neurônios serotonérgicos

desempenham um papel na regulação do humor, sono, atividade sexual, atividade motora, função neuroendócrina, e cognição.

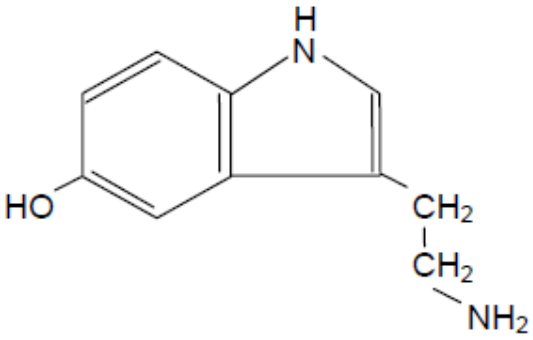

Figura 2: Estrutura química da serotonina.

Fonte: National Toxicology Program, Center for the Evaluation of Risks to Human Reproduction (2003).

De acordo com as revisões feitas por Wong et al. (1995) e por Stokes e Holtz (1997), as seguintes evidências obtidas a partir de vários estudos sugerem que a 5-HT desempenha um papel na depressão e levou ao desenvolvimento da fluoxetina: (a) níveis reduzidos de 5-HT e do ácido 5-hidroxindolacético (5-HIAA) em tecido cerebral ou líquido cefalorraquidiano de vítimas de suicídio; (b) efeitos antidepressivos, após tratamento com triptofano ou 5-hidroxitriptofano, isolado ou em combinação com inibidores da monoamina oxidase; e (c) tendência de pacientes deprimidos terem defeito no transporte de 5-HT e na atividade do receptor 5-HT_2 em plaquetas.

Em neurônios serotonérgicos, a 5-HT é sintetizada através da hidroxilação do triptofano para 5-hidroxitriptofano, que é então descarboxilado. A 5-HT produzida é por sua vez armazenada em vesículas até a sua liberação para a fenda sináptica através de um impulso nervoso. Quando liberada, a 5-HT pode ativar um dos vários subtipos de receptores pré- ou pós-sinápticos de serotonina (por exemplo, $5\text{-HT}_{1A,\ B,\ D,\ E\ ou\ F}$, $5\text{-HT}_{2A,\ C,}$ 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6 e 5-HT_7). A ação da 5-HT termina quando esta se liga ao transportador pré-sináptico para sua recaptação no terminal do nervo pré-sináptico onde ocorre a conversão da 5-HT a 5-HIAA pela monoamina oxidase A (Figura 3).

Quando uma droga ISRS é administrada, o efeito imediato é o bloqueio do transportador de recaptação da 5-HT. Essa ação causa um aumento súbito da 5-HT predominantemente nas áreas somatodendríticas (fenda sináptica em torno dos dendritos do corpo celular), e com isso a maior disponibilidade de 5-HT para receptores serotonérgicos em determinadas regiões do cérebro. Entretanto, nos terminais axônicos, onde presumivelmente a 5-HT é necessária para exercer sua ação terapêutica, este aumento de 5-HT é mais tardio. Essa maior disponibilidade de 5-HT imediata seria responsável pela maioria dos efeitos colaterais desta classe de drogas (efeitos gastrintestinais e sexuais relacionados com os receptores 5-HT_3, agitação relacionada a receptores 5-HT_2C). O aumento nos níveis de 5-HT no início do tratamento antidepressivo faz com que ocorra uma inibição da liberação neuronal da 5-HT, como também da atividade da enzima triptofano-hidroxilase (retroalimentação negativa). Entretanto, se o medicamento ISRS é administrado cronicamente, o aumento sustentado de 5-HT na área somatodendrítica causa uma dessensibilização dos auto-receptores 5-HT_{1A} dessa área. Em função da sua dessensibilização, estes receptores não detectam as altas quantidades de 5-HT aí existentes e enviam uma mensagem para os terminais axônicos liberarem mais 5-HT na fenda sináptica, desinibindo a neurotransmissão serotoninérgica. Essa dessensibilização dos receptores 5-HT_{1A} faz com que ocorra um aumento da atividade, produção e liberação pré-sináptica de 5-HT. Essa ativação é tardia quando comparada ao aumento de 5-HT nas áreas somatodendríticas dos neurônios serotoninérgicos. O tempo despendido pode ser o responsável pela ação terapêutica não imediata das drogas ISRSs. Outras alterações secundárias incluem a infrarregulação gradual dos receptores 5-HT_{2A} pós-sinápticos, que pode contribuir para os efeitos antidepressivos (Figura 3).

A fluoxetina e o seu principal metabolito, a norfluoxetina, têm uma elevada afinidade pelo transportador de 5-HT e ligam-se seletivamente ao transportador de acordo com um processo saturável que requer sódio. Em contraste, a fluoxetina tem baixa afinidade para locais de captação de noradrenalina (NA) e receptores de neurotransmissores, tais como os histaminérgicos, α_1-adrenérgicos, α_2-adrenérgicos, β-adrenérgicos, dopaminérgicos, muscarínicos, opiáceos, gabaérgicos, e benzodiazepínicos. A fluoxetina também tem uma afinidade relativamente baixa para a maioria dos

receptores serotonérgicos, incluindo 5-$HT_{1A, B, D}$, 5-HT_{2A} e 5-HT_3. Apesar da baixa atividade para o transportador de NA, os ISRSs diminuem a atividade da dopamina hidroxilase, que é a enzima passo limitante na velocidade de síntese de NA.

É importante ressaltar que existem proteínas transportadoras de 5-HT na placenta (revisto por Nguyen et al., 1999). No entanto, a interação da fluoxetina com estes transportadores ainda não foi esclarecida.

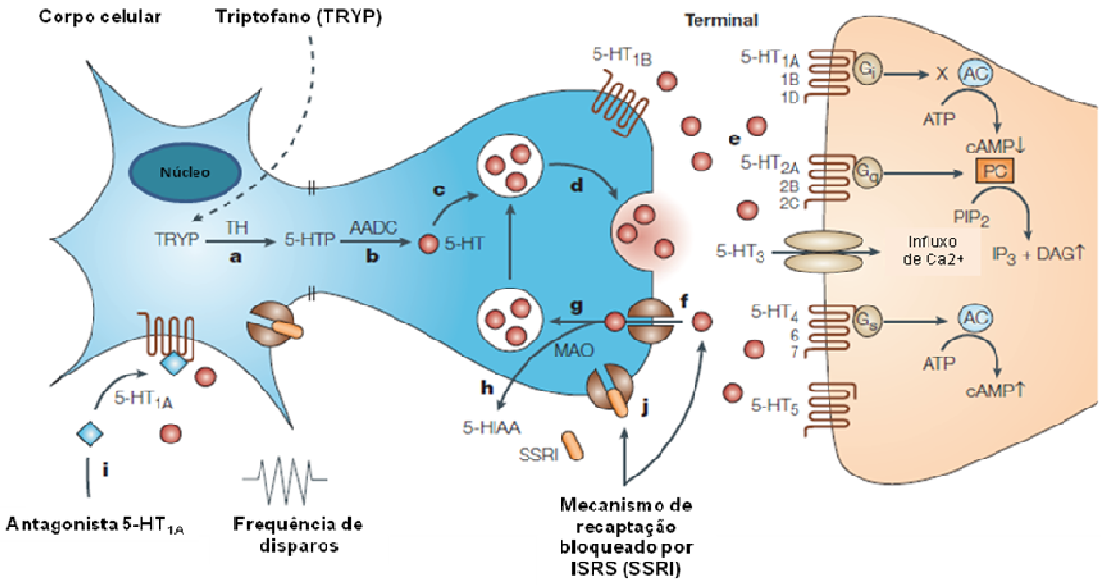

Figura 3: Esquema de neurotransmissão serotonérgica e alvo farmacológico da fluoxetina. a: triptofano-hidroxilase (TH) catalisa a conversão do triptofano (TRYP) a 5-hidroxitriptofano (5-HTP). **b**: descarboxilase de aminoácidos aromáticos (AADC) catalisa a conversão de 5-HTP a 5-hidroxitriptamina (5-HT, serotonina). **c**: 5-HT é acondiconada em vesículas de armazenamento. **d:** 5-HT é liberada a partir das vesículas de armazenamento para a fenda sináptica. **e:** 5-HT pode ativar os subtipos de famílias de receptores 5-HT (1, 2, 3, 4, 5, 6 e 7), os quais estão acoplados a seus respectivos sistemas de transdução de sinal no interior do neurônio pós-sináptico. **f**: 5-HT é levada para dentro dos terminais serotonérgicos pré-sinápticos pelo transportador de 5-HT. **g, h:** dentro dos terminais serotonérgicos pré-sinápticos a 5-HT será ou absorvida pelas vesículas de armazenamento ou degradada pela monoamina oxidase (MAO). **I**: 5-HT ativa o autoreceptor somatodendritico pré-sináptico 5-HT_{1A}, que pode ser bloqueado por antagonistas seletivos 5-HT_{1A}. **j:** inibidores seletivos de recaptação de serotonina, incluindo a fluoxetina, inibem o transportador de 5-HT. 5-HIAA, ácido 5-hidroxiindolacético; AC, adenilato ciclase; DAG, diacilglicerol; IP_3, inositol-1,4,5-trifosfato; PIP_2, fosfatidilinositol-4 ,5-bifosfato.

Fonte: Figura traduzida a partir de Wong et al. (2005).

2.2. DEPRESSÃO DURANTE A GESTAÇÃO E PÓS-PARTO

2.2.1. Características clínicas

O manual diagnóstico e estatístico de transtornos mentais, quarta edição (DSM-IV) não distingue os transtornos do humor durante a gestação e no pós-parto daqueles que acontecem em outros períodos, exceto como especificador, por exemplo “com início no pós-parto”, quando o início dos sintomas ocorre no período de quatro semanas após o parto. O DSM-IV define o diagnóstico de depressão usando os mesmos critérios para homens e mulheres, embora estudos mostrem algumas variações da apresentação no sexo feminino. O diagnóstico de TDM deve incluir a existência de humor deprimido ou irritável ou incapacidade de sentir prazer. Além disso, quatro dos seguintes sintomas devem estar presentes: (*i*) sentimentos de culpa, desesperança e inutilidade; (*ii*) distúrbios do sono (insônia ou hipersonia); (*iii*) alterações do apetite ou peso; (*iv*) dificuldades de atenção ou concentração; (*v*) diminuição da energia ou fadiga inexplicável; (*vi*) agitação ou retardo psicomotor; e, em casos graves, (*vii*) pensamentos de suicídio (Manual diagnóstico e estatístico de transtornos mentais, 1995). As mulheres ainda podem apresentar sintomas de depressão atípica (por exemplo, hipersonia, hiperfagia, desejo de carboidratos, ganho de peso, sensação de peso nos braços e pernas, piora dos sintomas à noite e insônia inicial) (Manual diagnóstico e estatístico de transtornos mentais, 1995). Muitos desses sintomas se sobrepõem com as mudanças físicas e mentais vivenciadas durante a gravidez, tornando-os difíceis de distinguir, e, portanto, muitas vezes estes sintomas acabam sendo desconsiderados (Kumar e Robson, 1984).

2.2.2. Epidemiologia

A depressão é um grave problema de saúde pública com altas taxas de prevalência ao longo da vida. Segundo a Organização Mundial de Saúde, estima-se que em 2020 a depressão maior unipolar será a principal causa de incapacidade em mulheres (World Health Organization, 2009). De acordo com estudos epidemiológicos, o TDM é aproximadamente duas vezes mais prevalente em mulheres que em homens (Angst et al, 2002;. Organização Mundial da Saúde, 2009), e a gestação e o pós-parto constituem os períodos

de maior risco para o desenvolvimento e/ou recorrência de episódios depressivos. Estima-se uma prevalência de depressão na gravidez da ordem de 7,4% no primeiro, 12,8% no segundo e 12% no terceiro trimestre (Bennett et al., 2004). Nas adolescentes foi verificada prevalência entre 16% e 44%, quase duas vezes mais elevada que nas gestantes adultas, o que pode estar relacionado à falta de maturidade afetiva e de relacionamentos dessas pacientes, bem como ao fato de grande parte delas terem que abandonar seus estudos em razão da maternidade (Szigethy e Ruiz, 2001).

A disforia no pós-parto inclui sintomas depressivos leves e pode ser identificada em 50% a 85% das puérperas, dependendo dos critérios diagnósticos utilizados. Um estudo com 1.558 mulheres detectou 17% das gestantes com sintomas significativos de depressão na gestação tardia, 18% no puerpério imediato e 13% entre a sexta e a oitava semanas do puerpério. O mesmo valor (13%) foi encontrado no sexto mês do puerpério (Josefsson et al., 2001). De maneira semelhante, uma metanálise de 59 estudos mostrou uma estimativa de prevalência de Depressão Pós-Parto (DPP) na ordem de 13% (O'Hara e Swain, 1996).

2.2.3. Depressão na gestação e pós-parto - possível relação com hormônios gonadais

O período de maior risco para as mulheres desenvolverem depressão é durante a fase reprodutiva (Kessler, 2003; Marcus, 2009), quando os hormônios esteróides e peptídicos variam drasticamente. Na verdade, o pós-parto é considerado o período de maior risco para a mulher desenvolver depressão (Drevets e Todd, 2005) e acredita-se que as flutuações hormonais durante a gravidez e o pós-parto podem desempenhar um papel importante no estabelecimento dos sintomas depressivos.

Tem sido sugerido que as flutuações hormonais no sexo feminino desempenham um papel na etiologia da depressão, em particular durante as fases reprodutivas (Studd e Panay, 2009). No entanto, até o momento nenhuma associação forte entre os estrogênios, a progesterona ou hormônios da gravidez e a depressão foram relatados (revisado por Zonana e Gorman, 2005). Isto pode ser, em parte, devido ao fato de que muitos estudos não levam sempre em conta as variáveis de confusão, tais como a idade, a

paridade, ou a amamentação. Por exemplo, a amamentação pode alterar os níveis hormonais de cortisol e ocitocina (Uvnas-Moberg e Eriksson, 1996; Tu et al., 2006) e, assim, é importante determinar a hora da última sessão de amamentação antes dos níveis plasmáticos destes hormônios serem dosados em lactantes. No entanto, o fato do risco para as mulheres desenvolverem distúrbios de humor ser maior durante o período pós-parto e durante a transição para a menopausa (Freeman et al., 2004; Woods et al., 2008) aponta para o envolvimento de hormônios gonadais na etiologia da depressão feminina.

Uma hipótese sobre a causa da DPP é a "hipótese da retirada dos esteróides ovarianos" (Hendrick et al., 1998; Bloch et al., 2000; Galea et al., 2001). Em mulheres os níveis de estrogênios sobem pouco antes do parto para mais de cem vezes de seus valores normais e em seguida caem drasticamente após o nascimento com a expulsão da placenta (Bloch et al., 2003). Acredita-se que esta queda brusca nos níveis hormonais possa ter um papel importante na disforia pós-parto, um fenômeno que é observado em 80% das mulheres logo após o nascimento e que pode se transformar em DPP (Hendrick et al., 1998; Bloch et al., 2003). Estes achados sugerem que declínios nos níveis de estradiol e/ou de progesterona podem predispor as mulheres ao transtorno depressivo. Isto é consistente com evidências de que mulheres que sofrem da síndrome de ovário policístico, que está associada a baixos níveis de estrogênios, têm um risco aumentado para transtornos de humor (Kerchner et al., 2009). Além disso, a incidência e gravidade da depressão aumenta durante a perimenopausa e menstruação, quando os hormônios ovarianos diminuem drasticamente e flutuam de maneira proeminente (Woods et al., 2008). Bloch et al. (2000) testaram a hipótese de que a retirada dos hormônios ovarianos poderia desencadear sintomas depressivos em seres humanos. Estes autores induziram hipogonadismo em mulheres (dando um agonista do hormônio liberador de gonadotrofinas) com ou sem história de depressão no pós-parto e reintroduziram doses suprafisiológicas de estradiol e progesterona por oito semanas. Depois de retirar ambos os esteróides, 62,5% das mulheres com história de depressão no pós-parto desenvolveram sintomas de humor depressivos significativos, enquanto nenhuma mulher do grupo controle desenvolveu tais sintomas. Este resultado sugere que a retirada dos hormônios

ovarianos pode induzir sintomas depressivos em mulheres suscetíveis. Além disso, um estudo mostrou que além dos estrogénios, a retirada de progesterona também pode contribuir para o desenvolvimento da DPP (Beckley e Finn, 2007).

Em contraste, a prevalência de depressão diminui ligeiramente em mulheres após a idade de cinquenta anos (a idade média da menopausa) de tal forma que aos sessenta anos, o risco de desenvolver depressão nas mulheres em relação aos homens cai para cerca de 1,5 vezes, em comparação com 2 ou 3 durante os anos reprodutivos (Gutierrez-Lobos et al., 2002). Isso sugere que os níveis mais altos de depressão em mulheres durante a idade fértil estão associados com os hormônios gonadais e que as mulheres com uma predisposição para a depressão podem ser mais sensíveis a alterações de humor de acordo com as flutuações nos níveis hormonais.

2.2.4. Consequências da depressão não tratada na gestação e pós-parto

A depressão durante a gestação é considerada uma das condições mais sub-reconhecidas e sub-tratadas na clínica médica (Marcus, 2009). Além disso, a situação torna-se mais complicada, pois mesmo que a depressão seja diagnosticada, muitas mulheres são relutantes em tomar a medicação antidepressiva devido ao fato de que não se sabe muito sobre as consequências a longo prazo da exposição precoce do feto e do recém-nascido aos antidepressivos (Brummelte e Galea, 2010).

A depressão não identificada e não tratada pode levar a efeitos prejudiciais sobre a mãe e o feto ou recém-nascido. Mulheres deprimidas são mais propensas a participar de práticas pouco saudáveis durante a gravidez, tais como o tabagismo e o abuso de substâncias ilícitas. Essas mulheres têm taxas mais altas de má nutrição, em parte devido à falta de apetite, levando ao baixo ganho de peso durante gravidez com risco aumentado para retardo de crescimento intrauterino. Além disso, mulheres deprimidas são menos complacentes com o pré-natal e menos comprometidas nos cuidados com a gravidez. Finalmente, mulheres que sofrem de depressão têm dores e desconfortos aumentados durante a gravidez, relatando piores náuseas, dores de estômago, falta de ar, sintomas gastrointestinais, taquicardia, e tonturas em comparação com mulheres não deprimidas (Zuckerman et al., 1989).

A depressão não tratada durante a gravidez tem sido associada com parâmetros gestacionais e neonatais prejudicados tais como pré-eclâmpsia, baixo peso ao nascimento, aumento do risco de parto prematuro, menor circunferência da cabeça, aumento de intervenções cirúrgicas ao nascimento, baixos escores no teste de Apgar, e mais entradas neonatal em unidades de terapia intensiva (revisado por Marcus e Heringhausen, 2009). Estudos sugerem que a depressão materna leva a alterações no eixo Hipotálamo-Ptuitária-Adrenais (HPA) da mãe e no fluxo sanguíneo uterino, os quais podem contribuir para o parto prematuro, baixo peso ao nascimento, e pré-eclampsia (Wadhwa et al., 1996; Teixeira et al., 1999).

Bebês de mães que sofreram de depressão durante a gravidez têm níveis de cortisol e catecolaminas elevados ao nascimento. Essas crianças choram com mais frequência e são mais difíceis consolar do que bebês nascidos de mulheres não deprimidas (Marcus e Heringhousen, 2009). Quando a depressão continua no período pós-parto podem existir riscos de efeitos a longo prazo sobre a criança, tais como pobre afeto mãe-bebê, atraso nas habilidades cognitivas e linguísticas, desenvolvimento emocional prejudicado, e alterações comportamentais (Marcus e Heringhousen, 2009). Um estudo reportou que se um bebê é exposto a um ambiente de depressão materna durante os quatro primeiros meses de vida, mesmo que a mãe receba tratamento mais tarde, o atraso no desenvolvimento da criança permanece (Forman et al., 2007). De fato, estudos tem mostrado que filhos de mães com DPP têm um risco aumentado de desenvolver depressão, transtornos emocionais e anti-social e apresentar deficiência cognitiva, motora e no desenvolvimento social (Pilowsky et al., 2006; Deave et al., 2008; Hay et al., 2008). Assim, o estado materno durante a gestação e pós-parto pode influenciar decisivamente o emocional e os resultados cognitivos da descendência e também pode contribuir para uma maior vulnerabilidade a distúrbios neuropsiquiátricos. Além disso, mulheres com depressão no período pós-natal são mais propensas a abusar de seus filhos, cometer suicídio e infanticídio (Pilowsky et al, 2006; Goodman, 2007).

2.2.5. Fluoxetina durante a gestação e lactação – Considerações gerais

Estudos clínicos

Existe uma grande controvérsia na literatura quanto ao uso de antidepressivos durante a gestação e lactação. A maioria dos primeiros estudos que analisaram a utilização de antidepressivos durante a gestação sugere que a exposição a antidepressivos ISRSs seja improvável de contribuir para grandes anomalias congênitas, acima do valor basal de risco de 1% a 3% observada na população em geral (Altshuler et al., 1996; Nulman e Koren, 1996; Goldstein, Corbin e Sundell, 1997; Ericson et al., 1999; Addis e Koren, 2000). No entanto, alguns estudos sugerem que a exposição aos ISRSs no primeiro trimestre de gestação possa contribuir para a prematuridade, restrição de crescimento fetal e malformações menores (Hendrick et al., 2003; Källén, 2004; Chambers, 2006), e exposições mais longas aos ISRSs foram associadas com aumento para o risco de prematuridade (Oberlander et al., 2008). Chambers et al. (2006) relataram risco aumentado para hipertensão pulmonar persistente em recém-nascidos de gestantes que utilizavam ISRSs no último trimestre gestacional. Além disso, o uso da fluoxetina na gestação foi associado a um pequeno aumento no risco de malformações cardiovasculares (Reis e Källén, 2010).

Após o nascimento, Laine et al. (2003) reportaram que crianças expostas à fluoxetina durante a gravidez apresentaram escores mais elevados para sintomas serotonérgicos nos quatro primeiros dias de vida. Os sintomas mais proeminentes nestes recém-nascidos incluíram tremor, agitação e rigidez (Laine et al., 2003). Além disso, vários estudos sugerem que a exposição prenatal aos ISRSs pode estar associada à síndrome de retirada neonatal, e, sintomas motores, respiratórios, gastrointestinais e no sistema nervoso central têm sido relatados em recém-nascidos. Foi reportado também que estes sintomas geralmente apresentam-se de maneira moderada e desaparecem dentro de duas semanas após o nascimento (Moses-Kolko et al., 2005; Sanz et al., 2005).

Padrões alterados no eixo HPA em resposta ao estresse têm sido relatados em bebês cujas mães usaram um ISRS (Oberlander et al., 2008). Recentemente, um estudo demonstrou que a exposição pré-natal a ambos,

humor materno deprimido e ISRSs, foi associada com o aumento de comportamentos de internalização durante a infância (Oberlander et al., 2010), indicando que os distúrbios comportamentais infantis relacionados a ansiedade materna não são evitados pelo tratamento com ISRSs durante a gravidez. A longo prazo, a exposição pré-natal a drogas ISRSs foi associada com o embotamento de respostas somatossensoriais (Oberlander et al., 2005), e prejuízo no desenvolvimento psicomotor (Casper et al., 2003).

Estudos pré-clínicos

Estudos pré-clínicos com animais, não têm atribuído efeitos teratogênicos aos ISRS (Byrd e Markham, 1993). Por outro lado, como a 5-HT desempenha várias funções durante o início do desenvolvimento fetal e durante a morfogênese crânio-facial no período de organogênese, acredita-se que o uso destas drogas possa aumentar o risco de defeitos ao nascimento (Chambers et al., 1996, Goldstein et al., 1997).

Vorhees et al., (1994) mostraram que o tratamento com fluoxetina entre os dias 7-21 de gestação causou uma redução no ganho de peso das ratas prenhas, um menor peso ao nascimento, e um maior índice de mortalidade pré- e pós-natal. Além disso, Noorlander et al. (2008), reportaram que a maioria dos descendentes (ratos) expostos à fluoxetina durante a gestação morreram após o nascimento de insuficiência cardíaca grave causada por cardiomiopatia dilatada. Efeitos semelhantes foram encontrados em ratos expostos a paroxetina durante a última semana de gestação, o que levou a uma redução no tempo gestacional, menor peso ao nascimento e a um aumento de dez vezes na mortalidade neonatal (van den Hove et al., 2008). Acredida-se que as malformações cardiovasculares possam ocorrer pela exposição aos ISRSs principalmente no início gravidez (Bar-Oz et al., 2007), enquanto a exposição tardia foi mais associada a sintomas de abstinência da 5-HT (Mosess-Kolko et al., 2005).

Em relação aos efeitos comportamentais pela exposição prenatal a antidepressivos, Hansen et al. (1997) foi o primeiro a mostrar que a exposição a um ISRS durante os dias 8 a 21 pós-natal em ratos resultou em aumento no tempo de imobilidade no teste de natação forçada durante a idade adulta. Em 2004, Ansorge e colegas relataram que ratos neonatos tratados com

fluoxetina (entre os dias 4-21 pós-natal) exibiram fenótipo de exploração reduzido e fenótipo de ansiedade aumentado durante a vida adulta, resultados muito semelhantes ao fenótipo de ratos geneticamente deficientes na expressão do transportador de 5-HT. A exposição à fluoxetina em ratos durante estágios iniciais de desenvolvimento também afetou os comportamentos de exploração, de alimentação em novos ambientes, e comportamentos frente ao estresse (Ansorge et al., 2004). Estudos posteriores revelaram que a exposição precoce a um ISRS resultou em aumento da atividade locomotora, prejuízo no comportamento sexual (Maciag et al., 2006), redução da agressividade (Manhães de Castro et al., 2001), aumento da duração da fase de sono REM (*Rapid Eye Movement:* movimento rápido dos olhos) e provocou anedonia (Popa et al., 2008). Como em seres humanos, a exposição a fluoxetina neonatal também induziu um embotamento de respostas somatossensoriais em ratos púberes (Lee, 2009). Lisboa et al., reportaram que ratos expostos à fluoxetina *in utero* e lactação apresentaram sintomas depressivos no teste de natação forçada (Lisboa et al., 2007). Porém, além desses resultados negativos, a exposição perinatal a ISRSs induziu também alguns resultados positivos em ratos, tais como a redução na impulsividade (Lisboa et al., 2007), e melhora na aprendizagem e memória (Bairy et al., 2007).

A amamentação é um meio de exposição direta do lactente aos psicofármacos. Dados sobre o uso dos ISRSs na amamentação são limitados. Dois estudos encontraram menos de 10% da dose terapêutica em crianças amamentadas por mães em uso de fluoxetina. Essa pequena dose de fluoxetina não foi relacionada a efeitos adversos nos lactentes (Wisner e Wheeler, 1994). No entanto, outro estudo que avaliou 190 lactentes expostas à fluoxetina encontrou dosagem sérica elevada em uma criança de seis semanas. Esse achado alerta para o potencial de acúmulo da droga, uma vez que sua meia-vida é longa. Nesse mesmo estudo foram relatados efeitos colaterais da droga, como cólica, irritabilidade, diarréia, vômitos e diminuição do sono em dez lactentes.

Evidências de atividade estrogênica e/ou antiandrogênica da fluoxetina

Estudos que avaliaram os efeitos reprodutivos da exposição à fluoxetina em organismos aquáticos mostraram alterações sobre a fecundidade (Fong,

Huminski e D'Urso, 1998; Fong, 1998; Flaherty, Kashian e Dodson, 2001; Brooks et al., 2003ab; Gust et al., 2009; Lister et al., 2009; Sánchez-Argüello, Fernández e Tarazona, 2009), os níveis circulantes de hormônios e na expressão dos seus genes (Brooks et al., 2003a; Foran et al., 2004; Gust et al., 2009; Lister et al., 2009; Mennigen et al., 2010), nas características sexuais, e nos tecidos reprodutivos (espessura) (Henry e Black, 2008; Gust et al., 2009). Estes resultados indicam que a fluoxetina é capaz de alterar o sistema endócrino em animais aquáticos.

Estudos em roedores mostraram que o tratamento com fluoxetina afetou o ciclo estral, a receptividade sexual (Matuszczyk, Larsson e Eriksson, 1998; Uphouse et al., 2006; Maswood, Sarkar e Uphouse, 2008), os níveis de hormônios gonadais (Taylor et al., 2004), diminuiu o número de corpos lúteos e implantes (Tabacova, 2001), além de diminuir o tamanho da ninhada e aumentar as perdas espontâneas (Bauer et al., 2010). Além disso, foram reportadas redução da fertilidade masculina (Hoyt et al., 1989; Tabacova, 2001) e da população de células de Sertoli (Silva et al., 2008) em roedores tratados com fluoxetina.

Estudos clínicos mostraram que a exposição *in utero* à fluoxetina aumentou a taxa de nascimento prematuro (Simon, Cunningham e Davis, 2002), e efeitos colaterais, como hiperplasia endometrial e menorragia foram observadas em crianças expostas a fluoxetina (National Toxicology Program, Center for the Evaluation of Risks to Human Reproduction, 2003). Relatos de casos mostraram que mulheres que tinham ciclos menstruais anovulatórios passaram a ter ciclos ovulatórios após a terapia com fluoxetina (Strain, 1994). De maneira semelhante, duas mulheres com hipogonadismo hipogonadotrófico associada com a síndrome de Prader-Willis desenvolveram episódios de sangramento genital (semelhante à menstruação) com o uso de fluoxetina (Warnock et al., 1995). Além disso, muitos relatos de casos têm descrito que adolescentes que receberam fluoxetina desenvolveram galactorréia, e, em pelo menos dois destes casos hiperprolactinemia foi também relatada (Iancu et al., 1992; Arya e Taylor, 1995; Egberts et al., 1997). Finalmente, ensaios clínicos mostraram alterações do ciclo menstrual em mulheres que estavam usando fluoxetina (Menkes et al., 1993; Steiner et al., 1997).

Estudos em homens sugerem que os ISRSs podem prejudicar a integridade do DNA espermático e, assim, afetar negativamente a fertilidade (Safarinejad, 2008a), e menores níveis séricos de gonadotrofinas e testosterona foram encontrados em homens deprimidos tratados com ISRSs em comparação com homens saudáveis (Safarinejad, 2008b).

O conjunto destes resultados indica uma possível interferência com o sistema endócrino pela fluoxetina, e várias dessas alterações são descritas como efeitos de substâncias com ações estrogênicas ou anti-androgênicas. Embora estas informações na literatura sugiram um possível efeito desregulador endócrino da fluoxetina em diferentes espécies, até o momento não havia nenhum estudo específico desenhado para investigar tal hipótese.

2.3. CLASSIFICAÇÃO DE RISCO TERATOGÊNICO DE PSICOFÁRMACOS E DROGAS INIBIDORAS DA RECAPTAÇÃO DE SEROTONINA

Até o presente momento nenhuma medicação psicotrópica foi considerada segura para o uso durante a gestação e lactação pela FDA, órgão norte-americano que controla alimentos e fármacos. Este órgão estabeleceu uma classificação para os psicofármacos que se baseia no risco reprodutivo, e em efeitos adversos no desenvolvimento, comparados com os benefícios da opção da terapia medicamentosa (Camacho et al., 2006). Esta classificação está abaixo descrita:

- Risco A: estudos controlados não demonstram risco. Estudos adequados e bem controlados em gestantes não têm demonstrado ou evidenciado nenhum risco ao feto.

- Risco B: sem evidência de risco em humanos. Ou os achados em animais demonstram risco, mas os achados em humanos não, ou se estudos adequados em humanos não têm sido realizados, achados em animais são negativos.

- Risco C: risco não pode ser excluído. Faltam estudos em humanos, e os estudos em animais são positivos para o risco fetal ou estão ausentes também. Contudo, potenciais benefícios podem justificar o risco.

- Risco D: evidência positiva de risco. Dados de investigação ou relatados, posteriormente, mostram risco ao feto. Ainda assim, potenciais benefícios podem ter mais valor que o risco em potencial.

- Risco X: contra-indicação absoluta na gestação. Estudos em animais ou humanos de investigação, ou relatados posteriormente, mostram um risco fetal que claramente suplanta qualquer possível benefício à paciente.

Os ISRSs, em geral, tem tido a sua segurança estabelecida ao longo do tempo, embora tenham menor tempo de mercado que os tricíclicos e os inibidores da monoaminoxidase. A fluoxetina, a sertralina e o citalopram têm risco B. A paroxetina está sendo reclassificada como risco D após um estudo conduzido por Williams e Wooltorton (2005) que apontaram risco teratogênico para essa droga e, a partir deste estudo, tem-se evitado o uso da paroxetina durante a gestação. A fluvoxamina assim como o escitalopram têm classificação de risco C (Camacho et al., 2006). A atual classificação das drogas ISRSs denota que o risco da exposição durante a gestação a estes antidepressivos não pode ser inteiramente desconsiderado em humanos, devido a insuficientes ensaios clínicos ou ausência de estudos em animais, ou em alguns casos, não há evidência de risco em animais.

Devido à dificuldade de interpretação acerca dos riscos reprodutivos e no desenvolvimento que as drogas psicotrópicas podem causar (American Academy of Pediatrics Committee on Drugs, 2000), a Sociedade de Teratologia propôs ao FDA uma substituição desta classificação por resumos descritivos, contendo dados de estudos sobre o potencial tóxico reprodutivo da cada psicofármaco (American Academy of Pediatrics Committee on Drugs, 2000; Viguera et al., 2002).

2.4 DESREGULADORES ENDÓCRINOS

2.4.1 Alvos para substâncias desreguladoras endócrinas

O sistema endócrino é uma rede complexa de glândulas e hormônios que regula muitas funções do corpo, incluindo o crescimento, o desenvolvimento e a maturação, bem como o funcionamento de vários órgãos. As glândulas endócrinas, incluindo a hipófise, tireóide, pâncreas, adrenais, ovários e testículos, liberam quantidades cuidadosamente reguladas de hormônios na corrente sanguínea, que agem como mensageiros químicos naturais, distribuindo-se para diferentes partes do corpo, a fim de controlar e ajustar muitas funções vitais.

Tem sido relatado que uma variedade de substâncias químicas estruturalmente diferentes pode interferir com o sistema endócrino e perturbar a função normal dos tecidos e órgãos, particularmente aqueles do trato reprodutivo. Dadas as suas diferenças físico-químicas e distintos efeitos biológicos, há um grande número de alvos através dos quais as substâncias desreguladoras endócrinas (*Endocrine-Disrupting Chemicals* - EDCs) podem modular o sistema endócrino e potencialmente causar efeitos adversos à saúde. Embora cada espécie possua características únicas, crescentes evidências indicam que existe uma preservação substancial nos sistemas moleculares, celulares, e fisiológicos associados com a reprodução nos vertebrados. Por exemplo, a sinalização de estrogênio, androgênio e tireóide são essenciais para o desenvolvimento embrionário normal e atividade reprodutiva em todos os vertebrados estudados até o momento (Boareto, Müller e Dalsenter, 2008).

Muitas das EDCs alteram a sinalização de estrogênios, androgênios, e de hormônios da tiróide através de respostas mediadas por receptores, incluindo a ligação do hormônio ao seu receptor na superfície da célula, ou nuclear, seguido por uma complexa série de eventos que levam a alterações na expressão dos genes. Os principais receptores nucleares envolvidos na ação das EDCs são receptores estrogênicos α e β, receptores androgênicos, receptores de hidrocarbonetos aromáticos, receptores tireoidianos, receptores de glicocorticóides, receptores pregnane-X, e receptores androstane constitutivos. Atenção também tem sido focada sobre receptores de

progesterona, os quais parecem ser mais sensíveis do que receptores estrogênicos e são um alvo para muitas EDCs (Boareto, Müller e Dalsenter, 2008).

Substâncias desreguladoras endócrinas também podem alterar a síntese, a ligação às proteínas plasmáticas, a biotransformação hepática e a depuração dos hormônios. Estas substâncias também são capazes de desregular as vias de sinalização neurais e imunes, e ainda podem alterar a regulação da expressão de genes (ex: interferindo na metilação do DNA, estabilidade do RNA ou na degradação de proteínas) (Figura 4) (Boareto, Müller e Dalsenter, 2008).

Em relação a síntese de hormônios, muitas pesquisas têm sido conduzidas sobre os inibidores de aromatase, os quais podem prevenir a conversão de androgênios a estrogênios através de um sistema aromatase do citocromo P450, que é altamente preservada entre as espécies. Tem sido mostrado que vários fungicidas azóis bem como alguns compostos organoestânicos podem causar inibição da enzima aromatase. Além disso, existe uma maior consciência de que os sistemas de múltiplos receptores agem em conjunto para regular funções biológicas. Por exemplo, *crosstalk* entre os receptores estrogênicos e receptores de fatores de crescimento parece ser necessário para a sinalização estrogênica para divisão ou diferenciação nas células mamárias. Outro exemplo de um efeito multi-fatorial mediado por desreguladores endócrinos é sobre o eixo Hipotálamo-Pituitária-Gônadas (HPG). O herbicida atrazina exerce efeitos reprodutivos em roedores, os quais se manifestam por alterações no ciclo estral, retardo do início da puberdade, desenvolvimento de glândulas mamárias em fêmeas expostas no período pré/pós-natal, aumento das perdas pré- e pós-implantação, indução de tumores mamários, bem como aceleração do envelhecimento reprodutivo.

Acredita-se que todos estes efeitos possam estar relacionado com uma alteração mais direta no eixo HPG, em particular na supressão de hormônio luteinizante (LH) causada por inibição do hormônio liberador de gonadotrofinas (GnRH) e prolactina. Além disso, devido as EDCs também atuarem sobre o sistema neuroendócrino, que desempenha funções de regulação e homeostasia em diferentes espécies, a exposição a estas substâncias pode ter implicações mais amplas para a saúde (Boareto, Müller e Dalsenter, 2008).

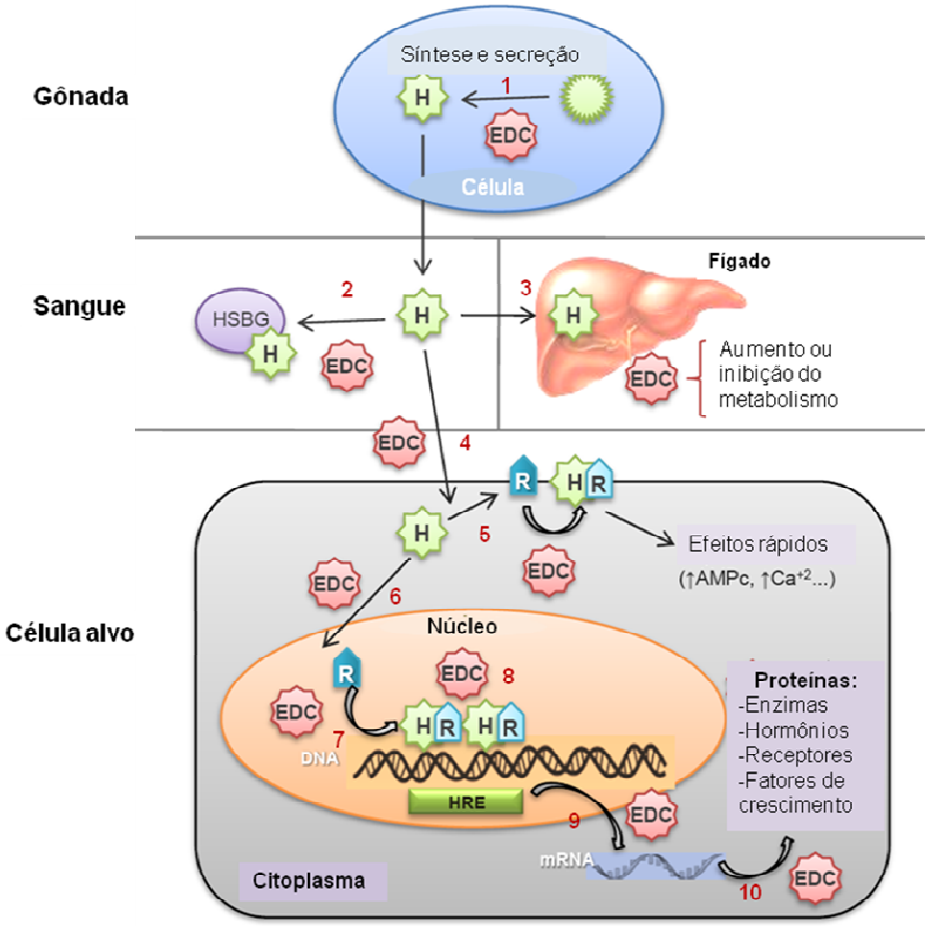

Figura 4. Possíveis alvos para substâncias desreguladoras endócrinas (EDCs). 1: síntese e secreção de hormônios esteroidais (H) a partir de células gonadais; **2:** afinidade de ligação do H pela globulina de ligação de hormônios sexuais (SHBG); **3:** alteração na função hepática pelo aumento ou inibição do metabolism do H; **4:** difusão para dentro da célula alvo; **5:** ligação do H com receptor (R) de superfície celular ou citoplasmático; **6:** difusão para dentro do núcleo; **7:** ligação do H em receptor nuclear e alteração conformacional do receptor formando homodímeros; **8:** formação de um complexo transcricional pela ligação do homodímero em sequências específicas do DNA, elementos responsivos a hormônios (HRE); **9:** transporte do RNA mensageiro (mRNA) para o citoplasma; **10:** Síntese e/ou degradação de proteínas.
Fonte: Figura modificada a partir de Boareto, Müller e Dalsenter, 2008.

2.4.2 Janelas de suscetibilidade

Os efeitos das EDCs dependerão não apenas da via de exposição e da dose, mas também da suscetibilidade do indivíduo ao composto químico. Sexo, idade e genótipo também podem influenciar a suscetibilidade à doenças, anormalidades anatômicas, e doenças por exposições. Por exemplo, sabemos

que as crianças não são adultos pequenos, pois elas têm comportamento, metabolismo e respostas aos desafios infecciosos e ambientais diferentes dos adultos. Dados experimentais indicam que a exposição pré-natal e/ou perinatal a EDCs pode levar a efeitos a longo prazo sobre a reprodução e o desenvolvimento que pode tornar-se evidente mais tarde durante a maturidade sexual e/ou na fase adulta. A identificação e caracterização das janelas de suscetibilidade para substâncias desreguladoras endócrinas ainda representa um desafio para os cientistas e os avaliadores de risco. Sabe-se que o desenvolvimento das gônadas e a diferenciação sexual de mamíferos ocorre durante uma janela de tempo relativamente estreita. Por exemplo, a produção de testosterona e outros hormônios pelos testículos fetais durante este período são fatores críticos para o correto desenvolvimento do trato reprodutivo masculino. Exposição *in utero* a substâncias químicas que perturbam a sinalização androgênica durante esta janela pode resultar em alterações reprodutivas tais como redução da distância anogenital, aumento da retenção de mamilos, agenesia do epidídimo, redução no peso de glândulas sexuais acessórias, criptorquidia, hipospádia e redução na fertilidade (Boareto, Müller e Dalsenter, 2008).

Uma janela crítica de suscetibilidade é um intervalo de tempo sensível durante o desenvolvimento quando a exposição a contaminantes ambientais pode desregular ou interferir com a fisiologia de uma célula, tecido ou órgão. É um período caracterizado por proliferação celular marcante e desenvolvimento, e várias mudanças na capacidade metabólica do organismo. A exposição a xenobióticos durante esta janela pode resultar em efeitos adversos muitas vezes permanentes que podem ter impactos ao longo da vida e até mesmo entre as gerações. Exposições durante as janelas de susceptibilidade podem ainda afetar o desenvolvimento ou resultar em eventuais doenças na vida adulta. Como o desenvolvimento continua após o nascimento, janelas críticas ocorrem no período perinatal (antes, durante e logo após a fertilização), durante a gravidez, primeira infância, infância, e puberdade (Boareto, Müller e Dalsenter, 2008).

3. HIPÓTESES E PREDIÇÕES

Nós propomos que a fluoxetina possa interferir com o sistema endócrino, mais especificamente com os hormônios gonadais, e que esta atividade seria a responsável pelos efeitos reprodutivos observados em vários estudos *in vivo*. Se esta hipótese for verdadeira, esperamos observar alterações no peso de órgãos estrógenos- e/ou andrógenos-dependentes nos testes de triagem para desreguladores endócrinos.

Se a fluoxetina for realmente capaz de interferir com a ação de hormônios gonadais, confirmando a primeira hipótese, então propomos que esta droga seria capaz de produzir efeitos reprodutivos a curto- e a longo-prazo. Se esta hipótese for verdadeira, esperamos observar alterações nos parâmetros gestacionais e lactacionais, assim como nos níveis hormonais das progenitoras tratadas com fluoxetina nesses períodos. Além disso, esperamos observar mudanças em marcos do desenvolvimento sexual dos descendentes expostos a esta droga *in utero* e lactação que são dependentes da ação dos hormônios gonadais.

A terceira parte deste estudo teve por objetivo investigar se a exposição à fluoxetina em períodos de neurodesenvolvimento poderia causar alterações comportamentais e neuroquímicas a longo-prazo através de dosagens de neurotransmissores e seus metabólitos em estruturas cerebrais relacionadas com ansiedade e depressão e no padrão comportamental dos descendentes submetidos ao campo aberto, labirinto em cruz elevado, natação forçada e preferência à sacarose. Se esta hipótese for verdadeira, esperamos observar alterações nos níveis de neurotransmissores e de seus metabólitos no córtex pré-fronatal e hipocampo, além de possíveis alterações em comportamentos que expressam locomoção, ansiedade e depressão.

4. OBJETIVOS

4.1. OBJETIVO GERAL

O presente estudo teve como objetivo investigar uma possível atividade desreguladora endócrina da fluoxetina, os possíveis efeitos adversos sobre a gestação e lactação, sobre o desenvolvimento sexual da progênie, assim como sobre alterações neuroquímicas e comportamentais dos descendentes expostos a esta droga *in utero* e lactação.

4.2. OBJETIVOS ESPECÍFICOS

- Investigar possível ação estrogênica e/ou antiestrogênica da fluoxetina através do teste uterotrófico e do ensaio do gene reporter.
- Investigar possível ação androgênica e/ou antiandrogênica da fluoxetina através do teste Hershberger.
- Investigar possíveis efeitos adversos sobre a gestação e lactação, além de possíveis alterações hormonais nas progenitoras tratadas com fluoxetina durante a gestação e lactação.
- Investigar possíveis alterações no desenvolvimento sexual da progênie feminina e masculina exposta à fluoxetina *in utero* e lactação.
- Investigar variáveis que indiquem toxicidade ou alteração em órgãos nas progenitoras e na progênie expostos à fluoxetina durante a gestação e lactação.
- Investigar possíveis alterações comportamentais através de testes que avaliam ansiedade, depressão e atividade locomotora da progênie durante a idade adulta exposta à fluoxetina *in utero* e lactação.
- Investigar os níveis de 5-HT, NA e dopamina (DA), assim como seus metabólitos, no córtex pré-frontal e hipocampo dos descendentes na idade adulta que foram expostos à fluoxetina *in utero* e lactação.

5. MATERIAIS, MÉTODOS E RESULTADOS

O medicamento investigado foi o cloridrato de fluoxetina em três diferentes doses. A primeira dose de fluoxetina foi baseada na dose terapêutica para humanos, calculada como a média das doses mais comumente utilizadas para depressão (20 e 40 mg/kg). A segunda dose investigada foi calculada por alometria e corresponde a dose terapêutica para ratos. A terceira dose foi calculada multiplicando-se a dose terapêutica para ratos por 10, e representa o fator de segurança devido às diferenças dentro da própria espécie. Os cálculos estão abaixo descritos:

- **Primeira dose – dose terapêutica para humanos**

Fluoxetina: 20 mg - 40 mg = (20 + 40) : 2 = 30 mg/70kg = **0,4 mg/kg**

- **Segunda dose – dose terapêutica para ratos calculada por alometria**

$$\frac{\text{Dose total para o modelo x Peso metabólico do animal modelo (PMAA)}}{\text{Peso metabólico do modelo (PMM)}}$$

Para proceder a conversão de massa (kg) em peso metabólico (kcal), elevou-se a massa do animal à potência 0,75 (Nevill, 1994). Considerando o modelo para seres humanos uma pessoa de 70 kg, e a média de peso para uma rata prenha como 250 g, obteve-se o seguinte resultado:

Fluoxetina

$\frac{30 \times 0{,}25^{0,75}}{70^{0,75}} = \frac{30 \times 0{,}35}{24{,}9}$ = 0,42 mg/250g peso animal = **1,7 mg/kg**

- **Terceira dose – fator de segurança da dose terapêutica para ratos**

A fim de se obter uma curva dose resposta, a terceira dose foi calculada através da aplicação do fator de segurança (vezes 10) à dose terapêutica para ratos.

Fluoxetina: 1,7 x 10 = **17 mg/kg**

Todo o material utilizado, a metodologia e as técnicas empregadas, bem como os resultados e a discussão específica de cada um dos três estudos que compõem esta tese estão descritos nos três manuscritos apresentados nos itens 6, 7 e 8.

6. ARTIGO 1

Artigo científico publicado na revista Reproductive Toxicology, doi: http://dx.doi.org/10.1016/j.reprotox.2012.04.001 (páginas 50 a 73 seguintes).

In vivo and *in vitro* estrogenic activity of the antidepressant fluoxetine

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Abstract

Recent years have seen an increase in the use of antidepressant drugs, especially fluoxetine (FLX), in sensitive populations, such as pregnant and lactating women. Although some evidence suggests a possible endocrine action of FLX, no specific studies have been performed to investigate this hypothesis. In the present study, we investigated the possible (anti)androgenic and (anti)estrogenic actions of FLX using Hershberger, uterotrophic (0.4, 1.7, and 17 mg/kg), and reporter gene (7.6-129 µM) assays. In the Hershberger assay, no differences were observed in androgen-dependent organ weights. However, the uterotrophic and gene reporter assays indicated a possible estrogenic action of FLX. Uterine weight increased in the 1.7 and 17 mg/kg/day groups in the 3-day uterotrophic assay in immature rats. Additionally, noncytotoxic concentrations of FLX induced estrogenic responses and increased the estrogenic response of estradiol in MCF-7 breast cancer cells transfected with luciferase.

Keywords: Depression, Fluoxetine, Endocrine disruption, Uterotrophic assay, Hershberger assay, Reporter gene assay, Estrogenic activity.

1. Introduction

Depression is among the most common disorders in women worldwide, and it is estimated that at least one in four women will manifest depression at some point in their lives [1]. Additionally, depressive episodes are more frequent during reproductive age, and approximately 10% of women will experience depression during pregnancy [2,3], and 20% of women will experience depression after childbirth [4].

Despite the principle of avoiding drugs during pregnancy and lactation, the clinical view of the treatment of depression during these periods has changed in recent years. The use of antidepressant medications is currently acceptable for pregnant and lactating women in situations where the risks to the mother and child of not treating the disease are greater than the risks associated with exposure to the antidepressant drug [5,6]. This change in opinion has been reflected by an increase in the use of antidepressant drugs during pregnancy and lactation, especially the use of selective serotonin reuptake inhibitors (SSRIs). Among the SSRIs, fluoxetine (FLX) has been reported to be the most commonly used drug during pregnancy [7-9]. Thus, clinicians who treat childbearing women must have information available to guide their treatment decisions as they negotiate the delicate balance between the risks of FLX during pregnancy and lactation and the risks produced by depressive illness and relapse.

Studies that evaluated FLX exposure and reproductive effects in aquatic organisms showed effects on fecundity [10-13], circulating hormone levels, gene expression [12,14-17], reproductive success [15,16,18,19], sexual characteristics, and the thickness of reproductive tissues [15,20]. These results indicate that FLX may alter the endocrine system in aquatic animals.

Additionally, much experimental and epidemiological data have demonstrated adverse endocrine and reproductive effects of FLX exposure. Studies in rodents reported that FLX treatment affected estrous cyclicity, sexual receptivity [21-23], and gonadal hormone levels [24], decreased the number of corpora lutea and implants [25], decreased litter size and increased spontaneous losses [26], decreased male fertility [25,27] and decreased sertoli cell population [28]. Clinical studies found preterm birth after *in utero* exposure

to FLX [29] and side effects, such as endometrial hyperplasia and menorrhagia, in children who were exposed to FLX [30]. Strain (1994) [31] presented two case reports of women who had anovulatory menstrual cycles and became ovulatory after FLX therapy. Similarly, two women with hypogonadotropic hypogonadism associated with Prader-Willis syndrome developed menstrual-like episodes of genital bleeding with the use of FLX [32]. Many case reports have described teenagers on fluoxetine who developed galactorrhea. In at least two of these cases, hyperprolactinemia was also reported [33-35]. Moreover, clinical trials showed menstrual cycle changes in women who were using FLX [36,37]. Studies in men have suggested that SSRIs may damage normal sperm DNA integrity and thereby adversely affect fertility [38]. Finally, lower serum gonadotropin and testosterone levels were found in depressed men treated with SSRIs compared with healthy men [39]. These results indicate the possible interference with the endocrine system, and several of these changes are described as the effects of substances with estrogenic or anti-androgenic actions [40-42].

Although this information in the literature suggests a possible endocrine-disrupting effect in different species, no specific studies have been designed to specifically investigate this hypothesis. According to Level 1 of the Organisation for Economic Co-operation and Development Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals [43], when scientifically relevant data on a substance indicate an endocrine-disrupting action, such as the case with FLX, the same must be selected and prioritized for assessment. Moreover, the high incidence of major depression during pregnancy and lactation associated with the use of FLX during these periods highlights the need for further research on the possible endocrine effects of this drug and reproductive outcomes.

Because of the numerous confounding factors and ethical and methodological limitations of clinical trials [44,45], conducting preclinical, well-controlled studies becomes necessary. Currently, specific protocols have been validated by regulatory agencies to screen the endocrine-disrupting effects of chemicals. Thus, the present study investigated whether FLX has (anti)estrogenic or (anti)androgenic effects using *in vivo* and *in vitro* assays. The *in vivo* endocrine-disrupting screening assays were developed using the

protocols of the Organisation for Economic Co-operation and Development [46-48].

2. Materials and methods

2.1 Animals

Wistar rats were obtained from Federal University of Parana and maintained under controlled conditions of 22 ± 2°C and a constant 12 h/12 h light/dark cycle. The animals were housed in collective polypropylene cages (414 mm × 344 mm × 168 mm), four animals per cage, and were maintained in the reproductive toxicology laboratory for 1 week for acclimatization before beginning the experiments. Standard pellet food (Nuvital, Curitiba, PR, Brazil) and tap water were available *ad libitum*. All of the experiments were approved by the Ethics Committee on Animal Experimentation (protocol no. 347) and were elaborated and developed based on the principle of the three R's (Refine, Reduce, and Redesign). The animals were weighed, ranked by weight, and randomly assigned to each of the treatment and control groups.

General clinical observations were made once per day, and all of the animals were observed for mortality, morbidity, and general clinical signs, such as changes in behavior (e.g., agitation, lethargy, and hyperactivity), neurological changes (e.g., convulsions, tremors, muscle rigidity, and hyperreflexia), and autonomic signs (e.g., lacrimation, piloerection, pupil size, and unusual respiratory patterns).

2.2 Substances, doses, and routes of administration

2.2.1 *In vivo* tests

Fluoxetine hydrochloride ($C_{17}H_{18}F_3NO \cdot HCl$) was supplied by Pharma Nostra Comercial (Rio de Janeiro, Brazil). For the *in vivo* tests, three doses of FLX were used, and the choice of doses was based on the following criteria: (*i*) human therapeutic dose calculated as the average therapeutic range indicated for depression (30 mg) for 70 kg human adult patients, (*ii*) the extrapolated

“therapeutic dose” for rats calculated by allometry [49], and (*iii*) the “therapeutic dose” for rats multiplied by 10 (i.e., the safety factor attributable to intra-specific differences). Thus, the three doses used for the *in vivo* studies were 0.4, 1.7, and 17 mg/kg/day, respectively. These doses were administered by gavage after dissolving FLX salt in distilled water.

For the uterotrophic assay, estradiol (17-α-ethynylestradiol, 95% pure; $C_{20}H_{24}O_2$) was obtained from Sigma-Aldrich (Steiheim, Germany), dissolved in canola oil (*Brassica napus*), and administered by gavage at a dose of 10 µg/kg/day. Tamoxifen (tamoxifen citrate, 99.2% pure; $C_{26}H_{29}NO$) was obtained from Galena Laboratory (Curitiba, Brazil), dissolved in distilled water, and administered by gavage at a dose of 10 mg/kg/day [46,48].

In the Hershberger assay, testosterone propionate (97% pure; $C_{22}H_{32}O_3$) was obtained from Fluka Chemika-Sigma-Aldrich (Buchs, Switzerland), dissolved in canola oil (*Brassica napus*), and administered subcutaneously at a dose of 0.25 mg/kg/day. Flutamide (99.2% pure; $C_{11}H_{11}F_3N_2O_3$) was obtained from Galena Laboratory (Curitiba, Brazil), dissolved in distilled water, and administered by gavage at a dose of 5 mg/kg/day [47]. The volumes of administration were 5.0 ml/kg orally and 1.0 ml/kg subcutaneously.

2.2.2 In vitro test

Carbon dioxide was obtained from Air Liquide (Liège, Belgium). Acetonitrile was obtained from Biosolve (Valkenswaard, Netherlands). Adenosine triphosphate (ATP), Dulbecco’s modified Eagle's medium (DMEM), MEM without phenol red, fetal bovine serum, and trypsin were obtained from Fisher Bioblock Scientific (Tournai, Belgium). 17β-Estradiol, charcoal, and dextran were obtained from Sigma-Aldrich (Bornem, Belgium). $_D$-luciferin (potassium salt) was obtained from Synchem OHG (Kassel, Germany).

Fluoxetine hydrochloride (Pharma Nostra Comercial, Rio de Janeiro, Brazil) was dissolved in acetonitrile, and concentrations of 7.6, 11.4, 17.0, 25.5, 38.3, 57.5, 86.2, and 129 µM were tested.

2.3 Immature rat uterotrophic assay

Nine experimental groups of immature female rats, 21 ± 1 days old, were used (10 animals per group) to investigate the possible (anti)estrogenic activity of FLX. To this end, immature female rats were treated daily for 3 consecutive days with different substances, which represent the experimental groups. Estradiol served as the positive control for estrogenicity, and its vehicle, canola oil, served as the negative control. Tamoxifen treatment in estradiol-treated rats was used as a positive control for anti-estrogenicity. Fluoxetine was administered alone (0.4, 1.7, and 17 mg/kg) for the evaluation of estrogenic activity, and FLX treatment in estradiol-treated females was used for the evaluation of anti-estrogenicity. Twenty-four hours after the last treatment, the animals were weighed and sacrificed by manual cervical dislocation according to the American Veterinary Medical Association Guidelines on Euthanasia (i.e., manual cervical dislocation is a humane technique for the euthanasia of rats that weigh < 200 g) [50]. Uteri were excised, trimmed free of fat, pierced, and blotted to remove fluid. The body of each uterus was cut just above its junction with the cervix and at the junction of the uterine horns with the ovaries. Wet uterus weight was measured and is expressed as absolute weight (g) and relative weight (wet uterus weight $\times$ 100/body weight).

2.4 Hershberger assay

For this test, male pubertal rats, aged 49 days, were castrated [47]. After a 7 day recovery period, the animals were separated into nine experimental groups (9-11 animals per group) to investigate the possible (anti)androgenic activity of FLX. The castrated male rats were treated daily for 10 consecutive days with different substances. Testosterone served as the positive control for androgenicity, and its vehicle, canola oil, served as the negative control. Flutamide administered to testosterone-treated rats served as the positive control for anti-androgenicity. Fluoxetine (0.4, 1.7, and 17 mg/kg) was administered alone for the evaluation of androgenic activity, and FLX administered to testosterone-treated rats was used for the evaluation of anti-androgenicity. Twenty-four hours after the last treatment, the animals were

weighed and euthanized by deep anesthesia with 100 mg/kg pentobarbital intraperitoneally. The ventral prostate, seminal vesicle (with coagulating glands), glans penis, and levator ani muscle/bulbocavernosus muscle (LABC) were removed and carefully dissected to remove the surrounding connective tissue. Organ weights were measured and are expressed as absolute weights (g) and relative weights (organ weight $\times$ 100/body weight). The ventral prostate was weighed without the prostatic capsule and seminal vesicle without its contents.

2.5 Cell-based assays for estrogen receptor-mediated activity

MCF-7 human mammary tumor cells, which express both estrogen receptor α and β, were stably transformed with a reporter vector formed by the luciferase gene under the control of the vitellogenin promoter to obtain an estrogen-responsive cell line [51]. These cells were grown in 75 cm^2 culture flasks in DMEM supplemented with 10% heat-inactivated fetal bovine serum at 37°C under 5% CO_2.

The cell-based assays for estrogen receptor (ER) -mediated activity were performed as follows. Confluent cells maintained at least 24 h in DMEM without phenol red (supplemented with 10% fetal bovine serum previously treated with charcoal-dextran) were unstuck from the culture flask using 1.5 ml trypsin (0.5 g/L). The cells were then suspended in 10 ml of fresh culture medium, and this suspension was diluted twice. One hundred microliters of diluted cells were seeded in 96-well culture plates, which were incubated overnight at 37°C under 5% CO_2. Afterward, the cells were incubated with 17β-estradiol, FLX, or both for 24 h. The final volume in one well was 200 µl. After incubation, cell viability was verified under a microscope. Subsequently, the medium was removed, and the cells were lysed with 50 µl of lysis solution. After the addition of luciferin and ATP, luciferase activity was determined using a luminometer Orion II (BRS, Drogenbos, Belgium) and is reported as relative light units (RLU). The maximal response observed for 17β-estradiol was arbitrarily set to 100%, and the subsequent responses are expressed as a percentage of the maximal response

(relative response). Estradiol equivalents were determined by linear extrapolation from calibration curves obtained after exposure to 17β-estradiol.

2.6 Statistical analyses

For the *in vivo* tests, parametric data were analyzed by analyses of variance (ANOVAs), and differences between groups were assessed by the Bonferroni test. Nonparametric data were analyzed by the Kruskal-Wallis test followed by Dunn's test. Differences were considered statistically significant at a probability level of 5% ($p < 0.05$). The statistical analyses were performed using Prism version 5.0 (GraphPad, San Diego, CA, USA).

Cell-based assays for ER-mediated activity data were processed with Slide Write version 6 software. Reference curves were fitted using the sigmoid dose-response curve equation $Y = a_0/(1 + (x/a_1)^{a^2})$, in which x is the concentration of 17β-estradiol or FLX, Y is the relative response, a_1 is the concentration of the half-maximal response (EC_{50}), and a_2 is the slope of the linear part of the curve.

3. Results

3.1 Uterotrophic assay

All of the animals showed signs of good health and did not show signs of toxicity or serotoninergic syndrome (characterized by behavioral, neurological, and autonomic changes).

In the uterotrophic assay, the body weights of immature female rats on the necropsy day did not differ significantly among the treatment groups (data not shown).

The animals that received estradiol (positive control for estrogenicity) showed a significant increase in absolute and relative uterine weights compared with the vehicle group (canola oil; Fig. 1 and 2). Similarly, the animals that received estradiol and tamoxifen simultaneously, representing the positive control for anti-estrogenic activity, showed a statistically significant reduction in

absolute and relative uterine weights compared with the animals that received only estradiol (Fig. 1 and 2). These results confirmed the reliability of this study.

When we investigated the possible anti-estrogenic effect of FLX, we found that the absolute and relative uterine weights did not differ between the groups that received estradiol + 0.4, 1.7, and 17 mg/kg/day FLX compared with immature females rats that received only estradiol. None of these FLX doses were able to inhibit the classic uterotrophic effect produced by estradiol (Fig. 1 and 2).

However, when we evaluated the estrogenic potential of FLX, we found that the absolute and relative uterine weights of the animals that received 1.7 and 17 mg/kg FLX significantly increased compared with the canola oil group (i.e., negative control for estrogenicity). These animals showed an increase in uterine weights of approximately 50% and 56% for relative weight and 74% and 81% for absolute weight, respectively (Fig. 1 and 2).

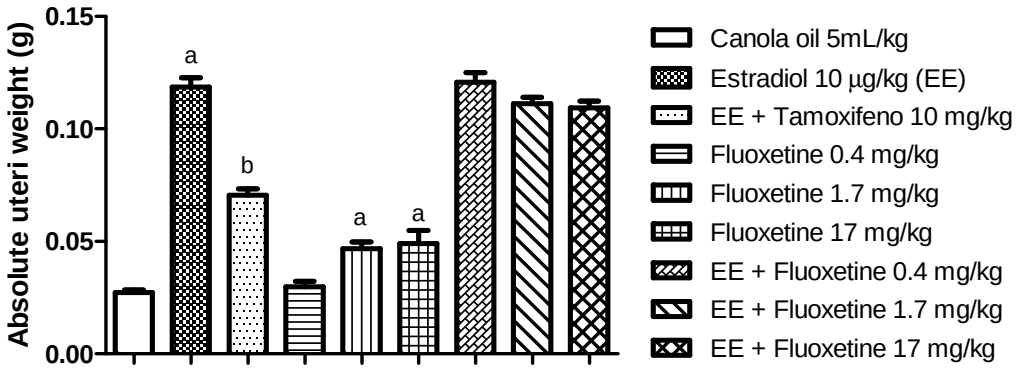

Fig. 1. The columns represent mean ± SE absolute uterus weights of immature female rats in the 3-day uterotrophic assay. The mass of the uterus is reported without fluid. *n* = 10 animals per group. [a]*p* < 0.05, compared with canola oil (vehicle). [b]*p* < 0.05, compared with estradiol.

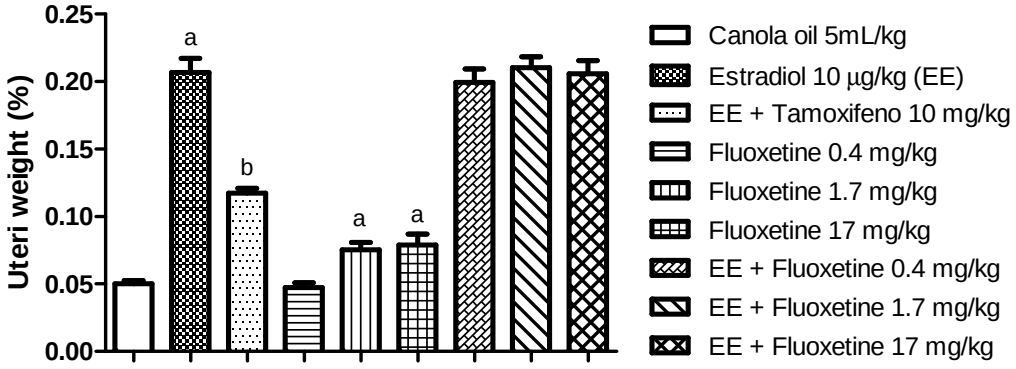

Fig. 2. The columns represent mean ± SE relative uterus weights of immature female rats in the 3-day uterotrophic assay. The mass of the uterus is reported without fluid. n = 10 animals per group. [a]$p < 0.05$, compared with canola oil (vehicle). [b]$p < 0.05$, compared with estradiol.

3.2 Hershberger assay

The results of the Hershberger assay are shown in Table 1. The body weights of male rats on the necropsy day were not statistically different, and any animal showed signs of toxicity or serotoninergic syndrome during the test.

The animals that received testosterone (positive control for androgenicity) showed a significant increase in the relative weights of ventral prostate, seminal vesicle, glans penis, and LABC compared with the vehicle group (canola oil). Similarly, the animals that received testosterone + flutamide (positive control for antiandrogenicity) showed a statistically significant reduction in the relative weights of these organs compared with the animals that received only testosterone. These results show the classic effects of testosterone in androgen-dependent organs, and flutamide, an androgen receptor antagonist, was able to inhibit this trophic effect. These results confirm the reliability of this study.

The Hershberger assay evaluates the ability of a chemical to elicit biological effects consistent with androgen agonists or antagonists. When we investigated the possible anti-androgenic effects of FLX, the relative weights of the ventral prostate, seminal vesicle, glans penis, and LABC did not differ between the animals that received testosterone + 0.4, 1.7, and 17 mg/kg/day

FLX compared with the rats that received only testosterone. These results show that FLX did not inhibit the weight gain in androgen-dependent organs produced by testosterone.

We then evaluated whether FLX has androgenic effects, and no significant changes were detected in androgen-dependent organ weights in the animals that received only 0.4, 1.7, and 17 mg/kg/day FLX compared with the canola oil group (Table 1).

Table 1. Body weight (g) and relative androgen-dependent organ weight (mg/100 g body weight) of pubertal castrated male rats after 10 days of treatment in the Hershberger assay.

Experimental group	Body weight	Prostate	Seminal Vesicle	Glans penis	LABC*
Canola oil	271.9 ± 6.7270	0,005 ± 0.0005	0.017 ± 0.0011	0.016 ± 0.0009	0.165 ± 0.0127
Testosterone (T)	292.6 ± 12.400	0.029 ± 0.0021[a]	0.071 ± 0.0052[a]	0.023 ± 0.0011[a]	0.444 ± 0.0342[a]
T + Flutamide	290.4 ± 7.6470	0.006 ± 0.0005[b]	0.019 ± 0.0009[b]	0.017 ± 0.0005[b]	0.204 ± 0.0135[b]
Fluoxetine 0.4 mg/kg	287.4 ± 10.450	0.005 ± 0.0005	0.018 ± 0.0023	0.015 ± 0.0012	0.214 ± 0.0228
Fluoxetine 1.7 mg/kg	275.8 ± 9.4360	0.005 ± 0.0004	0.018 ± 0.0011	0.015 ± 0.0008	0.178 ± 0.0093
Fluoxetine 17 mg/kg	271.7 ± 7.5260	0.004 ± 0.0006	0.017 ± 0.0018	0.016 ± 0.0007	0.197 ± 0.0135
T + Fluoxetine 0.4 mg/kg	301.2 ± 9.8230	0.031 ± 0.0019	0.068 ± 0.0031	0.022 ± 0.0010	0.451 ± 0.0149
T + Fluoxetine 1.7 mg/kg	298.0 ± 10.320	0.032 ± 0.0022	0.072 ± 0.0039	0.023 ± 0.0015	0.452 ± 0.0248
T + Fluoxetine 17 mg/kg	289.8 ± 10.300	0.035 ± 0.0035	0.075 ± 0.0035	0.024 ± 0.0011	0.451 ± 0.0219

Note: The data represent mean ± SE. Organ weights are expressed as relatives weights (organ weight × 100/body weight). * = levator ani muscle/bulbocavernosus muscle. The ventral prostate was weighed without the prostatic capsule, and the seminal vesicle was weighed without its contents. n = 9-11 animals per group. [a]$p < 0.05$, compared with canola oil (vehicle); [b]$p < 0.05$, compared with testosterone propionate.

3.3 Gene reporter assay

After exposing MCF-7-ERE cells to increasing concentrations of 17β-estradiol (E2), sigmoid dose-response curves were obtained (Fig. 3).

To detect the possible estrogenic activity of FLX *in vitro*, MCF-7-ERE cells were exposed for 24 h to increasing concentrations of this molecule diluted

in culture medium. Concentrations of FLX lower than 11.4 µM produced slight luciferase expression. Concentrations of FLX higher than 25.5 µM provoked cytotoxic effects (observed under a light microscope). The maximum luciferase expression was obtained when MCF-7-ERE cells were exposed to 17.0 µM of FLX (Fig. 4). At this concentration, FLX produced 20.3 ± 3.0% of a relative response, corresponding to $4.1 \times 10^{-6} \pm 7.4 \times 10^{-7}$ µM estradiol equivalents.

Reporter gene assays using MCF-7-ERE cells are also able to detect possible antagonist actions provoked by chemicals. Medium that contained a fixed amount of 17β-estradiol (1.8×10^{-5} µM) was supplemented with increasing concentrations of FLX and incubated with MCF-7-ERE cells for 24 h. MCF7-ERE cells exposed to FLX did not reveal any anti-estrogenic activity but displayed an increase in the cellular response (up to 33.8 ± 3.8%) when exposed simultaneously with 17β-estradiol, indicating a possible additive effect. Similar to the test that detected the estrogenic activity of FLX, concentrations higher than 25.5 µM provoked cytotoxic effects (observed under a light microscope; Fig. 5).

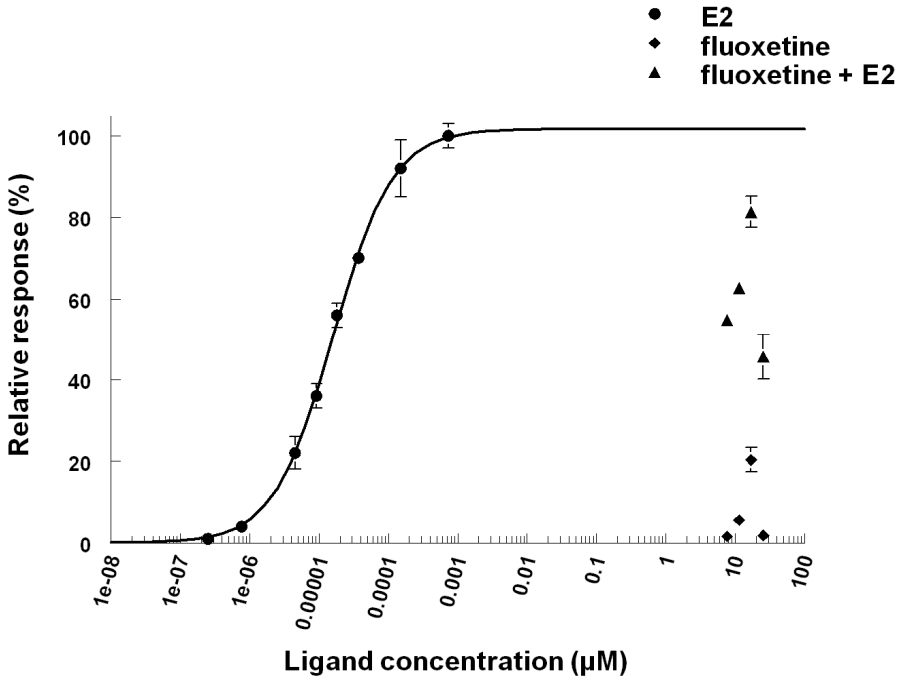

Fig. 3. Dose-response curve of increasing concentrations of E2 and estrogen receptor-mediated activity elicited by different concentrations of fluoxetine in MCF-7-ERE cells when exposed alone or simultaneously with E2 (1.8 × 10-5 µM). The data represent the mean ± SD. The maximal response observed for E2 was arbitrarily set to 100%.

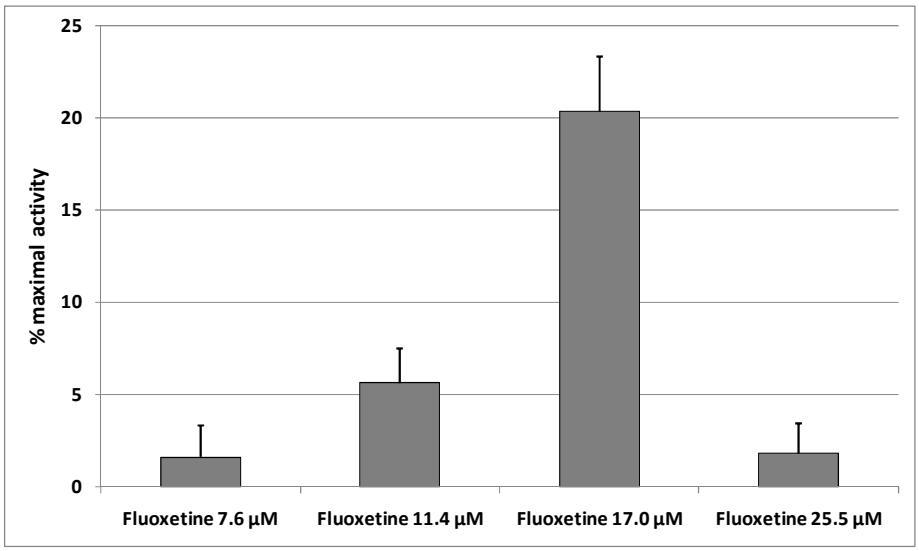

Fig. 4. Estrogen receptor-mediated activity elicited by different concentrations of fluoxetine in MCF-7-ERE cells. The results are expressed as a percentage of the maximal activity induced by E2 (data not shown).

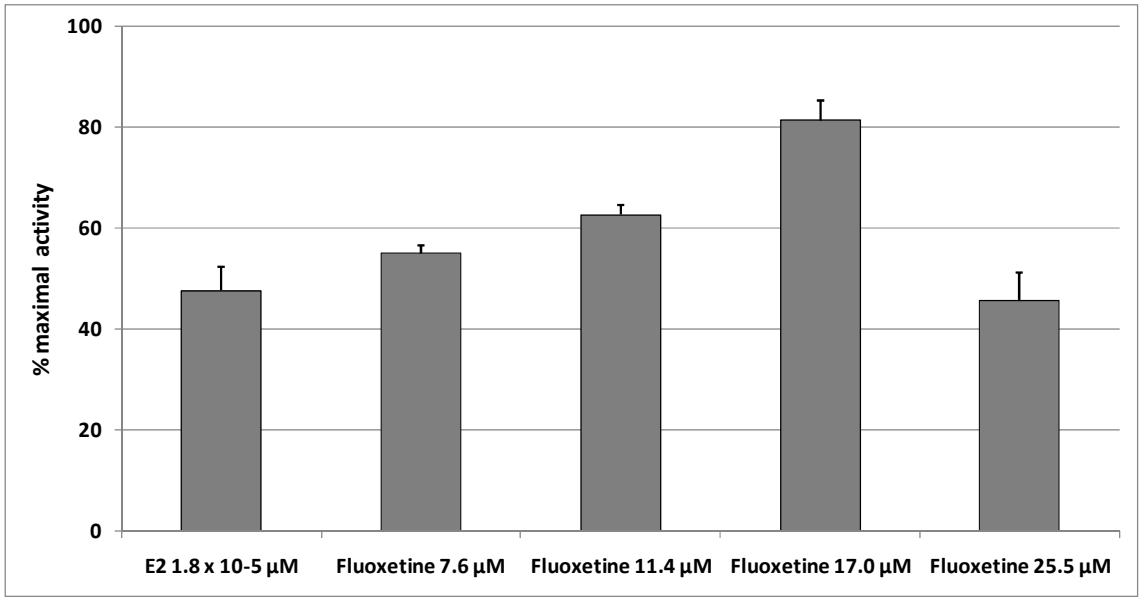

Fig. 5. Estrogen receptor-mediated activity elicited by different concentrations of fluoxetine in MCF-7-ERE cells when exposed simultaneously with E2. The results are expressed as a percentage of the maximal activity induced by E2 (data not shown).

4. Discussion and conclusion

In the present study, the *in vivo* (anti)estrogenic and (anti)androgenic effects of FLX were evaluated using the uterotrophic and Hershberger assays, respectively. These two short-term *in vivo* tests are considered gold standards for identifying substances with the potential to interact with the endocrine system [47,48]. Endocrine-disrupting chemicals can act via a wide range of mechanisms, including receptor-dependent and -independent processes. Additionally, they can demonstrate species-, tissue-, and cell-specific effects and be influenced by metabolism. Therefore, complementary *in vivo* and *in vitro* tests are essential for comprehensively assessing the potential endocrine-disrupting effects of a substance [52,53].

The results of the uterotrophic assay indicated a possible *in vivo* estrogenic effect of FLX. A significant increase in uterine weight was found in rats treated with 1.7 and 17 mg/kg/day FLX compared with the vehicle control. However, the uterus has also been shown to respond to non-estrogenic substances, such as progesterone, testosterone, and epidermal growth factor, which could lead to confounding results [54,55]. In the present study, the results of the gene reporter assay are consistent with the interpretation that FLX evoked ER-mediated effects. Non-cytotoxic concentrations of FLX were able to activate MCF7-ERE cells and increase the estrogenic activity of estradiol after the simultaneous treatment of cultured cells. Thus, the results of the *in vitro* gene reporter assay are consistent with the *in vivo* effects observed in the uterotrophic assay, indicating that FLX has the potential to disrupt estrogenic-mediated pathways. The *in vitro* results also indicated that FLX can elicit estrogenic responses without metabolic conversion. However, it remains to be determined whether active FLX metabolites, such as norfluoxetine, may also elicit *in vitro* and *in vivo* estrogenic effects.

Although the results found in the *in vitro* MCF-7 cell assay suggest that FLX exerts an agonist action on human ER, additional studies should be performed, such as the demonstration that estrogenic responses can be blocked in MCF-7 cells using ERs antagonists, or binding studies to confirm these results. Besides that, the estrogenic effect of FLX was observed at a concentration (17 µM) that was close to cytotoxic level (25 µM). In this way, it is

essential to consider other mechanisms of action that may be responsible or co-responsible for the estrogenic effect observed in the uterotrophic assay, such as effects on the synthesis of hypothalamic releasing hormones. It is known that pituitary hormone release is controlled by hypothalamic neurons, many of which are innervated by serotonergic (5HT) nerve terminals originating in the midbrain raphe nuclei. Thus, the class of SSRIs antidepressants, like FLX, by acting in 5HT re-uptake and receptors can modulate synapses with hypothalamic neurons producing indirect effects on hormonal control [56]. An investigation about the levels of estradiol and gonadotropins to determine whether the synthesis of these hormones was increased would be enlightening.

Another point to consider is that uterotrophic effect observed *in vivo* did not show dose-dependent characteristic, since the two higher doses produced a very similar effect. New research using intermediate doses would be interesting. Therefore, demonstrations of clear concentration - and dose-response curves are also important to confirm the *in vitro* and *in vivo* studies.

The results of the Hershberger assay indicated a lack of *in vivo* androgenic and anti-androgenic effects of FLX at the doses tested. As is well known that both aromatisable and non-aromatisable androgens are also able to increase uterine weight, the negative results for androgenicity suggest the absence of indirect androgenic activity in the uterotrophic effects of FLX. Notably, however, some endocrine disruptors may not be detected by classic screening tests. For example, substances that inhibit the biosynthesis of androgenic steroids, such as certain phthalate esters, can result in negative results in both *in vitro* and *in vivo* assays that are designed to detect receptor-mediated responses [57,58]. Moreover, the castrated peripubertal rat does not have an intact hypothalamic-pituitary-gonadal axis, and chemicals that act through disruption of this endocrine axis may not be adequately detected.

Substances with possible endocrine-disrupting action have been routinely screened and investigated by many research groups, and they may interfere with normal hormonal regulation through different mechanisms and cause several adverse effects by affecting development, differentiation, reproduction, and behavior. The fetus and neonate are also more susceptible to adverse reproductive effects. During this sensitive developmental period, exposure to endocrine-disrupting chemicals (EDCs) can interfere with the

physiology of cells, tissues, and organs [59,60]. Preclinical studies indicated that early prenatal or perinatal exposure to EDCs may result in irreversible reproductive effects that can have lifelong and even intergenerational impacts on health [61]. In this context, FLX crosses the placenta [62], reaching similar levels in maternal and fetal serum, and it is secreted into breast milk during lactation [63,64]. Therefore, a substantial number of unborn and newborn children are exposed to FLX during critical phases of development.

Importantly, uterotrophic, Hershberger, and reporter gene assays are pharmacological screening tests that are designed to detect the potential endocrine-disrupting effects of drugs and environmental chemicals. The assessment of the adverse effects that result from endocrine disruption must be performed using protocols that include sensitive endpoints and critical windows of exposure and consider the route of administration and number and relevance of the doses [43].

Overall, our results only indicate a possible estrogenic action of FLX. Moreover, conducting further studies are necessary to assess whether exposure to this antidepressant is able to result in adverse endocrine-mediated effects. Investigating whether FLX exposure interferes with the normal processes of sexual differentiation, development, and maturation is particularly important.

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7. ARTIGO 2

Artigo científico submetido à publicação na revista Toxicology (páginas 75 a 101 seguintes).

Pregnancy outcomes and sexual development of Wistar rats exposed to fluoxetine *in utero* and lactation

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Abstract

The present study evaluated the reproductive effects of FLX by *in utero* and lactational assay. Pregnant Wistar rats were treated daily with FLX (0.4, 1.7, and 17 mg/kg/day) or distilled water by gavage from gestation day (GD) 7 to lactation day (LD) 21. A significant reduction in maternal body weight was observed during pregnancy and lactation in dams exposed to 17 mg/kg FLX. Progestagen and glucocorticoid metabolites on GD 15 and estrogen and progestagen metabolites on LD 7 were increased. Besides that, was observed an increase in the weight of the adrenal glands and a reduction in uterine weight in dams exposed to higher dose of FLX. Finally, pup birth weight and the viability and weaning indices also were reduced in animals exposed to 17 mg/kg FLX. In conclusion, exposure to 17 mg/kg/day FLX during pregnancy and lactation caused maternal and fetal toxicity and hormonal changes in dams.

Keywords: Pregnancy, lactation, depression, fluoxetine, *in utero* and lactational assay, toxicity, sexual development, hypothalamic-pituitary-adrenal axis.

1. Introduction

Major depressive disorder is one of the most widespread and debilitating forms of mental illness, characterized by behavioral, affective, cognitive, and somatic symptoms [1]. Epidemiological studies show that depression is among the most common disorders in women worldwide [2], with an increased prevalence during reproductive age, especially during pregnancy (10%) and after childbirth (20%) [3,4].

The past decade has seen a large increase in the use of selective serotonin reuptake inhibitors (SSRIs) antidepressants during pregnancy and lactation. Among these, fluoxetine (FLX) was the most widely used antidepressant during pregnancy [5,6]. In this context, it is important to consider that FLX crosses the placenta [7], reaching similar levels in maternal and fetal serum. In addition, FLX is also secreted into breast milk during lactation [8,9]. The possibility of perinatal exposure to fluoxetine has raised significant concerns about its possible adverse effects in mothers and infants. The pre- and early postnatal development of the reproductive system is under complex integrative control of the central nervous system (CNS), pituitary, gonads, and genital tract, all of which are potential targets of transplacental and lactational chemical exposure [10]. Therefore, understanding the effects of FLX on the reproductive system of the developing fetus/neonate and pregnant and nursing women is important.

Investigations of the negative consequences of SSRI antidepressants during pregnancy and lactation are promising, but still with a lack of significant information on the spectrum of adverse effects and mode of action of these substances at different stages of animal and human development. According to the review by Ellfolk and Malm [11], clinical studies have revealed limited and often controversial information about the risks of using this class of drugs. Besides that, some preclinical studies showed that the use of SSRIs is safe during pregnancy and lactation, whereas other authors found many developmental and reproductive adverse effects of SSRIs during these periods [12].

A recent study carried out by Müller et al. [13] showed a possible estrogenic action of FLX by performing *in vitro* and *in vivo* screening tests. The

possible estrogenic activity of FLX could affect the sexual differentiation and maturation of individuals exposed to FLX *in utero* and during lactation. Because the effects associated with endocrine disruption may be latent or may not appear until the reproductive system matures [10], the present study evaluated the pregnancy outcomes and the long-term adverse reproductive effects of FLX in Wistar rats. The endpoints investigated included possible changes during pregnancy and lactation, fetal development, the hormone levels of dams, and the effects on the sexual development of male and female rat offspring exposed to FLX *in utero* and during lactation.

2. Materials and methods

2.1. Animals and conditions

Wistar rats were obtained from Federal University of Parana and maintained under controlled conditions at 22 ± 2°C and a constant 12 h/12 h light/dark cycle. The animals were maintained in the reproductive toxicology laboratory for 1 week for acclimatization before beginning the experiments. Standard food pellets (Nuvital, Curitiba, PR, Brazil) and tap water were available *ad libitum*. All of the experiments were approved by the Committee on Animal Research and Ethics of the Federal University of Parana (protocol no. 347), which is in accordance with national and international guidelines of animal welfare. With the exception of dams, all of the animals were euthanized by decapitation because we used the brains of animals for the determination of neurotransmitters in brain structures for a parallel study.

2.2. Fluoxetine: experimental groups

Fluoxetine hydrochloride ($C_{17}H_{18}F_3NO \cdot HCl$) was supplied by Pharma Nostra Comercial (Rio de Janeiro, Brazil). Pregnant rats were assigned to four experimental groups that received 5 ml/kg of distilled water (control group) or three different doses of FLX orally (gavage). The doses of FLX were chosen based on (*i*) the human therapeutic dose, calculated as the average therapeutic

range indicated for depression (30 mg) for human adult patients who weight 70 kg, (*ii*) the extrapolated “therapeutic dose” for rats, calculated by allometry [14], and (*iii*) the “therapeutic dose” for rats multiplied by 10 (i.e., the safety factor attributable to intra-species differences). Thus, the three doses were 0.4, 1.7, and 17 mg/kg/day, respectively. Fluoxetine salt was dissolved in distilled water daily, and the volume of administration was 5.0 ml/kg.

2.3. In utero and lactational assay

Nulliparous females (90 days old) were placed in contact with adult males (90 days old) for mating during the dark phase of the light/dark cycle at a female:male ratio of 3:1 and subjected to vaginal smear analysis. The day of sperm detection in the vaginal smear was considered day 0 of pregnancy (gestation day [GD] 0), and these females were randomly separated into the treatment and control groups and were housed individually in polypropylene cages (414 $\times$ 344 $\times$ 168 mm) until weaning. The day of birth was considered postnatal day (PND) 1, and the pups were weaned on PND 21. Pregnant rats (9-15 pregnant rats per group) received FLX (0.4, 1.7, and 17 mg/kg) or distilled water daily from GD 7 (after the period of embryo implantation) until lactation day (LD) 21.

2.3.1. Pregnancy outcomes

Maternal body weight was monitored daily during gestation and lactation. Additionally, the weight of the dams on GD 21 was corrected by subtracting the sum of the weights of the newborns on PND 1. Using this calculation, we could better estimate the possible maternal and/or fetal toxicity. During these periods, general clinical observations were made once per day. All of the animals were evaluated for mortality, morbidity, and general clinical signs, such as behavioral changes (e.g., agitation, lethargy, hyperactivity, and cannibalism), neurological changes (e.g., convulsions, tremors, muscle rigidity, and hyperreflexia), and autonomic signs (e.g., lacrimation, piloerection, pupil size, and unusual respiratory pattern). Additionally, the following data were recorded: pregnancy length, number of implantation sites, litter size, pup birth weight, and pup weaning weight. Postimplantation loss ([number of implants - number of live

offspring] × 100/number of implants), the live birth index ([number of live offspring/number of offspring delivered] × 100), the viability index ([number of live offspring on PND 4/number of live offspring delivered] × 100), the weaning index ([number of live offspring on PND 21/number of live offspring delivered] × 100), the male index ([number of male offspring/total number of pups per litter] × 100), and the anogenital distance (AGD) of female and male pups on PND 1 were evaluated. The AGD was defined as the distance between the center of the anus and center of the genital bud. The distances were measured with a digital caliper (Carrera Precision 6-Inch Titanium Digital LCD Caliper Micrometer) and were corrected for the cubic root of body weight (AGD/[body weight]$^{1/3}$). At weaning, dams were weighed and euthanized by deep anesthesia with 100 mg/kg pentobarbital intraperitoneally, and the liver, kidneys, adrenal glands, uterus, and ovaries were dissected and weighed. Organ weights are reported as absolute weights in grams. Dams that did not give birth until GD 25 were euthanized to assess the possible presence of fetuses, number of implantations, and post-implantation loss. The sexual development of the offspring was assessed as described below in sections 2.3.3 and 2.3.4.

2.3.2. Hormone analysis of dams

The hormone analysis was performed using a noninvasive method, in which hormone metabolites were measured from fecal extracts of dams. The samples were collected directly from the animals' cages on days 1, 8, 15, and 21 of pregnancy and 7, 14, and 21 of lactation for all of the treatment groups. For each animal, all fecal pellets obtained within a 24 h period were collected and stored in hermetic plastic bags at -20°C until analysis.

Fecal steroids were extracted according to the procedure previously described by Touma and Palme [15]. Briefly, an aliquot of approximately 0.5 g (± 0.05 g) of the well-mixed wet fecal sample was placed into a glass tube that contained 5.0 ml of 80% ethanol and 20% distilled water and vigorously shaken using a Multi Pulse vortexer (Glas-Col, Terre Haute, IN) for 30 min. Each sample was centrifuged at 1000 × *g* for 15 min. The supernatant was recovered and diluted at a 1:1 ratio with a phosphate-buffered solution.

Fecal extracts were analyzed for metabolites of progestagens, estrogens, and glucocorticoids by enzyme immunoassay [16]. Antibodies against pregnane (CL425; 1:10000), estradiol (R0008; 1:12000), and corticosterone (CJM006; 1:12000) and the respective horseradish peroxidase conjugates were obtained from Coralie Munro at the University of California, Davis (Davis, CA, USA). Serial dilutions of pooled fecal extracts produced displacement curves parallel to those of the standards for each assay. The intra- and interassay coefficients of variation were less than 10%. The data were corrected for dilution and are expressed as µg/g or ng/g of wet feces.

2.3.3. Sexual development of female pups

All of the females were evaluated daily for vaginal opening from PND 33. The day of complete vaginal opening and body weight on that day were recorded. Beginning on PND 60, the estrous cycle was monitored daily whenever possible in four pups per litter (17-32 females per group) by observing the changes in vaginal smear cytology during three complete cycles. The evaluation of vaginal smears was based on the following cytological criteria for estrous cycle staging: predominance of nucleated epithelial cells (proestrus), predominance of cornified epithelial cells (estrus), presence of both cornified epithelial cells and leukocytes (metestrus), predominance of leukocytes (diestrus) [17]. The mean cycle length (days) and number of animals with prolonged estrus (> 1 day) and prolonged diestrus (> 3 days) were evaluated. At the third estrus, the females were euthanized by decapitation, and the liver, kidneys, adrenal glands, uterus, and ovaries were removed and weighed.

2.3.4. Sexual development of male pups

All of the males were evaluated daily for preputial separation by manual retraction of the prepuce from PND 33. The day of complete preputial separation and body weight on that day were recorded. Afterward, whenever possible two male pups per litter (11-16 males per group) were randomly selected for the investigation of the reproductive effects of FLX in adulthood. Male offspring were euthanized by decapitation on PND 90 when the animals reached sexual maturity. The body was weighed, and the liver, kidneys, adrenal glands, testis, epididymis, ventral prostate, seminal vesicle, and levator

ani/bulbocavernosus muscle (LABC) were removed and weighed. The seminal vesicle was weighed without fluid.

2.3.5. Daily sperm production

To determine daily sperm production, the tunica albuginea of the right testicle was removed (the left testicle was used for histology), minced, and homogenized in 10 ml of saline (0.9% NaCl) that contained 0.5% Triton X-100 at medium speed in a tissue homogenizer (POTTER S tissuemizer) for 1 min. The homogenate was diluted 10 times in saline for the microscopic counting of the number of homogenization-resistant spermatids (i.e., spermatids in stages 17 to 19) in a Bürker hemocytometer chamber. The number of spermatids per animal, obtained by counting the right testicle multiplied by two, was divided by 6.1 for the conversion to daily sperm production [18]. This divisor (i.e., 6.1) corresponds to the number of days of the seminiferous epithelium in which the spermatids in stages 17 to 19 are present. Daily sperm production per gram was calculated to determine the efficiency of the process [19]. The left testes of these animals were used to make permanent slides and evaluate testicular morphometry as described below.

2.3.6. Testicular morphometry

The left testes of two males per litter (11-16 rats per group) were removed and immediately immersed in Metacan fixative solution (60% methanol, 30% chloroform, and 10% glacial acetic acid) for 3 h. The capsule was cut at the poles to allow the penetration of the fixative. After this preliminary fixation, the testis was cut transversally at the smaller diameter in two slices that were placed again into the fixative for an additional 3 h. After fixation, the slices were dehydrated in increasing concentrations of alcohol, diaphanized with xylene bathing solution, and impregnated with and embedded in paraffin. Sections (5 µm thickness) were obtained, placed onto glass slides, and stained with hematoxylin and eosin. The testis sections were used for testicular morphometry and cell counting.

The diameter of the seminiferous tubules was measured in 20 randomly selected round tubular sections per animal at 100× magnification using Scion image software, version 1.62 (Scion Corporation, Frederick, MD). The volume

density of the seminiferous tubules was determined by point counting [20]. A 300-intersection grid was superimposed on the captured images of the testicular sections. Ten randomly chosen fields were analyzed for each animal at 100× magnification. Points that fell over the seminiferous tubules, comprising the tunica albuginea, epithelium, and lumen, were scored, and the volume density was recorded, expressed as the ratio of the number of points over the seminiferous tubules to total points analyzed. The absolute volume of the seminiferous tubules was obtained by multiplying the volume density by the testis volume. For this calculation, the specific gravity of the testis was assumed to be 1.0. The total length of the seminiferous tubules was obtained by dividing the absolute volume of the seminiferous tubule by the squared radius of the tubule times the π value [21].

Sertoli cell nucleoli were counted in 20 round seminiferous tubule cross-sections chosen at random from each animal at 1000× magnification [21]. These counts were corrected for section thickness and the nucleolar diameter using Abercrombie's formula [22]. The total number of Sertoli cells per testis was determined from the corrected counts of Sertoli cell nucleoli per tubule cross-section and total length of seminiferous tubules according to Hochereau-de Reviers and Lincoln [23].

2.4. Statistical analyses

Parametric data were analyzed by analysis of variance (ANOVA), and differences between groups were assessed using the Bonferroni test. Nonparametric data were analyzed using the Kruskal-Wallis test followed by Dunn's test. Cannibalism behavior, cycle length, persistent estrus, and prolonged diestrus were analyzed using the Fisher test. Differences were considered significant at a probability level of 5% ($p < 0.05$). The analyses were performed and the graphs were made using GraphPad Prism software, version 5.0. With the exception of cannibalism behavior, cycle length, persistent estrus, and prolonged diestrus, all of the statistical analyses were performed by considering the litter as the statistical unit.

3. Results

3.1. Pregnancy outcomes

The dams exposed to 17 mg/kg FLX exhibited a reduction in body weight gain during pregnancy and a reduction in body weight during early lactation (Fig. 1 and 2). Similarly, the corrected body weight on GD 21 in the dams exposed to 17 mg/kg FLX was significantly reduced compared with the control group (Fig. 1).

Pregnancy length, the number of implantations sites, litter size, postimplantation loss, the live birth index, and the male index were unaffected by FLX exposure (Table 1). Pup birth weight was significantly reduced in pups exposed to 17 mg/kg FLX, but a recovery of body weight was observed at weaning (Table 1). The viability index and weaning index were also significantly reduced in dams exposed to 17 mg/kg FLX compared with the control group (Table 1).

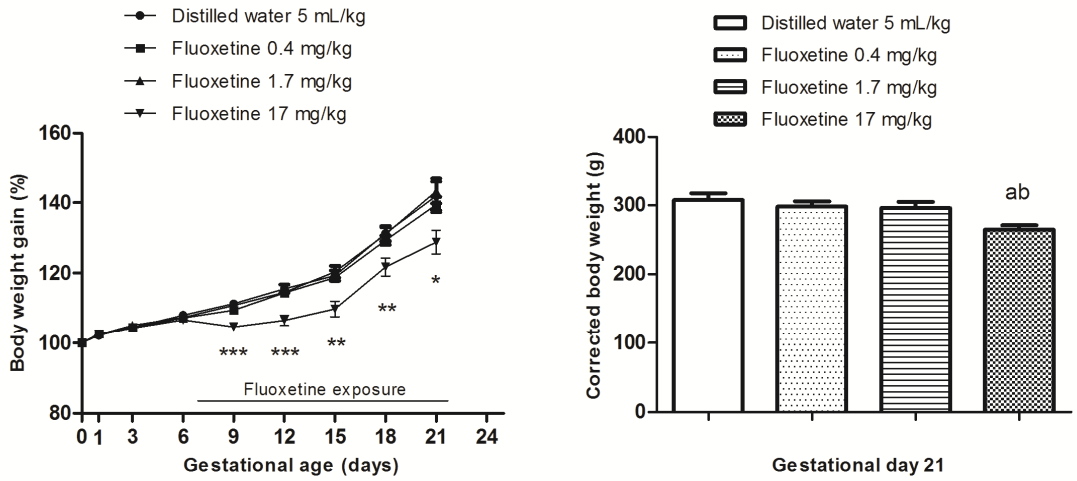

Fig. 1. Body weight gain in dams during pregnancy (left) and corrected body weight on GD 21 (right). The dams were treated with the antidepressant fluoxetine during pregnancy and lactation from GD 7 to PND 21. Body weight gain was determined by considering the body weight of the dams on GD 0 as 100%. The body weight of the dams on GD 21 was corrected by subtracting the sum of the weights of the pups on PND 1. The data are expressed as mean ± standard error. *$p < 0.05$, $^{a}p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ when compared with control group; $^{b}p < 0.05$ when compared with 0.4 mg/kg FLX (ANOVA followed by Bonferroni test). Number of animals per group = 9-15.

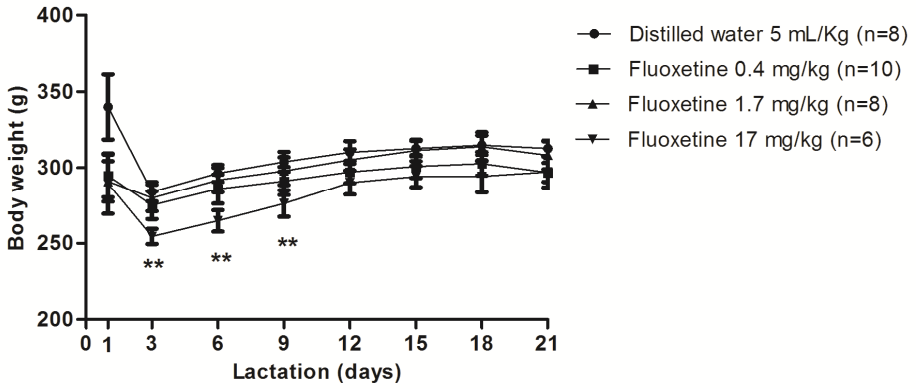

Fig. 2. Body weight of dams during lactation. These dams were treated with the antidepressant fluoxetine during pregnancy and lactation from GD 7 to PND 21. The data are expressed as mean ± standard error. ***p* < 0.01, compared with control group (ANOVA followed by Bonferroni test).

A significant increase in the absolute weight of the adrenal glands and a reduction in uterus weight were detected in dams exposed to the highest FLX dose (17 mg/kg). However, the absolute weight of the liver, kidneys, and ovaries in the dams was not affected by FLX exposure (Table 2). However, the relative organs weight did not differ between treatment groups (data not shown).

After GD 17, muscle rigidity and piloerection were observed in all of the dams exposed to 17 mg/kg FLX. Agitation and cannibalism behavior were observed after delivery in two dams exposed to 17 mg/kg FLX, in which the dams killed all of their pups. No other signs of toxicity were observed in the dams or their pups.

Besides the loss of these two litters, we also had losses in all treatment groups for dystocia and/or death of all pups (stillborn or postnatal death). Thus, the number of dams in the end of lactation was reduced due to these losses.

Table 1. Pregnancy outcomes of dams treated with fluoxetine from GD 7 to PND 21.

	Maternal fluoxetine dose (mg/kg/day)			
Parameter	Control	0.4	1.7	17
Number of dams	12	15	09	12
Pregnancy length (days)	22.09 ± 0.28	22.27 ± 0.18	22.13 ± 0.30	22.08 ± 0.31
Number of implantation sites	12.00 ± 0.81	10.87 ± 0.79	10.78 ± 1.44	12.08 ± 1.15
Postimplantation loss (%)	37.87 ± 13.06	41.93 ± 11.60	26.84 ± 11.52	35.91 ± 12.10
Live birth index (%)	72.78 ± 13.16	63.10 ± 11.69	91.83 ± 6.27	67.22 ± 12.52
Litter Size	9.50 ± 1.24	8.47 ± 1.07	10.50 ± 1.34	9.42 ± 1.37
Pup birth weight (g)	5.99 ± 0.21	5.55 ± 0.30	5.98 ± 0.11	5.08 ± 0.20 [abc]
Pup weaning weight (g)	36.78 ± 2.09	36.68 ± 2.32	34.17 ± 1.93	29.95 ± 0.93
Viability index (%)	100.00 ± 0.00	95.45 ± 4.55	100.00 ± 0.00	88.67 ± 4.31 [abc]
Weaning index (%)	92.58 ± 5.10	90.00 ± 9.05	99.04 ± 0.96	74.73 ± 13.47[abc]
Male index (%)	50.26 ± 4.57	52.50 ± 6.37	57.04 ± 5.17	50.19 ± 8.65
Dams that exhibited cannibalism behavior[#]	0/12 (0%)	0/15 (0%)	0/9 (0%)	2/12 (17%)

Note: Values are expressed as mean ± standard error.

[a] $p < 0.05$, compared with control group.

[b] $p < 0.05$, compared with 0.4 mg/kg FLX.

[c] $p < 0.05$, compared with 1.7 mg/kg FLX.

[#] Dams that killed all its pups by cannibalism.

Table 2. Absolute organ weights of dams exposed to fluoxetine during pregnancy and lactation

	Maternal fluoxetine dose (mg/kg/day)			
Parameter	Control	0.4	1.7	17
Number of dams	08	10	08	06
Body weight (g)	310.90 ± 5.58	301.00 ± 6.28	306.90 ± 7.54	306.00 ± 7.74
Liver weight (g)	16.72 ± 0.73	15.37 ± 0.98	16.03 ± 1.00	16.81 ± 0.76
Kidney weight (g)	1.11 ± 0.04	1.03 ± 0.03	1.05 ± 0.03	1.13 ± 1.26
Adrenal weight (mg)	28.06 ± 3.58	27.95 ± 2.38	31.46 ± 2.37	38.37 ± 2.18 [ab]
Uterus weight (g)	0.288 ± 0.033	0.215 ± 0.023	0.224 ± 0.017	0.182 ± 0.008 [ac]
Ovary weight (mg)	43.69 ± 2.99	42.75 ± 3.12	45.00 ± 1.54	39.67 ± 1.41

Note: Values are expressed as mean ± standard error (Kruskal-Wallis followed by Dunn's test).

[a] $p < 0.05$, compared with control group.

[b] $p < 0.05$, compared with 0.4 mg/kg FLX.

[c] $p < 0.05$, compared with 1.7 mg/kg FLX.

3.2. Hormonal analysis of dams

Maternal hormone levels determined during FLX treatment are illustrated in Fig. 3 and 4. The fecal concentrations of progestagen and glucocorticoid metabolites significantly increased on GD 15 in the dams exposed to 17 mg/kg FLX (Fig. 3). Besides that, during the lactation period, a significant increase in the fecal metabolites of estrogens and progestagens was detected on LD 7 in the same group of dams (Fig. 4). Additionally, the dams exposed to 17 mg/kg FLX exhibited a trend toward an increase in glucocorticoid metabolites on LD 7, but this difference was nonsignificant ($p = 0.11$). A significant increase in fecal estrogen metabolites was also observed on LD 7 in dams exposed to 0.4 mg/kg FLX (Fig. 4). A proper analysis of steroid hormone metabolites on GD 21 was impaired because on this day some of the dams had already given birth, while others did not.

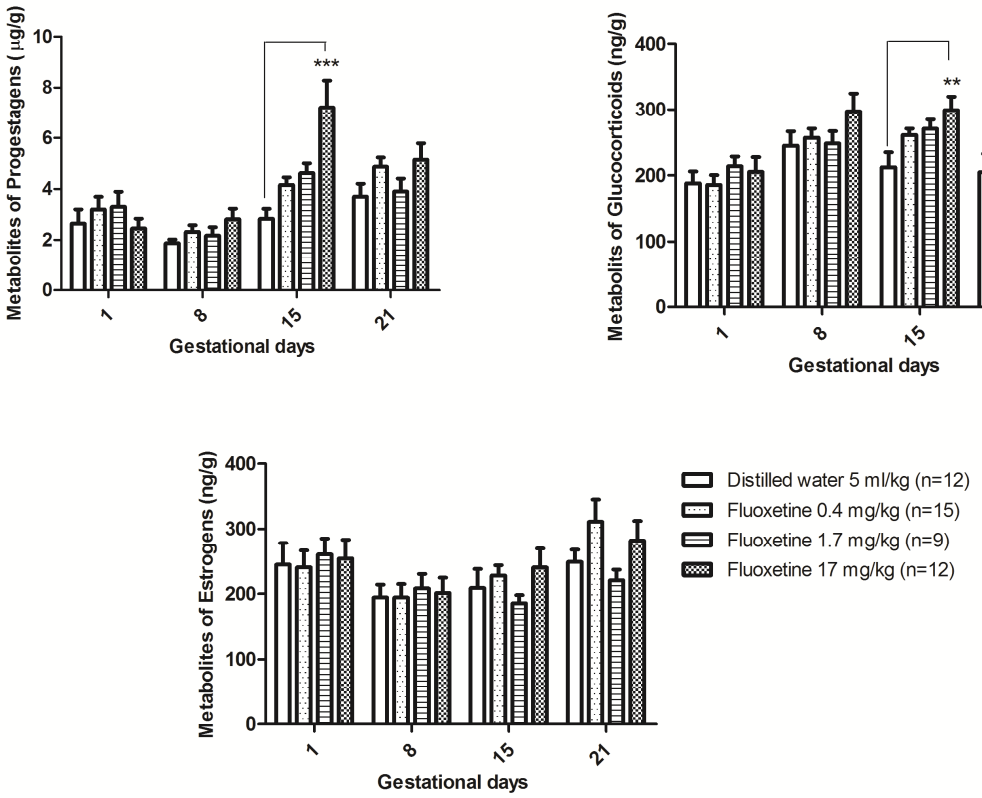

Fig. 3. Effects of fluoxetine exposure *in utero* and during lactation on fecal hormone metabolites (progestagens, glucocorticoids, and estrogens) during pregnancy. The dams were treated with the antidepressant fluoxetine during pregnancy and lactation from GD 7 to LD 21. Columns represent mean ± standard error. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with control group (Kruskal-Wallis followed by Dunn's test).

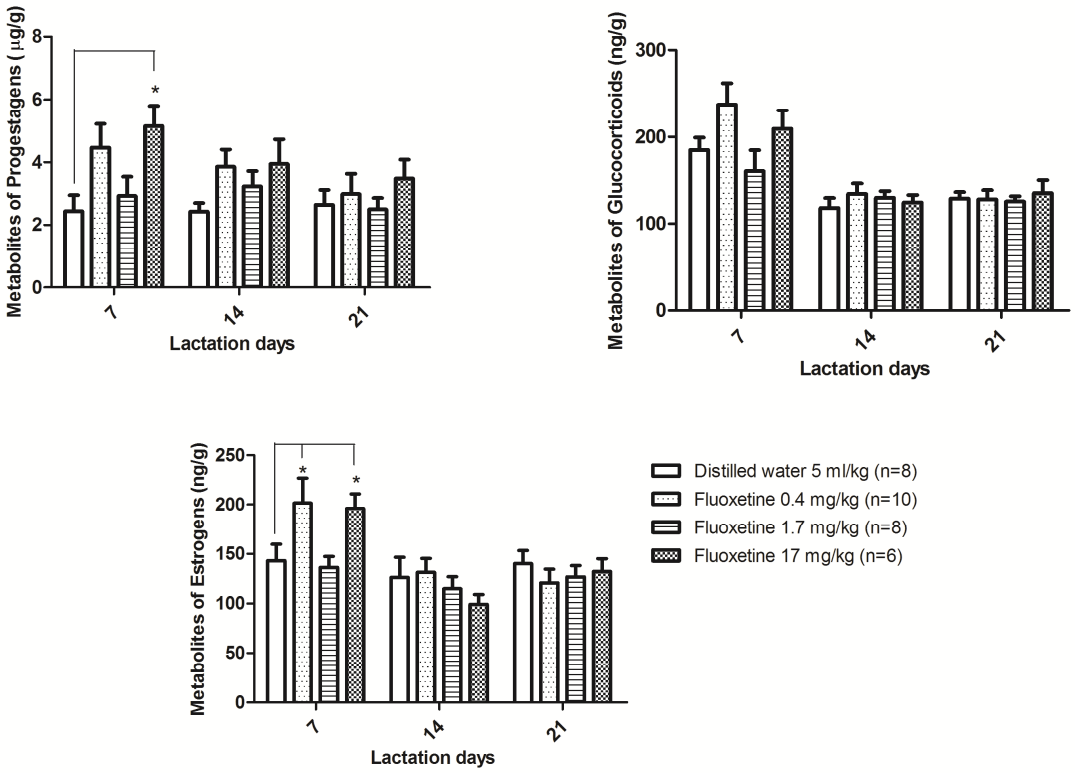

Fig. 4. Effects of fluoxetine exposure *in utero* and during lactation on fecal hormone metabolites (progestagens, glucocorticoids, and estrogens) during the lactation period. The dams were treated with the antidepressant fluoxetine during pregnancy and lactation from GD 7 to LD 21. Columns represent mean ± standard error. *$p < 0.05$, compared with control group (Kruskal-Wallis followed by Dunn's test).

3.3. Sexual development of female pups

No adverse effects were observed on sexual development in females exposed to FLX *in utero* and during lactation. Body and organ weights (i.e., uterus, ovaries, liver, kidneys, and adrenal glands) did not differ from the control group (data not shown). The age at vaginal opening, body weight on that day, cycle length, and the number of animals with prolonged estrus or diestrus did not differ from the control group (Table 3).

Table 3. Sexual development of female offspring exposed to fluoxetine *in utero* and during lactation.

Parameter	Control	Maternal fluoxetine dose (mg/kg/day)		
		0.4	1.7	17
No. of animals (litter)	38 (8)	44 (10)	29 (8)	26 (5)
Age at vaginal opening (days)	34.95 ± 1.01	37.08 ± 1.16	36.81 ± 1.14	38.61 ± 1.87
Body weight at vaginal opening (g)	94.46 ± 4.58	98.72 ± 3.73	97.64 ± 4.98	96.68 ± 5.21
No. of animals (litter)	26 (8)	32 (10)	27 (8)	17 (5)
Mean cycle length (days)	4.97 ± 0.28	4.81 ± 0.17	4.78 ± 0.28	4.56 ± 0.15
Cycle length				
< 4 days	0/26 (0%)	0/32 (0%)	1/27 (3.7%)	0/17 (0%)
4-5 days	21/26 (81%)	23/32 (72%)	20/27 (74%)	15/17 (88%)
> 5 days	5/26 (19%)	9/32 (28%)	6/27 (22%)	2/17 (12%)
Animals with prolonged estrus [a]	10/26 (38%)	12/32 (38%)	9/27 (33%)	8/17 (47%)
Animals with prolonged diestrus [b]	5/26 (19%)	5/32 (16%)	5/27 (19%)	0/17 (0%)

Note: Values are expressed as mean ± standard error.

[a]estrus > 1 day.

[b]diestrus > 3 days.

3.4. Sexual development of male pups

Exposure to FLX *in utero* and during lactation did not affect preputial separation and body weight on that day compared with control animals. Rats exposed to 17 mg/kg FLX showed a trend toward a reduction (although nonsignificant) in daily sperm production ($p = 0.09$) and testicular efficiency ($p = 0.15$; Table 4). Body and organ weights (i.e., liver, kidneys, adrenal glands, testis, epididymis, ventral prostate, seminal vesicle, and LABC) in the groups exposed to FLX did not differ from the control group (data not shown). Likewise, the testicular morphometric analyses were not affected by maternal exposure to FLX (Table 4).

Table 4. Sexual development and testicular morphometric analysis of male offspring exposed to fluoxetine *in utero* and during lactation.

		Maternal fluoxetine dose (mg/kg/day)		
Parameter	Control	0.4	1.7	17
No. of animals (litter)	36 (8)	45 (10)	44 (7)	31 (6)
Age at complete preputial separation (days)	46.90 ± 0.54	47.07 ± 0.55	47.35 ± 0.69	47.54 ± 0.53
Body weight at complete preputial separation (g)	163.5 ± 5.66	172.9 ± 6.13	165.0 ± 3.60	152.6 ± 4.27
No. of animals (litter)	12 (7)	16 (10)	14 (7)	11 (6)
Daily sperm production ($\times 10^6$)	77.49 ± 7.39	79.00 ± 4.66	71.40 ± 4.65	59.12 ± 5.43
Testicular efficiency	46.60 ± 3.41	49.89 ± 2.90	46.06 ± 3.69	38.86 ± 2.97
Mean diameter of seminiferous tubule (µm)	228.1 ± 1.66	232.9 ± 2.88	228.6 ± 4.58	237.4 ± 5.65
Relative density of seminiferous tubule (%)	0.640 ± 0.007	0.638 ± 0.006	0.625 ± 0.009	0.651 ± 0.009
Absolute density of seminiferous tubule (cm^3)	1.055 ± 0.041	1.014 ± 0.034	0.980 ± 0.042	0.991 ± 0.046
Total length of seminiferous tubule (m)	25.90 ± 1.300	23.88 ± 1.007	23.81 ± 0.371	22.51 ± 1.237
Sertoli cell/tubule cross-section	13.54 ± 0.324	13.78 ± 0.440	13.97 ± 0.564	13.91 ± 0.905
Sertoli cells/testis ($\times 10^6$)	69.78 ± 2.604	65.77 ± 3.289	66.56 ± 3.003	61.61 ± 1.195

Note: Values are expressed as mean ± standard error.

4. Discussion and conclusions

The developing organism is exquisitely sensitive to endocrine effects, and any mammalian assay or test must include exposure to the test compound *in utero* and during lactation to fully evaluate its effects on subsequent growth and development. Perinatal exposure demonstrates the life-stage sensitivity of

reproductive systems, allowing the evaluation of offspring beyond weaning to at least puberty and detection of endocrine-sensitive endpoints in rodents [10].

In the present *in utero*-through-lactation assay, it was observed a reduction in body weight gain during pregnancy and lactation in dams exposed to 17 mg/kg FLX. Additionally, a reduction in dam body weight on GD 21, corrected for newborn weight, was also found in this group. These results indicate that this dose of FLX caused both, fetal toxicity, because the pups had a lower birth weight, and maternal toxicity, because even when subtracting the weights of the pups on PND 1, the dams still had lower body weights on GD 21 [24]. Moreover, the dams exposed to 17 mg/kg FLX presented clinical signs of toxicity, manifested by neurological and autonomic changes, including piloerection and muscle rigidity. Two dams in this group also exhibited agitation and cannibalism, further supporting the previously described maternal toxicity.

The fetal toxicity produced by higher dose of FLX, characterized by the restriction of intrauterine growth, was reflected by a lower birth weight in the offspring at the same gestational age. Laboratory investigations have shown that serotonin had a strong vasoconstriction effect on human umbilical arteries [25,26]. Thus, it is possible that the increased serotonin level that resulted from the maternal ingestion of SSRIs may increase the risk of adverse outcomes that are sensitive to the placental blood flow, such as intrauterine growth retardation, which has been observed consistently in various studies [27-29]. In addition, many authors have shown that low birth weight is related to increased mortality rates and developmental problems [30-33]. In the present study, a reduction in the viability and weaning indices in the pups of the dams treated with 17 mg/kg FLX during pregnancy and lactation were observed. These findings clearly showed a higher mortality in the offspring in this group, showing once again that FLX at the highest dose caused fetal and neonatal toxicity. In contrast, Byrd and Markhan [34] reported maternal toxicity in pregnant rats exposed to 12.5 mg/kg FLX without affecting the birth weight and viability index of pups.

Hormonal analysis showed that dams exposed to 17 mg/kg FLX presented increased levels of fecal glucocorticoid metabolites on GD 15. Studies have shown that both serotonin receptor agonists and SSRIs, such as FLX, may increase the secretion of adrenocorticotropic hormone (ACTH) by increasing the secretion of hypothalamic corticotropin-releasing hormone (CRH)

and vasopressin (antidiuretic hormone) and acting directly on the adrenal gland [35-39]. The possible stimulation of the hypothalamic-pituitary-adrenal (HPA) axis may also have indirectly increased the synthesis of progesterone because this is an intermediate in the pathway of glucocorticoid synthesis from cholesterol. Additionally, increased levels of estrogen metabolites observed in early lactation may also be caused by hyperactivation of the HPA axis. The increased activity of this axis produces higher levels of androgens by the adrenal gland. In turn, these androgens are then converted to estrogens by the aromatase enzyme.

The possible hyperstimulation of the HPA axis is supported by the increased weight of the adrenal glands observed in the group treated with 17 mg/kg FLX. Meltzer et al. [40] showed that FLX but not tricyclic antidepressants potentiated the increase in plasma cortisol secretion mediated by 5-hydroxytryptophan in subjects with major depression or obsessive compulsive disorder. Additionally, a clinical study conducted by Bschor et al. [41] showed an increase in plasma cortisol levels after 28 days of treatment with citalopram, another SSRI antidepressant. However, because increased levels of fecal estrogen metabolites were also observed in the dams exposed to 0.4 mg/kg FLX, which did not display adrenal gland hypertrophy, additional pathways could also be involved in the increased synthesis of estrogens observed, such as effects on the synthesis of hypothalamic releasing hormones by serotonergic stimulation in hypothalamic neurons.

It is important to note that the HPA axis undergoes significant changes during pregnancy. There are evidences that cortisol, ACTH, CRH and corticosterone binding globulin levels are altered significantly during pregnancy and postpartum. Stress hormone levels rise during pregnancy, reach a peak before birth and drop after birth in rats and humans [42]. However, the dams exposed FLX 17 mg/kg/day showed statistically higher levels of glucocorticoid metabolites on GD 15 compared with the control group. Thus, the excess in glucocorticoids synthesized in late pregnancy is the cause of concern about possible negative effects on the fetus and neonate.

Hyperactivity of the HPA axis is a common symptom in psychiatric disorders, such as major depressive disorder, and this condition can cause lower birth weight and affect the development of the conceptus [42-44]. The

pharmacological treatment of depression during pregnancy aims to alleviate the symptoms of the disease, including reducing stress and improving maternal and fetal conditions. However, contradictorily, our results show that FLX caused increased levels of stress hormones during pregnancy. These results highlight the importance of antidepressant dose selection during pregnancy and lactation because high doses of FLX might actually increase maternal glicocorticoid levels. Furthermore, there are many evidences in the literature showing that estrogenic substances or others that are able to disrupt the endocrine system can alter the sexual development of offspring exposed during critical periods of development [45,46]. Thus, both the estrogenic effect of 17 mg/kg FLX observed in a previous study by Müller et al. [13] assessed by uterotrophic and reporter gene assays, and the hormone imbalance observed in the present study at the same dose of FLX (17 mg/kg) could cause changes in the sexual development of offspring exposed to FLX *in utero* and during lactation. However, in the present study, no significant long-term reproductive effects were observed in the parameters of sexual development of male and female offspring.

The literature shows that FLX can disrupt the hormonal cycle. Clinical trials showed menstrual cycle changes in women who were using FLX [47,48]. Additionally, dysregulation of the estrous cycle (i.e., an elongated cycle or a complete cessation of cyclicity) was observed in adult, regularly cycling Fischer rats exposed daily to 10 mg/kg FLX [49]. Similarly, a transient disturbance of the estrous cycle was observed in regularly cycling Sprague-Dawley rats exposed to 10 mg/kg FLX [50]. However, the changes in the estrous cycle were observed in mature rats during FLX treatment, whereas in the present study, the female rats were exposed to FLX only via the placenta and maternal milk. Thus, we conclude that exposure to FLX did not cause irreversible changes in the HPG axis in females exposed to this drug *in utero* and during lactation. In contrast, dams treated with 17 mg/kg FLX presented a reduction in uterine weight on LD 21, indicating that FLX is able to induce changes in reproductive organs in mature female rats.

The reduction in daily sperm production and testicular efficiency in male rats exposed to 17 mg/kg FLX *in utero* and during lactation, although not significant, deserves attention, mainly because it is an effect commonly caused

by estrogenic and anti-androgenic substances. Studies in rodents showed that FLX treatment decreased male fertility [51,52]. Silva et al. [53] observed a reduction in final Sertoli cell number in Wistar rats treated intraperitoneally with 10 mg/kg FLX during lactation. Additionally, studies in men have suggested that SSRIs may damage normal sperm DNA integrity, and decrease testosterone and gonadotropin levels affecting fertility [54,55]. Thus, further investigations are needed on the possible effects of FLX on male sexual development.

Importantly, the majority of adverse effects were observed by FLX exposure at a dose of 17/mg/kg, and this dose represents the therapeutic dose for rats multiplied by 10 and served as the safety factor, attributable to intra-species differences. Overall, the present results showed that the treatment of rats with 17 mg/kg FLX during pregnancy and lactation induced maternal and fetal toxicity and caused changes in the hormonal profile of the dams, probably by hyperstimulation of the HPA axis.

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8. ARTIGO 3

Artigo científico que será submetido à revista Neurotoxicology and Teratology (páginas 103 a 132 seguintes).

Evaluation of Long-term Behavioral and Neurochemical Effects of Wistar Rats Exposed to Fluoxetine *In Utero* and Lactation

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Abstract

The use of the antidepressant fluoxetine (FLX) by pregnant and lactating women has increased in recent years, accompanied by limited and controversial information about the long-term effects on brain development and behavior. The present study evaluated the effects of FLX exposure *in utero* and lactation on behaviors of locomotion, anxiety and depression and the amount of neurotransmitters in brain structures related to anxiety and depression in adulthood. Pregnant Wistar rats were treated daily with FLX (0.4, 1.7, and 17 mg/kg/day) or distilled water by gavage from gestation day (GD) 7 to lactation day (LD) 21 and male and female offspring were evaluated in adulthood. FLX caused increase in locomotor activity in offspring (17 mg/kg FLX) in the elevated plus maze, but did not cause anxiety- and depression-like behaviors in adult rats assessed by open field, elevated plus maze, sucrose preference and forced swimming tests. Neurochemical analysis showed a significant reduction in NA, DOPAC and 5-HIAA in prefrontal cortex of females (0.4 mg/kg FLX) and males (1.7 and 17 mg/kg FLX) offspring exposed to FLX *in utero* and lactation. In addition, NA (0.4 mg/kg FLX) and 5-HIAA (1.7 mg/kg FLX) were significantly decreased within the hippocampus of male offspring exposed to FLX in the same periods. Neurochemical changes modulated by early-life exposure to FLX was sexually dimorphic and varied with the dose. These results indicate that FLX can cause long-term neurochemical and behavioral effects when administered at critical periods of neurodevelopment.

Keywords: Depression, fluoxetine, *in utero* and lactational assay, neurochemical analysis, behavior.

1. Introduction

Major depressive disorder is among the most common neuropsychiatric disorders worldwide, with an incidence of two-three times higher in women than in men (Sloan and Kornstein, 2003; World Health Organization, 2009). Furthermore, depressive episodes are more frequent during reproductive age, and gestation and postpartum periods are considered the time of greatest risk for women to develop depression (Bennett et al., 2004; Moraes et al., 2006).

The use of antidepressant medications is currently acceptable for pregnant and lactating women in situations where the risks to the mother and child of not treating the disease are greater than the risks associated with exposure to the antidepressant drug (Marcus and Heringhausen, 2009; APA, 2010). The past decade has seen a large increase in the use of selective serotonin reuptake inhibitors (SSRIs) antidepressants during pregnancy and lactation. Among these, fluoxetine (FLX) was reported as the most widely used antidepressant during pregnancy (Cooper et al., 2007; Andrade et al., 2008). FLX readily cross the placenta (Heikkine et al., 2002) reaching similar levels in maternal and fetal serum, and is also secreted into breast milk during lactation (Hendrick et al., 2001; Suri et al., 2002).

The possibility of perinatal exposure to FLX has raised significant concerns about its possible adverse effects on newborn and child development. Despite the predominant use of SSRIs are due in part to their limited side-effects in comparison to tricyclic, side-effect testing has only been well established in adults, while safety at critical periods of development is less clear, with many conflicting results (Ellfolk and Malm, 2010). Major concerns of prenatal exposure to SSRI drugs include neonatal neurologic symptoms and possible adverse effects on the developing central nervous system. However, few studies were conducted to evaluate the long-term behavioral effects of early life exposure to SSRIs. The clinical evaluation of long-term effects is limited (Homberg, Schubert and Gaspar, 2010; Ellfolk and Malm, 2010) and complicated due to time constraints associated with human research and many confounding variables (Altshuler et al, 1996; American Academy of Pediatrics, 2000; Olivier et al., 2010). In this regard, rodent studies may provide further insights on long-term effects.

It is known that serotonin (5-HT) plays an important role in neuroplasticity during critical embryonic developmental stages (Gaspar, Cases and Maroteaux, 2003; Homberg, Schubert and Gaspar, 2010). Besides that, some studies have shown that drugs or genetic manipulations that increase 5-HT neurotransmission can modify the fine wiring of brain connections and produce permanent changes in adult behavior when administered during the critical developmental periods (Gingrich and Hen, 2001; Whitaker-Azmitia, 2001; Gross et al., 2002). In addition, a recent study conducted by Müller et al., (2012) showed estrogenic action of FLX by performing *in vitro* and *in vivo* screening tests. It is known that exposure to estrogenic substances is also able to interfere with synaptic remodeling and to produce long-term behavioral changes (Palanza et al., 1999; Hajszan and Leranth, 2010). Besides that, another study found increased levels of glucocorticoids in progenitors treated with FLX during pregnancy and lactation (Manuscript submitted for publication). It is well recognized that the hippocampus contains a large number of glucocorticoid receptors and is vulnerable to the effects of stress (Buss et al., 2007; Pruessner et al., 2009), and corticosterone (McEwen, 2008), and many animal models of depression use chronic stress or stress hormone manipulation (e.g. chronic mild stress or elevated high corticosterone) to induce depressive-like symptoms in animals (Brummelte and Galea, 2010). Similarly, it has been shown that hormones that stimulate the production of glucocorticoids, such as the corticotrophin-releasing factor also to influence anxiety responses (Magalhães et al., 2010).

These results indicate that FLX exposure in early life stage could alter the neurodevelopment mediated by 5-HT. Additionally, the high levels of maternal glucocorticoids found in dams treated with FLX during pregnancy as well as the possible estrogenic activity of FLX also could affect the brain differentiation and maturation. Thus, this work aimed to evaluate whether exposure to FLX *in utero* and during lactation could produce permanent behavioral changes and correlate these results with levels of neurotransmitters in brain structures involved in depression and anxiety. The endpoints investigated included possible behavioral changes assessed by open field, elevated plus maze, modified forced swimming and sucrose preference test, and possible changes in the amount of dopamine (DA), noradrenaline (NA),

serotonin (5-HT) and their metabolites in prefrontal cortex and hippocampus of male and female rat offspring exposed to FLX *in utero* and during lactation.

2. Materials and Methods

2.1. Animals and conditions

Wistar rats were obtained from Federal University of Parana and maintained under controlled conditions at 22 ± 2°C and a constant 12 h/12 h light/dark cycle. The animals were maintained in the reproductive toxicology laboratory for 1 week for acclimatization before beginning the experiments. Standard food pellets (Nuvital, Curitiba, PR, Brazil) and tap water were available *ad libitum*. All of the experiments were approved by the Committee on Animal Research and Ethics of the Federal University of Parana (protocol no. 347), which is in accordance with national and international guidelines of animal welfare. With the exception of dams, all of the animals were euthanized by decapitation because we used the brains of animals for the determination of neurotransmitters in brain structures.

2.2. Fluoxetine: experimental groups

Fluoxetine hydrochloride ($C_{17}H_{18}F_3NO \cdot HCl$) was supplied by Pharma Nostra Comercial (Rio de Janeiro, Brazil). Pregnant rats were assigned to four experimental groups that received 5 ml/kg of distilled water (control group) or three different doses of FLX orally (gavage). The doses of FLX were chosen based on (*i*) the human therapeutic dose, calculated as the average therapeutic range indicated for depression (30 mg) for human adult patients who weight 70 kg, (*ii*) the extrapolated “therapeutic dose” for rats, calculated by allometry (Nevill, 1994), and (*iii*) the “therapeutic dose” for rats multiplied by 10 (i.e., the safety factor attributable to intra-species differences). Thus, the three doses were 0.4, 1.7, and 17 mg/kg/day, respectively. Fluoxetine salt was dissolved in distilled water daily, and the volume of administration was 5.0 ml/kg.

2.3. In utero and lactational assay

Nulliparous females (90 days old) were placed in contact with adult males (90 days old) for mating during the dark phase of the light/dark cycle at a female:male ratio of 3:1 and subjected to vaginal smear analysis. The day of sperm detection in the vaginal smear was considered day 0 of pregnancy (gestation day [GD] 0), and these females were randomly separated into the treatment and control groups and were housed individually in polypropylene cages ($168 \times 344 \times 414$ mm [height, width and depth]) until weaning. The day of birth was considered postnatal day (PND) 1, and the pups were weaned on PND 21. The administration of FLX (0.4, 1.7, and 17 mg/kg) and distilled water for dams began after the period of embryo implantation on GD 7 and was performed daily until lactation day (LD) 21.

2.3.1. Behavioral tests of the offspring

Four tests were conducted to investigate behaviors of anxiety, depression and changes in locomotion of male and female offspring in adulthood using wherever possible two pups per litter (all animals were evaluated in the interval between PND 90 and PND 110). Females were tested in the period of diestrus (predominance of leukocytes in the vaginal smear) to minimize the behavioral changes produced by hormonal variations. Always on the same days in which females were evaluated, their male siblings were also tested. The animals were subjected to behavioral tests in the following order: open field, elevated plus maze and modified forced swimming test. Furthermore, the sucrose preference test was also performed. Because the time needed to perform this test is approximately 15 days, it was used males and females descendants of the same litter, but different from those used in behavioral tests previously made. With the exception of sucrose preference test, all experiments were videotaped and later analyzed. All experiments were carried out between 14:00-18:00 pm to avoid the influence of circadian rhythms in emotional response (Eidmann et al., 1990).

Open-field test

The female and male offspring were evaluated for locomotor activity in adulthood through the open field test (Kelly, 1993; Dombrowski et al., 2006). The open-field used was a square arena (60 × 60 cm), built from wood (walls and floor). The floor was divided by lines into 16 small squares. The rats were placed individually in the center of the open-field and locomotion (number of squares crossed) and time spent in the center of the open field was recorded for 5 min. A crossed square was defined as the rat placing its two forepaws in the next square and moving forward. The number of units crossed in the open field was used as a primary index of locomotor activity. The open-field was cleaned with a 10% alcohol/water solution prior to behavioral testing to avoid possible bias due to odors and/or residues left by previously tested rats.

Elevated Plus Maze

The elevated plus maze consisted of two open arms (50 cm × 10 cm [height, width]) surrounded by a wooden edge (0.5 cm of height), and two closed arms with (40 cm × 10 cm × 50 cm [height, width and length]) mounted at an angle of 90°. The whole apparatus was made of wood and was 50 cm above the floor. The rats were individually placed at the center of the plus maze facing one closed arm and the behaviors were recorded for a 5-min period. After each animal, the maze floor was cleaned with a 10% alcohol solution. The parameters observed were number of entries in the open and closed arms and time of permanence in the open arms. The first and the last parameters were expressed in percentage. A rat was considered to have entered an arm when all four legs were on the arm. The number of entries in the closed arms was considered as the locomotor activity index and the percentage of the time spent and percentage of entries on the open arms as the anxiety index (Lister, 1987; Andreatini and Leite, 1994).

Sucrose preference test

The method to determine sweet preference was performed as described previously by Lilienthal et al. (2006). Starting on PND140, males and females were housed in single cages and each was given two bottles filled with tap

water during 7 days for adaptation. After this period, each rat was given a bottle filled with tap water and another bottle filled with 0.25% sucrose solution in water (Fluka, Seelze - Germany). Position of the bottles was counterbalanced in each group and changed on every second day to prevent position preferences. Solutions were prepared daily, and tap water was also changed each day during the test period of 5 days. Food was available ad libitum throughout the testing time. Weight of rats was determined on the day before and on the day after the test. The bottles were weighed every day before and after consumption and the weight difference was considered to be the rat intake from each bottle. The sum of water and sucrose intake was defined as total intake and the sucrose preference was expressed as the percentage of sucrose intake from the total intake (% sucrose preference = sucrose intake $\times$ 100/ total intake). The test was conducted by an investigator who was unaware of the exposure conditions.

Modified forced swimming test

The procedure developed by Lucki and co-workers (Detke, Rickels and Lucki, 1995; Lucki, 1997; Page et al., 1999; Cryan, Page and Lucki, 2005), that was a modification of the Porsolt test (Porsolt et al., 1978) was employed. Briefly, rats were placed in an opaque plastic cylinder (50 cm $\times$ 20 cm [height and diameter]) containing 60 cm of water (24±1 °C). There were no training sessions and the animals did not receive any drug treatment in the day of the test (Cryan, Page and Lucki, 2005). The behaviors registered during the test session were: immobility (when the rat stopped all active behaviors and remained floating in the water with minimal movements, with its head just above the water), swimming (movements throughout the swim cylinder) and climbing (upward directed movements of the forepaw along the cylinder walls). Each 5-s period of the 300-s of the test, the rater recorded the predominant behavior: (1) immobility, (2) climbing or (3) swimming. The water was changed and the cylinder rinsed with clean water after each rat.

2.3.2. Neurochemical analysis of the offspring

Determination of dopamine, noradrenaline, serotonin and metabolites concentrations

After the animals have been tested in the open field, elevated plus maze and modified forced swimming tests, the male and their female siblings were euthanized by decapitation approximately one week after the last test, in the same day (females were euthanized at the same stage of the estrous cycle). The prefrontal cortex and hippocampus structures were rapidly dissected and stored at −80 °C until the neurochemical quantification. The endogenous concentrations of noradrenaline (NA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) were assayed by reverse-phase high performance liquid chromatography (HPLC) with electrochemical detection (ED). Briefly, the system consisted of a Synergi Fusion-RP C-18 reverse-phase column (150×4.6 mm i.d., 4 µm particle size) fitted with a 4×3.0 mm pre-column (Security Guard Cartridges Fusion-RP); an electrochemical detector (ESA Coulochem III Electrochemical Detector) equipped with a guard cell (ESA 5020) with the electrode set at 350 mV and a dual electrode analytical cell (ESA 5011A); a LC-20AT pump (Shimadzu) equipped with a manual Rheodyne 7725 injector with a 20 µL loop. The column was maintained inside in a temperature-controlled oven (25 °C; Shimadzu). The cell contained two chambers in series: each chamber including a porous graphite coulometric electrode, a double counter electrode and a double reference electrode. Oxidizing potentials were set at 100 mV for the first electrode and at 450 mV for the second electrode. The tissue samples were homogenized with an ultrasonic cell disrupter (Sonics) in 0.1 M perchloric acid containing sodium metabisulfite 0.02% and internal standard. After centrifugation at 10,000×g for 30 min at 4 °C, 20 µL of the supernatant was injected into the chromatograph. The mobile phase, used at a flow rate of 1 mL/min, had the following composition: 20 g citric acid monohydrated (Merck), 200 mg octane-1-sulfonic acid sodium salt (Merck), 40 mg ethylenediaminetetraacetic acid (Sigma), 900 mL HPLC-grade water. The pH of the buffer running solution was adjusted to 4.0 then filtered through a 0.45 µm filter. Methanol (Merck) was added to give a final composition of 10%

methanol (v/v). The neurotransmitters and metabolites concentrations were calculated using standard curves that were generated by determining in triplicate the ratios between three different known amounts of the internal standard. The unit was expressed as ng/g of wet weight.

2.3. Statistical analyses

Parametric data were analyzed by analysis of variance (Two-way ANOVA), and differences between groups were assessed using the Bonferroni test. Nonparametric data were analyzed using the Kruskal-Wallis test followed by Dunn's test. The analyses were performed using Statistica 7.0. All of the statistical analyses were performed by considering the litter as the statistical unit and differences were considered significant at a probability level of 5% ($p < 0.05$).

3. Results

3.1. Behavioral tests of the offspring

All behavioral tests were first compared between males and females in order to determine whether there were differences in behavior between genders. The only test that showed sexually dimorphic behavior was an open field, in which females showed a higher locomotor activity than males. In this test all the parameters were evaluated separately considering males and females as different groups. The other tests were analyzed considering both, the sexes as different groups and as a single group. The analyzes showed different results only for the number of entries into the closed arms and number of total entries in the elevated plus-maze, which showed to be different from the control group when males and females were considered as a same group (Fig. 1; described below). All other results were described as separate groups according to the sex.

The results of behavioral tests are presented in Table 1 and 2. In the open field test there were no differences in locomotor activity, in time spent in

the periphery and in time spent in the central area of females and males exposed to FLX *in utero* and lactation compared with the control group (Table 1).

In the elevated plus-maze test, two-way ANOVA indicated a significant increase in the number of entries in the closed arms [$F(3, 25) = 3$, $p < 0.02$] and in the total arms entries [$F(3, 70) = 3$, $p < 0.01$] (Fig. 1) when males and females exposed to 17 mg/kg FLX were considered a single group. The percentage of time spent in the open arms and the percentage of entries in the open arms were not different from control group in animals exposed to FLX in both genders compared alone and/or grouped (Table 1).

The forced swimming test revealed no differences in the frequencies of the behaviors of climbing, swimming and immobility between treatment groups when animals were subjected to 15 min test period (1-5, 6-10, and 11-15 min; Table 2).

Finally, the examination of the sucrose preference test revealed no differences in the percentage of sucrose intake in any day of the test in adult males and females exposed to FLX *in utero* and lactation compared with the control group (Table 1).

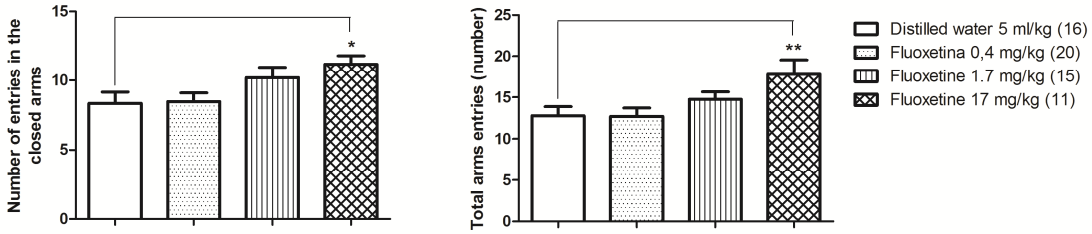

Fig. 1. Elevated plus maze. Number of entries in the closed arms (left) and number of total arms entries (right). The dams were treated with the antidepressant fluoxetine during pregnancy and lactation from GD 7 to PND 21. In this analysis males and females offspring were considered as a same group. The data are expressed as mean ± standard error. *$p < 0.05$ and **$p < 0.01$ when compared with control group (Two-way ANOVA - Bonferroni test).

Table 1. Behavioral response of the offspring in the open field, elevated pluz maze and sucrose preference tests.

Parameter	Maternal fluoxetine dose (mg/kg/day)							
	Female				Male			
	Control	0.4	1.7	17	Control	0.4	1.7	17
No. of animals (litter)	14 (8)	17 (10)	14 (8)	9 (5)	16 (8)	17 (10)	14 (7)	11 (6)
Open field								
Locomotor activity (No.) [a]	86.56 ± 8.39	104.5 ± 7.08	102.9 ± 8.95	115.5 ± 9.07	78.63 ± 6.88	90.60 ± 10.3	72.97 ± 6.32	95.88 ± 10.5
Time in the center (s)	20.38 ± 3.05	20.80 ± 4.04	12.69 ± 2.67	28.40 ± 3.71	14.13 ± 1.91	11.75 ± 2.12	8.97 ± 2.53	14.50 ± 1.67
Time in the periphery (s)	279.6 ± 3.05	279.2 ± 4.04	287.3 ± 2.67	271.6 ± 3.71	285.9 ± 1.91	288.3 ± 2.12	291.0 ± 2.52	285.5 ± 1.67
Elevated pluz maze								
Entries in the open arms (%)	35.76 ± 5.49	31.72 ± 2.43	32.91 ± 3.60	34.94 ± 7.15	31.58 ± 2.74	32.42 ± 3.03	29.37 ± 3.75	32.77 ± 4.61
Time spent in the open arms (%)	22.20 ± 1.81	20.96 ± 2.76	23.46 ± 3.15	26.82 ± 7.15	14.81 ± 3.33	19.01 ± 3.22	17.29 ± 2.98	26.23 ± 5.54
Entries in the closed arms (No.)	9.44 ± 1.34	8.93 ± 0.81	10.50 ± 0.85	11.20 ± 0.56	7.25 ± 0.81	8.00 ± 0.97	9.86 ± 1.32	11.17 ± 1.07
Total arms entries (No.)	14.75 ± 1.44	13.19 ± 1.21	15.69 ± 1.09	18.20 ± 2.42	10.81 ± 1.39	12.20 ± 1.62	13.71 ± 1.43	17.42 ± 2.72
No. of animals (litter)	7 (7)	8 (8)	7 (7)	5 (5)	8 (8)	8 (8)	7 (7)	5 (5)
Sucrose preference								
Sucrose intake mean (%) [b]	95.16 ± 3.56	96.82 ± 1.38	95.49 ± 1.91	84.38 ± 9.11	94.59 ± 1.68	89.44 ± 4.46	92.51 ± 2.91	74.82 ± 18.72

Note: Values are expressed as mean ± standard error. [a] = number of lines crossed. [b] = sucrose intake × 100/ total intake.

Table 2. Behavioral response of the offspring in the forced swim test.

Parameter	Maternal fluoxetine dose (mg/kg/day)							
	Female				Male			
	Control	0.4	1.7	17	Control	0.4	1.7	17
No. of animals (litter)	14 (8)	17 (10)	14 (8)	9 (5)	16 (8)	17 (10)	14 (7)	11 (6)
Forced swim test								
0-5 min								
Climbing	15.50 ± 1.15	15.95 ± 2.11	15.81 ± 1.76	13.20 ± 0.75	17.75 ± 1.83	15.25 ± 2.15	15.43 ± 1.25	12 .00 ± 1.48
Swimming	9.56 ± 0.68	10.00 ± 1.44	10.56 ± 2.06	6.60 ± 2.23	9.69 ± 1.70	11.30 ± 1.39	7.64 ± 0.67	9.08 ± 2.15
Immobilitty	34.94 ± 1.61	34.05 ± 3.12	33.63 ± 2.51	40.20 ± 2.36	32.56 ± 2.72	33.55 ± 2.79	36.93 ± 1.73	38.83 ± 3.07
6-10 min								
Climbing	1.19 ± 0.74	0.45 ± 0.26	1.63 ± 0.81	1.20 ± 0.97	0.56 ± 0.56	0.85 ± 0.49	2.00 ± 1.38	0.92 ± 0. 42
Swimming	11.44 ± 2.22	10.70 ± 2.22	11.94 ± 2.40	7.00 ± 3.93	10.69 ± 2.33	8.45 ± 1.66	7.14 ± 1.35	8.92 ± 2.80
Immobilitty	47.38 ± 2.52	48.85 ± 2.27	46.50 ± 2.36	51.80 ± 3.60	48.56 ± 2.10	50.70 ± 1.77	50.86 ± 2.27	50.17 ± 2.70
11-15 min								
Climbing	0.31 ± 0.25	0.20 ± 0.20	1.13 ± 0.72	0.50 ± 0.50	0.44 ± 0.22	0.55 ± 0.34	0.36 ± 0.21	0.58 ± 0. 42
Swimming	8.56 ± 1.85	9.65 ± 2.01	10.88 ± 1.32	5.90 ± 2.85	10.63 ± 2.11	6.10 ± 1.50	6.21 ± 1.36	7.00 ± 1.76
Immobilitty	51.13 ± 1.78	50.15 ± 2.00	58.00 ± 1.48	53.60 ± 2.94	49.00 ± 2.16	53.35 ± 1.58	53.43 ± 1.35	52.42 ± 1.54

Note: Values are expressed as mean ± standard error of predominant behavior in each 5-s period.

3.2. Neurochemical analysis of the offspring

The prefrontal cortex and hippocampus concentrations of NA, DA, 5-HT and metabolites DOPAC and 5-HIAA are presented in Table 3, 4, 5 and 6. NA, DOPAC and 5-HIAA were significantly reduced in prefrontal cortex of females exposed to 0.4 mg/kg FLX compared to the control group (Table 3). Likewise, males exposed to 1.7 and 17 mg/kg FLX presented a significant reduction of the NA, DOPAC and 5-HIAA in prefrontal cortex (Table 5).

The analyses of the neurotransmitters and metabolites within the hippocampus in male offspring showed that NA was significantly decreased in rats exposed to 0.4 mg/kg FLX, and that 5-HIAA was significantly decreased in rats exposed to 1.7 mg/kg FLX when these groups were compared to the control group (Table 6). However, the amount of hippocampal neurotransmitters and metabolites were not different in females exposed to any dose of FLX when compared to the control group (Table 4).

Table 3. Neurotransmitters and metabolites amount in prefrontal cortex of adult female rats exposed to fluoxetine *in utero* and lactation.

FEMALE	MATERNAL FLUOXETINE DOSE				(df) H (p)
	Control	0.4	1.7	17	
No. animals (litter)	8 (8)	10 (10)	8 (8)	6 (6)	
Prefrontal cortex					
NA	1103.9 (473.1-1266.2)	337.3 ***** (257.3-446.6)	584.7 (436.0-632.1)	503.1 (411.2-564.4)	(3.31)1.6 (0.0089)
DA	26.2 (22.3-30.3)	10.8 (6.9-18.6)	30.8 (27.2-49.6)	33.2 (27.7-41.5)	(3.32)14.4 (0.0024)
DOPAC	23.9 (20.0-27.7)	8.5 ***** (5.1-11.6)	15.2 (12.9-17.5)	22.6 (15.2-27.9)	(3.32)16.8 (0.0008)
5-HT	246.6 (207.6-269.8)	140.4 (95.5-214.2)	344.0 (231.0-478.1)	234.2 (225.0-290.9)	(3.32)11.4 (0.0097)
5-HIAA	303.0 (255.0-412.7)	132.3 ***** (101.6-212.4)	233.8 (202.2-258.3)	272.7 (199.5-304.9)	(3.32)13.1 (0.0045)

Note: Values are ng/g of wet tissue expressed as median (lower quartile - upper quartile). Kruskal-Wallis ANOVA followed by Dunn's test. *= $p < 0.05$, reduced values when compared with control group.

Table 4. Neurotransmitters and metabolites amount in hippocampus of adult female rats exposed to fluoxetine *in utero* and lactation.

FEMALE	MATERNAL FLUOXETINE DOSE				(df) H (p)
	Control	0.4	1.7	17	
No. animals (litter)	8 (8)	9 (9)	8 (8)	6 (6)	
Hippocampus					
NA	671.1 (622.7-735.0)	720.6 (657.1-869.2)	673.1 (621.0-710.6)	623.3 (503.5-647.5)	(3.31) 4.4 (0.2164)
DA	22.2 (16.5-46.9)	32.8 (18.6-35.7)	68.1 (44.8-95.2)	31.2 (29.0-50.0)	(3.31) 6.2 (0.1042)
DOPAC	16.9 (13.1-21.8)	28.4 (20.2-39.2)	25.5 (16.5-36.7)	15.6 (9.4-34.8)	(3.31) 2.7 (0.4421)
5-HT	216.0 (192.1-377.6)	355.7 (341.9-473.2)	406.9 (316.3-502.5)	260.7 (183.1-283.1)	(3.31) 9.0 (0.0289)
5-HIAA	407.1 (372.7-486.3)	408.9 (372.9-447.2)	392.7 (365.3-459.5)	402.9 (308.1-470.8)	(3.31) .64 (0.8873)

Note: Values are ng/g of wet tissue expressed as median (lower quartile - upper quartile). Kruskal-Wallis ANOVA followed by Dunn's test.

Table 5. Neurotransmitters and metabolites amount in prefrontal cortex of adult male rats exposed to fluoxetine *in utero* and lactation.

MALE	**MATERNAL FLUOXETINE DOSE**				(df) H (p)
	Control	0.4	1.7	17	
No. animals (litter)	8 (8)	10 (10)	7 (7)	6 (6)	
Prefrontal cortex					
NA	1131.9 (761.2-1204.8)	693.5 (629.1-797.0)	621.5 ***** (532.8-668.5)	497.6 ***** (460.8-639.5)	(3.30)14.8 (0.0020)
DA	29.5 (23.3-36.0)	26.7 (21.3-32.7)	36.9 (30.4-40.2)	35.2 (21.4-45.7)	(3.31) 2.6 (0.4569)
DOPAC	36.8 (32.5-49.6)	29.2 (23.1-31.7)	23.4 ***** (16.7-27.5)	17.0 ***** (14.4-24.4)	(3.31) 16.0 (0.0012)
5-HT	301.9 (242.7-382.0)	372.5 (333.1-404.1)	409.9 (389.2-496.2)	260.8 (214.7-377.5)	(3.31) 6.9 (0.0736)
5-HIAA	522.7 (475.5-613.2)	328.3 (268.8-404.2)	281.7 ***** (237.4-374.7)	200.4 ***** (180.3-304.1)	(3.31) 14.4 (0.0024)

Note: Values are ng/g of wet tissue expressed as median (lower quartile - upper quartile). Kruskal-Wallis ANOVA followed by Dunn's test. *= $p < 0.05$, reduced values when compared with control group.

Table 6. Neurotransmitters and metabolites amount in hippocampus of adult male rats exposed to fluoxetine *in utero* and lactation.

MALE	**MATERNAL FLUOXETINE DOSE**				(df) H (p)
	Control	0.4	1.7	17	
No. animals (litter)	7 (7)	9 (9)	7 (7)	6 (6)	
Hippocampus					
NA	919.2 (788.6-942.0)	639.9 ***** (558.3-680.6)	633.2 (600.2-713.4)	689.5 (686.7-697.8)	(3. 27) 12.1 (.0071)
DA	17.8 (16.3-35.5)	19.0 (14.8-59.1)	26.2 (14.3-54.5)	25.6 (15.6-36.9)	(3.29) .12 (.9890)
DOPAC	25.7 (10.9-33.7)	16.4 (6.5-22.5)	10.2 (6.3-14.3)	8.3 (4.2-12.7)	(3. 29) 5.8 (.1225)
5-HT	308.9 (250.2-440.6)	371.3 (291.8-416.4)	405.6 (371.3-500.7)	415.7 (398.2-442.3)	(3. 29) 3.0 (.3863)
5-HIAA	464.0 (446.8-681.4)	376.7 (340.9-416.6)	362.8 ***** (290.6-434.5)	361.9 (322.2-454.9)	(3. 29) 10.0 (.0185)

Note: Values are ng/g of wet tissue expressed as median (lower quartile - upper quartile). Kruskal-Wallis ANOVA followed by Dunn's test. *= $p < 0.05$, reduced values when compared with control group.

4. Discussion and conclusion

FLX inhibit the function of the serotonin transporter (5-HTT), leading to an accumulation of 5-HT in the extracellular space and thus increasing the magnitude and duration of the activity of 5-HT on pre- and postsynaptic 5-HT receptors (Homberg, Schubert and Gaspar, 2010). Despite the effect of SSRIs on the 5-HT system is comparable in fetuses and adults (Olivier et al., 2011), the possible neurodevelopmental changes mediated by serotonergic stimulation in early life SSRI exposure are unknown. Therefore this study aimed to evaluate the possible long-term behavioral and neurochemical effects in animals exposed to FLX in periods of neurodevelopment.

In the present study, exposure of rats to the antidepressant FLX *in utero* and lactation did not alter the behavioral patterns when subjected to open field test. This result is consistent with those observed by Hansen et al. (1997) who demonstrated no differences in locomotor activity in mice treated with Lu 10-134-C (a SSRI) in rats between PND 8-21 in the open field test. Besides that, Karpova et al. (2009) also did not observe differences in total distance travelled and in percentage of time in central area in the open field in mice treated with FLX 10 mg/kg i.p. between PND 4-21. In addition, Coleman et al. (1999) showed that prenatal paroxetine exposure (two weeks before conception and throughout gestation) did not affect locomotor and exploratory activities in adult mice (activity test and radial 8-arm maze). Vorhees et al. (1994) also showed that prenatal (GD 7-21) FLX by gavage exposure (1, 5 and 12 mg/kg) did not affect locomotor activity (activity test), and learning and memory (acoustic startle, spontaneous alternation, passive avoidance and complex learning in a water maze) in adult rats. Similarly, Vartazarmian et al. (2005) did not observed differences in acoustic startle responses of adult guinea pig exposed to FLX by osmotic pump during pregnancy.

In the present results, *in utero* and lactation FLX exposure did not cause depression-like behaviors in adult rats when these animals were tested in the sucrose preference and forced swimming tests. Likewise, Coleman et al. (1999) did not observe differences in the parameters of the forced swimming test in adult

mice exposed to paroxetine in the prenatal period. However, there have been reports showing that perinatal administration of SSRIs caused anxiety- and depression-like behaviors in rodents, which persisted into adulthood (Homberg, Schubert and Gaspar, 2010; Olivier et al., 2011). Hansen et al. (1997) showed that exposure to SSRI Lu 10-134-C in rats from PND 8 to PND 21 resulted in increased immobility and reduced swimming time in the forced swim test during adulthood. Later studies also demonstrated that early life SSRIs exposure resulted in symptoms of behavioral despair in the forced swim test (increased immobility time) (Lisboa et al., 2007; Popa et al., 2008), in the tail suspension test (increased immobility time) (Popa et al., 2008) and in the sucrose preference test (anhedonia: loss of appetitive motivation) (Popa et al., 2008). However, in these studies the animals were exposed to SSRIs only during lactation, unlike the present study in which animals were exposed during pregnancy and lactation. Therefore, it is possible that different neuroadaptation may have occurred in animals of our study due to longer exposure to FLX. In contrast, Karpova et al. (2009) showed a reduction in total immobility time in forced swim test in adult mice treated with FLX 10 mg/kg i.p. between PND 4-21, indicating that FLX “decreased” behavioural despair in adulthood.

Anxiety-like behaviors were demonstrated by Ansorge et al. (2004) who reported that neonatal (PND 4-21) mice treated with FLX displayed increased anxiety-related phenotypes in the novelty suppressed feeding paradigm (prolonged the latency to begin feeding), in adulthood. This test reflects anxiety- and depression-related behaviors because chronic antidepressant and anxiolytics administration reduce the latency to begin feeding and because animal models of depression and anxiety present an increase in the latency to begin feeding (Ansorge et al., 2004). These authors also showed a significant impairment in shock avoidance, a paradigm that assesses behavioral responses to stress. However, although the authors have not observed changes in locomotor activity during the intershock intervals, they showed a statistical reduction in locomotor activity in the open field (total distance traveled, total ambulatory time and rearing) and in the elevated plus maze (decrease in the total number of arm entries) tests.

In the present study, no significant changes in parameters that reflect anxiety-like behavior in the elevated plus maze were observed in adult rats exposed to FLX *in utero* and lactation. These results are in agreement with Karpova et al. (2009) who did not found statistical differences in parameters evaluated in the light-dark box test (percentages of distance travelled, time spent and number of entries into the light compartment) in adult mice treated with FLX between PND 4-21. Likewise, Popa et al. (2008) showed no anxiety-like behaviors in the light-dark box test and in the elevated plus maze in adult mice treated with excitalopram from PND 5 to PND 19. In addition, Coleman et al. (1999) did not observe differences in parameters of the elevated plus maze test in adult mice exposed to paroxetine in the prenatal period.

A reduction in ambulation of male rats treated with FLX 7.5 mg/kg during pregnancy and lactation was reported by Lisboa et al. (2007). In contrast, despite the open field test did not show differences in locomotor activity between the groups in the present results, the number of entries in the closed arms and the total arms entries in the elevated plus-maze was increased in offspring exposed to 17 mg/kg FLX compared with the control group. This result indicates that exposure to FLX *in utero* and lactation caused an increase in locomotion of these animals in adulthood (Cruz, Frey and Graeff, 1994; Rodgers and Johnson, 1995; Anseloni and Brandão, 1997). This result is in agreement with Maciag et al. (2006), who also observed an increased locomotor activity in adult male rats, treated from PND 8 to PND 21 subcutaneously with excitalopram 5 mg/kg (another SSRI).

Some studies using knockout mice for the 5-HTT have shown to result in anxiety- and depression-like behaviors, which were similar to some found in wild type rodents (Ansorge et al., 2004; Popa et al., 2008; Kalueff et al., 2010). Indeed, Hansen et al. (1997) reported that developmental SSRI exposure causes a reduction in 5-HTT expression in adults animals that imitates, in part, the genetic inactivation of 5-HTT. However, despite the use of knockout mice to be an interesting tool to compare the possible effects of early exposure to SSRI drugs, there are evidences that other pathways may be altered by exposure to these antidepressants in addition to possible permanent reduction in 5-HTT, such as

changes in hippocampus brain-derived neurotrophic factor (Karpova et al., 2009) or pathways related to other targets of FLX such as neurotrophins and calcium or sodium channels (Mostert et al., 2008). Besides that, it is known that the monoaminergic system is widely spread throughout the brain and underlies several psychological functions such as cognition and learning processes, arousal, mood and reward (Femenía et al., 2012). Therefore, unlike a wild type animal exposed to SSRIs in the neonatal period, knockout mice develop to adulthood with the absence of 5-HTT, and then behavioral changes in these animals may represent a loss in a variety of signaling pathways of many serotonergic innervations areas.

So far, investigations on the possible long-term behavioral effects from SSRIs exposure during critical periods of development, together with the results of this study, show how delicate is to predict possible changes through experimental models. It is observed that there is not a linearity of these results, suggesting that the possible long-term behavioral effects caused by FLX can be modulated by a number of factors, such as the specie, the range of exposure and especially the dose of these drugs.

Well-documented anxiolytic and antidepressant properties of drugs that act primarily on monoaminergic systems have implicated 5-HT, NA, and DA in the pathogenesis of mood and anxiety disorders (Martin el at., 2009). Prefrontal cortex and hippocampus are structures that receive significant serotonergic innervations and therefore may be affected by serotonergic hyperstimulation during development. However, few authors have investigated possible monoaminergic changes in brain structures caused by early exposure to SSRIs. In the present study, neurochemical analysis showed a significantly reduction in NA, DOPAC and 5-HIAA in prefrontal cortex of females (0.4 mg/kg FLX) and males (1.7 and 17 mg/kg FLX) offspring exposed to FLX *in utero* and lactation. In addition, NA (0.4 mg/kg FLX) and 5-HIAA (1.7 mg/kg FLX) were significantly decreased within the hippocampus of male offspring exposed to FLX in the same periods. Similarly, *ex vivo* studies showed that chronic fluoxetine postweaning (10 mg/kg i.p.) decreased hippocampal 5-HIAA and 5-HIAA/5-HT ratio in grouped animals (Bianchi et al., 2009). Besides that, Hilakivi et al. (1995) showed a significant increase of 5-HT

metabolism in the rat brainstem and cortex in adult rats neonatally treated (PND 7-21) with a SSRI (Zimelidine). These results suggest a permanent decreased reuptake of 5-HT and therefore reduced metabolism of 5-HT to 5-HIAA by intraneuronal monoamine oxidase A in the hippocampus and prefrontal cortex. Bianchi et al. (2009) did not observe decrease in hippocampal NA or DA metabolism in rats treated with FLX, however, in this study rats were exposed to FLX after the critical periods of brain development, because the author's purpose was to analyze the neurochemical and behavior changes in isolation or grouped animals. Another study conducted by Cabrera-Vera et al. (1997) showed a significant decrease only in midbrain 5-HT content in adult male rats exposed to FLX *in utero* between GD 13-20. However, the authors did not observe differences in 5-HT, 5-HIAA, NA and DA contents or in density of 5-HT uptake sites in the prefrontal cortex, hippocampus, hypothalamus and striatum of these animals. It is possible that this discrepancy between the results is mainly due to the period of exposure to FLX, which was much smaller in the experiment conducted by Cabrera-Vera et al. (1997) than in the present study. The possible mechanism responsible for the reduction in NA content and in the DA metabolism observed in prefrontal cortex of females and males offspring and for reduction in NA content observed only in hippocampus of male offspring is unknown. Likewise, the possible behavioral consequences that these neurochemical changes could produce are also of concern.

Overall, the present results showed a decrease changes in levels of NA, 5-HIAA and DOPAC in the prefrontal cortex and hippocampus of adult rats exposed to FLX *in utero* and during lactation. FLX also caused increase in locomotor activity in the offspring, but did not cause anxiety- and depression-like behaviors in male and female adult rats. Neurochemical changes modulated by early-life exposure to FLX was sexually dimorphic and varied with the doses. These results indicate mainly that FLX can cause long-term neurochemical and behavioral effects when administered at critical periods of neurodevelopmental. The potential functional consequences and clinical implications of these alterations in brain structures remain to be elucidated.

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9. DISCUSSÃO GERAL

Os três estudos que constituem esta tese formam uma sequência de experimentos que se complementam, iniciando com a investigação de uma possível atividade (anti)estrogênica e/ou (anti)androgênica do antidepressivo fluoxetina seguido de investigações acerca dos possíveis efeitos nos parâmetros gestacionais e lactacionais além dos possíveis efeitos reprodutivos, comportamentais e neuroquímicos a longo prazo que a fluoxetina poderia produzir em ratos Wistar.

No primeiro estudo, ambos os resultados, do teste uterotrófico e do ensaio do gene repórter, indicaram uma possível atividade estrogênica da fluoxetina. O efeito *in vivo* mostrado pelo aumento relativo e absoluto na massa uterina foi observado em ratas tratadas com fluoxetina nas doses de 1,7 e 17 mg/kg/dia. Apesar do ensaio do gene repórter ser consistente com o achado *in vivo*, e ter mostrado um efeito estrogênico mediado pela ação da fluoxetina em receptores estrogênicos, não podemos refutar a idéia de que outros mecanismos também possam estar envolvidos no efeito estrogênico observado *in vivo*. A hiperestimulação serotonérgica produzida pela fluoxetina poderia modular sinapses hipotalâmicas que por sua vez podem desencadear efeitos sobre o controle hormonal, ou seja, produzindo uma estimulação do eixo HPG. Assim, dosagens de gonadotrofinas e de hormônios gonadais seriam esclarecedores para determinar se a síntese destes hormônios poderia ser aumentada pela fluoxetina. Além disso, são necessários outros testes para avaliar se a norfluoxetina também seria passível de atuar como substância estrogênica, já que a mesma possui também atividade antidepressiva.

Os resultados do segundo estudo mostraram que a fluoxetina na dose de 17 mg/kg provocou toxicidade fetal e materna, além de reduzir os índices de viabilidade e de desmame, que expressam claramente um maior percentual de mortalidade pós-natal. Estudos com animais, assim como em humanos já haviam mostrado resultados semelhantes de baixo peso ao nascimento e aumento das perdas pós-natal (Vorhees et al., 1994; Chambers et al., 1996), e acredita-se que

a redução do crescimento intrauterino causada pela fluoxetina possa estar relacionada com o efeito vasoconstritor desta droga sobre as artérias da placenta (Bjoro e Stray-Pedersen, 1986; Haugen, 1996). Além disso, efeitos como redução na massa uterina e aumento dos metabólitos hormonais fecais de glicocorticóides, progestogênios e estrogênios foram encontrados em progenitoras que receberam 17 mg/kg de fluoxetina durante a gestação e a lactação. Estas mesmas progenitoras apresentaram um aumento no peso das glândulas adrenais, sugerindo que o aumento na síntese hormonal poderia estar relacionado a uma hiperatividade do eixo HPA. De fato, alguns estudos clínicos mostraram que a fluoxetina e outras drogas ISRSs são capazes de aumentar a síntese de glicocorticóides em adultos (Meltezer et al., 1997; Bschor et al., 2011). Além disso, Oberlander et al. (2008) relataram padrões alterados no eixo HPA em resposta ao estresse em bebês cujas mães usaram ISRSs. No entanto, níveis aumentados de metabólitos de estrogênios fecais foram também observados nas progenitoras expostas a 0,4 mg/kg de fluoxetina, as quais não exibiram hipertrofia das supra-renais. É possível que a hiperestimulação das adrenais, isto é, a síntese aumentada de hormônios, não necessariamente seja acompanhada de um aumento significativo no peso destas glândulas. Assim, os níveis aumentados de metabólitos de estrogênios fecais neste grupo de tratamento também podem ter ocorrido por uma hiperestimulação do eixo HPA. Entretanto, outras vias podem também estar envolvidas na síntese aumentada de estrogênios, tais como a estimulação da síntese de hormônios liberadores hipotalâmicos. Como discutido anteriormente, esta via poderia ser a responsável pela atividade estrogênica da fluoxetina observada *in vivo*.

Nenhum efeito reprodutivo a longo-prazo foi observado nos descendentes fêmeas e/ou machos expostos à fluoxetina *in utero* e lactação. Porém, como a exposição fetal e/ou perinatal de machos a agentes químicos com atividade estrogênica pode, teoricamente, alterar o processo de masculinização, maior atenção deve ser dada aos parâmetros de desenvolvimento sexual da progênie masculina, pois apesar de não termos encontrado resultados significativos, houve

uma tendência de redução na contagem espermática diária e na eficiência testicular em filhotes adultos expostos à 17 mg/kg de fluoxetina.

Reunindo os resultados dos dois primeiros estudos, não foi possível determinar com segurança qual ou quais mecanismos exercidos pela fluoxetina foram responsáveis por desencadear as alterações hormonais e nos pesos das supra-renais e do útero nas progenitoras, porém os resultados apontam para uma possível atividade estrogênica, e uma hiperestimulação do eixo HPA. Se estes efeitos ocorrem por vias diferentes ou se ambos efeitos seriam desencadeados primariamente por uma hiperestimulação serotonérgica hipotalâmica, são questões que ainda precisam ser esclarecidas. É importante ressaltar que a maioria dos efeitos adversos reprodutivos foram observados pela exposição à fluoxetina na dose de 17 mg/kg, e esta dose representa a dose terapêutica para ratos multiplicada por dez, e serviu como fator de segurança, atribuível as diferenças intra-espécies. Outra questão a ser considerada é se o efeito estrogênico observado seria um efeito específico da fluoxetina, ou se outras drogas da mesma classe também apresentariam tal efeito.

Finalmente, os resultados do terceiro estudo mostraram que a exposição à fluoxetina *in utero* e lactação a ratos Wistar produziu efeitos neuroquímicos e aumento na locomoção sem causar alterações em comportamentos que expressam ansiedade e depressão na vida adulta. Podemos inferir que tais efeitos foram a longo prazo, desde que os animais foram expostos apenas no período de gestação e lactação e foram avaliados após o dia 100 pós-natal. Estes efeitos foram sexualmente dimórficos e variaram de acordo com a dose de fluoxetina administrada. Os níveis reduzidos de NA, do 5-HIAA e do ácido 3,4-dihidroxifenilacético (DOPAC) no córtex pré-frontal e hipocampo observados neste estudo indicam que houve uma redução do metabolismo da 5-HT e da DA, além da redução da síntese de NA nestas regiões. As consequências funcionais e implicações clínicas destas alterações nas estruturas cerebrais permanecem a ser elucidadas.

É importante deixar claro que o grande interesse em pesquisar os possíveis efeitos adversos do uso da fluoxetina em períodos de gestação e lactação não

teve como objetivo fazer críticas ao uso de antidepressivos por mulheres gestantes e lactantes que necessitam de tratamento farmacológico, mas sim buscar o máximo de informação científica com o intuito de auxiliar a conduta clínica. Os possíveis efeitos adversos, as doses que causaram estes efeitos e/ou que demonstraram ser tóxicas, relacionados com o período de administração desta droga são informações valiosas para a compreensão dos possíveis riscos do uso da fluoxetina, assim como para a tomada de decisões pelos clínicos.

10. CONCLUSÕES

Concluímos que a fluoxetina demonstrou atividade estrogênica, causou toxicidade fetal e materna, além de induzir alterações hormonais em progenitoras tratadas com esta droga durante a gestação e lactação. A maioria destas alterações reprodutivas foi observada apenas com a maior dose de fluoxetina investigada (17 mg/kg). A exposição à fluoxetina *in utero* e lactação produziu alterações a longo-prazo nos níveis de neurotransmissores e estas alterações variaram de acordo com o sexo e as doses de fluoxetina. Além disso, a exposição *in utero* e lactação à fluoxetina na dose de 17 mg/kg produziu aumento no comportamento de locomoção dos animais na vida adulta.

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ANEXO: Certificado de análise de controle de qualidade do cloridrato de fluoxetina utilizado no desenvolvimento dos protocolos experimentais.

Farma Nostra

NOSTRA

Superação. Nossa principal matéria-prima.

CERTIFICADO DE ANÁLISE

INSUMO:	CLORIDRATO DE FLUOXETINA**	**DATA DE ANÁLISE:**	23/01/2009
ORIGEM/PROCEDÊNCIA:	CHINA	**LOTE FABRICANTE:**	IF-FL-080409
LOTE PHARMA NOSTRA:	08102996B	**DATA DE VALIDADE:**	Abril/2011
DATA DE FABRICAÇÃO:	Abril/2008		
CONDIÇÕES DE ARMAZENAGEM:	TEMPERATURA ENTRE 15°C E 30°C		
FM: $C_{17}H_{18}F_3NO.HCL$	CAS: 59333-67-4		
PM: 345,79	DCB: 04177		
DATA DE EMISSÃO:	28/01/2009 **NF:** 3-008.867	**ORDEM FRACIONAMENTO:**	168-09

TESTES	ESPECIFICAÇÕES	RESULTADOS	REFERÊNCIAS
DESCRIÇÃO*	Pó cristalino branco a quase branco.	Pó cristalino quase branco.	USP - 31
SOLUBILIDADE*	Facilmente solúvel em metanol e em álcool; Ligeiramente solúvel em água e cloreto de metileno; Praticamente insolúvel em éter.	Conforme.	USP - 31
IDENTIFICAÇÃO*	IV - O espectro da amostra está de acordo com o padrão	Positivo.	USP - 31
	Teste positivo para cloreto.	Positivo	USP - 31
METAIS PESADOS*	≤ 0.003%	< 0.003%	USP - 31
UMIDADE (KF)	≤ 0.5%	0.20%	Met.Geral FB IV
DENSIDADE APARENTE*	Informativo (sem compactação)	0.49 g/ml	
SUBSTÂNCIAS RELACIONADAS*	2-metilamino EtilBenzenometanol: ≤ 0.25%	Não detectado	
	Fluoxetina Comp. Relacionado B: ≤ 0.25%	Não detectado	
	Fluoxetina Comp. Relacionado A: ≤ 0.15%	Não detectado	USP - 31
	Outras Impureza ≤ 0.1%	Não detectado	
	Impurezas Total ≤ 0.5%	Não detectado	
IMPUREZA ORGÂNICA VOLÁTIL*	Clorofórmio ≤ 60 ppm	Não detectado	USP - 31
	1,4-Dioxano: ≤ 380 ppm	Não detectado	
	Cloreto de metileno ≤ 600 ppm	Não detectado	
	Tricloroetileno: ≤ 80 ppm	Não detectado	
TEOR (sob base anidra) *	98 % - 102%	101.35%	USP - 31

*Resultados obtidos em análises realizadas no Laboratório de Controle de Qualidade Pharma Nostra (UNIDADE ANÁPOLIS) . Os demais foram transcritos conforme certificado de análise do fabricante.

** Portaria 344-Lista C1 /***Feq = 1,12 (Manual de Equivalência da Anfarmag)

LEGENDA DAS REFERÊNCIAS: FB (Farmacopeia Brasileira) / USP (United States Pharmacopoeia) / EP (European Pharmacopoeia) / BP (British Pharmacopoeia) / JP (Japanese Pharmacopoeia) / MG (Método Geral farmacopeico) / Fabricante (especificação e metodologia conforme o fabricante do insumo) / Informativo (resultado fornecido como informativo pelo LCQ Pharma Nostra) .

CONCLUSÃO: (X) Aprovado ( ) Reprovado

Responsável pelo Lab. Controle de Qualidade
Danielle Rocha Barbosa - CRF-GO Nº 9222

Responsável Técnico
Amin Gabriel Gebrim - CRF-GO Nº 1829

SAIBA MAIS SOBRE O PADRÃO DE QUALIDADE PHARMA NOSTRA.

QUALIDADE