



UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”
Campus de Botucatu



UNIVERSIDADE ESTADUAL PAULISTA

“Júlio de Mesquita Filho”

INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

Exposição ao antidepressivo sertralina associado ou não ao estresse/ou
probiótico em diferentes períodos do desenvolvimento: Uma
abordagem sobre os impactos reprodutivos e neurocomportamentais
em roedores

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Tese apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Doutora no Programa de Pós-Graduação em Biologia Geral e Aplicada, área de concentração Biologia Celular, Estrutural e Funcional.

Orientadora: Profa. Dra. Wilma De Grava Kempinas

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Dedicatória

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“Nenhuma árvore alcança os céus sem a força de suas raízes”

Desconhecido

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“Cada pessoa que passa em nossa vida, passa sozinha, é porque cada pessoa é única e nenhuma substitui a outra! Cada pessoa que passa em nossa vida passa sozinha e não nos deixa só porque deixa um pouco de si e leva um pouquinho de nós. Essa é a mais bela responsabilidade da vida e a prova de que as pessoas não se encontram por acaso”.

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Epígrafe

“No caminho aprendemos, no caminho florescemos, no caminho nos tornamos. Nos tornamos quando mudamos de acordo com as evoluções que a vida nos permite experimentar. Nos tornamos quando aprendemos a ser mais gentis, mais flexíveis e corajosos para surfar as ondas gigantes da vida. É sobre aprender a fazer o melhor que puder, com o que tiver, aonde estiver. É sobre ser gentil com as nossas limitações. É sobre entender que haverá luz e haverá escuridão, e aprender a se equilibrar entre estes extremos é tudo o que podemos fazer. Tornar-se é sobre ser um pouco mais de nós a cada dia. Ouvindo, respeitando e seguindo a nossa intuição, os nossos sentimentos, os nossos instintos. Tornar-se é sobre a marca que deixamos em cada coração que tocamos e cada pessoa que encontramos.”

Wandy Luz

Resumo

A depressão, o estresse e a ansiedade são comumente observados na população, e como consequência o consumo de antidepressivos tem aumentado exponencialmente nos últimos anos. A sertralina (SE), classificada como inibidor seletivo de recaptção de serotonina (ISRS), é um antidepressivo/ansiolítico utilizado por pessoas com distintos transtornos psiquiátricos e diferentes faixas etárias. Os ISRS podem provocar graves efeitos colaterais, no entanto existem poucos relatos sobre os impactos da SE em aspectos reprodutivos e comportamentais, tampouco há tratamentos adjuvantes que amenizem seus efeitos indesejáveis. Sabendo-se dos benefícios dos probióticos no eixo microbiota-intestino-cérebro e sua ação benéfica na saúde reprodutiva, o presente estudo investigou os impactos da exposição à SE associada ou não ao estresse/ ou probiótico, em diferentes períodos do desenvolvimento de roedores. Testes estatísticos utilizados: ANOVA (seguido pelo teste de Tukey) ou Kruskal-Wallis (seguido pelo teste de Dunn) ($p \leq 0.05$). Para o experimento I e II, ratas Wistar prenhes foram distribuídas nos grupos ($n=20-18$ ratas/grupo): CO - receberam água filtrada; SE – receberam 20 mg/kg de SE; ST - animais submetidos ao estresse por contenção e receberam água filtrada; ST/SE - animais submetidos ao estresse e receberam 20 mg/kg de SE. O tratamento foi realizado por via gavagem entre os dias gestacionais (DG) 13 ao 20. Parte das fêmeas prenhes foram eutanasiadas no DG 20 para análise dos fetos, e o restante foi mantida até o final da lactação. No experimento I, a maturação somática, o desenvolvimento de reflexos, e o neurocomportamento foram investigados na prole masculina. No experimento II, foram analisados no DG 20, e nos dias pós-natais (DPN) 21, 45 e 110, a histologia dos testículos e epidídimos, bem como a imunohistoquímica testicular do antígeno nuclear de proliferação celular (PCNA) e proteína do tumor de Wilms (Wt1). A morfologia, motilidade e vitalidade espermáticas foram observadas no DPN 110, assim como a produção diária de espermatozoides e seu tempo de trânsito epididimário. Os resultados do experimento I mostraram que a exposição gestacional à SE provocou redução do peso corporal e sangramento vaginal nas mães. Já a prole masculina do grupo SE apresentou atrasos na erupção dos incisivos, no aparecimento de pelos e na realização de geotaxia negativa. Além disso, o grupo SE foi menos exploratório (personalidade ansiosa) em comparação com os grupos CO e ST. No experimento II, observou-se que os testículos fetais apresentaram grande número de células acidófilas, que representam células em processo de morte celular, nos grupos expostos à SE. O grupo ST/SE também apresentou diminuição do volume nuclear das células de Leydig. Este mesmo grupo mostrou baixa expressão de PCNA e WT1, diminuição do peso dos testículos e epidídimo, menor distância anogenital (DAG) e atraso na instalação da puberdade. Os animais adultos, expostos *in útero* à SE apresentaram alterações na morfologia e motilidade espermática. O experimento III foi conduzido de forma a investigar as repercussões reprodutivas e comportamentais da exposição lactacional à SE. Para tanto, 30 ratas Wistar lactantes foram distribuídas em 3 grupos experimentais ($n=10$ /grupo): grupo CO recebeu água filtrada, grupos S10 e S20 receberam, respectivamente, 10 e 20 mg/kg/dia de SE. O tratamento foi realizado por via gavagem do 1º ao 20º dia de lactação. Nesse período, observou-se o desenvolvimento de reflexos e somáticos da prole masculina, bem como o comportamento materno. No dia 21, as mães foram eutanasiadas, e nos dias 21, 45 e 100, um macho de cada ninhada foi eutanasiado. Além das metodologias realizadas no experimento I e II, também foram observados o comportamento sexual e fertilidade natural. Os resultados mostraram que a prole masculina exposta à SE apresentou menor peso corporal, menor DAG e alterações no desenvolvimento dos reflexos, além de alterações histológicas nos testículos. Na idade adulta, a maioria dos parâmetros analisados foram normalizados, porém, a qualidade espermática mostrou-se alterada, sem comprometimento da fertilidade natural. O experimento IV investigou os efeitos da SE nos parâmetros reprodutivos de camundongos adultos, bem como verificou se o probiótico *Lactobacillus rhamnosus* (Lr) reduz os efeitos colaterais desse



antidepressivo. Assim, duas etapas consecutivas foram desenvolvidas. Na 1ª etapa, quatro grupos experimentais (n=6/grupo) foram formados: Grupo CO recebeu água filtrada (veículo); e grupos S10, S20 e S40 receberam respectivamente 10, 20 e 40 mg/Kg de SE diluída em veículo. O tratamento ocorreu via gavagem por 30 dias. Ao final do tratamento, foram analisados a qualidade espermática, o peso de órgãos e a histologia dos testículos e epidídimos. Na 2ª etapa, 32 camundongos foram distribuídos em 4 grupos experimentais (n=8/grupo): Grupo CO recebeu água filtrada (veículo); grupo S – recebeu 20 mg/Kg de SE; grupo P- recebeu o probiótico Lr (1×10^9 UFC), diluído em veículo; e grupo SP que recebeu tanto LR quanto SE. Ao final do tratamento, por via gavagem, foram analisadas a histologia dos testículos e epidídimos, qualidade espermática, verificação da fertilidade natural. De forma complementar, ensaio *in vitro* foram realizados com o intuito de identificar os efeitos da SE sobre a reação acrossômica durante a capacitação e fertilização *in vitro*. Os resultados da 1ª etapa demonstraram que os animais dos grupos S10 e S20 apresentaram diminuição do peso das glândulas seminais em relação ao grupo CO ($p < 0.05$). Na 2ª etapa, observou-se que o grupo S também teve uma menor glândula seminal. Esta, no grupo SP, manteve-se com peso semelhante ao grupo CO. Embora um atraso no tempo de trânsito espermático tenha sido observado no grupo SP, a fertilidade natural não foi comprometida. Além disso, esse mesmo grupo apresentou maior número de fetos e menor número de reabsorções comparado ao grupo S ($p < 0.05$). Nos testes neurocomportamentais, observou-se que os perfis dos animais dos grupos P e S foram diferentes entre si ($p < 0.05$), sendo este último menos exploratório e mais ansioso. Assim, os quatro experimentos aqui desenvolvidos demonstram que a SE tem impacto no sistema reprodutor e no neurocomportamento de roedores em diferentes fases da vida. E pela primeira vez, demonstrou-se que o probiótico foi capaz de atenuar estes efeitos colaterais.



Abstract

Depression, stress, and anxiety are commonly observed in the population, and as a consequence, the consumption of antidepressants has increased exponentially in recent years. Sertraline (SE), classified as a selective serotonin reuptake inhibitor (SSRI), is an antidepressant/anxiolytic used by people with different psychiatric disorders and different ages. SSRIs can cause serious side effects, however, there are few reports on the impacts of SE on reproductive and behavioral aspects, nor are there adjuvant treatments that alleviate its undesirable effects. Knowing the benefits of probiotics on the microbiota-gut-brain axis and its beneficial action on reproductive health, the present study investigated the impacts of SE associated or not with stress/or probiotic, in different periods of rodent development. Statistical tests used: ANOVA (followed by Tukey's test) or Kruskal-Wallis (followed by Dunn's test) ($p \leq 0.05$). For experiment I and II, pregnant Wistar rats were distributed into groups ($n=20-18$ rats/group): CO - received filtered water; SE - received 20 mg/kg of SE; ST - animals subjected to restraint stress and received filtered water; ST/SE - animals submitted to stress and received 20 mg/kg of SE. The treatment was carried out by gavage between gestational days (GD) 13 to 20. Part of the pregnant females was euthanized on GD 20 for analysis of the fetuses, and the rest were maintained until the end of lactation. In experiment I, somatic maturation, reflex development, and neurobehavior were investigated in male offspring. In experiment II, on GD 20 and on postnatal days (PND) 21, 45 and 110, the histology of the testes and epididymis were analyzed, as well as the testicular immunohistochemistry of the proliferating cell nuclear antigen (PCNA) and Wilms tumor protein (Wt1). Sperm morphology, motility, and vitality were observed on PND 110, as well as daily sperm production and epididymal transit time. The results of experiment I showed that gestational exposure to SE caused a reduction in body weight and vaginal bleeding in mothers. The male offspring of the SE group showed delays in the eruption of the incisors, in fur development, and in the performance of negative geotaxis. Furthermore, the SE group was less exploratory (anxious personality) compared to the CO and ST groups. In experiment II, it was observed that the fetal testes had a large number of acidophilic cells in the groups exposed to SE. The ST/SE group also showed a decrease in the nuclear volume of Leydig cells. This same group showed low expression of PCNA and WT1, decreased weight of the testes and epididymis, shorter anogenital distance (AGD), and delayed onset of puberty. Adult animals exposed *in utero* to SE showed changes in sperm morphology and motility. Experiment III was conducted in order to investigate the reproductive and behavioral repercussions of lactational exposure to SE. For this purpose, 30 lactating Wistar rats were divided into 3 experimental groups ($n=10$ /group): CO- group received filtered water, S10 and S20 groups received, respectively, 10 and 20 mg/kg/day of SE. The treatment was carried out by gavage from the 1st to the 20th day of lactation. During this period, the reflex and somatic development of male offspring was observed, as well as maternal behavior. On day 21, the mothers were euthanized, and on days 21, 45 and 100, one male from each litter was euthanized. In addition to the methodologies carried out in experiment I and II, sexual behavior and natural fertility were also observed. The results showed that the male offspring exposed to SE had lower body weight, lower AGD, and changes in the development of reflexes, in addition to histological alterations in the testes. In adulthood, most of the parameters analyzed were normalized, however, sperm quality was altered without compromising natural fertility. Experiment IV investigated the effects of SE on the reproductive parameters of mice, as well as verified whether the probiotic *Lactobacillus rhamnosus* (Lr) alleviates the side effects of this antidepressant. Thus, two consecutive steps were developed. In the first step, four experimental groups ($n=6$ /group) were formed: Group CO received filtered water (vehicle); and groups S10, S20, and S40 received respectively 10, 20 and 40 mg/Kg of SE diluted in the vehicle. The treatment occurred by gavage for 30 days. At the end of treatment, sperm quality, organ weight, and histology of testes



and epididymis were analyzed. In the second step, 32 mice were distributed into 4 experimental groups (n=8/group): Group CO received filtered water (vehicle); Group S – received 20 mg/kg of SE; Group P- received the probiotic Lr (1×10^9 CFU), diluted in vehicle; and SP group that received both Lr and SE. At the end of treatment, the histology of the reproductive organs and sperm quality were analyzed. Natural fertility was also verified. Complementarily, *in vitro* tests were carried out in order to identify the effects of SE on the acrosomal reaction during capacitation and *in vitro* fertilization. The results of the the first step demonstrated that the animals in the S10 and S20 groups presented a decrease in the weight of the seminal glands in relation to the CO group ($p < 0.05$). In the second step, it was also observed that the S group had a smaller seminal vesicle. This organ, in the SP group, maintained a similar weight to the CO group. Although a delay in sperm transit time was observed in the SP group, natural fertility was not compromised. Furthermore, this same group had a higher number of fetuses and a lower number of resorptions compared to the S group ($p < 0.05$). In the neurobehavioral tests, it was observed that the profiles of the animals in the P and S groups were different from each other ($p < 0.05$), the latter being less exploratory and more anxious. Thus, the four experiments demonstrate that SE has an impact on the reproductive system and on the neurobehavior of rodents at different life stages. And for the first time, we demonstrated that the probiotic was able to attenuate these side effects.



Introdução

1- Sistema Reprodutor

O desenvolvimento dos mamíferos é caracterizado por uma série de períodos críticos, durante os quais o ambiente fetal pode influenciar a vida pós-natal. A saúde reprodutiva é determinada já na fase embrionária e a diferenciação sexual genital envolve eventos pelos quais o embrião adquire progressivamente características masculinas ou femininas (Rey *et al.*, 2000).

A constituição cromossômica, determinará o sexo genético e levará a gônada primitiva a se diferenciar em testículo ou ovário. Sob a orientação do gene SRY no cromossomo Y, a produção de testosterona resulta na regressão do trato genital feminino e no desenvolvimento dos órgãos reprodutores masculinos e na programação de um fenótipo permanente (Mäkelä *et al.*, 2019).

As células germinativas primordiais (CGP) são observadas próximas à base do alantoide em desenvolvimento na parede do saco vitelino. À medida que o embrião se dobra, a parte do endoderma extraembrionário que é habitada pelas CGP é incorporada ao embrião e forma o intestino médio e o intestino posterior. Por meio de migração e por deslocamentos causados pelos movimentos de crescimento embrionário, as CGP saem do saco vitelino e atingem as gônadas bipotenciais (Mäkelä *et al.*, 2019) (Figura 1).

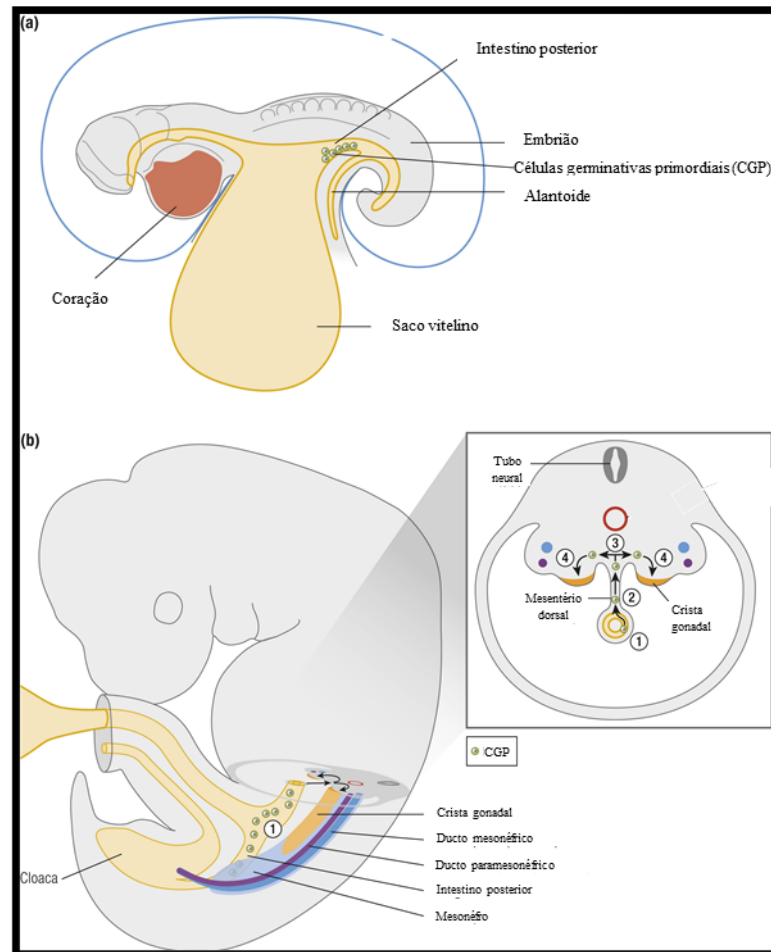


Figura 1. Formação inicial das gônadas indiferenciadas. Local inicial das células germinativas primordiais (a) e seu deslocamento (b). Etapas 1,2,3 e 4 referem-se à migração celular até a crista gonadal (Mäkelä *et al.*, 2019).

Os fatores que estão envolvidos com a diferenciação da gônada incluem o fator esteroidogênico 1 (Sf1), o supressor tumoral de Wilms (WT1) e a proteína homeobox de Lim (LHX-9). Durante a diferenciação gonadal, o WT1 é expresso no epitélio celômico e posteriormente nas células de Sertoli e granulosa. O SF1 também é um receptor nuclear expresso na hipófise e nas gônadas. O WT1, através da interação com CITED2 e LHX-9 regulam a expressão de SF1 e a montante da cascata de desenvolvimento gonadal. Fatores da família GATA4 e SOX também regulam a expressão de SF1 na gônada (Wilhelm e Englert, 2002).

Durante a diferenciação do testículo, os cordões seminíferos e o interstício são formados. As células de Sertoli originam-se a partir das células epiteliais do celoma embrionário e envolvem os gonócitos para formar os cordões seminíferos. Formam também

uma importante barreira através de junções especializadas que protegem as células germinativas e permitem a ocorrência da espermatogênese (O'Donnell *et al.*, 2022).

Os cordões dão origem aos túbulos seminíferos adultos, enquanto as células de Leydig e outros tipos celulares se diferenciam na região do interstício. Os precursores das células de Leydig fetais têm sido propostos como células migratórias do epitélio celômico, do mesonefro ou da crista neural ou células residentes presentes no primórdio adrenogonadal. Estas células produzem altos níveis de androgênio fundamental para a diferenciação da genitália masculina e masculinização do cérebro, assim como manutenção do processo de espermatogênese (Zirkin e Papadopoulos, 2018). Em ratos, as células de Leydig adultas se desenvolvem a partir de células de Leydig tronco que estão presentes no testículo pré-púbere, sendo essenciais para as funções fisiológicas do sistema reprodutor masculino (Chen *et al.*, 2017).

Dependendo da espécie analisada, os dias para o surgimento da crista genital e demais etapas do desenvolvimento reprodutivo podem ser diferentes, como observado na Figura 2.

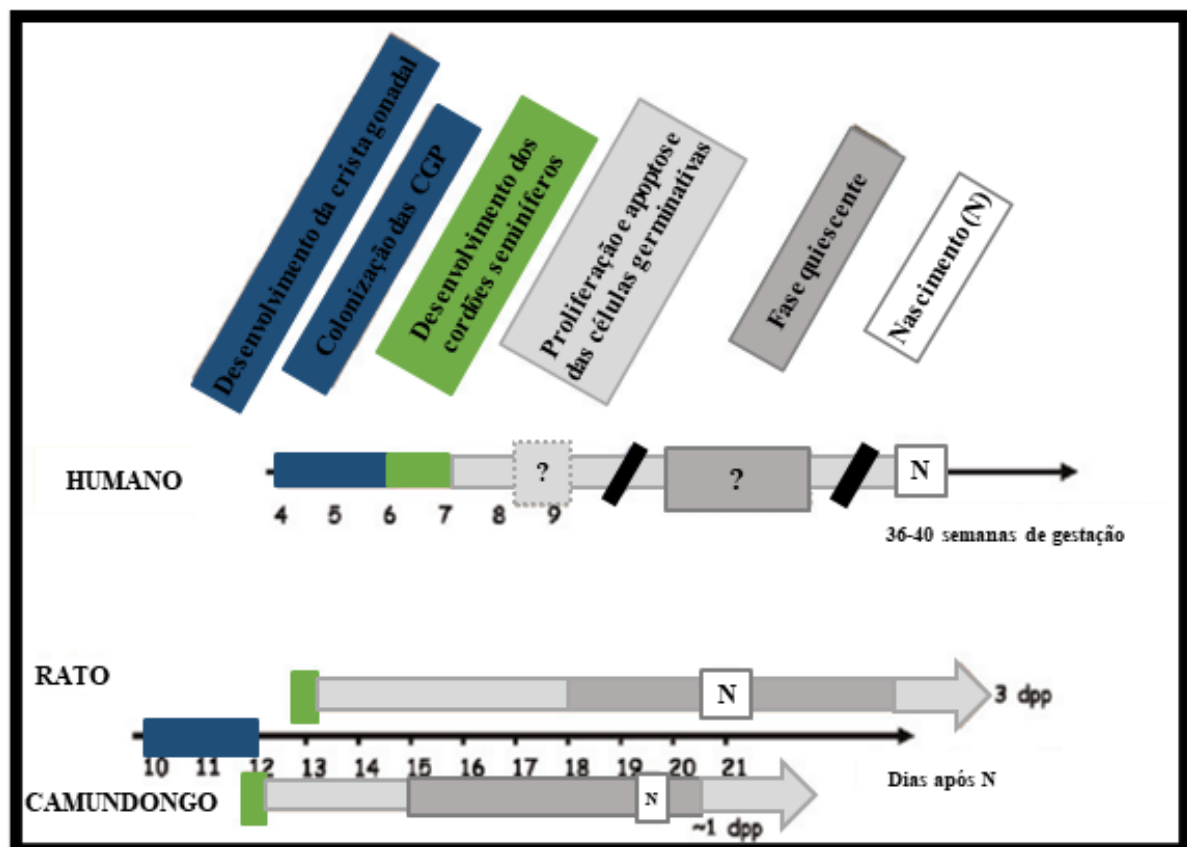


Figura 2. Cronologia comparativa do desenvolvimento de testículos fetais de humanos, ratos e camundongos (Rouiller-Fabre *et al.*, 2009).

Após o nascimento de roedores, uma subpopulação de pré-espermatogônias se diferenciam em espermatogônias indiferenciadas (tronco e progenitoras). Um outro grupo de

pré-espermatogônias diferencia-se diretamente em espermatogônias A1 e A2 e inicia o primeiro ciclo da espermatogênese. As pré-espermatogônias estão localizadas no centro do cordão seminífero e interagem com células de Sertoli imaturas (Yan *et al.*, 2020).

As espermatogônias (diploides, 2C) se desenvolvem em espermatócitos pré-leptótenos (4C) após a replicação pré-meiótica do DNA. A primeira divisão meiótica gera espermatócitos secundários que possuem duas cópias de cada gene (células 2C). Estas células sofrem a segunda divisão meiótica e originam espermatídes haploides (células 1C) que se diferenciarão em espermatozoides. A duração da espermatogênese varia de espécie para espécie, em camundongos por exemplo, a duração é de 35 dias, enquanto no rato é 54 dias e no humano é 74 dias (Perrard *et al.*, 2016).

Os túbulos seminíferos convergem para formar a rete testis, que por sua vez dá origem aos ductos eferentes que convergem para formar um único ducto altamente enrolado, o epidídimo que varia de 1 m em camundongos, 3 m em ratos, e de 3 a 6 m no homem. Anatomicamente pode ser dividido em quatro regiões, o segmento inicial, a cabeça, o corpo e a cauda. Durante o trânsito espermático pelo epidídimo a capacidade fertilizante e a motilidade são adquiridas (Robaire *et al.*, 2006).

2- Sistema serotoninérgico

Os neurônios serotoninérgicos estão entre os primeiros neurônios a serem gerados, e a serotonina (5-hidroxitriptamina ou 5-HT) é liberada pelos axônios em crescimento antes que as sinapses convencionais sejam estabelecidas. A 5-HT, foi isolada pela primeira vez do sangue na década de 1940, e somente durante a década de 1950 evidenciou-se sua ação no sistema nervoso central (Lasiuk e Hegadoren, 2007). Posteriormente descobriu-se seu papel modulador em uma série de eventos biológicos, incluindo divisão celular, migração neuronal, diferenciação celular e sinaptogênese (Azmitia, 2001; Gaspar *et al.*, 2003).

A 5-HT se distribui amplamente pelas principais regiões do cérebro, influenciando o estado afetivo, o aprendizado, a memória, a atenção, o sono, a agressão, além de ter papel significativo no período da maternidade (Pawluski *et al.*, 2019). Sabe-se que cerca de 2% da 5-HT está no sistema nervoso e o restante está na periferia em plaquetas, mastócitos e células do intestino. Além de seu papel como neurotransmissor cerebral, esta monoamina é um importante fator regulador no trato gastrointestinal, tanto que é o intestino responsável por produzir mais de 90% do 5-HT do corpo (Yano *et al.*, 2015). Também está presente em órgãos do sistema reprodutor, como no testículo que fora encontrada na cápsula testicular e nas células de Leydig

(Campos *et al.*, 1990; Frungieri *et al.*, 2002). Estudos *in vitro* e *in vivo* tem apontado seu papel sobre a esteroidogênese testicular e produção espermática (Frungieri *et al.*, 2002; Díaz-Ramos *et al.*, 2018).

A diminuição da atividade serotoninérgica, atribuível a menor liberação de 5-HT ou a uma menor quantidade de receptores de serotonina ou alterações na transdução de sinal mediada por receptores de serotonina, está relacionada ao surgimento de depressão (Mann, 1999). Sugere-se que o humor deprimido, a característica principal da depressão, seja regulado em parte pela função anormal da 5-HT. A diminuição de seus estoques, depleção de seus precursores (triptofano) ou inibição da sua síntese, precipita sintomas de depressão em indivíduos saudáveis e em indivíduos deprimidos em remissão (Staley *et al.*, 1998).

No sistema nervoso, o receptor 5-HT_{1A} está localizado principalmente no córtex frontal e na rafe do tronco cerebral. Desempenha papel importante na depressão e no transtorno de ansiedade generalizada, já que fatores genéticos e ambientais foram associados à ligação alterada de 5-HT_{1A}. Assim, este receptor tornou-se alvo de medicamentos, por exemplo, a buspirona, que é um agonista do receptor 5-HT_{1A} e é comumente usada para o tratamento de distúrbio de ansiedade. Já a vilazodona também atua como agonista parcial do 5-HT_{1A} (Köhler *et al.*, 2016).

A hipótese monoaminérgica propõe que a depressão também possa ser ocasionada por depleção nos níveis de serotonina, norepinefrina e/ou dopamina no sistema nervoso central. Assim inúmeros antidepressivos, tais como os inibidores seletivos da recaptação da serotonina (ISRS), foram elaborados de forma a elevar os níveis 5-HT, norepinefrina e/ou dopamina (Delgado, 2000).

3- Depressão e estresse

A depressão fora classificada como um transtorno mental que remonta ao século XIX, quando os primeiros esforços foram realizados para coletar informações estatísticas sobre sua incidência (Payne e Maguire, 2019). De acordo com o Manual de Diagnóstico e Estatística para Transtornos Mentais, um indivíduo atende aos critérios para depressão maior se apresentar humor deprimido ou perda de interesse em atividades normalmente prazerosas, juntamente com outros sintomas como alterações do peso corporal, retardo psicomotor ou agitação, diminuição da concentração e/ou pensamentos recorrentes de morte ou ideação suicida (Vahia, 2013).

O diagnóstico de depressão é recorrente em mulheres, sendo 70% mais propensas do que os homens, e tem sido associada ao ciclo reprodutivo e à síndrome pré-menstrual. Outros

eventos reprodutivos incluem a depressão pós-parto (DPP) e durante a transição da menopausa (Vahiaini, 2016).

A DPP refere-se a episódios depressivos que são prevalentes após o parto. Impacta negativamente a mãe, sendo o suicídio responsável por aproximadamente 20% das mortes pós-parto. Além disso, a depressão materna também tem efeitos adversos no desenvolvimento comportamental, emocional e cognitivo infantil (Payne e Maguire, 2019). Atualmente a DPP é classificada como "Transtorno Depressivo Maior, e seus sintomas iniciam durante a gravidez ou nas primeiras quatro semanas após o parto. Estudos ainda apontam que estes sintomas podem iniciar após 6 meses após o parto (O'Hara e McCabe, 2013).

O estresse é um dos desencadeadores da depressão já que altera a homeostasia do organismo. Além disso, outros agentes também contribuem com essa patologia, como a predisposição genética, efeitos colaterais de medicamentos, drogas de abuso e eventos traumatizantes (Bailey *et al.*, 2019).

O organismo frente a estímulos estressores prioriza mecanismos de sobrevivência sobre funções fisiológicas menos essenciais, incluindo crescimento e reprodução. Como parte da adaptação, o eixo hipotálamo-hipófise-adrenal medeia as funções do eixo hipotálamo-adrenal-gonadal, que é responsável pela maturação e função dos órgãos reprodutivos. Sabe-se que altos níveis de glicocorticoides têm efeitos inibitórios sobre os neurônios GnRH, nos gonadotrofos hipofisários e nas gônadas (Joseph e Whirledge, 2017).

Como resposta sistêmica à condição estressante, o hipotálamo libera o hormônio liberador de corticotropina (CRH), que sinaliza à glândula pituitária para secretar o hormônio adrenocorticotrófico (ACTH). O ACTH estimula o córtex adrenal a liberar corticosteróides, preferencialmente cortisol. Este, por sua vez, pode alterar a secreção de CRF (fator de liberação de corticotrofina) e modular a liberação de hormônios hipofisários pelo GnRH. Em mulheres, por exemplo, a ovulação pode ser comprometida por esta alteração hormonal (Figura 3).

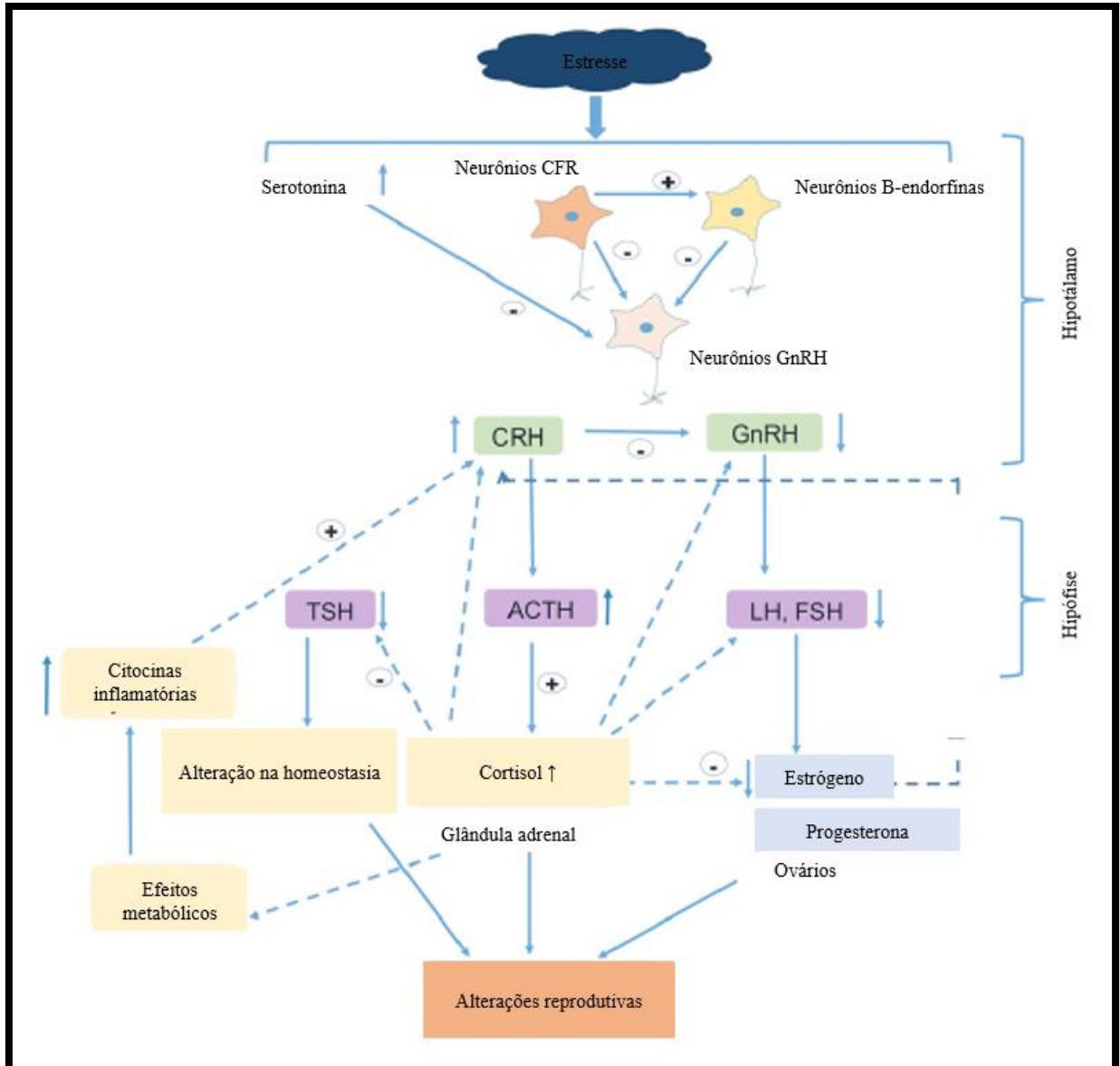


Figura 3. Impacto do estresse no eixo hipotálamo- hipófise-adrenal. Adaptado de Bala *et al.* (2021).

O estresse tem importante influência na homeostasia e pode afetar muitos aspectos da fisiologia reprodutiva, através de desregulações diretas do processo espermatogênico, alterações da expressão da proteína reguladora aguda esteroidogênica (StAR) e da enzima de clivagem da cadeia lateral do citocromo P450, assim como aumentar a produção de oxidantes que causam a morte das células de Leydig (Bräuner *et al.*, 2020; Haider, 2004).

Em modelos experimentais, a indução de estresse por contenção produz estímulos não nociceptivos, aumenta a atividade do eixo hipotálamo-hipófise-adrenal (HHA) e a expressão de Rfrp3 hipotalâmico que está associado a níveis circulantes elevados de corticosterona (Buechel *et al.*, 2014; Glavin *et al.*, 1994; Geraghty *et al.*).

Em camundongos adultos, o estresse crônico induz alterações espermáticas, incluindo a contagem, motilidade, morfologia e integridade do DNA (Guo *et al.*, 2020). Em fêmeas, ocasiona níveis elevados de glicocorticoides que desencadeiam apoptose pronunciada nas tubas uterinas. Além disso, evidências apontam que o estresse reduz o número de blastocistos e altera o desenvolvimento embrionário (Zheng *et al.*, 2016).

O estresse pré-natal produz altos níveis de glicocorticoides que provocam redução do peso fetal, predisõem os descendentes a doenças cardiometabólicas, perturbações do eixo HHA e distúrbios afetivos (Joseph e Whirledge, 2017). De maneira geral, gestantes que apresentam transtornos psiquiátricos representam um desafio complexo para os médicos, pois devem equilibrar a saúde e o bem-estar da mãe, a potencial toxicidade dos medicamentos antidepressivos para o feto e os riscos da não intervenção medicamentosa. Em decorrência dos impactos da depressão materna não tratada tanto para a mãe quanto para o feto, o início (ou ajuste) da terapia antidepressiva é essencial (Zheng *et al.*, 2022).

4- Antidepressivos

Os primeiros antidepressivos foram descobertos ao acaso quando pacientes com tuberculose administraram medicamentos antimicrobacterianos, tais como a isoniazida e iproniazida, e tiveram uma melhora em seu humor e sensação de bem-estar. O mecanismo de ação desses agentes foi inibir a enzima monoamina oxidase, demonstrando que a depressão tinha efeito neuroquímico (López-Muñoz e Alamo, 2009).

As monoamina oxidases foram as primeiras drogas aprovadas para depressão no final da década de 1950, e foram utilizadas em diferentes transtornos psiquiátricos ao longo dos anos. Os antidepressivos tricíclicos, criados inicialmente como anti-histamínicos funcionam bloqueando a recaptação da norepinefrina e serotonina. Já as classes de antidepressivos desenvolvidas mais recentemente e mais conhecidas são os ISRS e os inibidores da recaptação da serotonina-norepinefrina (Duarte *et al.*, 2021; Schneider *et al.*, 2019; Sabella, 2018).

Os ISRS ligam-se ao transportador de serotonina, bloqueando a reabsorção de serotonina pelo neurônio pré-sináptico e aumentam os níveis de serotonina na fenda sináptica (Millard *et al.*, 2017) (Figura 4).

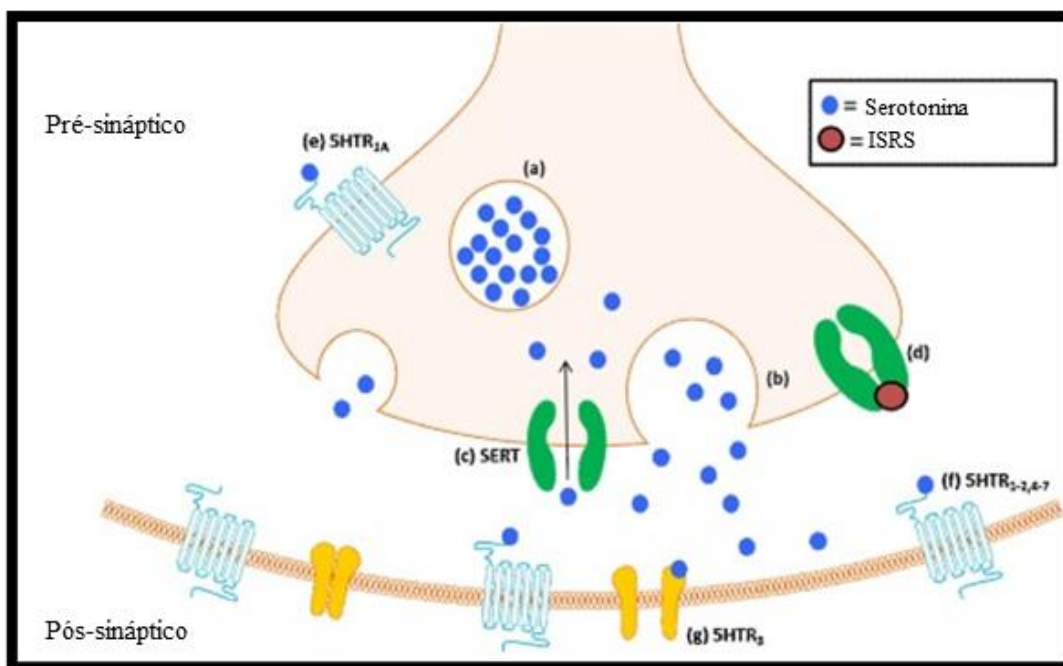


Figura 4. Mecanismo de ação dos inibidores seletivos de recaptação de serotonina (ISRS) (Millard *et al.*, 2017).

Atualmente os ISRS, tais como o citalopram, escitalopram, fluoxetina, fluvoxamina, paroxetina, e a sertralina (SE), estão entre os medicamentos mais comumente usados para controlar a depressão e são considerados o tratamento de primeira linha, já que são mais seguros e apresentam menos efeitos adversos. Estes efeitos podem ser nervosismo, agitação, sedação, fadiga, dor de cabeça, insônia, disfunção sexual, desconforto gastrointestinal, perda ou ganho de peso (Sabella, 2018; Yohn *et al.*, 2017).

Embora os ISRS causem elevações agudas nas concentrações de serotonina, acredita-se que as propriedades antidepressivas sejam também desencadeadas por aumentos na expressão do fator neurotrófico derivado do cérebro (FNDC) e neurogênese subsequente no córtex pré-frontal e hipocampo (Martinowich e Lu, 2008). O sistema serotoninérgico e outros efetores como o FNDC, também são críticos para o neurodesenvolvimento normal, levantando preocupações sobre a exposição do feto em desenvolvimento e recém-nascidos aos ISRS. Estes são os mais amplamente prescritos para mulheres grávidas (cerca de 80% dos casos) e foram considerados moderadamente seguros para uso pré-natal (Millard *et al.*, 2017).

As consequências a longo prazo da exposição aos ISRS, incluindo o risco de desenvolver distúrbios do neurodesenvolvimento, não são bem compreendidas. Vários estudos apontam correlações positivas entre o aumento da prevalência de transtornos do espectro autista. Alguns trabalhos apontam que crianças expostas a estes antidepressivos no início da

vida apresentam mudanças no comportamento afetivo, como aumento da ansiedade e depressão (Liu *et al.*, 2021).

A SE foi aprovada para uso nos Estados Unidos em 1991 e continua em amplo uso, com quase 40 milhões de prescrições sendo preenchidas anualmente. É o segundo ISRS mais potente da recaptação de serotonina e o segundo bloqueador mais seletivo da recaptação de serotonina em relação à noradrenalina, sendo o único ISRS que se liga a transportadores de dopamina (Hiemke e Härtter, 2000).

Indicada para diferentes condições psiquiátrica, a SE pode ser usada em casos de depressão maior, transtorno obsessivo-compulsivo, transtorno do pânico, estresse pós-traumático, transtorno de ansiedade generalizada, transtorno disfórico pré-menstrual, neuropatia diabética. É também considerada o ISRS de escolha para depressão durante a amamentação em decorrência de sua meia-vida relativamente curta e concentração sérica infantil muitas vezes indetectável (Cuomo *et al.*, 201a). De maneira geral, os efeitos colaterais comumente relatados da SE são dores de cabeça, dispneia, sonolência, náusea, aumento da sudorese e disfunção sexual (Livertox, 2012).

A absorção da SE pelo trato gastrointestinal é quase completa, atingindo concentrações plasmáticas máximas aproximadamente 4 a 8 horas após a administração de uma dose única de 100 mg. Sua meia vida ocorre de 22 a 37 horas, e o metabolismo hepático é a via de eliminação mais importante, com apenas 0,2% de uma dose oral sendo excretada inalterada na urina (Murdoch e McTavish, 1992; Hiemke e Härtter, 2000). A N-desmetilsertralina (Figura 5), é o principal produto metabólico no processo de biotransformação, e possui apenas 5-10% da potência inibidora da recaptação de serotonina da SE, não tendo, portanto, uma contribuição clínica (Dziurkowska e Wesolowski, 2018).

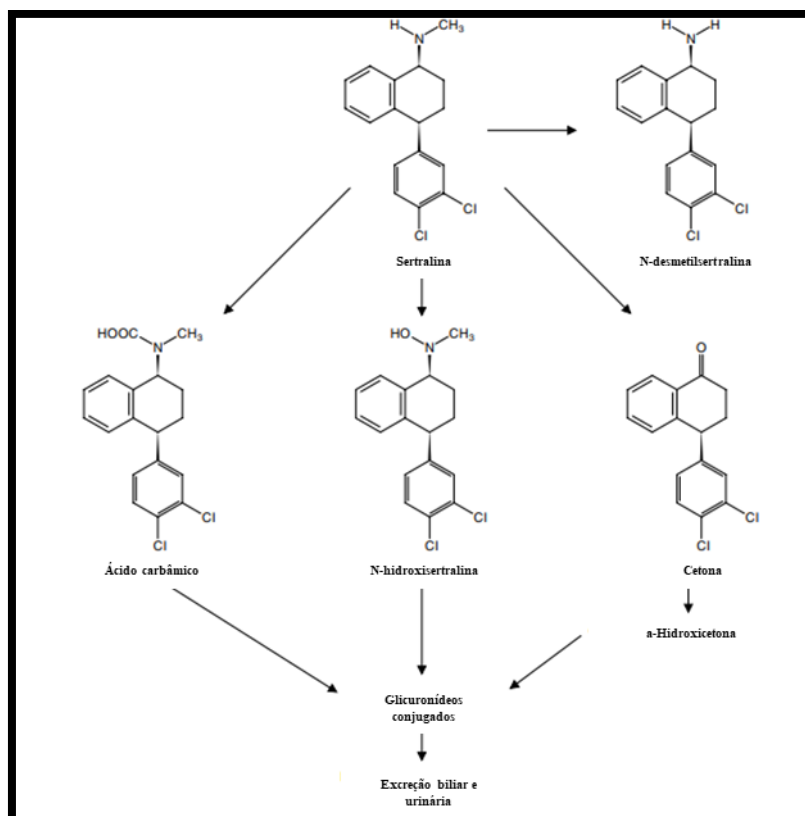


Figura 5. Vias metabólicas da sertralina (DeVane *et al.*, 2002).

5- Probióticos

Os probióticos são leveduras e bactérias regulamentadas como suplementos dietéticos (comprimidos e cápsulas) e alimentos (iogurte e leite). Dentro desse amplo grupo de microrganismos (Tabela 1), os mais utilizados incluem as bactérias do ácido lático, especificamente as espécies *Lactobacillus* e *Bifidobacterium*, e a levedura *Saccharomyces boulardii* (Williams, 2010).

Tabela 1. Microrganismos usados como probióticos

<i>Lactobacillus</i>	<i>Bifidobacterium</i>	Ouros
<i>L. acidophilus</i>	<i>B. adolescentis</i>	<i>Bacillus cereus</i>
<i>L. bulgaricus</i>	<i>B. animalis</i>	<i>Enterococcus faecalis</i>
<i>L. casei</i>	<i>B. bifidum</i>	<i>Enterococcus faecium</i>
<i>L. crispatus</i>	<i>B. breve</i>	<i>Escherichia coli</i> Nissle
<i>L. fermentum</i>	<i>B. infantis</i>	<i>Streptococcus thermophilus</i>
<i>L. gasseri</i>	<i>B. lactis</i>	<i>Saccharomyces boulardii</i>
<i>L. johnsonii</i>	<i>B. longum</i>	
<i>L. lactis</i>		
<i>L. plantarum</i>		
<i>L. reuteri</i>		
<i>L. rhamnosus</i> GG		

Adaptado de Williams (2010).

O microbioma regula a permeabilidade intestinal, a composição e a função da camada mucosa, a função das células epiteliais, a imunidade inata e adaptativa e os processos de neurotransmissão por meio da secreção de várias substâncias (Jandhyala *et al.*, 2015).

Os probióticos têm sido usados para o tratamento de várias disfunções intestinais tais como doença de Crohn, colite ulcerativa, síndrome do intestino irritável e infecções por *Helicobacter pylori* (Jakubczyk *et al.*, 2020; Goderska *et al.*, 2018; Shi *et al.*, 2019). Também são utilizados em outras doenças não associadas ao trato gastrointestinal, como problemas urogenitais, respiratórios e imunológicos (Borges *et al.*, 2014; López-Moreno e Aguilera, 2021; Li *et al.*, 2019). Estudos recentes apontam que os probióticos reduzem sintomas ansiosos e depressivos por regular diretamente o eixo microbiota-intestino-cérebro (Pinto-Sanchez *et al.*, 2017; Li *et al.*, 2019).

A produção de ácidos (como o ácido lático, acético e propiônico), peróxido de hidrogênio, bacteriocinas e biosurfactantes, permitem que haja uma redução do pH intestinal e supressão do crescimento de várias bactérias patogênicas, restabelecendo assim o equilíbrio da flora intestinal (Doron e Gorbach, 2006). Há evidências que os probióticos podem modular diretamente a resposta imune, através da atividade fagocitária e produção de citocinas e imunoglobinas (Cortes-Perez *et al.*, 2021).

Alterações na microbiota intestinal podem promover ativação do sistema imunológico e desencadear inflamações testiculares e epididimárias. Além de induzir resistência à insulina juntamente com hormônios gastrointestinais, como leptina e grelina, que por sua vez afeta a secreção de vários hormônios sexuais, como LH, FSH e testosterona (Wang e Xie, 2022).

Foi demonstrado que a suplementação por *Lactobacillus rhamnosus* aumentou a porcentagens de espermatozoides progressivamente móveis, além de modular os níveis séricos



de testosterona, LH e FSH de camundongos normais e obesos (Dardmeh *et al.*, 2017). Além disso, os *Lactobacillus spp* e *Bacillus spp* tiveram atividade antioxidante e efeito protetor contra danos espermáticos induzidos pela dieta rica em gordura (Chen *et al.*, 2013).

Um trabalho realizado por Ibrahim *et al.* (2012), demonstrou que a suplementação de probióticos e selênio inorgânico diminuiu significativamente os efeitos adversos da hiperlipidemia, aumentando o nível sérico de testosterona e melhorando a qualidade espermática.

Guo *et al.* (2020) verificaram que a administração oral de *Lactobacillus rhamnosus*, reduziu as alterações provocadas por estresse crônico, no que se refere a contagem, morfologia, motilidade e integridade do DNA de espermatozoides. Além disso, a alteração do nível de testosterona induzida pelo estresse foi evitada pela regulação do probiótico nas expressões de StAR e P450scc nos testículos. O probiótico também aumentou as atividades da catalase, glutatona peroxidase e superóxido dismutase, e diminuiu os níveis de produtos oxidativos, bem como os níveis de interleucina (IL)-1 β , IL-6, e fator de necrose tumoral- α .



Justificativa

Sabendo-se que a SE é um ISRS muito recomendado na clínica médica, faz-se necessário investigar possíveis efeitos colaterais sobre o sistema reprodutor e neurocomportamental de indivíduos em diferentes períodos da vida.

É importante também que tratamentos associados possam ser utilizados para amenizar impactos provocados por este e outros antidepressivos. Uma vez que os probióticos proporcionam benefícios a saúde humana e tem demonstrado papel positivo em sinalizações serotoninérgicas e reprodutivas, o presente estudo utilizou o *Lactobacillus rhamnosus* como tratamento associado à SE.



Objetivo geral

Investigar os efeitos da SE associada ou não ao estresse/ou probiótico sobre parâmetros reprodutivos e neurocomportamentais de roedores em diferentes períodos do desenvolvimento.



Objetivos específicos

- ✓ Investigar o desenvolvimento somático, de reflexo e neurocomportamental de ratos expostos à SE (associada ou não ao estresse) durante a gestação.
- ✓ Evidenciar os impactos causados pela SE associada ou não ao estresse no desenvolvimento do sistema reprodutor de ratos machos.
- ✓ Verificar a saúde materna e esclarecer o efeito da SE no desenvolvimento neurocomportamental e reprodutivo de ratos machos expostos durante à lactação.
- ✓ Avaliar se a exposição à SE compromete o sistema reprodutor e neurocomportamento de camundongos adultos e analisar se o probiótico *Lactobacillus rhamnosus* ameniza seus efeitos colaterais.

O presente trabalho foi dividido em quatro experimentos, descritos nos capítulos subsequentes. Os animais utilizados foram mantidos de acordo com os Princípios Éticos em Experimentação Animal, adotados pela Sociedade Brasileira de Ciência em Animais de Laboratório junto à Comissão de Ética em Experimentação Animal do Instituto de Biociências de Botucatu (Protocolos dos experimentos: 1169, 3597170322 e 3548071220).

Para o experimento I e II (Figura 6), ratas Wistar prenhes foram distribuídas nos grupos (n=20-18 ratas/grupo): CO - receberam água filtrada; SE – receberam 20 mg/kg de SE; ST - animais submetidos ao estresse por contenção e receberam água filtrada; ST/SE - animais submetidos ao estresse e receberam 20 mg/kg de SE. O tratamento foi realizado por via gavagem entre os dias gestacionais (DG) 13 ao 20. Parte das fêmeas prenhes foram eutanasiadas no DG 20 para análise dos fetos, e o restante foi mantido até o final da lactação. No experimento I, a maturação somática, o desenvolvimento de reflexos, e o neurocomportamento foram investigados na prole masculina. No experimento II, foram analisados a histologia dos testículos e epidídimos, bem como a imuno-histoquímica testicular do antígeno nuclear de proliferação celular (PCNA) e proteína do tumor de Wilms (Wt1), no DG 20, e nos dias pós-natais (DPN) 21, 45 e 110. A morfologia, motilidade e vitalidade espermáticas foram observadas no DPN 110, assim como a produção diária de espermatozoides e seu tempo de trânsito epididimário.

O experimento III (Figura 6) foi conduzido de forma a investigar as repercussões reprodutivas e comportamentais da exposição lactacional à SE. Para tanto, 30 ratas Wistar lactantes foram distribuídas em 3 grupos experimentais (n=10/grupo): grupo CO- recebeu água filtrada, grupos S10 e S20 receberam, respectivamente, 10 e 20 mg/kg/dia de SE. O tratamento foi realizado por via gavagem do 1º ao 20º dia de lactação. Nesse período, observou-se o desenvolvimento de reflexos e somáticos, bem como o comportamento materno. No dia 21, as mães foram eutanasiadas, e nos dias 21, 45 e 100, um macho de cada ninhada foi eutanasiado. Além das metodologias realizadas no experimento I e II, também foram observados o comportamento sexual e fertilidade natural.

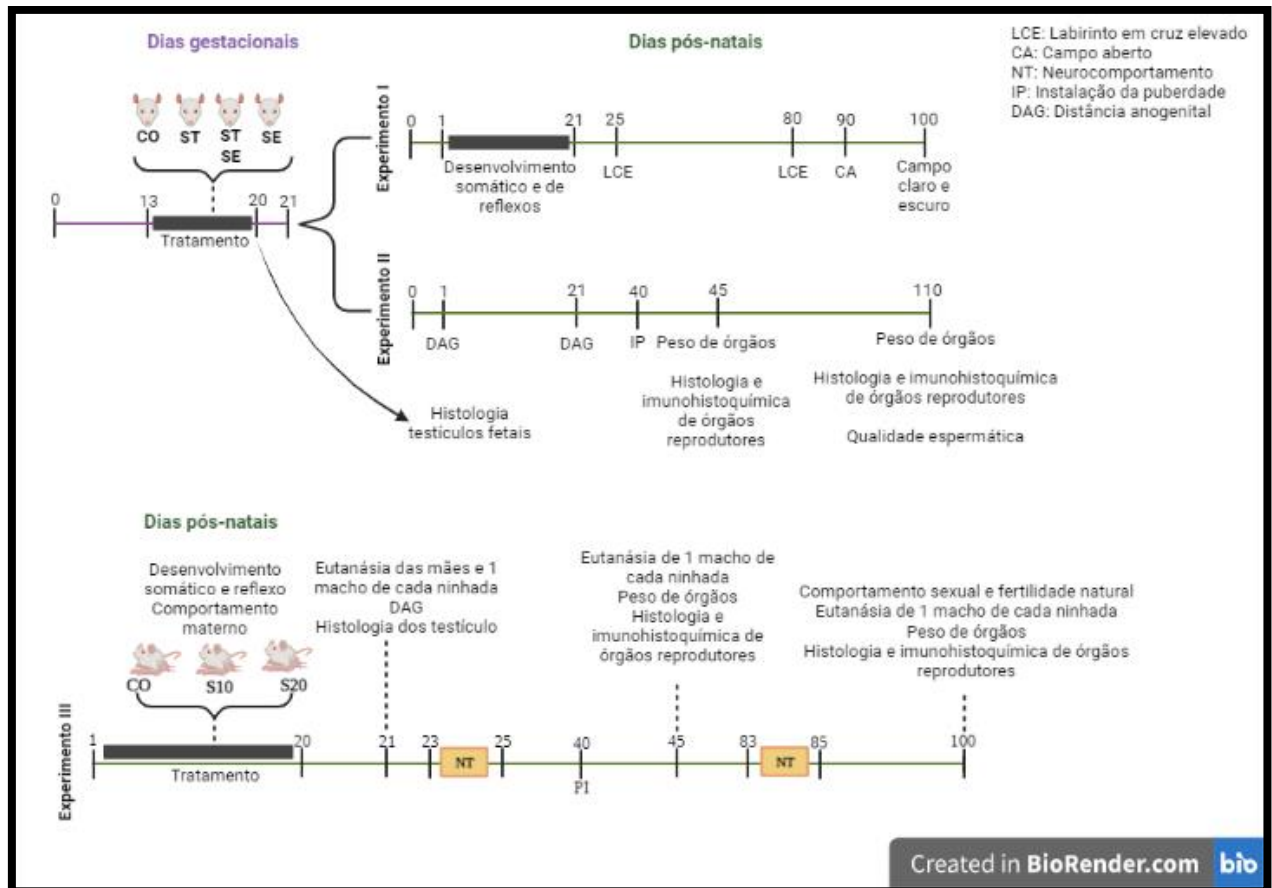


Figura 6. Desenho experimental I, II e III.

O experimento IV (Figura 7), investigou os efeitos da SE sobre aspectos reprodutivos de camundongos, bem como verificou se o probiótico *Lactobacillus rhamnosus* (Lr) ameniza os efeitos colaterais deste antidepressivo. Assim, duas etapas consecutivas foram desenvolvidas, na primeira, 4 grupos experimentais ($n=6/\text{grupo}$) foram formados: Grupo CO recebeu água filtrada (veículo); e grupos S10, S20 e S40 recebeu respectivamente 10, 20 e 40 mg/Kg de SE diluída em veículo. O tratamento ocorreu por via gavagem por 30 dias e durante este período a evolução do peso corporal foi observada. Ao final do tratamento, foram analisadas a qualidade espermática (motilidade e morfologia), o peso de órgãos e a histologia dos testículos e epidídimos. Após identificar a dose de SE capaz de provocar alterações reprodutivas, a etapa 2 foi conduzida. Para tanto, 32 camundongos foram distribuídos em 4 grupos experimentais ($n=8/\text{grupo}$): Grupo CO recebeu água filtrada (veículo); grupo S – recebeu 20 mg/Kg de SE; grupo P- recebeu o probiótico Lr (1×10^9 UFC), diluído em veículo; e grupo SP que recebeu tanto Lr quanto SE. Ao final do tratamento, por via gavagem, foram analisadas a histologia dos órgãos reprodutores, qualidade espermática, e a fertilidade natural.

Estudos *in vitro* foram também executados. Primeiramente foi verificado a reação acrossomal de espermatozoides expostos ou não a diferentes concentrações de SE (0,5, 5, 50, 500 $\times 10^{-5}$ mg/mL) durante o processo de capacitação. A concentração de 500 $\times 10^{-5}$ mg/mL de SE foi elegida para a condução da segunda etapa. Nesta, foi verificado o desenvolvimento embrionário após a exposição de SE em diferentes períodos, tais como a capacitação, fertilização e desenvolvimento embrionário. Assim, os seguintes grupos experimentais foram formados: SSS- Exposto durante todas as etapas à SE; CCC: Sem exposição à SE; SCC: Exposto à SE somente durante a capacitação; CSC- Exposto à SE somente durante a fertilização; SSC- Exposto à SE durante a capacitação e fertilização; CCS- Exposto à SE durante o desenvolvimento embrionário.

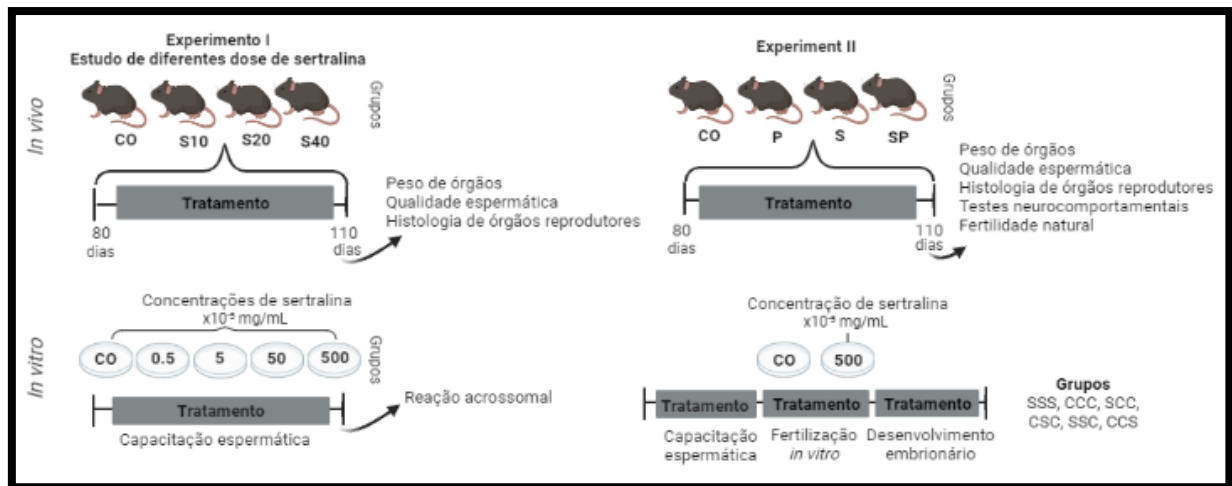


Figura 7. Desenho experimental IV.



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Capítulo 1

O experiment I deu origem ao manuscrito “Exposure of pregnant rats to stress and/or sertraline: Side effects on maternal health and neurobehavioral development of male offspring” que foi publicado na revista Life Sciences (Fator de Impacto = 6.78). Doi: 10.1016/j.lfs.2021.119960.



Exposure of pregnant rats to stress and/or sertraline: side effects on maternal health and neurobehavioral development of male offspring

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ABSTRACT

Aims: Sertraline (SE) is one of the most prescribed medications for treating gestational depression, anxiety and stress. However, little is known about its effects on nervous-system development in offspring. Therefore, this study investigated the somatic, reflex and neurobehavioral development of rats exposed to SE during pregnancy, associated or not with stress.

Main Methods: Pregnant Wistar rats were assigned to the following groups (n=10-8 rats/group): CO - control animals administered filtered water by gavage; SE - animals administered 20 mg/kg SE by gavage; ST - animals subjected to restraining stress and administered filtered water; ST/SE - animals subjected to restraining stress and administered 20 mg/kg SE. The treatment was administered between gestational days (GD) 13 to 20. Somatic and reflex developments were investigated in the male offspring from postnatal day (PND) 1 to 21. The elevated plus maze was performed on PND 25 and 80. The open field and light/dark box test were performed on PND 90 and 100, respectively.

Key findings: Body weight reduction and vaginal bleeding were observed in pregnant rats exposed to SE. The male offspring of the SE group showed delay in incisor eruption, fur development and negative geotaxis. In addition, the SE group was less exploratory (anxious personality) compared to the CO and ST groups.

Significance: The results obtained in the present study demonstrate that sertraline not only impairs maternal health, but also, associated or not with stress, can compromise the somatic, reflex and neurobehavioral development of male rats.

Keywords: Gestational depression; gestational stress; sertraline; vaginal bleeding; neurosomatic development; rat



1. Introduction

Psychiatric disorders, such as depression, anxiety and stress, have been commonly observed in the population in recent years, and constitute a global public health problem, as they cause severe losses in quality of life [1,2]. The pandemic caused by the new coronavirus (COVID-19) accentuated stress, not only due to deaths, but also due to social and economic impacts. Women are the most affected by these disorders, as they are overloaded with housework, children and professional work [3]. Pregnant women in particular are the most vulnerable, as these psychiatric disorders can have consequences for fetal development [4].

Depression, currently considered the most debilitating disorder, affects more than 350 million people worldwide. Many people are not properly diagnosed and are not treated, as a result of not only the lack of adequate health resources and effective therapies, but also the stigma of the disorder [5,6].

It is known that women are more susceptible than men, and can be affected during periods of pregnancy and lactation [7]. About 6.5% to 12.9% of women are diagnosed with major and minor depression during pregnancy and 19.2% in the postpartum period. Underreporting is considered significant, since the symptoms of this disorder may overlap with those that normally occur in a normal pregnancy [6,8].

Depression and stress are associated with premature births, low birthweight and changes in brain development (which affect the neural and behavioral activities of the offspring) [9,10,11]. These alterations may occur due to a decrease in the placental barrier (uterine artery resistance), reprogramming of the hypothalamic-pituitary-adrenal (HPA) axis, diminished levels of dopamine and serotonin, and increased levels of glucocorticoid / cortisol [12, 13].

The use and clinical recommendation of antidepressants/anxiolytics have increased significantly in recent decades, as a way to mitigate the alterations caused by gestational stress and depression. Selective serotonin reuptake inhibitors (SSRIs), such as sertraline (SE), fluoxetine, paroxetine, fluvoxamine and citalopram, are among the most recommended [11,12,14].

SSRIs inhibit serotonin reuptake and cause an increase in this neurotransmitter in the synaptic cleft. Although the drugs in this family have similarities with each other, they may produce different pharmacological and pharmacokinetic effects, caused by the form of interaction with CYP450 enzymes, as well as by interaction with other types of



receptors [15]. As an example, SE also has a weak affinity for $\alpha 1$ adrenergic receptors and dopamine transporters [16].

Serotonin plays an important role in several events of neuronal development, such as proliferation, differentiation, migration, apoptosis and synaptogenesis. Experimental models show that transient changes in serotonin homeostasis can affect brain connections and cause permanent damage to adult behavior [17].

Annually, thousands of children are exposed to serotonin-modulating agents through gestational and lactation exposure. Up to 30% of children exposed to SSRI and serotonin and norepinephrine reuptake inhibitors (SNRIs) are known to develop a spectrum of altered symptoms, including irritability, nervousness and lethargy [11]. It was observed that children exposed to SSRIs in a critical phase of development exhibit reduced social behavior, anxiety and even increased risk of depression [1].

Despite being considered safer than other psychotropic drugs and highly recommended, especially during pregnancy and lactation [18], SE is able to cross the placenta and enter the fetal circulation [19]. Thus, the present study aimed to investigate the somatic, reflex and neurobehavioral development of rats exposed to this medication (associated or not with stress) during pregnancy. The restraint stress model used in this study is classified as a psychosocial stress that produces non-nociceptive stimuli, being the most effective in rats to increase the activity of the hypothalamic-pituitary-adrenal axis [20, 21].

2. Material and methods

2.1. Animals and treatment

Male and female Wistar rats (45 days old) were obtained from the Central Biotherium of UNESP Botucatu and maintained under controlled conditions (22 °C, 30% air humidity, 12/12-h light/dark cycle) with food and water *ad libitum*. At 90 days of age, estrus females were mated during the dark cycle of the photoperiod. The presence of sperm in the vaginal smear was considered gestational day (GD) 0.

Pregnant rats were pseudo-randomly allocated into four experimental groups (n=10-8 animals/group), in order to avoid differences in initial body weight: CO – control animals administered filtered water by gavage; SE – animals administered 20 mg/kg of SE by gavage [22]; ST – animals subjected to restraining stress in an acrylic cylinder



(with variable diameter) for 1 h/day [23] and receiving filtered water; ST/SE – animals subjected to stress and administered 20 mg/kg of SE.

The treatment was carried out from GD 13 to 20, a critical period in which serotonergic development occurs in rats [24]. SE, purchased from Cruz Vermelha (Botucatu, Brazil), was diluted in filtered water and administered between 12-13 pm (light period of the light/dark cycle).

The concentration of 20 mg/kg of SE used in the present study provides antidepressant and anxiolytic effects in rats, and was previously used in other studies [22, 25, 26, 27].

At the end of the experiments, the animals were euthanized by CO₂ inhalation followed by decapitation. The experimental procedures used in this study were approved by the local Ethics Committee for the Use of Experimental Animals of the São Paulo State University (protocol number 1169) in accordance with the Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health).

2.2. Maternal data and offspring characteristics

Maternal body weight was recorded on GD 0 and 20. Postnatal day (PND) 0 refers to the day that pups were born. On PND 1, the number of live pups and their sex were noted.

2.3. Somatic, reflex and neurobehavioral analyses

After birth, the same proportion of males and females (4:4) was maintained throughout the lactation period, in order to standardize the size of all litters. Only males were assessed for the reflex, somatic and neurobehavioral development (Figure 1). The female offspring was evaluated in other study, not shown herein.

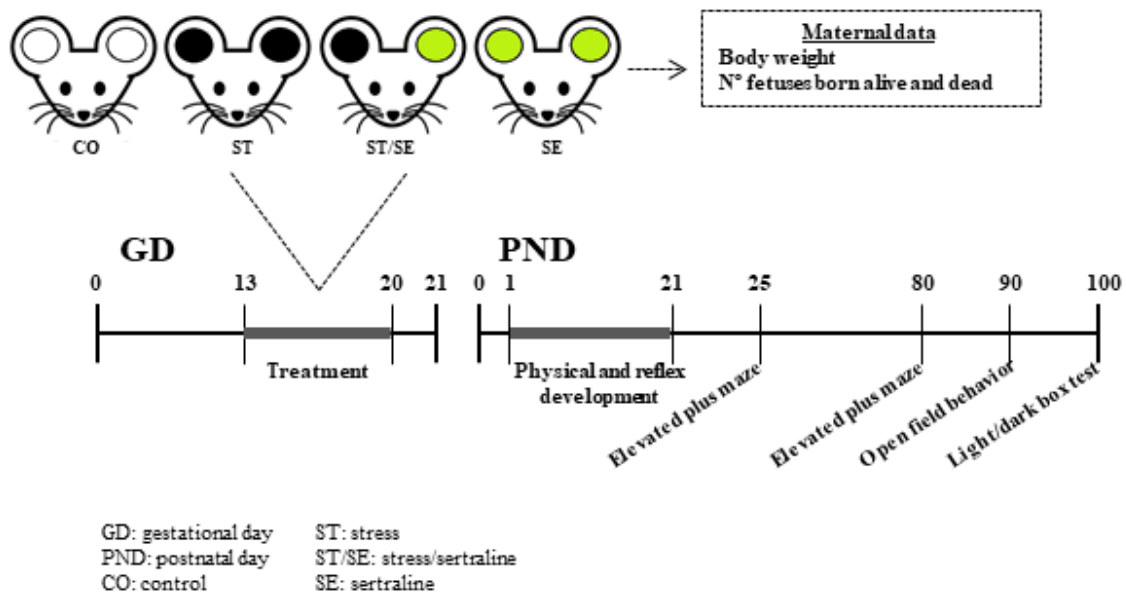


Figure 1. Experimental design of the study.

The data obtained from somatic and reflex maturation were expressed as the mean of the litter (the mean of four males per litter was taken and utilized to calculate the group mean). Neurobehavioral tests evaluated two male rats from each litter, as described by other studies [28], and the results were obtained by average.

All experiments were carried out in a quiet room during the light phase of the day/night cycle between 1pm and 4 pm.

Two observers, who were blind to the experimental group, used a camera and a stop watches to assess rat's behavior. All equipment used was cleaned with a 5% ethanol solution between each test.

Adverse environmental events or failure in the transmission of videos by the camera, were considered factors that excluded the results.

2.3.1. Somatic maturation and reflex development

Physical and reflex tests were carried out daily from the 1st to 21st PND. To evaluate somatic maturation, ear detachment, incisor eruption, fur development and eye opening were observed.

Reflex tests were performed according to previous studies [28, 29], with some modifications:



- Palmar grasp: The forelimbs were touched with a thin rod and the first day of not grasping onto the rod was recorded.
- Righting: Animals were placed in a supine position, and the PND in which they turned in a prone position was noted.
- Cliff avoidance: Rats were positioned with their snout and half of their anterior digits outside the edge of a platform (10 cm high). The test was considered positive when they moved away from the “cliff”.
- Negative Geotaxy: Rats were placed head down on an inclined platform (45°). The day they turned around (180°) was recorded.

2.3.2. Elevated plus maze

The elevated-plus-maze test was conducted on PND 25 and 80 using an apparatus consisting of two open and two enclosed arms of equal length and width (50 × 10 cm). The height of the closed arms was 40 cm, and the open arms had a curb with 1 cm high. The plus maze was elevated 50 cm above the floor. Each rat was placed in the center of the elevated plus maze facing one of the closed arms. The following parameters were observed for 3 and 5 min (PND 25 and 80, respectively): Number of entries and time spent (seconds) in the open and closed arms; number of rearing, stretching, grooming and head dipping movements [30].

2.3.3. Open field

To evaluate the behavior of the rats on PND 90, an open field was used, which consists of an arena with a diameter of 100 cm and a height of 40 cm. Each animal was placed in the central region of the arena and the following parameters were observed for 3 min: ambulation (number of sections inserted with all four legs, close to the arena wall); time near the arena wall; time in the center; freezing duration; latency until the first crossing and total number of crossings in the arena [31].

2.3.4. Light/dark box test



The light/dark box test was performed on PND 100. The device is a rectangular box divided in half into two chambers, one transparent and one opaque black (50 cm × 50 cm each), connected by a passage. The illuminated part of the box was illuminated with incandescent lamp from the top of the device. The dark part of the box was covered in black. The animal was placed in the passage, facing the dark field, and tracked for 5 min. The time in each box and the number of transitions were noted [32].

2.4. Statistical Analysis

Data are presented as mean ± standard error of mean (S.E.M.) and percentage. The results were compared among groups by Two-Way ANOVA followed Tukey's test or Chi-Square test. Differences were considered significant when $p \leq 0.05$. Statistical analyses were performed using the software GraphPad Prism (Version 8.0).

3. Results

3.1. Maternal body weight

All pregnant rats increased their body weight throughout pregnancy ($p < 0.05$) (Figure 2). However, on GD 20 maternal body weight was reduced in the ST/SE and SE groups when compared to the CO group ($F(1, 31) = 7.970$ $p < 0.05$).

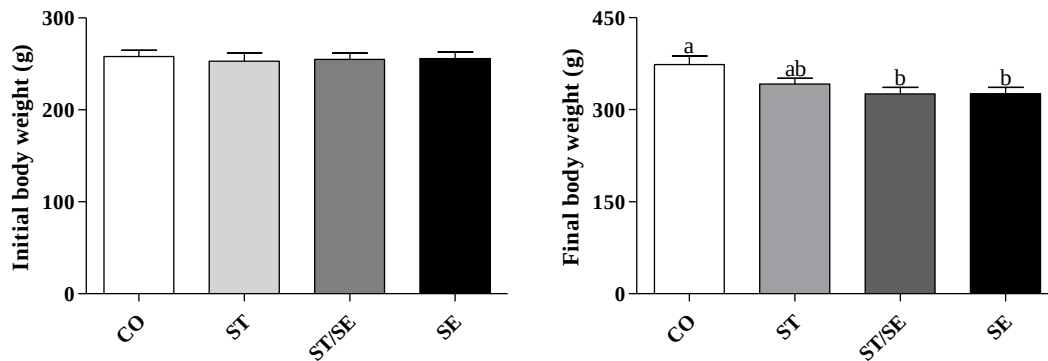


Figure 2. Initial and final body weight were observed on gestational days 0 and 20 respectively. Data are expressed as mean $\pm$ S.E.M. Repeated measures of two-way ANOVA with age as the repeat, followed Tukey's test (Different letters, "a" and "b", represent different results $p \leq 0.05$). CO: control (n=9); ST: stress (n=9); ST/SE: stress/sertraline (n=8); SE: sertraline (n=9).

3.2. Maternal vaginal bleeding

Between GD 15 and 17, approximately 30% of the rats in the ST/SE and SE groups (Figure 3) presented vaginal bleeding, which was not observed in the control and stress groups ($p < 0.0001$). This bleeding was not intense and was stopped on the same day.

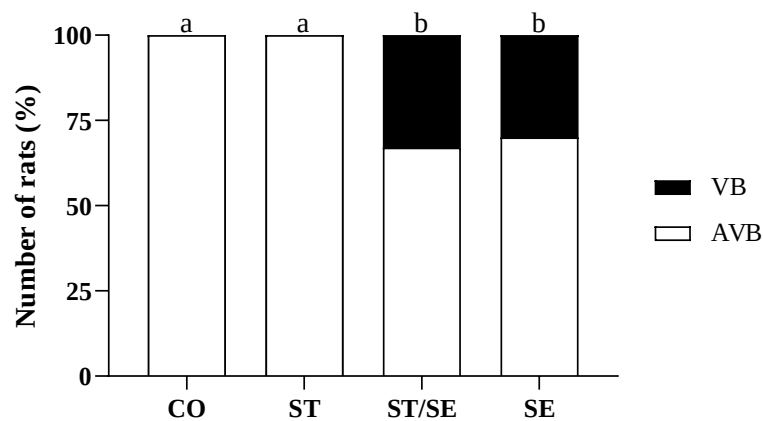


Figure 3. Number of rats (%) that had vaginal bleeding (VB) and absence of vaginal bleeding (AVB). CO: control (n=10); ST: stress (n=9); ST/SE: stress/sertraline (n=9); SE: sertraline (n=9). Values expressed as percentage (Chi-Square test; different letters, "a" and "b", represent different results $p < 0.0001$).

3.3. Litter features

The number of live pups did not differ between the experimental groups ($p > 0.05$). However, in the groups exposed to sertraline, 22-25% of litters presented 1 stillborn (Table 1). 1 female rat from the SE group and 1 from the ST/SE group did not give birth on the expected day, as a consequence, the pups were stillborn.

Table 1

Number of live and dead pups, observed on PND 1.

Features	CO (n=10)	ST (n=9)	ST/SE (n=8)	SE (n=8)
¹ Number of live pups	9.67±0.88	9.22±0.59	10.00±0.84	10.20±0.38
² Litter with dead pups (%)	0 ^a	0 ^a	25 ^b	22 ^b
¹ Sex ratio (M:F)	0.98±0.1	1.13±0.18	1.46±0.53	1.06±0.12

¹Values expressed as mean ± S.E.M. Two-Way ANOVA followed Tukey's test. ²Values expressed as percentage (Different letters, "a" and "b" represent different results, Chi-square test, $p < 0.0001$). CO: control; ST: stress; ST/SE: stress/sertraline; SE: sertraline.

3.4. Somatic maturation

The fur development and incisor eruption in the SE group were delayed when compared to the CO group ($F(1,30) = 4.761$ and 10.37 , respectively $p < 0.05$). The ST/SE group also showed a delay in the incisor eruption compared to the CO group ($p < 0.05$) (Figure 4).

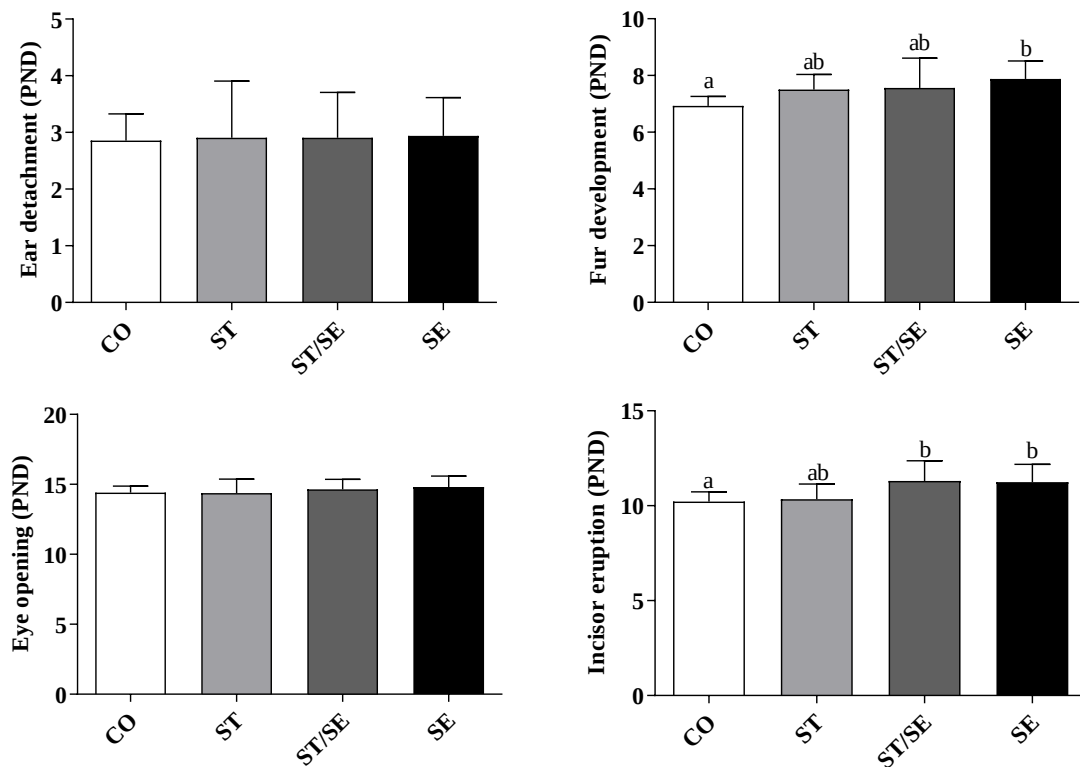


Figure 4. Maturation age (days) for physical features. Values expressed as mean $\pm$ S.E.M. Two-Way ANOVA followed Tukey's test (Different letters, "a" and "b", represent different results $p \leq 0.05$). CO: control (n=10); ST: stress (n=8); ST/SE: stress/sertraline (n=8); SE: sertraline (n=8).

3.5. Reflex development

The analysis of reflex development revealed that the SE group had a delay in negative geotaxis when compared to the CO group ($F(1,30) = 8.283$, $p < 0.05$) (Figure 5).

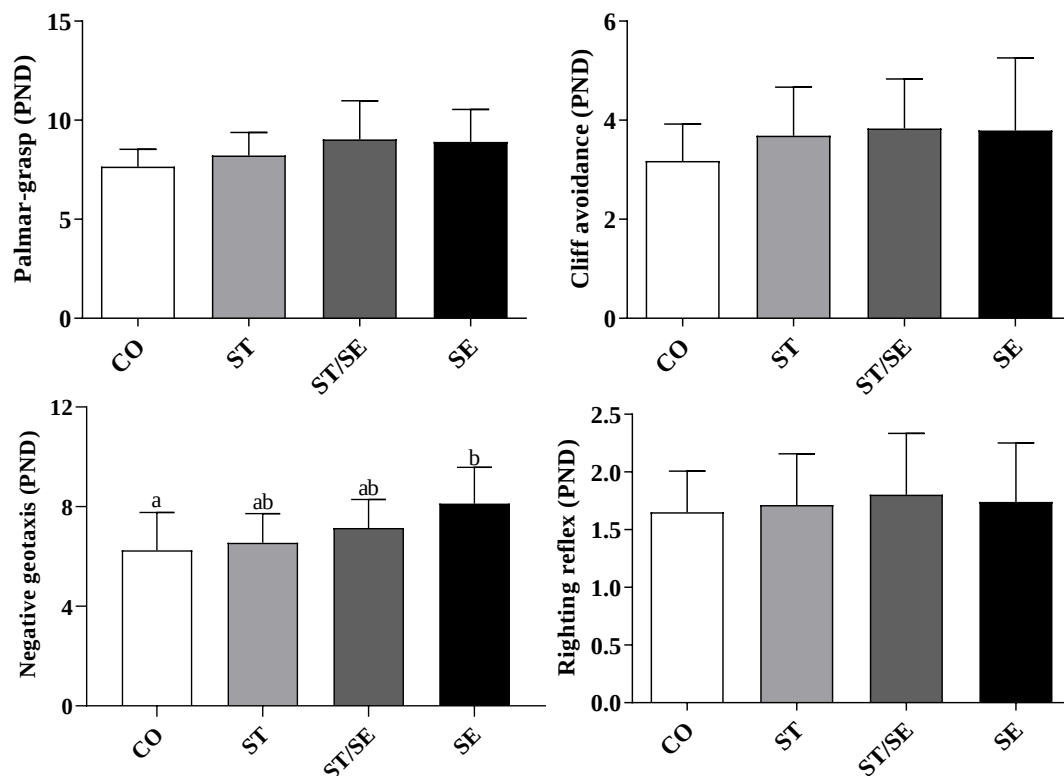


Figure 5. Maturation age (days) for reflex maturation. Values expressed as mean $\pm$ S.E.M. Two-Way ANOVA followed Tukey's test (Different letters, "a" and "b", represent different results $p \leq 0.05$). CO: control (n=10); ST: stress (n=8); ST/SE: stress/sertraline (n=8); SE: sertraline (n=8).

3.6. Elevated plus maze

The analysis of the elevated plus maze on PND 25 showed a decrease in the number of entries in the open arms and rearing in the SE group when compared to the ST group ($F(1,30) = 7.868$ and 5.536 respectively, $p < 0.05$).

There were no differences in the activities performed in the elevated plus maze between the experimental groups on PND 80 ($p > 0.05$) (Table 2).

Table 2

Elevated plus maze on PND 25 and 80.

Parameters	CO (n=10)	ST (n=8)	ST/SE (n=8)	SE (n=8)
<i>PND 25</i>				
Time in open arms (s)	39.20±11.06	34.75±6.95	31.63±11.71	22.31±5.77
Time in closed arms (s)	144.8±9.40	145.3±6.96	149.3±11.94	157.7±5.77
Open-arm entries (count)	3.55±0.76 ^{ab}	4.31±1.03 ^a	2.31±0.63 ^{ab}	1.3±0.43 ^b
Closed-arm entries (count)	7.95±0.42	8.31±0.68	7.25±1.21	5.06±0.95
Rearing (count)	13.10±0.47 ^{ab}	15.31±1.38 ^a	13.94±1.37 ^{ab}	10.88±1.23 ^b
Stretched (count)	10.45±1.15	11.56±0.49	10.38±1.13	8.25±1.27
Grooming (count)	2.45±0.27	2.56±0.35	2.18±0.51	3.44±0.43
Head dipping (count)	5.45±1.39	6.06±1.19	3.87±0.98	3.81±1.03
<i>PND 80</i>				
	CO (n=8)	ST (n=8)	ST/SE (n=8)	SE (n=8)
Time in open arms (s)	48.75±8.23	47.63±11.39	45.88±8.85	42.50±8.72
Time in closed arms (s)	251.3±8.23	247.8±13.74	259.1±7.38	257.5±8.72
Open-arm entries (count)	3.44±0.44	3.78±0.49	3.64±0.37	3.06±0.74
Closed-arm entries (count)	7.19±0.6	5.50±0.92	6.2±0.69	6.70±1.25
Rearing (count)	14.81±1.05	12.69±1.36	14.8±0.65	13.50±1.64
Stretched (count)	6.00±0.38	5.87±0.69	5.5±0.46	5.94±0.73
Grooming (count)	2.37±0.51	2.2±0.36	2.43±0.36	2.18±0.33
Head dipping (count)	5.62±0.68	5.25±1.27	7.93±1.17	5.75±1.19

Values expressed as mean ± S.E.M. Two-Way ANOVA followed Tukey's test (Different letters, "a" and "b", represent different results $p \leq 0.05$). CO: control; ST: stress; ST/SE: stress/sertraline; SE: sertraline.

3.7. Open field

The activity of rats in the open field showed that the ST group stayed longer in the central region compared to the CO group ($F(1, 30) = 4.476$, $p < 0.05$) (Table 3).

Table 3

Open field on PND 90.

Parameters	CO (n=10)	ST (n=8)	ST/SE (n=8)	SE (n=8)
Ambulation near the wall	39.6±4.36	49.13±2.32	47.44±4.25	48.50±4.39
Time near the wall (s)	153.6±3.33	158.6±2.58	150.8±5.82	157.7±4.09
Time in the center (s)	0.00±0 ^a	1.37±0.55 ^b	0.50±0.28 ^{ab}	0.62±0.42 ^{ab}
Freezing duration (s)	7.75±3.56	9.37±3.63	8.25±3.57	14.31±6.4
Latency until the first crossing (s)	40.75±10.11	39.31±9.91	35.88±9.65	42.25±15.45
Total crosses (count)	1.15±0.33	1.12±0.18	0.94±0.26	0.94±0.26

Values expressed as mean ± S.E.M. Two-Way ANOVA followed Tukey's test (Different letters, "a" and "b", represent different results $p < 0.05$). CO: control; ST: stress; ST/SE: stress/sertraline; SE: sertraline.

3.8. Light/dark box test

In the light/dark box the experimental groups did not differ in their behavior ($p > 0.05$) (Table 4).

Table 4

Light/dark box test on PND 100.

Parameters	CO (n=9)	ST (n=8)	ST/SE (n=8)	SE (n=8)
Latency to enter in the dark (s)	21.17±6.30	17.25±6.12	19.93±9.05	12.94±4.64
Transitions (count)	2.83±0.54	2.25±0.47	2.4±0.27	2.8±1.06
Time in the light (s)	49.14±10.56	53.00±10.79	53.03±7.90	46.63±17.32
Time in the dark (s)	126.3±13.41	127.0±10.79	131.1±8.01	133.4±17.32

Values expressed as mean ± S.E.M. Two-Way ANOVA followed Tukey's test ($p > 0.05$).

CO: control; ST: stress; ST/SE: stress/sertraline; SE: sertraline.



4. Discussion

The present study investigated the impact of gestational exposure to stress associated or not with sertraline on the neurobehavioral and reflex development of male offspring of rats. The impairment of maternal health was also verified. The results showed that sertraline causes delay in the somatic and reflex development of male offspring. In addition, the SE group was less exploratory compared to the CO and ST groups. Maternal health was also compromised in animals exposed to sertraline, as they showed reduced body weight and vaginal bleeding.

Depression, anxiety and stress are commonly seen in women during pregnancy, compromising maternal and fetal health [33]. Adverse events during intrauterine and postnatal periods not only may modify gene expression and development, but also can lead to permanent cognitive, neurobehavioral and motor changes in the offspring [23, 27, 34, 35].

Side effects caused by stress are related to atrophy and loss of neurons in the hippocampus [36] and to imbalance in the HPA axis, resulting in an increase in cortisol and catecholamines [11,37]. The HPA axis is one of the body's main physiological response pathways, allowing animals to respond appropriately to adverse conditions and maintain their homeostasis [38].

SSRIs are utilized to mitigate the impacts caused by mental disorders. They act by inhibiting the reuptake of serotonin, thereby increasing its concentration in the synaptic cleft. Thus, they normalize HPA axis function and hippocampal plasticity [39]. Several studies have shown the importance of serotonin in the proliferation, migration, apoptosis and synaptogenesis of neuronal cells [40].

Since SSRIs can cross the placenta and enter the fetal circulation, there is a concern that the changes in serotonin signaling could have important consequences for brain development. Sertraline, characterized as an SSRI (capable of modulating the levels of serotonin and also of other neurotransmitters such as dopamine), has been a recommended medication for pregnant and lactating women [16]. Because there are few reports in the literature about its action on brain development [41], the present study investigated possible impacts that this medication, associated or not with stress, has on the somatic, reflex and neurobehavioral development of male rat offspring.

Animal models such as rats and mice are commonly used in psychiatric studies because they have better etiological validity that induce pathological characteristics with



predictive validity. The brain development of humans and rats have some similarities regarding neurulation, neural differentiation, proliferation and cell migration, which allow a translational view of human exposure [13, 38].

The treatment period between GD 13 to 20 of the present study included the beginning of the appearance of serotonergic neurons in rats (GD 12 and 14) and the peak activity of these cells [24]. Serotonergic neurons are among the first neurons to be generated, and serotonin is released by growing axons before conventional synapses are established [43]. In rodents, the first 2 postnatal weeks are the critical period of neuronal maturation [35]. Therefore, in the present study our analysis of the development of somatic and reflexes started on the first postnatal day.

Physical landmarks such as eye opening, fur development and incisor eruption are characteristic of rats in the first days of life [44]. It was observed in the present study and also by Deiró et al. [45] that the animals treated with sertraline showed delay in the fur development and incisor eruption. This last landmark may have been influenced by alterations in serotonergic components, since serotonin plays an important role in the formation of craniofacial structures, including the maturation of the tooth germ [46].

The serotonergic system also plays a modulating role in the development of reflexes due to the hyperinnervation of serotonin in the primary sensory neocortex and α motor neurons [47, 48]. Reflex responses are characterized as involuntary movements commanded by the brainstem and spinal cord [49]. The analysis of reflex responses found that the SE group had a delay in performing negative geotaxis, as observed in studies using other types of SSRIs [50, 51, 52, 53]. This test, in addition to showing the degree of reflex development, also demonstrates the sensory, proprioceptive, and vestibular function activity [54].

Serotonergic changes, early in life, can modify the maturation of the neurotransmitter system and induce neurobehavioral alterations [55]. It is known that children exposed *in utero* to SSRIs are at risk of developing psychopathologies, including increased internalizing behavior, autism and depression during adolescence [1, 17].

Neurobehavioral analyses of rats are considered a key component in neurotoxicity tests, being observational and non-invasive measures that assess neurological, motor and functional integrity [56]. The use of elevated plus maze, open field and light / dark box tests allows the investigation of neurobehavioral development in animal models.



The elevated plus maze provides a measure of anxiety, fear and exploratory activity [57, 58]. In the present study, it was found on PND 25 that the SE group had a lower degree of exploration compared to the CO, and especially to the ST group. Rats with a profile found in the SE group are characterized as anxious, since they are less exploitative especially in potentially threatening regions. Previous studies of rats have also found that exposure to SSRI *in utero* induces reduced exploration in the offspring [57, 59, 60].

Although no significant behavioral differences were observed between the CO and ST groups in most analyses, the latter showed greater exploitation in regions considered “dangerous” for rodents. The study conducted by Kott et al. [61] reported that female offspring of mothers exposed to corticosterone before pregnancy entered more frequently in the open arms of the elevated plus maze than those just treated with oil. Szuran et al. [62] also observed a higher exploratory activity of male rats exposed to stress *in utero*. The increase in glucocorticoids caused by stress can be transferred from maternal to fetal circulation and provoke direct effects on brain development [37].

In the present study, the neurobehavioral changes observed in adulthood were not as significant as those observed in childhood. Other studies have shown that the use of SSRIs in pregnant women and young children can cause emotional alterations later in life. Experimental models that used fluoxetine [63] and citalopram [64] also showed neurobehavioral changes later in life. It is known that the period and time of exposure to sertraline and other antidepressants constitute a decisive factor in causing neurobehavioral changes in the offspring [61].

The open field test and light / dark test were employed to investigate the exploratory activity of animals in adulthood. The first test is determined by the conflict between the motivation to explore and the aversion to open spaces in the arena [65].

Rodents are known to be highly exploratory in both natural and captive situations and show preference for areas close to the periphery of the arena [66]. Corroborating this, all experimental rats in the present study spent a longer time in the peripheral region of the arena, reflecting an evolutionary behavior that avoids making them easy prey in open spaces.

The exploratory characteristic associated with the innate aversion of rodents to highly lit areas was investigated in the light / dark box test [67]. It was observed that all



experimental groups in adulthood presented similar results, which reflects a behavioral normalization between the ST and SE groups.

Interestingly, the ST/SE group showed unique characteristics in most of the tests performed (mainly in neurobehavioral analyses), being mostly similar to the CO group. Velasquez et al. [68] observed that prenatal exposure to stress and citalopram (SSRI) have different effects on fetal neurodevelopment. The antidepressant reversed the main effects of maternal gestational stress on the brain development. The authors found that the serotonin tissue content in the fetal forebrain increased in association with maternal stress, an increase that was reversed by citalopram.

Pregnant rats of the ST group and especially those exposed to sertraline, had their body weights reduced, corroborating previous studies [69, 70]. Despite the maternal body weight variation, the size of the litters remained unchanged. The ST group did not present litters with stillbirths, corroborating case and experimental studies [71, 72], although the groups exposed to sertraline showed 1 dead fetus in 22-25% of the litters. Van den Hove et al. [73] also observed an increase in neonatal deaths in the group exposed to paroxetine (SSRI) associated or not with stress, and did not find deaths in the group exposed only to stress. The use of SSRIs during pregnancy may increase the risk of premature birth and perinatal death, due to intrauterine growth retardation influenced by changes in placental blood flow [74].

In the present study some pregnant rats exposed to sertraline presented vaginal bleeding, as observed in case studies SSRIs can, in some rare clinical conditions, cause platelet dysfunctions by decreasing platelet content and capillary fragility even causing severe bleeding in patients [75, 76, 77].

Finally, although our results demonstrate the negative impact that sertraline has on maternal health and on the development of the nervous system, we reinforce the need for future studies to elucidate the molecular mechanisms involved in these alterations. In addition, we recognize the limitation in accessing a small number of puppies per litter. However, our results are extremely relevant to the scientific and medical community.

5. Conclusion

Our results show that pregnant rats exposed to sertraline presented decreased body weight and vaginal bleeding. Moreover, prenatal exposure to sertraline compromised the somatic, reflex and neurobehavioral development of the male offspring. Thus, our



findings reinforce the warning to the scientific and medical community that more experimental and clinical investigation are needed to assess the growing use of sertraline by pregnant women.

6. Declaration of competing interest

The authors declare that there are no conflicts of interest

7. Author contributions

Ana Flávia Quiarato Lozano: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Visualization, Writing-Original Draft.

Mayara Silva Moura and Bruna Marques Tavares: Methodology and Investigation

Wilma De Grava Kempinas: Conceptualization, Methodology, Formal analysis, Writing-Review and Editing, Project administration, Supervision.

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Capítulo 2

O estudo desenvolvido no experimento II, intitulado como “Reproductive Development of Male Rats Exposed In Utero to Stress and/or Sertraline” foi publicado na revista *Toxicological Sciences* (Fator de impacto= 4.84). Doi: <https://doi.org/10.1093/toxsci/kfac100>.



Reproductive development of male rats exposed *in utero* to stress and / or sertraline

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Abstract

Despite increased prescription of sertraline during pregnancy, little is known about its action on reproductive development. Therefore, the present study aimed to investigate the impact that stress, associated or not with sertraline, causes on the reproductive development of male rats. Pregnant Wistar rats were divided into 4 groups (n=16/group): CO – received filtered water; SE – received 20 mg/kg sertraline; ST – submitted to restraint stress and received filtered water; SS – submitted to restraint stress and received sertraline. The treatment was carried out from gestational days (GD) 13 to 20. The animals were euthanized on GD 20 (n=8/group), postnatal day (PND) 45 (n=8/group) and PND 110 (n=8/group). The testes and epididymis were analyzed histologically, and immunohistochemistry was performed on the testes by proliferating cell nuclear antigen (PCNA) and the Wilms tumor protein (Wt1). Sperm quality was also analyzed on PND 110. The evolution of body weight, anogenital distance (AGD), and puberty installation day were also verified. Statistical analysis: Two-way ANOVA or Kruskal-Wallis test ($p \leq 0.05$). Fetal testes presented a large number of acidophilic cells in the sertraline-exposed groups. The SS group also showed a decrease in the nuclear volume of Leydig cells. This same group showed low expression of PCNA and Wt1, decreased weight of the testes and epididymis, lower AGD, and delayed puberty installation. The adulthood groups exposed to sertraline presented alterations in sperm morphology and motility. The results demonstrated that prenatal exposure to sertraline compromises the development of the rat reproductive system.

Keywords: Gestational stress, sertraline, reproductive system, male rats.



1. Introduction

In recent years, depression has been one of the most disabling disorders in the world, affecting about 322 million people (World Health Organization, 2017). Pregnant women are often diagnosed with this condition and about 13 to 36% have a depressive disorder (Marcus *et al.*, 2012; Mikšić *et al.*, 2018). Factors such as stress, anxiety, lack of social support, low socioeconomic status, maternal age, unplanned (or unwanted) pregnancy and even seasonality contribute to the emergence of depression (Lancaster *et al.*, 2010; Bais *et al.*, 2018). Importantly, the pandemic caused by Severe Acute Respiratory Syndrome Coronavirus (COVID-19) is also associated with psychiatric implications, since immune dysregulation can lead to increased cortisol and reduced serotonin levels (Hamed and Hagag, 2020). Not only do biological mechanisms contribute to psychiatric worsening, but also stressors such as fear of the disease, uncertainty about the future and social isolation (Mazza *et al.*, 2020).

Depression and stress are related to maternal and neonatal morbidities, including premature birth, low birth weight, cognitive, emotional and behavioral problems (Bais *et al.*, 2018). Since the negative impacts of these psychiatric disorders can be greater than those caused by treatment with antidepressants/anxiolytics, drug therapy is usually prescribed (Latendresse *et al.*, 2017; Lenoir *et al.*, 2020).

Antidepressants, whose consumption has increased in recent years, are the third most recommended medication in the United States (Pratt *et al.*, 2017). Studies suggest that the prevalence of antidepressant use during pregnancy has increased 3 to 7 times in recent decades and that about 13% of pregnant women are exposed to these medications (Uguz, 2019).

Selective Serotonin Reuptake Inhibitors (SSRIs) are the most widely used and prescribed class of antidepressants (Pogliani *et al.*, 2019). Sertraline, one of the most recommended SSRIs, is used by pregnant and lactating women because it is quickly metabolized and is little transferred to milk (Cuomo, Maina, Stephen M. Neal, *et al.*, 2018). This antidepressant has a half-life of 26 hours, and about 98% of sertraline is bound to plasma proteins. During its metabolism, predominantly hepatic, sertraline gives rise to N-desmethylsertraline, which has a low affinity for the serotonin receptor and, therefore, is not associated with a clinical effect (Dziurkowska and Wesolowski, 2018).

Although some studies have not observed adverse effects of sertraline exposure during gestation and lactation, recent reports have shown serious impacts on fetal



development, by impairing the structure and functioning of the skeletal, cardiovascular and nervous systems. These alterations may be caused by the transmission of sertraline into the milk and across the placental barrier (Bérard *et al.*, 2015; Shen *et al.*, 2017; Lozano *et al.*, 2021; Dziurkowska and Wesolowski, 2018; Cuomo, Maina, Stephen M Neal, *et al.*, 2018).

A study by Atli *et al.* (2017) in adult rats showed that sertraline can increase testicular oxidative stress, alter sex hormone concentrations and compromise sperm quality. Although the impact of sertraline on the male reproductive system in adults is already described, reports on its effect during fetal development are scarce. Therefore, the present study investigated the impacts caused by this drug associated or not with stress on the development of the reproductive system of male rats.

2. Materials and methods

2.1. Animals and treatment

Sixty-four female rats and twenty male Wistar rats were obtained from the Central Biotherium of UNESP Botucatu-SP, Brazil, and kept under controlled conditions (22°C, 30% air humidity, 12/12-h light/dark cycle) in the Biotherium at the Center for Toxicological Assistance, with food and water provided *ad libitum*. The animals were kept in accordance with the Ethics Committee for the Use of Experimental Animals of São Paulo State University, protocol number 1169.

At 90 days of age, the rats mated. On the first day of pregnancy (determined by the presence of sperm in the vaginal smears) 64 females were divided into four experimental groups (16 animals/group: CO: Animals received filtered water (vehicle) via gavage, once a day. ST: Animals were restrained in an acrylic cylinder for 1 hour/day, and received vehicle. SS: Animals were restrained in an acrylic cylinder and received 20 mg/kg/day of sertraline hydrochloride, via gavage. SE group: Animals received sertraline hydrochloride 20 mg/Kg/day, via gavage. The treatment was carried out from gestational day (GD) 13 to 20, which is considered a critical period of male reproductive development, as there is augmented proliferation of Sertoli and germ cells, in addition to an increase in testosterone levels (Ward, 1972; Shima and Morohashi, 2017).

Stress was induced in rats by the restraint method, which is already recognized in the literature as one of the best methods to activate the hypothalamic-pituitary-adrenal axis, without causing nociceptive stimuli (Glavin *et al.*, 1994).



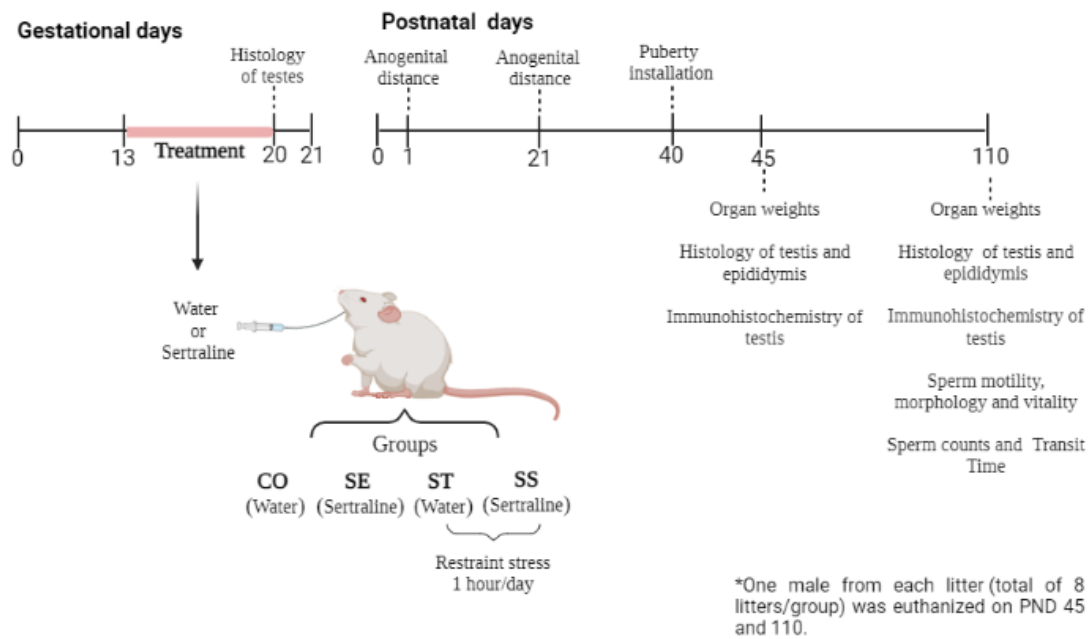
It is already established that the dose of sertraline used in the present study leads to an antidepressant effect in rats. The dose was considered moderate and had been used in previous studies (Kott *et al.*, 2018; Atli *et al.*, 2017; Aynaoglu Yildiz *et al.*, 2019). The pharmaceutical drug, purchased from Cruz Vermelha (Botucatu, Brazil), was diluted in filtered water and administered from 12:00 to 1:00 pm.

2.2. Experimental Design

On GD 20, 32 (n=8/group) pregnant dams were euthanized to collect the fetal gonads of male offspring.

The other 32 pregnant female rats (n=8/group) were kept until the end of the lactation, for evaluation of male offspring. It is important to note that the birthrate did not differ between male and female progenies. Thus, the same proportion of pups per litter (4 males and 4 females) was maintained. The female offspring had been evaluated in another study, not shown herein.

The evolution of body weight and the day of puberty onset were recorded. On PND 1 and 21 the anogenital distance (AGD) was verified, and on PND 45 and 110 one male rat per litter was euthanized by CO₂ asphyxiation followed by decapitation. Organs were collected and testes and epididymis were separated for histological analysis and sperm count. Sperm were also collected to assess morphology, motility and vitality. Immunohistochemistry of proliferating cell nuclear antigen (PCNA) and Wilms tumor protein (WT1) was also performed in the testes (Figure 1).



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Figure 1. Experimental design.

2.3. AGD and preputial separation

AGD was evaluated on PND 1 and 21 and was normalized with the cube root of body weight. From PND 40, puberty onset was evaluated, which occurs with complete preputium retraction and glans exposure.

2.4. Organ collection for histology

The left epididymis and testis were collected, fixed in Bouin's solution (75% picric acid, 25% formaldehyde, and 5% glacial acetic acid) for 24h and processed for histological analysis. The organs were embedded in Paraplast and cut at a thickness of 5µm. Subsequently, the sections were stained with hematoxylin and eosin (HE) for histological analysis. Only one animal from each litter (total of 6 litters/group) was evaluated for histological and immunohistochemical aspects (LINDER *et al.*, 1995; Borges *et al.*, 2020).

2.5. Histological and histopathological analyses of testes

On GD 20, PND 45 and PND 110, 100 seminiferous tubules/animal were histologically analyzed. Histological alterations were classified as: acidophilia, vacuoles,



multinucleated cells, retained spermatid and epithelial degeneration (Creasy *et al.*, 2012).

The interstitium was also analyzed and characterized for the presence of influx of defense cells, alterations in blood vessels, presence of acidophilic cells, and morphological changes in Leydig cells.

2.6. Sertoli cell and gonocyte count

The Sertoli cell nuclei and gonocytes from 20 seminiferous tubules/animal were counted. Subsequently, the numerical proportions of Sertoli cells and gonocytes were obtained (Toledo *et al.*, 2011)

2.7. Maturation degree

To evaluate the degree of maturation of the seminiferous epithelium (Toledo *et al.*, 2011), 100 cross-sections of seminiferous tubules/ animal were randomly evaluated, using the method of assigning values according to the most mature germ cell type present in the tubular epithelium. The degrees were rated from 1 to 7:

- 1: Presence of spermatocytes I or II;
- 2: Spermatids in stage 1 to 8 of spermiogenesis;
- 3: Spermatids in stage 9 to 14 of spermiogenesis;
- 4: Spermatids in stage 15 to 18 of spermiogenesis;
- 5: Spermatids in stage 19 of spermiogenesis in small quantity;
- 6: Spermatids in stage 19 of spermiogenesis in average amount;
- 7: Spermatids in stage 19 of spermiogenesis in larger quantity.

The number of seminiferous tubules in each degree was multiplied by its degree. The values were added and divided by 100, resulting in the average degree.

2.8. Spermatogenesis dynamics

In order to evaluate the dynamics of spermatogenesis, the relative frequency of stages was estimated as follows: I-VI (two generations of spermatids), VII-VIII (mature spermatids), IX-XIII (only one generation of spermatids), and XIV (secondary spermatocytes) in 100 cross-sections of seminiferous tubules per animal. The relative frequency of stages estimates the rate or duration of the spermatogenic process (Clermont and Harvey, 1965).



2.9. Type A spermatogonia count

On PND 45 and 110, type A spermatogonia were counted in 20 seminiferous tubules at maturation degree 3 and at stage VII of spermatogenesis, respectively (Vieira and Favareto, 2017).

2.10. Seminiferous tubule morphometry and Leydig cell volume

The morphometry of seminiferous tubules was analyzed on GD 20, PND 45 and 110. Ten sections of seminiferous tubules (classified as grade 3 on PND 45, and stage IX on PND 110) per animal were evaluated, at 400x magnification, using the software packages Leica Q-win Version 3 and ImageJ 1.48v.

For nuclear volume of Leydig cells, 50 nuclei that presented circular or elliptical shape were randomly chosen, in order to calculate the volume of these cells. The software Leica Q-win Version 3 for Windows TM was used to obtain the images and subsequently the measurement was performed with the aid of the software ImageJ 1.48v. The volume was determined using the formula: $V = [D \times \pi] / 6$, where D is the diameter (Borges *et al.*, 2020).

2.11. Immunohistochemistry for cell proliferative potential

The testicular sections on PND 45 and 110, previously fixed and embedded in paraffin, were deparaffinized and hydrated. Next, the sections were submitted to the antigen recovery procedure, by immersion in citrate buffer (0.1M, pH 6.0) for 20 min in a microwave and subsequently incubated with 3% hydrogen peroxide in PBS to block the endogenous peroxidase. Unspecific blocking was performed with 3% BSA in PBS (blocking solution) for 30 min before the incubation with the primary antibody anti-PCNA antibodies (PCNA PC10: sc-56, Monoclonal, Santa Cruz Biotechnology, CA, USA – 1:100) and anti-WT1 (4 µg/ml, SC215 393498 / H-1; Santa Cruz Biotechnology-1:100) overnight at 4°C. For negative controls, sections were incubated in the blocking solution, without the primary antibody.

Afterwards, the sections were washed with PBS and incubated with secondary antibodies (HRP anti-mouse goat; 1.6 µg/ml SC-2005; 218 Santa Cruz Biotechnology-1:200). Then, after washing with PBS, the sections were exposed to diaminobenzidine (DAB) associated with hydrogen peroxide for 4 minutes. Finally, the sections were



counterstained with hematoxylin, dehydrated in an increasing series of ethanol, cleared in xylol and mounted for analysis.

The semi-quantitative evaluation of immunohistochemistry was performed on 50 seminiferous tubules per animal, and the cellular immunostaining intensity was classified as weak or strong (de Barros *et al.*, 2018).

2.12. Epididymis histopathology

Qualitative analysis of the epididymis on PND 45 and 110 occurred throughout the entire length of the organ, using the software Leica Q-win Version 3 for Windows TM. The presence of sperm, acidophilia, vacuoles, cell debris and cribriform appearance was noted (Sun and Flickinger, 1979; De Grava Kempinas and Klinefelter, 2015).

2.13. Sperm parameters

Sperm motility

Sperm were released from the right proximal epididymal cauda in 1 ml of PBS and 1% of BSA. Sperm motility was evaluated in a 10 μ L aliquot of sperm suspension, which was immediately transferred to a Makler chamber and maintained at 36°C. One hundred sperm were counted and classified as: 1- mobile with progressive movement, 2- mobile without progressive movement, 3-immobile.

Sperm Morphology

A 100 μ L aliquot of the sperm suspension from sperm motility was added to 900 μ L of formol saline (810 μ L saline + 90 μ L formol). To analyze sperm morphology, smears were prepared on histological slides and 200 spermatozoa per animal were analyzed under a phase-contrast microscope (400X magnification). Abnormalities were classified for head morphology (without characteristic curvature or isolated head) and tail morphology (broken or rolled into a spiral). Sperm were also classified as to the presence or absence of the cytoplasmic droplet.

Sperm vitality

In a tube, 50 μ L of spermatozoa (diluted in PBS-BSA 1%), one drop of yellow eosin 3% and two drops of nigrosine 8% were added. The solution was homogenized after



which smears were made on slides. For each animal, two smears were performed; a total of 100 cells were counted; and the mean of the two results was obtained. Sperm were considered dead when stained red, and alive when unstained. Vitality was analyzed under phase microscopy at 400X magnification.

Sperm Counts and Sperm Transit Time

Testes were decapsulated, weighed and homogenized in 5 ml of NaCl 0.9% containing Triton X 100 0.5%, followed by sonication for 1 min. After a 10-fold dilution, one sample was transferred to Neubauer chambers (4 fields per animal), and mature spermatids (stage 19 of spermiogenesis) were counted. To calculate the daily sperm production (DSP), the number of spermatids at stage 19 was divided by 6.1, which is the number of days of the seminiferous cycle during which these spermatids are present in the seminiferous epithelium. The caput/corpus and cauda epididymides were cut into small fragments with scissors and homogenized. Sperm were counted as described for the testes. The sperm reservoir in the cauda region of the epididymis was calculated by combining the number of cells in the sperm suspension for sperm motility and the sperm count in the homogenized tissue. The sperm transit time through the epididymis was determined by dividing the number of sperm in each region of the epididymis by the DSP (Borges *et al.*, 2020).

2.14. Statistical analysis

The results were analyzed using two-way ANOVA parametric test, followed by the Tukey test, or the non-parametric Kruskal Wallis test. Differences were considered significant when $p \leq 0.05$. Statistical analysis was performed using the program GraphPad Prism (Version 8).

3. Results

3.1. Evolution of body weight

The body weight of animals was lower in the SE and SS groups than in the other experimental groups on PND 1 ($p < 0.001$). From PND 7 to 21, only the SE group continued to present a lower body weight compared to the CO and ST groups ($p < 0.05$).



On PND 45 and 110, no differences were observed among the experimental groups ($p>0.05$) (Figure 2).

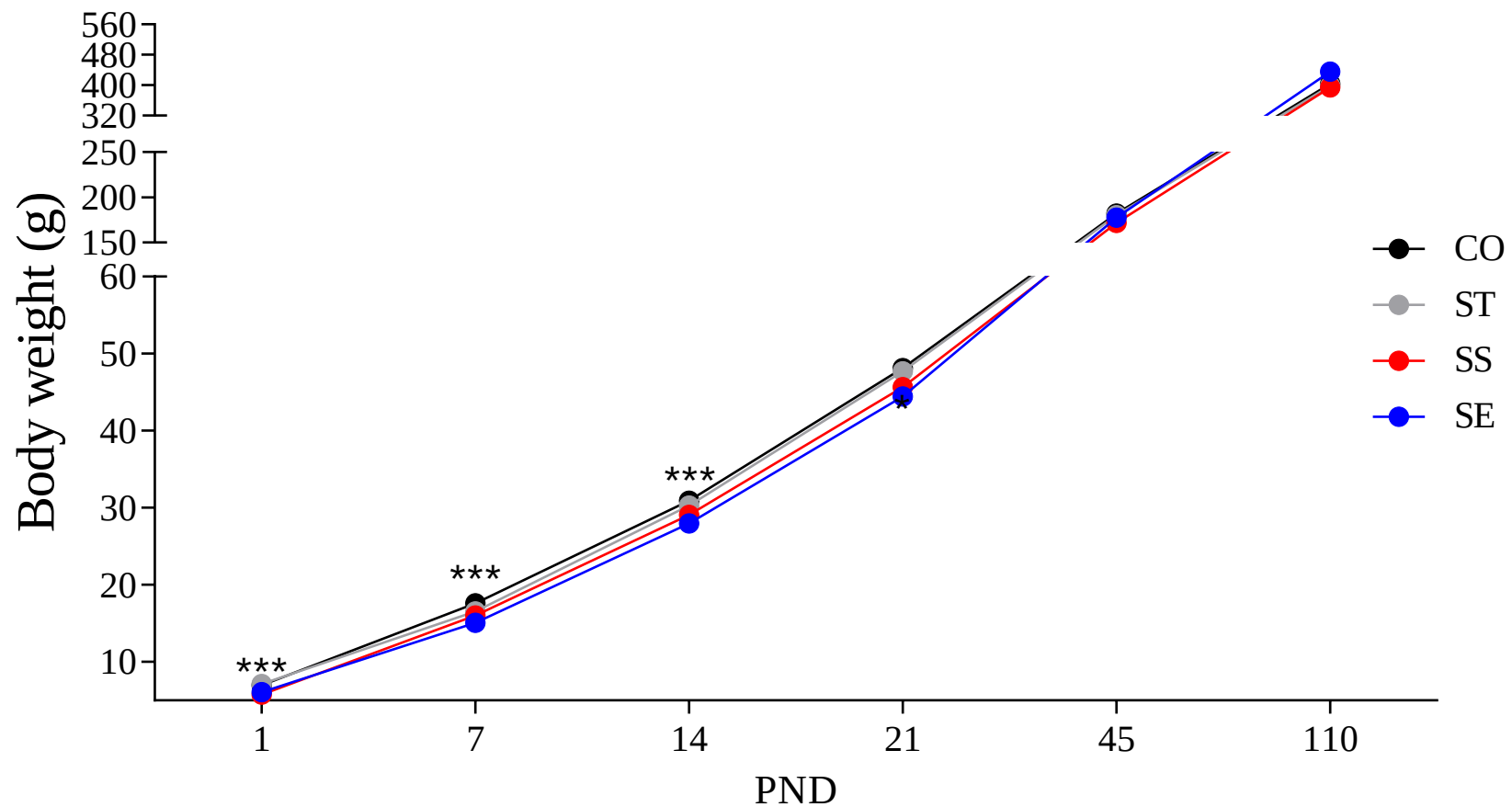


Figure 2. Evolution of body weight (n=8/group). Values expressed as mean $\pm$ S.E.M. Two-way ANOVA followed by Tukey's test, $p < 0.001$ (***), $p < 0.05$ (*). CO: control, ST: stress, SS: stress/sertraline, SE: sertraline, PND: postnatal day.

3.2. Organ weights

On PND 45, the SS group presented lower weights of the epididymis ($p=0.006$) and testes ($p=0.04$) when compared to the CO group (Table 1). On PND 110 the organ weights did not differ among the experimental groups ($p>0.05$).

Table 1. Organ weights

Organs	CO (n=8)	ST (n=8)	SS (n=8)	SE (n=8)
<i>PND 45</i>				
Testes (g)	0.95±0.03 ^a	0.92±0.03 ^{ab}	0.83±0.03 ^b	0.88±0.02 ^{ab}
Epididymis (mg)	136.8±5.0 ^a	132.4±4.0 ^{ab}	117.2±5.4 ^b	124.6±4.8 ^{ab}
Ventral prostate (mg)	97.0±6.2	102.1±7.5	74.5±8.6	101.6±7.0
Seminal vesicle (mg)	74.4±4.8	79.5±10.8	63.46±9.1	79.26±7.9
Liver (g)	9.4±0.3	9.8±0.3	8.96±0.3	9.77±0.5
Kidneys (g)	1.9±0.07	1.9±0.07	1.8±0.06	1.9±0.09
Brain (g)	1.8±0.03	1.8±0.02	1.79±0.03	1.80±0.02
Lungs (g)	1.2±0.09	1.2±0.05	1.2±0.07	1.3±0.09
Heart (g)	0.8±0.30	0.8±0.30	0.8±0.20	0.8±0.30
Adrenals (mg)	44.0±2.1	39.2±2.5	41.2±4.2	42.4±2.3
Thyroids (mg)	14.5±0.8	12.6±0.9	14.2±1.6	16.5±0.9
Pituitary (mg)	6.0±0.25	6.6±0.20	6.4±0.30	6.1±0.30
<i>PND 110</i>				
Testes (g)	1.7±0.04	1.6±0.07	1.5±0.04	1.5±0.05
Epididymis (mg)	625.3±17.8	584.6±30.1	607.0±18.2	589.6±21.7
Ventral prostate (mg)	441.4±19.0	447.8±20.1	401.6±39.7	472.3±25.8
Seminal vesicle (mg)	940.0±43.9	992.7±69.1	911.7±91.8	976.4±83.9
Empty seminal vesicle (mg)	445.3± 25.3	425.0±33.8	396.4±13.9	406.6±26.6
Liver (g)	1.4±0.5	1.3±0.4	1.3±0.6	1.6±0.2
Kidneys (g)	3.2±0.1	3.2±0.1	3.0±0.1	3.2±0.2
Brain (g)	2.0±0.4	2.0±0.2	2.0±0.3	1.9±0.7
Lungs (g)	1.7±0.04	1.6±0.08	15.0±0.09	15.8±0.08
Heart (g)	0.4±0.02	0.4±0.02	0.4±0.04	0.4±0.03
Adrenals (mg)	66.6±3.8	67.6±4.7	60.4±5.4	65.2±6.3
Thyroids (mg)	20.1±1.7	20.3±1.8	19.6±1.9	21.4±2.8
Pituitary (mg)	10.3±0.6	8.9±0.7	10.9±1.0	9.0±0.7

Data expressed as mean ± SEM (ANOVA followed Tukey's test). CO: control, ST: stress, SS: stress/sertraline, SE: sertraline. Different letters indicate $p<0.05$. One male from each litter (total of 8 litters/group) was euthanized on PND 45 and 110.

3.3.AGD and puberty onset

On PND 1, the AGD of the SE group was lower than in the CO group ($p=0.05$). On PND 21, SS was the only group that showed a significant decrease, compared to CO ($p=0.01$) (Figure 3).

The day of partial retraction of prepuce did not differ among the experimental groups ($p>0.05$). The total separation of the preputium was delayed in the SS group when compared to the CO group ($p = 0.02$) (Figure 3).

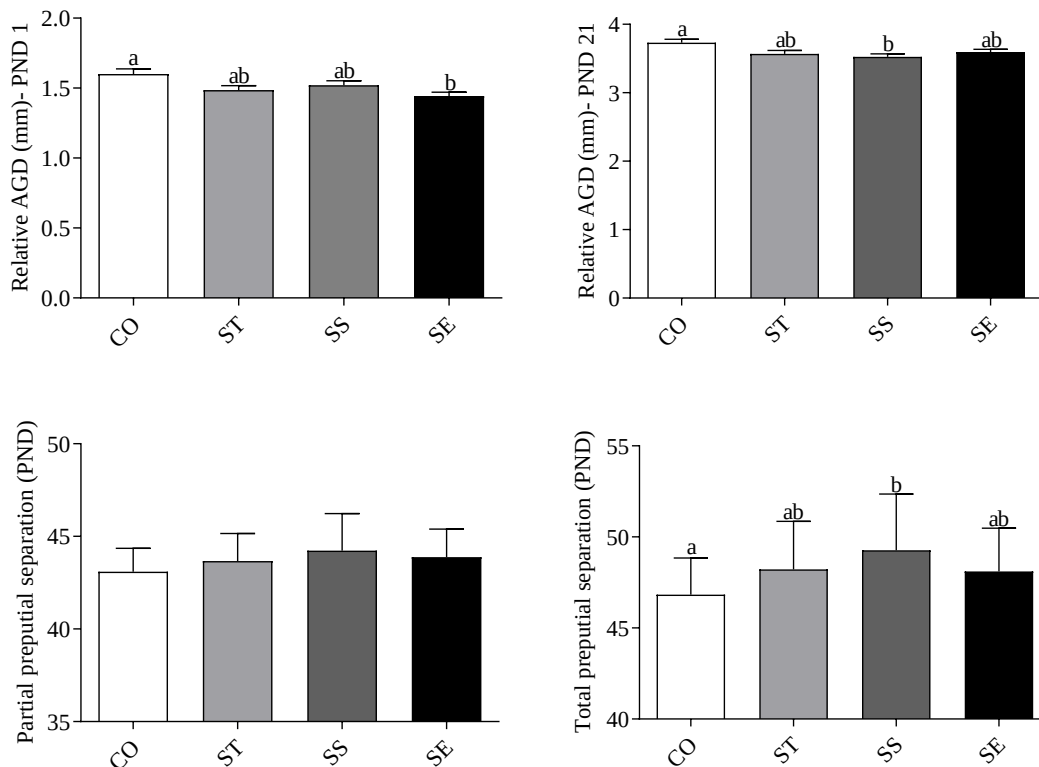


Figure 3. Anogenital distance (AGD) on PND 1 and 21, and partial and total preputial separation ($n= 8/\text{group}$). Values expressed as mean $\pm$ SEM (Two-way ANOVA followed the Tukey test). Different letters express $p < 0.05$. CO: control, ST: stress, SS: stress / sertraline, SE: sertraline, PND: post-natal day.

3.4. Histological analysis of the testes

3.4.1. Fetal testes

The respective numbers of seminiferous tubules, Sertoli cells and gonocytes classified as normal were similar among the experimental groups ($p>0.05$) (Table 2).

Histopathological alterations of the seminiferous tubules were quantified according to the level of severity, with vacuolization being classified as the most severe.

Animals in the ST group frequently presented acidophilia and vacuoles in the same tubule, thus they were classified as tubules with vacuoles. A smaller volume of Leydig cells was observed in the SS group compared to the CO group ($p=0.04$) (Table 2).

Table 2. Histology and morphometry of fetal testes

Parameter	CO (n=6)	ST (n=6)	SS (n=6)	SE (n=6)
¹ Normal tubules (%)	72.2±5.0	70.0±5.6	59.2±11.0	66.5±7.15
¹ Vacuolization	20.6±4.2	27.4±6.4	23.6±7.6	20.2±3.5
¹ Acidophilic cells	7.0±3.4	2.8±1.3	17.1±5.2	10.3±2.3
² Sertoli cells/tubule	12.1±0.3	11.9±0.6	11.2±0.4	11.7±0.4
² Gonocytes/tubule	4.0±0.3	3.8±0.2	3.4±0.2	3.4±0.1
² Sertoli cells/Gonocytes	3.2±0.2	3.4±0.2	3.4±0.3	3.6±0.2
² Tubular diameter (µm)	62.1±2.7	58.9±1.3	56.9±1.7	56.5±1.4
² Leydig cell volume (µm ³)	92.8±4.9 ^a	75.5±6.0 ^{ab}	73.8±7.3 ^b	80.8±6.12 ^{ab}

¹Values expressed as mean ± S.E.M (Kruskal-Wallis test). ²Values expressed as mean ± S.E.M (Two-way ANOVA followed by Tukey's test). Different letters indicate $p<0.05$. Testicular histology of one animal from each litter (total of 6 litters/group).

Analysis of the fetal testicular interstitium showed an influx of defense cells in the SS and SE groups. Acidophilic cells were also observed in both groups that received sertraline (Figure 4) when compared to the CO group.

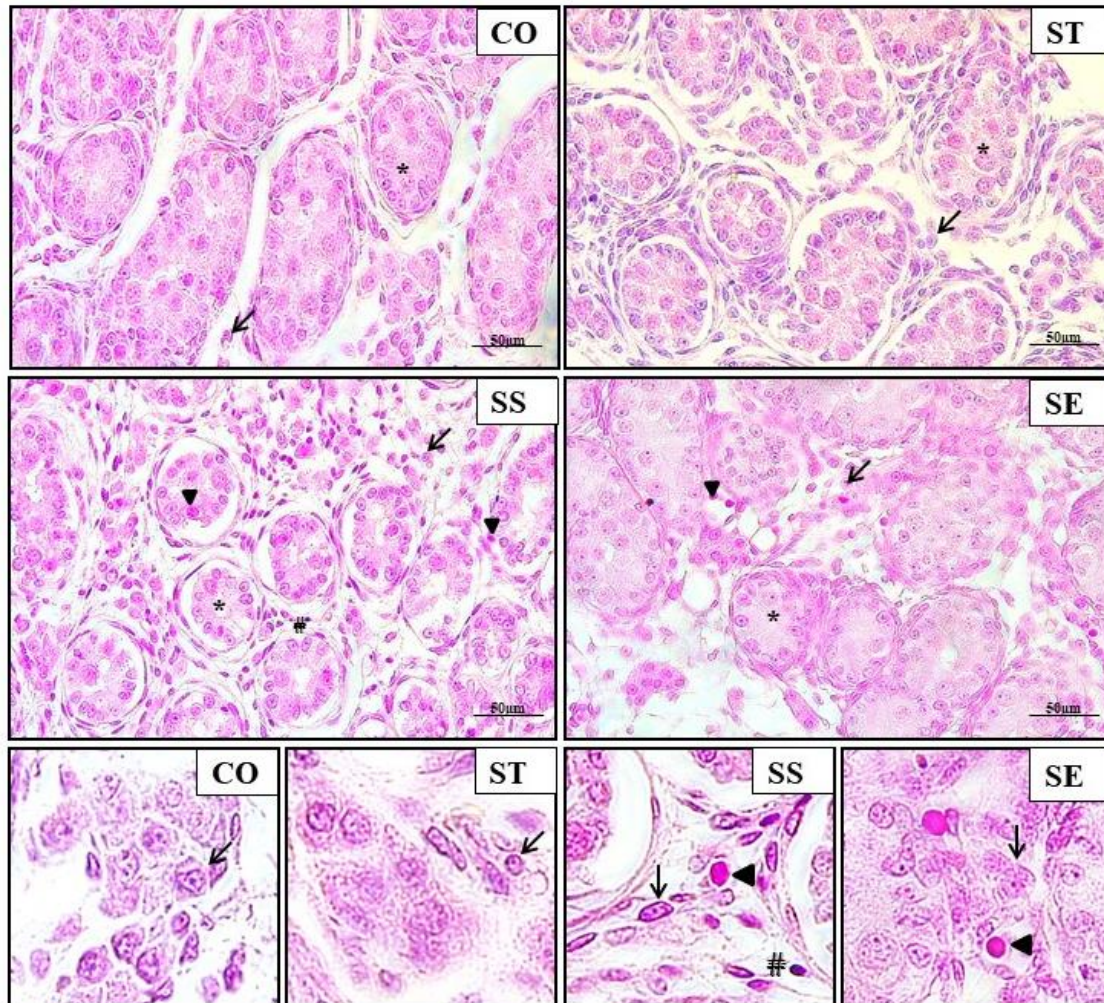


Figure 4. Cross-sections of fetal testis stained with HE. CO group, ST group, SS group, SE group. (*) Seminiferous tubule, (arrow) Leydig cells, (#) leukocyte infiltrate; (arrowhead) acidophilic cell. 400x magnification.

3.4.2. Testes on PND 45 and 110

On PND 45, no histopathological or morphometric differences were observed in the testes among the experimental groups ($p > 0.05$). The number of type A spermatogonia in grade 3 seminiferous tubules was lower in the SE group ($p = 0.05$) than in the ST group.

On PND 110, only the SS group showed an increase in acidophilic cells compared to the CO group ($p = 0.01$) (Table 3).

Table 3. Testicular histopathology and morphometry on PND 45 and 110

Parameter	CO (n=6)	ST (n=6)	SS (n=6)	SE (n=6)
<i>PND 45</i>				
¹ Normal tubules (%)	87±1.6	85±1.7	85±3.5	81±2.2
¹ Vacuolization	8.3±2.0	7.6±1.2	9.6±2.4	11.3±2.2
¹ Acidophilic cells	4.6±0.7	7.0±0.8	4.3±1.2	6.5±1.1
¹ Exfoliation	0.1±0.1	0.3±0.2	0.3±0.2	0.8±0.4
² Maturation degree	3.88±0.04	3.63±0.09	3.72±0.13	3.59±0.09
² Leydig cell volume (µm ³)	104.9±4.5	100.9±4.8	93.47±2.2	98.2±4.3
² Tubular diameter (µm)	218.6±28.6	239.5±63.2	231.2±79.0	227.2±60.1
¹ Spermatogonia type A	62.7±2.2 ^{ab}	64.6±1.8 ^b	58.0±1.4 ^a	57.8±1.7 ^a
² Epithelium height (µm)	79.7±1.7 ^a	91.1±2.6 ^b	86.9±2.2 ^{ab}	85.7±2.1 ^{ab}
<i>PND 110</i>				
¹ Normal tubules (%)	89±1	78.4±7.8	85.6±1.6	88.8±2.4
¹ Vacuolization (%)	1.4±0.5	2.0±0.6	1.4±0.2	1.6±0.5
¹ Acidophilic cells (%)	8.2±0.9 ^a	11.2±0.2 ^{ab}	13.8±0.5 ^b	9.2±2.0 ^{ab}
¹ Dynamics of spermatogenesis (%)				
I – VI	40.2±0.4	40.4±1.2	36.6±2.0	42.72±0.85
VII-VIII	30.4±1.2	28.6±2.3	30.2±2.0	28.0±1.1
IX-XIII	28.0±1.0	28.0±1.8	30.8±3.0	27.0±1.6
XIV	1.4±0.2	3.0±0.4	2.4±0.2	1.6±0.7
² Leydig cell volume (µm ³)	98.6±1.9 ^{ab}	89.3±5.8 ^{ac}	82.0±2.5 ^c	105.9±3.9 ^b
² Tubular diameter (µm)	276.0±5.1	280.4±3.6	279.9±6.8	275.8±8.2
² Epithelium height (µm)	92.1±2.7	97.9±3.2	93.9±2.6	95.9±2.1
¹ Spermatogonia type A	55.6±0.6	55.9±0.6	56.6±0.7	54.9±0.5

¹Values expressed as mean ± S.E.M (Kruskal-Wallis test followed by Dunn's test).

²Values expressed as mean ± S.E.M (ANOVA followed by Tukey's test). Different letters indicate p<0.05. CO: control; ST: stress; SS: stress/sertraline; SE: sertraline. Testicular histology of one animal from each litter (total of 6 litters/group).

Testicular histology on PND 45 and 110 (Figure 5).

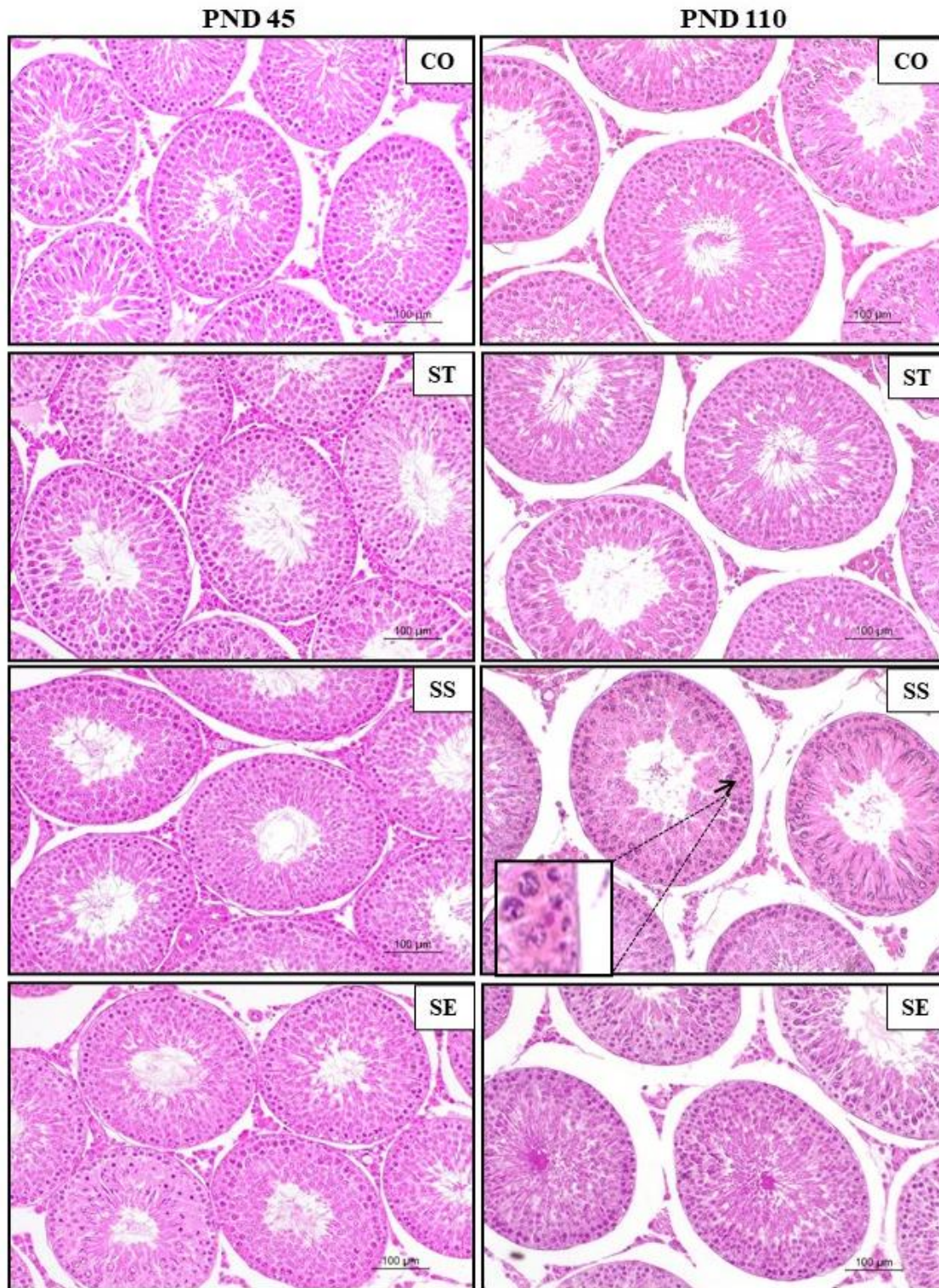


Figure 5. Cross-sections of testes on PND 45 and 110 stained with HE. CO group, ST group, SS group, SE group. (Arrow) Acidophilic cells.

3.5. Immunohistochemistry

3.5.1. PCNA

Qualitative analysis of PCNA immunohistochemistry on PND 45 revealed weak staining in sertraline-treated groups, especially in the SE group (Figure 6 and Table 4). On PND 110 there was no difference among groups (results not shown).

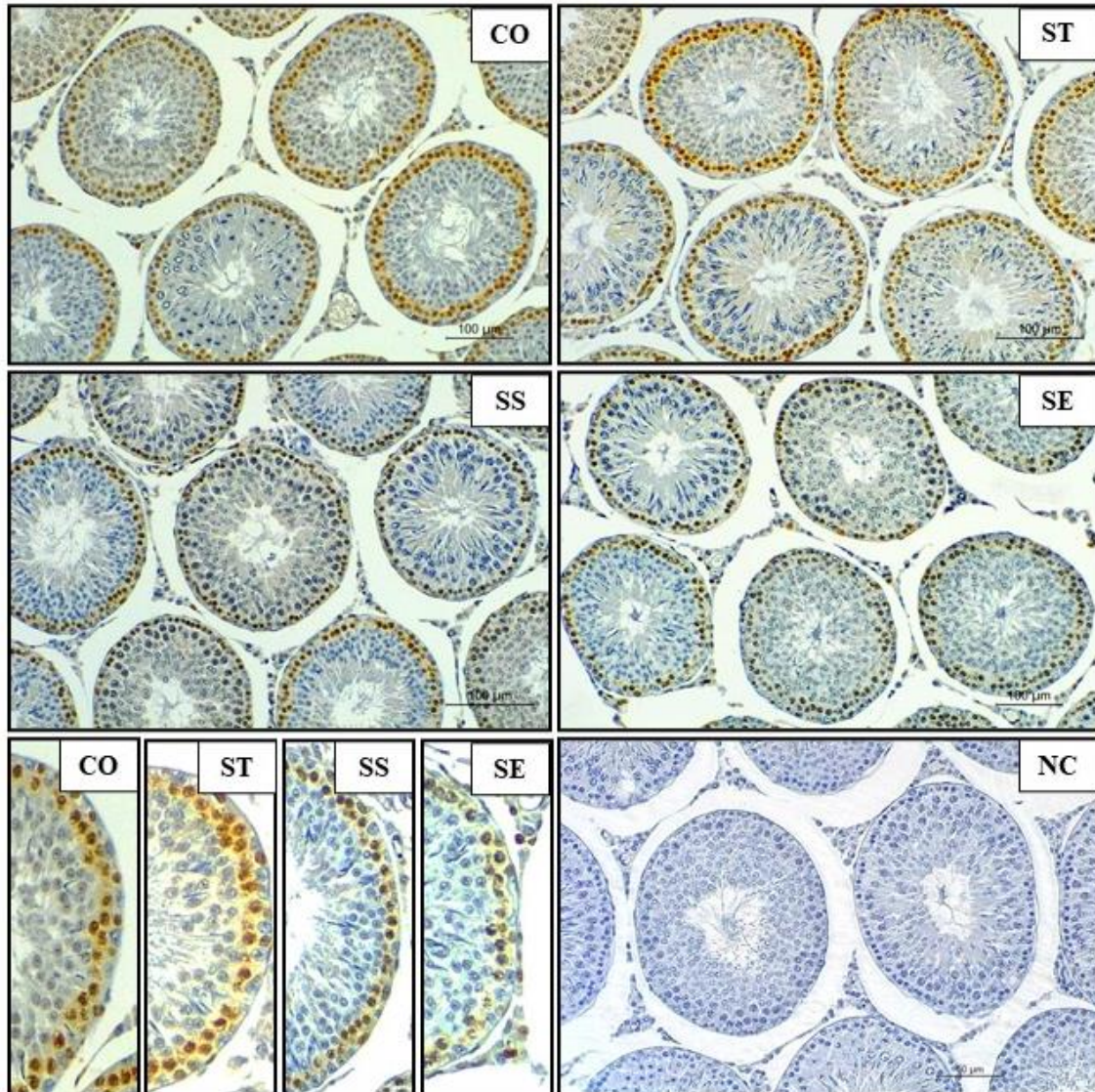


Figure 6. PCNA immunohistochemistry in cross-section of testes on postnatal day 45. CO group, ST group, SS group, SE group, Negative control (NC, without primary antibody).

3.5.2. WT1

Compared to the CO group, SS and ST had weaker cytoplasmic staining of WT1, especially SS (Figure 7 and Table 4). On PND 110 there was no difference among groups (results not shown).

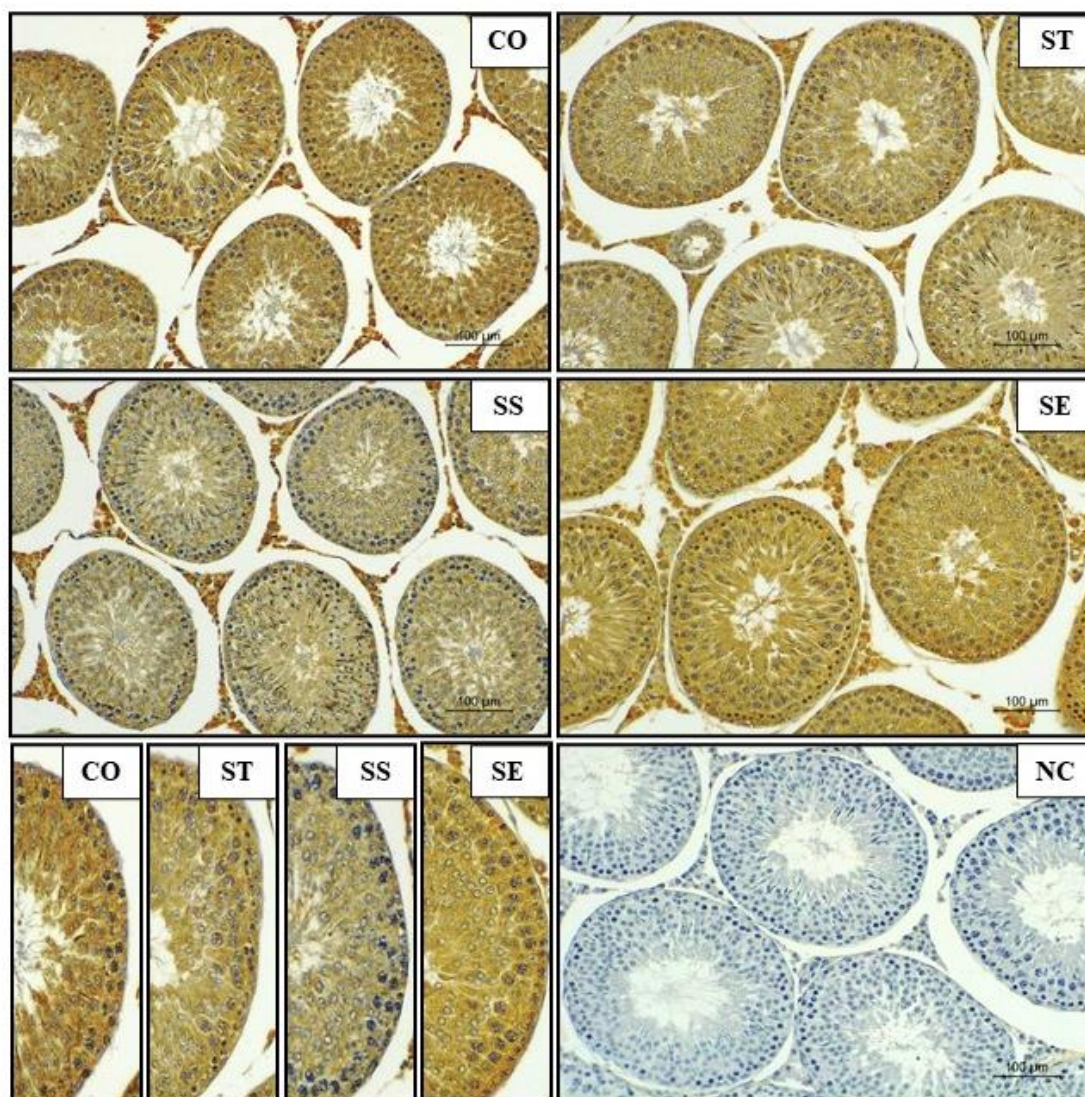


Figure 7. WT1 immunohistochemistry in testes on PND 45. CO group, ST group, SS group, SE group, Negative control (NC, without primary antibody).

3.5.3. Semi quantitative immunohistochemistry for PCNA and WT1

Semi-quantitative analysis for Wt1 on PND 45 revealed weak intensity in the SS and ST groups. PCNA, on the other hand, presented weak intensity in groups treated with sertraline (Table 4). On PND 110 there were no differences among the experimental groups (data not shown).

Table 4. Semi-quantitative evaluation of immunostaining in seminiferous tubules

Parameter	CO (n=6)	ST (n=6)	SS (n=6)	SE (n=6)
Degree of immunostaining for Wt1 (%)				
Strong	100 ^a	60 ^b	17 ^c	100 ^a
Weak	0 ^a	40 ^b	83 ^c	0 ^a
Degree of immunostaining for PCNA (%)				
Strong	100 ^a	100 ^a	50 ^b	50 ^b
Weak	0 ^a	0 ^a	50 ^b	50 ^b

Values expressed as percentage (Chi-square test). Different letters indicate $p < 0.05$. CO: control; ST: stress; SS: stress/sertraline; SE: sertraline. Teste's immunostaining of one animal from each litter (total of 6 litters/group).

3.6. Epididymal histology

3.6.1. Epididymis on PND 45

Spermatozoa in the epididymal lumen were detected only in the initial segment of half of the animals in all experimental groups.

The ST group showed an increase in cell debris, especially in the cauda. Although this alteration was also observed in a smaller amount in the animals of the SS group, the accentuated cribriform aspect of the cauda, especially in the 6B region, was characteristic of this group.

Animals treated with sertraline showed a significant increase in acidophilia and vacuoles throughout the epididymis, especially in the corpus and cauda regions (Figure 8).

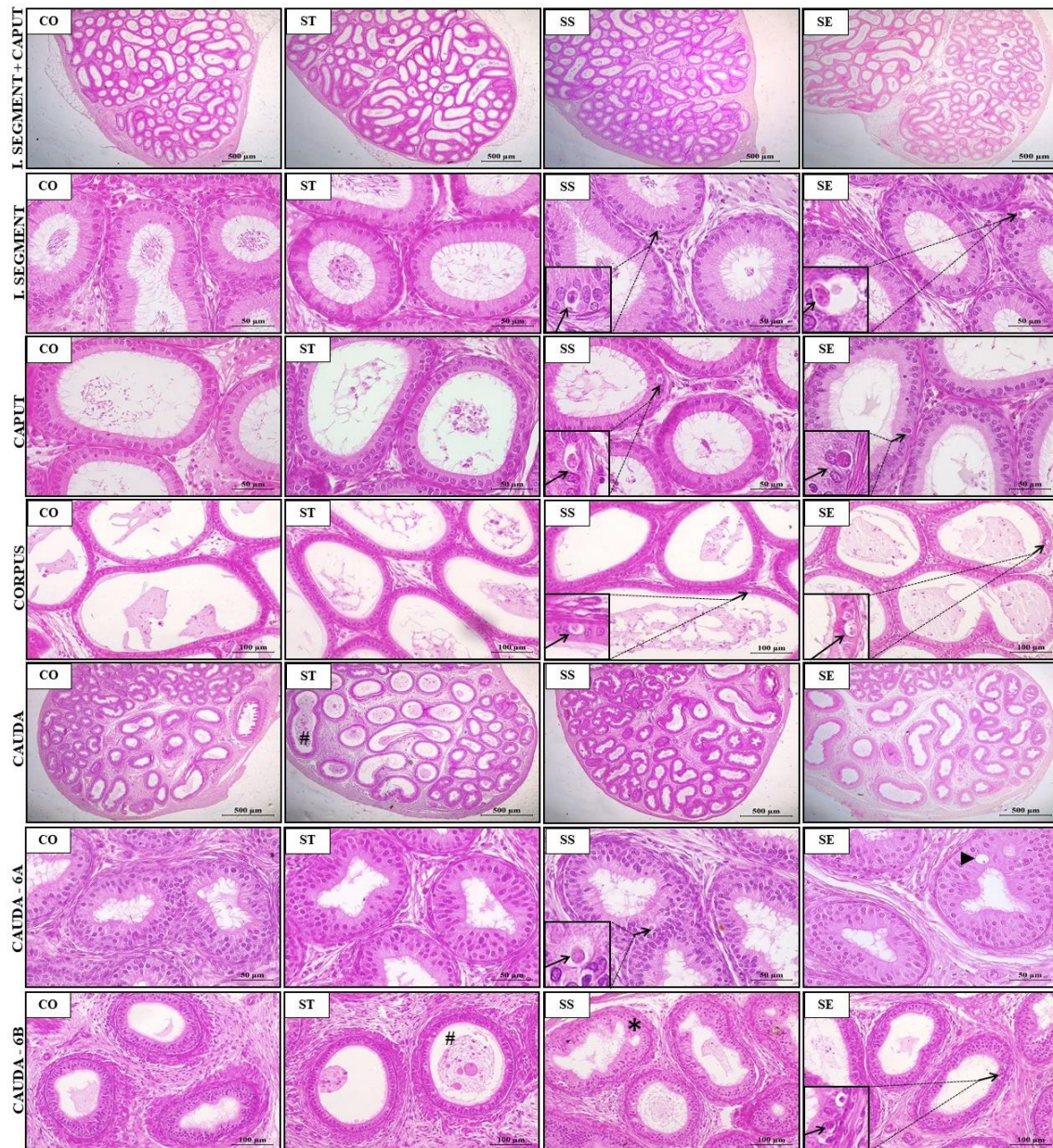


Figure 8. Epididymis on PND 45 stained with HE. CO group; ST group; SS group; SE group. Acidophilic cells (arrow), vacuole (arrowhead), cell debris (#), cribriform aspect (*).

3.6.2. Epididymis on PND 110

No significant histological alterations were observed in the epididymides on PND 110. However, the SS group showed a slight increase in acidophilic cells (Figure 9).

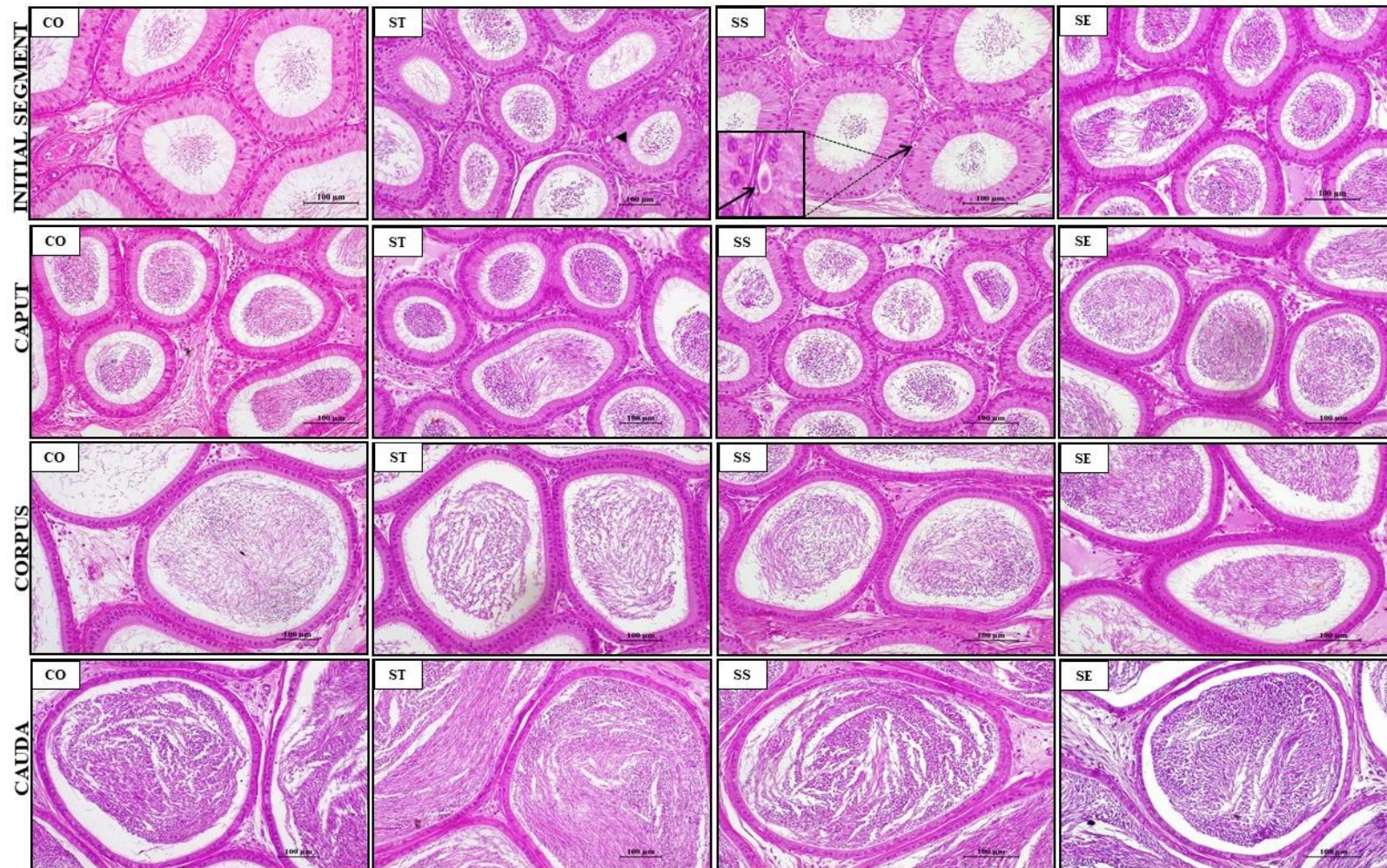


Figure 9. Epididymis on PND 110 stained with HE. CO group; ST group; SS group; SE group. Acidophilic cells (arrow) and vacuole (arrowhead).



3.7. Sperm quality

Animals in the SE group showed a decrease in sperm with normal morphology ($p=0.02$) and progressive motility ($p=0.008$). The SS group also showed a progressive reduction in motility ($p=0.04$). Alterations in sperm vitality and sperm count were not observed ($p>0.05$) (Table 5).



Table 5. Sperm quality of animals on PND 110

Parameters	CO (n=8)	ST (n=8)	SS (n=8)	SE (n=8)
¹ <i>Sperm morphology (%)</i>				
Normally shaped sperm	97.6 (96.0-99.0) ^a	96.5 (95.0-99.0) ^{ab}	96.5 (92.0-99.0) ^{ab}	95.2 (93.0-97.0) ^b
Abnormalities of the sperm flagellum	1.0 (0.0-2.0) ^a	2.8 (1.0-4.0) ^b	1.4 (0.0-2.0) ^{ab}	3.5 (1.0-6.0) ^b
Abnormalities of the sperm head	0.25 (0.0-1.0)	0.4 (0.0-2.0)	0.7 (0.0-3.0)	0.5 (0.0-2.0)
Sperm with cytoplasmic droplet	61.1 (41.0-74.0)	63.4 (44.0-77.0)	56.1 (43.0-77.0)	53.1 (40.0-69.0)
¹ <i>Sperm motility (%)</i>				
Mobile with progression	74.5 (72.2-76.7) ^a	66.0 (53.0-69.0) ^{ab}	59.0 (44.0-69.0) ^b	53.0 (38.0-64.0) ^b
Mobile without progression	17.0 (12.5-21.5) ^a	25.0 (17.0-32.0) ^{ab}	28.5 (16.2-41.7) ^{ab}	33.0 (24.5-47.5) ^b
Immobile	10.0 (9.0-14.0)	17.0 (8.0-20.5)	12.5 (10.2-14.7)	13.0 (10.5-18.0)
¹ <i>Sperm vitality (%)</i>				
Alive	79.0 (71.0-81.0)	75.5 (70.2-82.5)	72.0 (69.0-79.0)	71.0 (60.0-78.2)
Dead	21.0 (18.2-26.0)	24.5 (17.5-29.7)	28.0 (21.0-31.0)	29.0 (21.7-40.0)
² <i>Sperm counts</i>				
Mature spermatid number (10 ⁶ /testis)	183.0 (177.1-207.9)	183.3 (143.2-225.4)	151.5 (140.3-186.9)	170.7 (128.1-199.8)
DSP (10 ⁶ /testis/day)	29.8 (28.3-32.9)	30.0 (23.4-36.9)	24.8 (23.0-30.6)	26.5 (19.7-32.0)
SN in the caput/corpus (x10 ⁶ /organ)	72.4 (63.9-87.6)	70.6 (59.9-81.6)	67.2 (52.4-95.8)	71.3 (59.2-88.1)
SN in the cauda (x10 ⁶ /organ)	206.6 (142.2-244.1)	207.3 (193.2-240.6)	181.5 (159.8-242.5)	225.5 (206.7-247.6)
STT in the caput/corpus (days)	2.5 (2.2-2.9)	2.2 (1.8-2.8)	2.5 (1.78-4.27)	2.6 (2.3-3.2)
STT in the cauda (days)	5.5 (3.3-6.9)	5.4 (2.4-6.5)	5.920 (4.45-6.95)	6.2 (5.8-8.0)

¹Values expressed as median and interquartile intervals ± SEM (Kruskal-Wallis test followed by Dunn's test). ²Values expressed as median and interquartile intervals ± SEM (ANOVA followed Tukey's test). Different letters indicate p<0.05. DSP=Daily Sperm Production SN= Sperm Number; STT= Sperm Transit Time. Sperm quality of one animal from each litter (Total of 8 litters/group).



4. Discussion

During pregnancy, women go through numerous physical, hormonal and psychological changes that can negatively affect their mental health. Pregnant women with depression, anxiety and stress often experience metabolic and hormonal disruption, leading to changes in fetal development (Becker *et al.*, 2016; McGrady, 1984). Due to these psychiatric disorders, the use of an antidepressant is sometimes recommended, especially an SSRI such as sertraline, citalopram, escitalopram or fluoxetine (Balkowiec-Iskra *et al.*, 2017).

Information on the adverse effects of SSRI use during pregnancy or lactation is limited to concluding whether these medications should be discontinued. In recent years, studies have shown that some SSRI types cause congenital malformations and neonatal toxicity (Goracci *et al.*, 2015). However, the effects of sertraline in the perinatal period still need to be elucidated.

In the present study it was demonstrated that the offspring exposed to sertraline *in utero* had lower body weight. There is substantial evidence that prenatal exposure to SSRIs alters birth weight in humans and rodents (Hutchison *et al.*, 2018), a finding that may be related to an increase in serotonin levels, since this neurotransmitter alters the blood flow in the placenta and consequently restricts intrauterine growth (Vorhees *et al.*, 1994; Bairy *et al.*, 2007; Wen *et al.*, 2006).

On PND 45 the body weight and the weights of most organs were similar to those of the CO group. However, reproductive organs such as the epididymis and testes were lighter in the SS group. Histological analysis of the epididymis of the SS group revealed an enhanced cribriform aspect in the cauda region. Delays in the decline of these epithelial undulations reflect the immaturity of the organ (Sun and Flickinger, 1979).

Changes in the production of sex hormones, especially testosterone, directly affect the development of reproductive organs. Previous studies have shown that an increase in brain serotonin can alter GnRH, LH and FSH levels. Sertraline, when administered from juvenile to puberty ages, decreases the concentrations of LH, FSH, testosterone and 17 β -Hydroxysteroid dehydrogenase (17 β -HSD) (ElMazoudy *et al.*, 2020). On PND 110, the animals in the SS group showed normalization rather than a reduction of reproductive organ weights. Rayen *et al.* found that adult animals exposed to fluoxetine and/or to stress *in utero* did not show changes in testosterone concentrations (Rayen *et al.*, 2013).



Testosterone production is mainly carried out by Leydig cells, which have a large number of organelles involved in steroidogenesis, such as the smooth endoplasmic reticulum and mitochondria, which occupy 53% of the cytoplasm. It has already been established that the volume of these cells is directly related to the production of androgens (Sinha Hikim *et al.*, 1993; Zhu *et al.*, 1998). In the SS group the volume of Leydig cells was reduced on GD 20, but normalized on PND 45 and 110.

Animals in the SS group had a lower AGD on PND 21 and delayed puberty onset. Alterations of these reproductive markers demonstrate the occurrence of androgenic disturbance (Schwartz *et al.*, 2019; Korenbrot *et al.*, 1977).

Androgenic changes can also be caused by imbalances in WT1 production. This transcription factor, which is involved in the expression of steroidogenic enzymes and in the development of fetal Leydig cells (Chen *et al.*, 2014; Wen *et al.*, 2016), was weakly stained in Leydig and Sertoli cells in the SS group on PND 45. During development, WT1 is essential for the survival and proliferation of cells in the genital ridge, since its action, combined with other transcription factors (such as SOX9, SF1 and GATA4), is able to regulate the expression of anti-Müllerian hormone (Xu *et al.*, 2019).

In the present study, it was observed that the testes and epididymides of groups exposed to sertraline presented a large number of acidophilic cells, characteristic of cell death (Rock and Kono, 2008; Guazzone *et al.*, 2009). Although the type of interstitial cells with acidophilia was not identified, it is possible they are Leydig cells since they are the most abundant cells in the interstitium.

In recent years, studies have shown that SSRIs perform anti-proliferative and pro-apoptotic actions. Dapoxetine (SSRI), for example, enhanced pro-apoptotic Bax protein expression and also caspase-3 activity in rats with prostatic hyperplasia (Sayed *et al.*, 2016). Fluoxetine has also been shown to inhibit the proliferation of prostate cancer cells. Sertraline and paroxetine had cytotoxic effects on malignant T cells, by inducing apoptosis and inhibiting their proliferation, through the increase of caspase-3 activity (Amit *et al.*, 2009). Kuwahara *et al.* (2015) demonstrated that sertraline has greater potential to significantly increase caspase-3/7 activity when compared to other antidepressants.

To investigate the cell proliferative potential, PCNA expression was demonstrated in the testes. This is a cell cycle regulatory protein marker that plays critical roles in DNA synthesis, initiation of cell proliferation, chromosomal recombination and RNA



transcription (Zhao *et al.*, 2018; Boehm *et al.*, 2016). On PND 45 animals exposed to sertraline showed low PCNA staining and a lower spermatogonia population, especially in the SE group, reinforcing the hypothesis that this antidepressant produces antiproliferative action.

Most of the changes observed in the SE group also occurred in the SS group. However, the latter showed more severe reproductive impacts, possibly due to the synergistic action between stress and sertraline. Nevertheless, no significant presence of cellular debris in the epididymis was observed in the SS group as also occurred in the ST group on PND 45. Although the presence of cellular debris is normal in immature animals (Weiser *et al.*, 2020), its increase may reflect functional dysregulations.

The most common source of cellular debris in the epididymis lumen is from sloughed germ cells in the testes, or from direct toxicity in the epididymis (De Grava Kempinas and Klinefelter, 2015). The present study has found that testicular histology was normalized on PND 45 in all experimental groups. Therefore, stress may have directly impacted the epididymis.

An increase in cellular debris in the epididymal lumen may also be related to smooth muscle contractile alterations (Weiser *et al.*, 2020). In the cauda region there is a thick smooth muscle layer richly innervated by fibers of the sympathetic nervous system, and also by serotonergic fibers (Mueller *et al.*, 2019). Previous studies have shown that both sertraline and fluoxetine are able to increase epididymal smooth muscle contractility (Bezerra *et al.*, 2019). Thus, it is possible that sertraline normalized the effects of stress on the epididymis of animals in the SS group. However, further studies are needed to support this hypothesis.

In adulthood, the animals did not show significant histological changes in the epididymis or testes; however, the sperm quality in sertraline-exposed groups was compromised. Akasheh *et al.* showed that the exposure of adult men to sertraline for 3 months compromised the normal morphology of sperm in addition to fragmenting their genetic material (Akasheh *et al.*, 2014). An *in vitro* study demonstrated that sertraline acts on the CatSper channels, an important calcium channel involved in sperm function (Rahban, Rehfeld, Schiffer, Brenker, Egeberg Palme, Wang, Lorenz, Almstrup, Niels E Skakkebaek, *et al.*, 2021).

Some drug labels mention that doses equal to or greater than 80 mg/kg are capable of causing side effects on fertility; however, our results showed that 20 mg/kg of sertraline



is capable of provoking important alterations in reproductive aspects. Although our study has shown that sertraline associated or not with stress compromises male reproductive development, further studies are needed to clarify the action mechanism of this drug, hormone concentrations, and also to verify whether the fertilization process is affected by the treatment, which may impact offspring development.

5. Conclusion

The results obtained from this study demonstrate that sertraline associated or not with stress compromises the development of the male reproductive system in rats, with negative repercussions at older ages. These findings encourage further studies aiming to assess the safety of the use of this antidepressant by pregnant women.

6. Declaration of competing interest

The authors declare that they have no potential conflicts of interest with respect to the research, authorship or publication of this article.

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Capítulo 3

O experimento III, intitulado como “Is lactational Sertraline exposure safe for maternal health and the reproductive/neurobehavioral development of the descendants? A study in rats” foi publicado na revista Reproductive Toxicology (Fator de impacto: 3.14). Doi: 10.1016/j.reprotox.2023.108356



Is lactational Sertraline exposure safe for maternal health and the reproductive/neurobehavioral development of the descendants? A study in rats.

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ABSTRACT

Although sertraline is considered one of the safest antidepressants in the lactation period, there are still few studies that assess its impact on child development. Therefore, this experimental study aimed to clarify the effect of sertraline on the neurobehavioral and reproductive development of male rats. Thus, 30 lactating rats were divided into 3 experimental groups (n=10/group): CO- received filtered water, S10 and S20 groups that received, respectively, 10 and 20 mg/kg/day of sertraline. Treatment was performed by gavage, from postnatal days (PND) 1 to 20. During this period, the reflex and somatic development of rats were observed, as well as maternal behavior. On PND 21, mothers were euthanized and the organs were weighed. On PND 21, 45, and 100, one male from each litter was euthanized for histological and immunohistochemical (PCNA and WT1) analysis of the reproductive organs. The growth of body weight, the anogenital distance (AGD), the time to puberty, sperm quality, sexual behavior, neurobehavior, and natural fertility were also verified. Statistical analysis: One-way ANOVA or Kruskal-Wallis test ($p \leq 0.05$). The results showed that mothers in the S20 group had an increase in thyroid weight. The male offspring exposed to sertraline had lower body weight (PND 7), lower AGD (PND 7 and 14), and delay in reflex development, in addition to histological alterations in the testis (PND 21). In adulthood, sperm quality was altered, without compromising natural fertility. Therefore, the present study found important alterations in the reflex and reproductive development of male rats exposed to sertraline during lactation.

Keywords: Lactation, sertraline, reproductive system, neurobehavior, thyroid, rat.



1- INTRODUCTION

Breastfeeding is critical to the survival, health, and development of all mammals [1]. As a source of energy and nutrients, the World Health Organization recommends exclusive breastfeeding for the first six months of life and its continuation until two years of age, along with complementary feeding [2]. Great physiological changes occur in breastfeeding women and sometimes it becomes a vulnerable period for the manifestation of different psychiatric disorders. The onset of postpartum depression occurs in approximately 50% of patients who were diagnosed with depression before giving birth [3][4].

Evidence points out that depression affects about 10-20% of breastfeeding women, however, it is known that this percentage is even higher when considering undiagnosed cases [5]. Postpartum depression can last up to a year and sometimes maternal care can be impaired and affect the child's emotions, cognition, behavior, and social life [4][6]. There are numerous risk factors for the development of postpartum depression which can be prenatal anxiety/stress, lack of social support, financial and marital problems, as well as obstetric problems [7].

One of the first-line treatments for major depression is selective serotonin reuptake inhibitors (SSRIs). Although the recommendation of this drug to pregnant and lactating women is made with caution, studies show that newborn exposure to the drug through breast milk is much lower than exposure in the uterus [8]. Many authors recommend sertraline and paroxetine (SSRIs) as first-line antidepressants for breastfeeding women, as they have the highest safety profile among psychotropic drugs [9].

Sertraline has become in recent years an excellent therapeutic alternative for the treatment of depression and anxiety [10]. Because it binds to 98% of plasma proteins, there is a reduction in its transfer to milk [11]. The relative infant dose (infant dose [mg/kg]/maternal dose [mg/kg]) of sertraline is ~0.5% of the maternal dose and is well tolerated by infants [12].

The most widely reported side effects in babies exposed to antidepressants through breastfeeding are sedation, eating disorders and poor weight gain [13]. Previous studies carried out in our laboratory showed that rat gestational exposure to sertraline negatively impacted neurobehavioral and reproductive aspects, in addition to



compromising maternal health [14][15][16]. The present study aimed to investigate these neurobehavioral and reproductive parameters after lactational exposure.

2- METHODOLOGY

Experimental design

Thirty lactating Wistar rats were pseudo-randomly allocated into 3 experimental groups (n=10/group), in order to avoid differences in initial body weight: CO- received filtered water (vehicle), S10 and S20 that received, respectively, 10 and 20 mg/kg/day of sertraline (diluted in the vehicle). Treatment was performed by gavage, from postnatal days (PND) 1 to 20. During the lactation period, food intake and maternal body weight were verified. Four males and four females were kept per litter. Only male offspring were used in this study, the female offspring had been evaluated in another study, not shown herein. The concentrations of sertraline used in the studies, low and moderate doses for rodents [17][18]. It is important to point out that according to International Program on Chemical Safety Poisons Information, the LD 50 (oral) for rats is 1591 mg/kg (for males) and 1327 mg/kg (for females).

On PND 5 and 10, maternal behavior was assessed, and on PND 21 the mothers and 1 male rat/litter were euthanized for organ collection. On PND 23-25 and 83-85 neurobehavioral tests were performed with 2 male rats/litter. One male from each litter was euthanized on PND 45 and 100, by narcosis in a CO₂ chamber followed by decapitation for collection and analysis of biological material.

The growth of body weight, the anogenital distance (AGD), and the time of puberty onset were also verified. The evaluation of sexual behavior and natural fertility of 1 male/litter occurred on PND 100 (Figure 1).

During the experiment, the animals were kept under controlled conditions (12h of light /12h of dark, average temperature of 23°C) in the biotherium of CEATOX, with food and water *ad libitum*. The animals were kept in accordance with the Ethics Committee for the Use of Experimental Animals of the São Paulo State University, protocol number 3448071220.

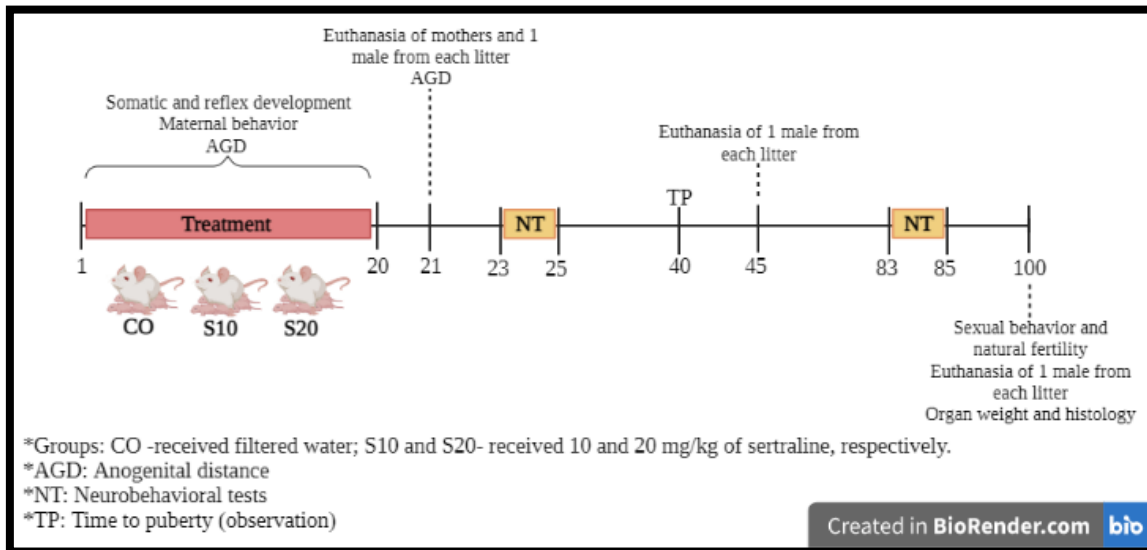


Figure 1. Experimental design.

Maternal behavior

Maternal behavior was evaluated according to Kiss et. al [19], with some modifications. On PND 5 and 10, all pups were removed from the cage. After 20 minutes, the nest was destroyed, the pups returned to the cage, and mother-pup interaction was recorded for 30 minutes. The number of rearing and grooming, latency to pup collection, pup-grooming time, and latency to crouching were recorded.

Analysis of somatic and reflex development

The day on which ear detachment, eye-opening, fur development, and incisor eruption occurred was recorded. For the analysis of reflexes, the following tests were carried out: Palmar grasp, righting, cliff avoidance, and negative geotaxis [14].

The data obtained from somatic and reflex maturation were expressed as the mean of the litter (the mean of four males per litter was taken and used to express the results).

Neurobehavioral analysis

The elevated plus maze and the open field were performed in juvenile (PND 23-25) and adult rats (PND 83-85). The elevated plus-maze test was performed using an apparatus consisting of two open and two closed arms (50 × 10 cm). The height of the closed arms was 40 cm, and the open arms had a curb 1 cm high. Each rat was placed in the center of the elevated plus-maze facing one of the closed arms. The analysis took place for 5 minutes and the following parameters were recorded: The number of rearing;



stretching; head dipping movements; grooming; the number of entries and time spent in the open and closed arms [14].

The open field consists of an arena of 100 cm in diameter and 40 cm in height. The analysis was initiated when each animal was placed in the central region of the arena. For 3 minutes, the following parameters were recorded: latency until the first crossing and total number of crossings in the arena; freezing duration; time near the arena wall; time in the center; ambulation near the arena wall [20].

Neurobehavioral tests evaluated two male rats from each litter and the results were obtained on average.

AGD and the onset of puberty

Rat pups were weighed and the AGD was measured on PND 1, 7, 14, and 21. AGD was normalized with the cube root of body weight [21]. The onset of puberty was determined when complete retraction of the preputium and glans exposure.

Organ collection

On PND 21, 45, and 100 one male from each litter was euthanized to collect reproductive organs. The testes and epididymis were immersed in Bouin's fixative solution. These materials were later histologically processed for inclusion in Paraplast and were cut to a thickness of 5µm. The sections were stained with hematoxylin and eosin (HE) for histopathological and morphometric analysis. All histopathological analyzes were performed by two investigators who were blinded to the experimental groups.

The mothers were euthanized on PND 21 and the organs were collected and weighed.

Histopathology of testes and epididymis

For histopathology analyses, 100 seminiferous tubules were analyzed, as previously described [22]. The analysis of the diameter was performed with seminiferous tubules at maturation grade 3 (PND 45) and at stage IX (PND 100). The thickness of the germinal epithelium was evaluated in 10 sections of seminiferous tubules per animal [23].

The epididymis was qualitatively analyzed, and alterations present in the initial segment, caput, corpus, and cauda were noted, as described by other studies [24].

Immunohistochemistry of testes



The testicular sections on PND 45 and 100 were submitted to the antigen recovery procedure, by immersion in citrate buffer (0.1 M, pH 6.0) and subsequently incubated with 3% hydrogen peroxide in PBS. Unspecific blocking was performed with 3% BSA in PBS (blocking solution) before the incubation with the primary antibody anti-PCNA antibodies (PCNA PC10: sc-56, Monoclonal, Santa Cruz Biotechnology, California, USA—1:100) and anti-WT1 (4 mg/ml, SC215 393498/H-1; Santa Cruz Biotechnology—1:100) overnight at 4°C. For negative controls, sections were incubated in the blocking solution, without the primary antibody. Afterward, the sections were incubated with secondary antibodies (HRP anti-mouse goat; 1.6 mg/ml SC-2005; 218 Santa Cruz Biotechnology—1:200). Then, the sections were exposed to diaminobenzidine associated with hydrogen peroxide. Finally, the sections were counterstained with hematoxylin [15]. The analysis of the material was carried out qualitatively.

Maturation Degree

On PND 45, the testes were analyzed by classifying them into 100 seminiferous tubules according to the most mature cell type: 1: Presence of spermatocytes I or II; 2: Spermatids in stages 1–8 of spermiogenesis; 3: Spermatids in stages 9–14 of spermiogenesis; 4: Spermatids in stages 15–18 of spermiogenesis; 5: Small amount of spermatids in stages 19; 6: Spermatids in stage 19 in medium quantity; 7: Spermatids in stage 19 in greater quantity.

The number of seminiferous tubules in each degree was multiplied by its grade (1-7), and the values were added and divided by 100.

Spermatogenesis dynamics

The relative frequency of the stages was performed according to Clermont and Harvey [25]. For this purpose, 100 seminiferous tubules were classified into stages I-VI: Two generations of spermatids; stage VII-VIII: Mature spermatids in the lumen; stage IX-XIII: Only one generation of spermatids; stage XIV: Presence of secondary spermatocytes.

Sperm motility and morphology

Spermatozoa collected from region 6A of the cauda of the epididymis were transferred to a solution of PBS and 1% BSA. Using a contrast microscope and Makler



chamber, 100 spermatozoa were counted and their motility was classified as Type A (mobile with progressive movement), Type B (mobile without progressive movement), or Type C (immobile).

For analysis of sperm morphology, 100 μ l of the solution containing spermatozoa was transferred to 900 μ l of 10% formalin-saline solution. Subsequently, the solution was placed on glass slides for analysis under a contrast microscope. One hundred sperm were analyzed per animal and morphological abnormalities were noted. In addition, 100 sperm were classified for the presence or absence of cytoplasmic droplets [15].

Calculation of daily sperm production and sperm transit time

The left testes after decapsulation were homogenized in a solution of NaCl and Triton x100 for 5 minutes and then in a sonicator for 30 seconds. After a 10-fold dilution, an aliquot was transferred to Neubauer chambers and mature spermatids were counted. To calculate sperm production per day (PDE), the number of spermatids in stage 19 was divided by 6.1 (the number of days in the cycle during which these spermatids are present in the seminiferous epithelium). The transit time of sperm through the epididymis was determined by dividing the number of sperm in each region by the PDE [26].

Sexual behavior and natural fertility

Male rats, during the dark phase of the lighting cycle, were placed in crystal polycarbonate boxes five minutes before the introduction of a receptive adult female. The animals were observed in the dark period of the cycle and during the subsequent 40 minutes the following parameters were analyzed: latencies for first mount, intromission, and ejaculation, number of intromissions until the first ejaculation, the latency of the first post-ejaculation intromission, number of post-ejaculate intromissions, and number of ejaculations. Males that did not mount in the first 10 minutes had their evaluation completed and were considered sexually inactive. After 12 hours the females were removed and vaginal swabs were collected to confirm insemination.

Twenty days later, the pregnant rats were euthanized to allow the assessment and calculation of fertility and reproductive performance parameters. After collecting the uterus and ovaries, the numbers of corpora lutea, implants, and resorptions were determined. From these results, the following parameters were calculated: fertility potential (implantation efficiency): $\text{implantation sites/corpora lutea} \times 100$, pre-



implantation loss rate: $(\text{number of corpora lutea} - \text{number of implants} / \text{number of corpora lutea}) \times 100$, and post-implantation loss rate: $(\text{number of implants} - \text{number of live fetuses}) / \text{number of implants} \times 100$.

Statistical analysis of results

Results were compared by ANOVA followed by Tukey's test, for parametric variables, or by Kruskal-Wallis followed by Dunn's test, for non-parametric variables. Differences were considered statistically significant when $p \leq 0.05$. Statistical analyzes were performed using the GraphPad Prism software (version 8.0).

3- RESULTS

Food intake and maternal body weight

There was no difference in food intake between the experimental groups ($p > 0.05$). However, the maternal body weight of the S20 group was lower on PND 14 compared to the CO group ($p = 0.02$) (Figure 2).

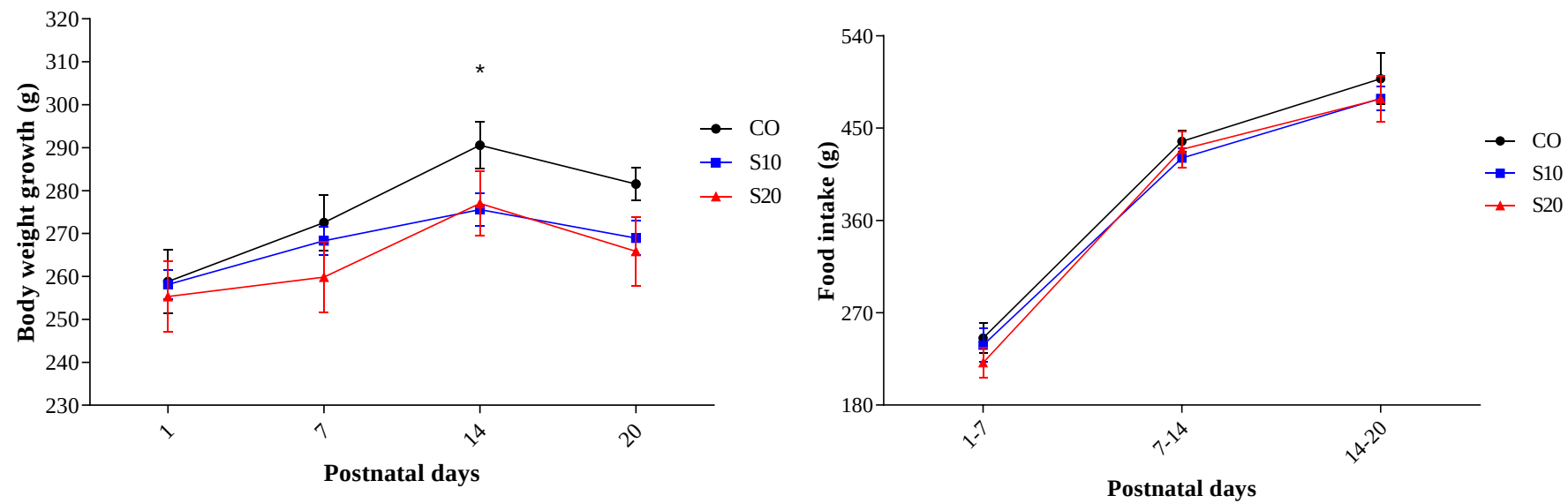


Figure 2. Food intake and body weight (n=10). Values expressed as mean $\pm$ S.E.M. ANOVA followed by Tukey's test. *p=0.02. CO -received filtered water; S10 and S20- received 10 and 20 mg/kg of sertraline.

Maternal organ weights

An increase in thyroid weight was observed in the S20 group compared to the CO group ($p=0.006$) (Table 1).

Table 1. Organ weights

Organs	CO (n=10)	S10 (n=10)	S20 (n=10)
Brain (g)	1.8±0.02	1.8±0.03	1.8±0.03
Pituitary (mg)	10.4± 0.3	9.9±0.6	10.0±0.5
Thyroids (mg)	13.9±0.9 ^a	12.6±0.5 ^a	16.9±0.9 ^b
Heart (g)	1.2±0.04	1.1±0.05	1.1±0.06
Liver (g)	16.7±0.8	15.6±0.7	15.0±0.4
Adrenals (mg)	103.1±3.3	110.8±5.2	114.2±7.5
Kidneys (g)	2.3±0.03	2.3±0.06	2.4±0.1
Ovary (mg)	81.3±4.6	77.5±2.7	83.7±3.6
Uterus (mg)	316.5±23.0	265.0±12.3	310.2±16.4

Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. Different letters express $p<0.05$. CO -received filtered water; S10 and S20- received 10 and 20 mg/kg of sertraline

Maternal behavior

The behavior analysis (Figure 3) showed that mothers from the S20 group had an increase in grooming compared to other groups ($p=0.001$). The other parameters were similar between the experimental groups ($p>0.05$).

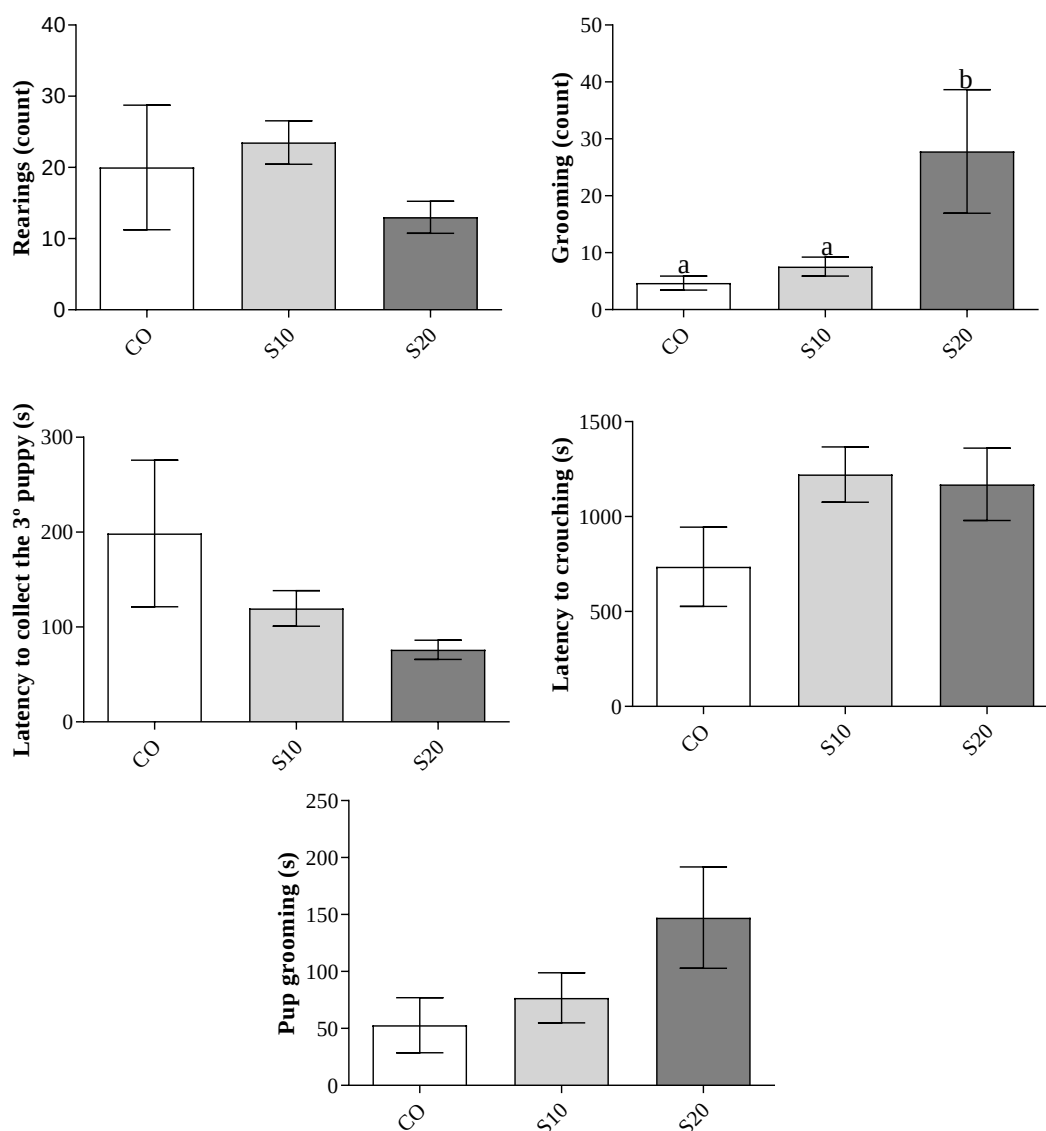


Figure 3. Maternal behavior (n=6-8). Values expressed as mean $\pm$ S.E.M. ANOVA followed by Tukey's test. Different letters express $p < 0.05$. CO -received filtered water; S10 and S20- received 10 and 20 mg/kg of sertraline.

Offspring body weight

During the treatment, lower body weight was observed in the S20 group, and this reduction was significant on PND 7 ($p=0.01$) (Figure 4).

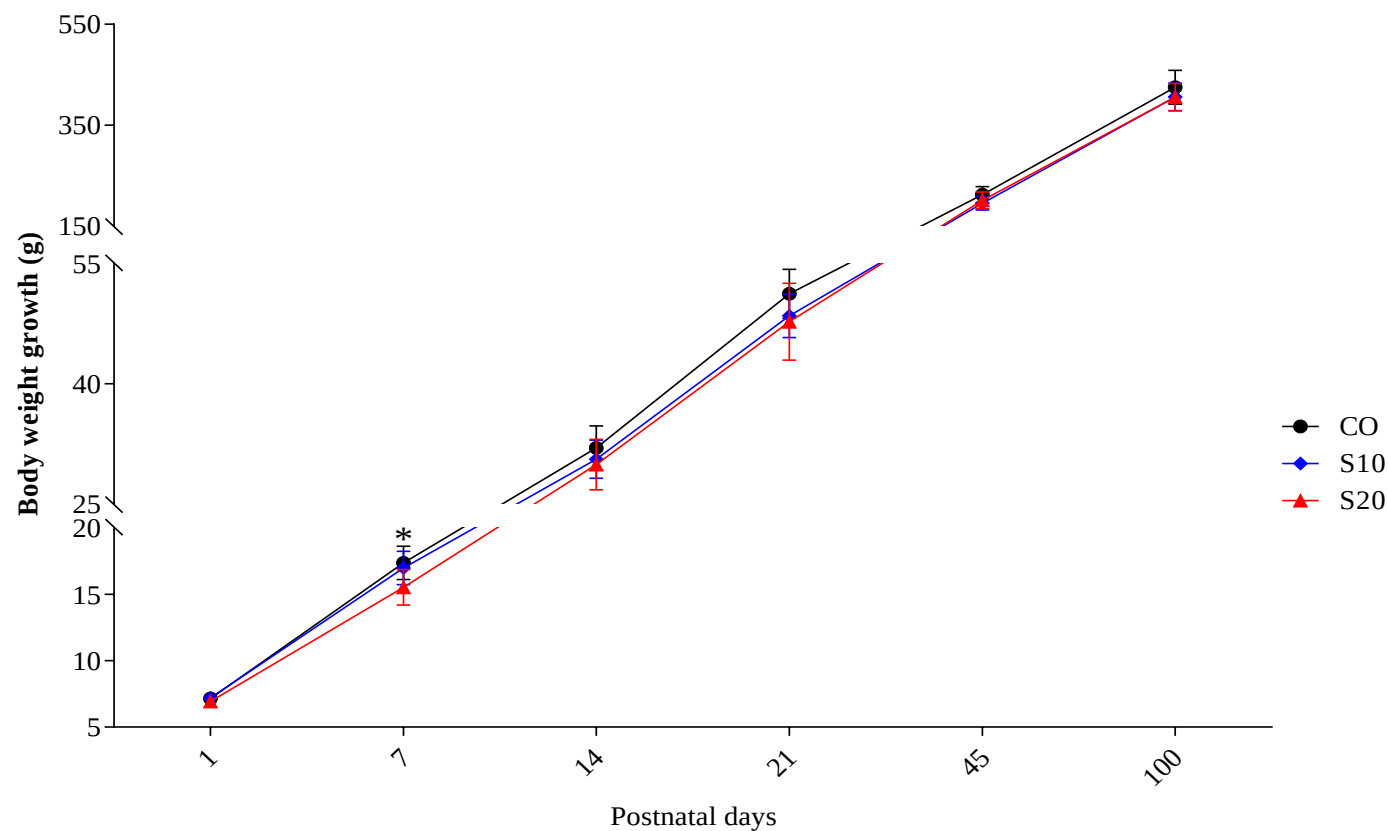


Figure 4. Body weight of male offspring on postnatal days 1, 7, 14, 21, 45, and 100 (n= average of all males from each litter; 10 litters/group). Values expressed as mean $\pm$ S.E.M. ANOVA followed by Tukey's test. * $p = 0.01$. CO -received filtered water; S10 and S20- received 10 and 20 mg/kg of sertraline.

Anogenital distance and time to puberty

A lower AGD was observed in the S20 group compared to the CO group on PND 7 ($p=0.03$) and 14 ($p=0.02$). The time to puberty was not different between the experimental groups ($p>0.05$) (Figure 5).

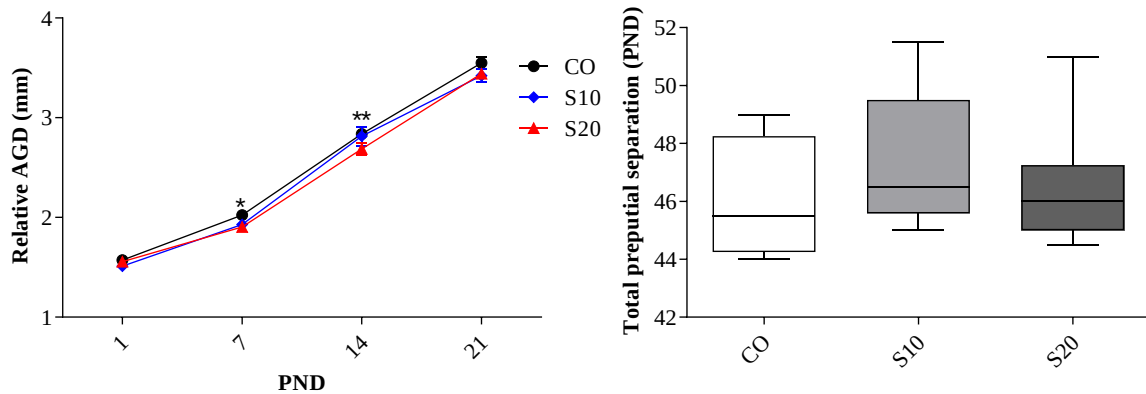


Figure 5. Relative anogenital distance (AGD) and total preputial separation (n = average of all males from each litter; 10 litters/group). PND= Postnatal days. Values expressed as mean $\pm$ S.E.M. ANOVA followed by Tukey's test. * $p = 0.03$; ** $p=0.02$. CO -received filtered water; S10 and S20- received 10 and 20 mg/kg of sertraline.

Somatic and reflex development

There was a delay in the cliff avoidance and negative geotaxis ($p=0.02$) in the S10 and S20 groups, respectively. Both sertraline-treated groups similarly had a delay in palmar grasp, which was unrelated to dose-response ($p=0.002$) (Table 2).

Table 2. Somatic and reflex development

Parameters (PND)	CO (n=10)	S10 (n=10)	S20 (n=9)
Righting reflex	1.97±0.14	2.40±0.17	2.50±0.17
Palmar grasp	5.50±0.12 ^a	6.57±0.13 ^b	6.42±0.34 ^b
Cliff avoidance	4.42±0.28 ^a	5.37±0.22 ^b	4.89±0.16 ^a
Negative geotaxis	6.17±0.1 ^a	7.02±0.23 ^a	7.25±0.39 ^b
Ear detachment	3.00±0.10	3.02±0.02	3.44±0.21
Incisor eruption	10.43±0.11	10.63±0.09	10.58±0.20
Fur development	7.00±0.00	7.00±0.00	7.11±0.08
Eye-opening	14.11±0.17	14.25±0.14	14.28±0.23

Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test ($p>0.05$).

Different letters express $p<0.05$. PND= Postnatal days. CO -received filtered water; S10 and S20- received 10 and 20 mg/kg of sertraline

Neurobehavior analysis

The neurobehavior analysis during childhood and adulthood, through the elevated plus-maze and the open field, showed no difference between the experimental groups ($p>0.05$) (Table 3).



Table 3. Elevated plus-maze and open field

Parameters	Childhood			Adulthood		
	CO (n=10)	S10 (n=10)	S20 (n=10)	CO (n=10)	S10 (n=10)	S20 (n=10)
<i>Plus-maze</i>						
Open-arm entries (count)	1.05±0.28	1.20±0.33	1.17±0.32	1.33±0.17	1.18±0.32	1.36±0.34
Closed-arm entries (count)	5.15±0.80	5.50±0.70	6.78±1.03	6.28±0.55	6.63±1.09	6.21±1.01
Time in open arms (s)	8.85±2.17	10.70±3.41	6.50±1.80	11.92±1.53	6.92±2.27	12.17±3.68
Time in closed arms (s)	291.2±2.17	289.3±3.14	293.5±1.80	288.1±1.53	290.4±3.27	287.8±3.68
Rearings (count)	8.15±0.74	8.55±0.66	7.33±0.60	10.21±1.51	13.88±1.65	9.50±0.82
Stretched (count)	7.55±0.96	8.05±0.83	8.67±1.23	10.43±1.08	12.50±1.01	8.64±1.55
Head dipping (count)	1.65±0.38	2.80±0.60	1.72±0.51	3.71±1.19	2.5±0.66	3.07±0.81
Time in the center	3.25±1.68	4.10±1.60	1.39±0.64	6.14±1.85	6.06±1.68	3.0±0.87
Grooming (count)	2.00±0.24	2.05±0.22	2.83±0.42	2.14±0.34	2.06±0.59	2.00±0.34
<i>Open field</i>						
Time near the wall (s)	161.5±3.84	154.0±3.93	159.4±3.61	161.5±12.45	141.9±7.94	151.7±5.80
Latency until the FI (s)	28.78±11.75	48.20±10.41	32.85±10.95	22.13±6.32	11.90±7.28	18.92±11.26
Total crosses (count)	0.77±0.24	0.85±0.21	0.72±0.21	0.50±0.15	0.25±0.11	0.27±0.11
Freezing duration (s)	6.61±4.30	1.05±0.83	1.17±0.77	15.00±4.12	18.83±8.36	2.33±0.87
AM near the wall (count)	57.19±2.15	53.25±2.28	61.0±6.14	27.56±3.15	25.08±5.16	37.33±3.78

Values expressed as mean ± S.E.M. ANOVA test followed by Tukey's (p>0.05). FI=first crossing; AM= Ambulation. CO -received filtered water;

S10 and S20- received 10 and 20 mg/kg of sertraline.



Offspring organ weights

The weight of the organs of the experimental groups did not show differences on PND 21, 45, and 100 ($p>0.05$) (Table 4).

Table 4. Organ weights

Organs	PND 21			PND 45			PND 100		
	CO (n=10)	S10 (n=10)	S20 (n=10)	CO (n=10)	S10 (n=10)	S20 (n=10)	CO (n=10)	S10 (n=10)	S20 (n=10)
Testis (g)	0.1±0.004 1±0.08(x10 ⁻²)	0.1±0.002 1±0.06(x10 ⁻²)	0.1±0.005 1±0.1(x10 ⁻²)	0.9±0.03 151.5±3.4	0.8±0.04 134.9±10.0	0.9±0.04 143.8±12.9	1.7±0.02 614.3±13.7	1.6±0.04 610.4±24.2	1.5±0.03 578.4±7.2
Epididymis (mg)									
Ventral prostate (mg)	2±0.2(x10 ⁻²)	2±0.1(x10 ⁻²)	2±0.1(x10 ⁻²)	122.4±6.8	89.5±13.1	112.5±7.6	428.5±21.9	390.6±30.5	384.1±37.9
Seminal vesicle (mg)	8±1(x10 ⁻³)	6±0.3(x10 ⁻²)	7±0.6(x10 ⁻²)	94.0±5.2	69.3±10.2	112.0±10.8	996.6±60.1	1127.0±60.8	953.8±65.8
Liver (g)	1.8±0.05	1.7±0.04	1.7±0.05	10.1±0.3	9.1±0.4	10.0±0.3	14.2±0.4	14.5±0.5	13.43±0.4
Kidneys (g)	0.5±0.01	0.5±0.01	0.5±0.02	2.2±0.05	1.9±0.08	2.1±0.06	3.1±0.1	3.2±0.1	3.1±0.1
Brain (g)	1.4±0.02	1.4±0.01	1.4±0.02	1.8±0.02	1.8±0.03	1.8±0.02	2.0±0.01	1.9±0.03	2.0±0.01
Lungs (g)	0.4±0.02	0.4±0.02	0.4±0.02	1.2±0.04	1.2±0.09	1.2±0.05	1.8±0.1	1.7±0.1	1.8±0.09
Heart (g)	0.3±0.01 1 ±0.7(x10 ⁻³)	0.2±0.1 1±0.7(x10 ⁻³)	0.2±0.01 1±0.8(x10 ⁻³)	0.9±0.02 45.4±2.6	0.8±0.02 47.4±2.3	0.9±0.02 45.0±2.5	1.4±0.02 73.7±5.7	1.3±0.05 60.2±2.8	1.3±0.04 72.1±5.4
Adrenals (mg)									
Thyroids (mg)	- 2±0.2 (x10 ⁻³)	- 1±0.2(x10 ⁻³)	- 1±0.1 (x10 ⁻³)	16.4±1.2 6.2±0.3	14.5±1.1 5.3±0.3	14.2±0.7 5.6±0.4	17.0±1.0 8.5±0.5	17.8±0.8 8.7±0.2	16.1±0.8 7.8±0.5
Pituitary (mg)									

Values expressed as mean ± S.E.M. ANOVA test followed by Tukey's test (p>0.05). On PND 21, thyroids were not collected. CO -received filtered water; S10 and S20- received 10 and 20 mg/kg of sertraline.

Histology of the testes

On PND 21, the S20 group compared to the CO group, showed a decrease in normal tubules ($p=0.02$), an increase in the number of acidophilic cells ($p=0.04$), in addition to a decrease in the volume of Leydig cells ($p=0.007$). In the other ages, no differences were detected ($p>0.05$) (Table 5 and figure 6).

Table 5. Testicular histopathology and morphometry on PND 21, 45 and 100.

Parameters	CO (n=6)	S10 (n=6)	S20 (n=6)
<i>PND 21</i>			
¹ Normal tubules (%)	90.1±1.4 ^a	86.5±2.2 ^{ab}	79.4±2.6 ^b
¹ Vacuolization (%)	1.0±0.3	1.1±0.4	1.1±0.3
¹ Acidophilic cells (%)	8.5±1.2 ^a	11.4±2.3 ^{ab}	18.1±2.3 ^b
² Leydig cell volume (μm^3)	89.05±5.6 ^a	71.98±4.3 ^b	67.93±3.1 ^b
² Tubular diameter (μm)	172.0±2.8	170.1±2.3	168.5±1.9
<i>PND 45</i>			
¹ Normal tubules (%)	90.8±1.9	95.2±1.4	93.0±0.9
¹ Vacuolization (%)	2.5±0.6	2.1±0.9	2.5±0.6
¹ Acidophilic cells (%)	6.3±1.6	3.4±0.8	4.5±0.9
² Degrees of maturation	4.0±0.03	3.9±0.03	3.9±0.06
² Leydig cell volume (μm^3)	222.3±8.8	206.4±16.2	215.9±8.7
² Tubular diameter (μm)	237.6±1.6	226.2±4.1	229.8±6.7
<i>PND 100</i>			
¹ Normal tubules (%)	85.6±1.3	86.4±1.4	92.2±2.2
¹ Vacuolization (%)	1.8±0.8	1.2±0.5	0.6±0.2
¹ Acidophilic cells (%)	12.0±1.1	11.8±1.3	7.0±2.3
¹ Dynamics of spermatogenesis (%)			
I–VI	36.8±2.2	41.0±1.4	42.4±1.2
VII–VIII	32.8±1.6	29.8±1.1	30.2±1.5
IX–XIII	28.2±3.1	27.0±1.8	25.2±2.5
XIV	2.2±0.6	2.1±0.6	2.2±0.5
² Leydig cell volume (μm^3)	258.6±6.4	259.2±7.4	261.8±9.2
² Tubular diameter (μm)	286.6±5.0	281.8±3.2	274.7±5.8

¹Values expressed as mean ± S.E.M (Kruskal-Wallis test followed by Dunn's test).

²Values expressed as mean ± S.E.M (ANOVA followed by Tukey's test). Different letters express $p<0.05$. CO -received filtered water; S10 and S20- received 10 and 20 mg/kg of sertraline.

In figure 6 it is possible to observe the histological aspect of the testes stained with HE on PND 21, 45, and 100.

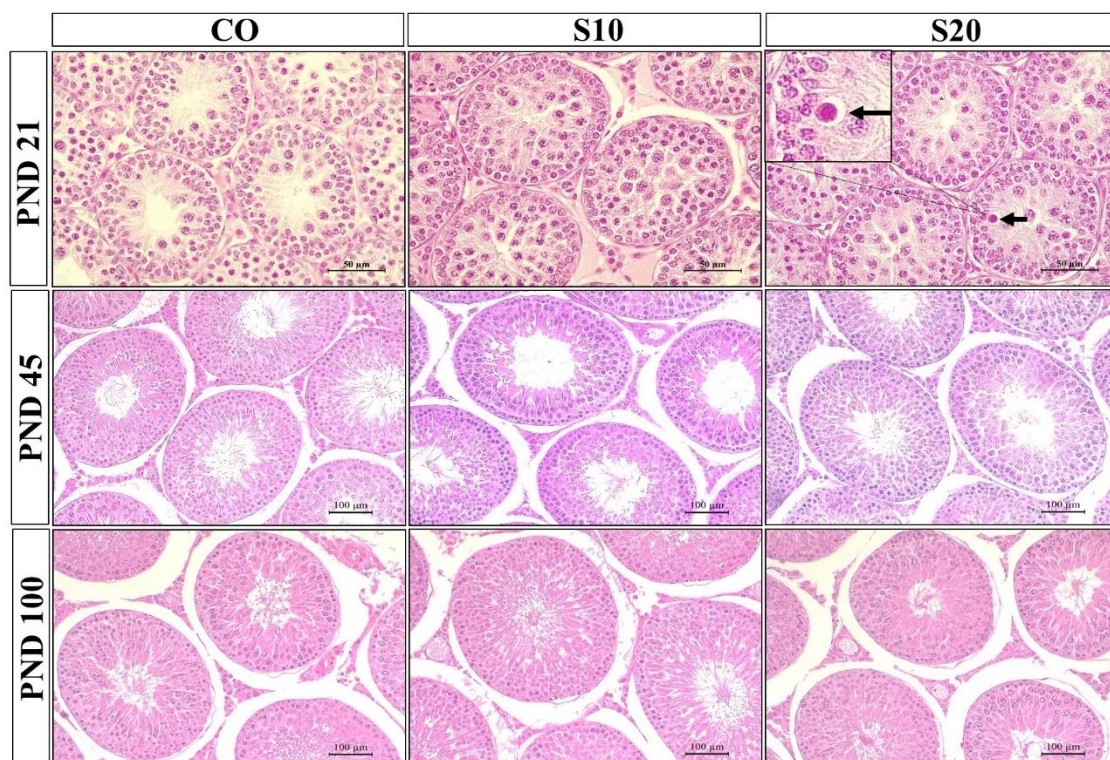


Figure 6. Cross-sections of testes stained with HE on PND 21, 45, and 100. Acidophilic cells (arrow). Final magnification: 400x and 200x. CO -received filtered water; S10 and S20- received 10 and 20 mg/kg of sertraline.

Immunohistochemistry

Qualitative analysis of PCNA on PND 45 (Figure 7) and 100 (results not shown), revealed no differences between experimental groups ($p>0.05$).

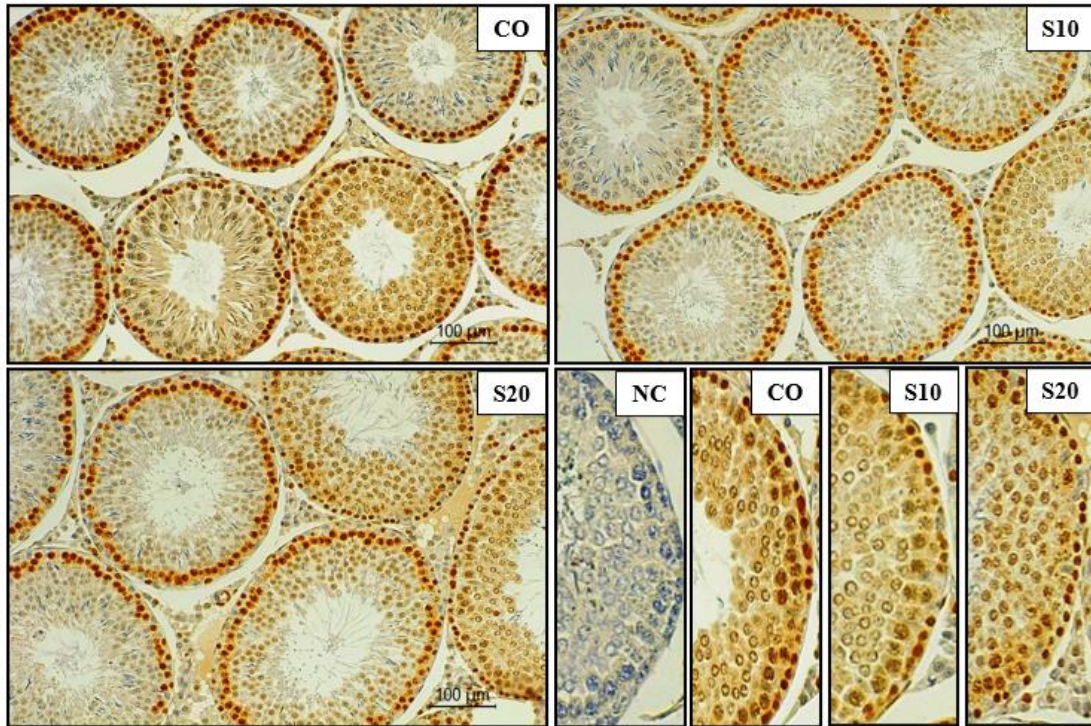


Figure 7. PCNA immunohistochemistry in cross-section of testes on PND 45. CO group, S10 group, S20 group, and negative control (NC, without primary antibody). CO - received filtered water; S10 and S20- received 10 and 20 mg/kg of sertraline.

Qualitative analysis of WT1 also showed no differences between the experimental groups ($p>0.05$), on PND 45 (Figure 8) and 100 (results not shown).

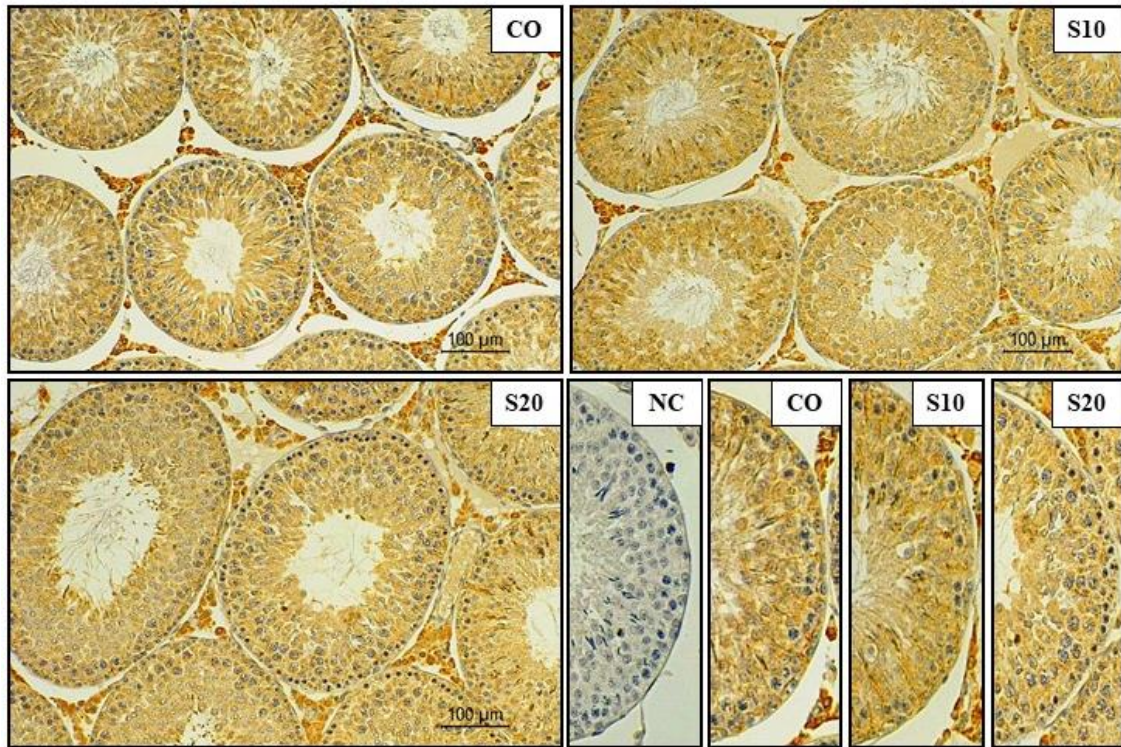


Figure 8. WT1 immunohistochemistry on PND 45. CO group, S10 group, S20 group, negative control (NC, without primary antibody). Final magnification: 200x. CO - received filtered water; S10 and S20- received 10 and 20 mg/kg of sertraline.

Histology of the epididymis

Histological analysis of the epididymis on PND 45 showed no differences between the experimental groups (Figure 9).

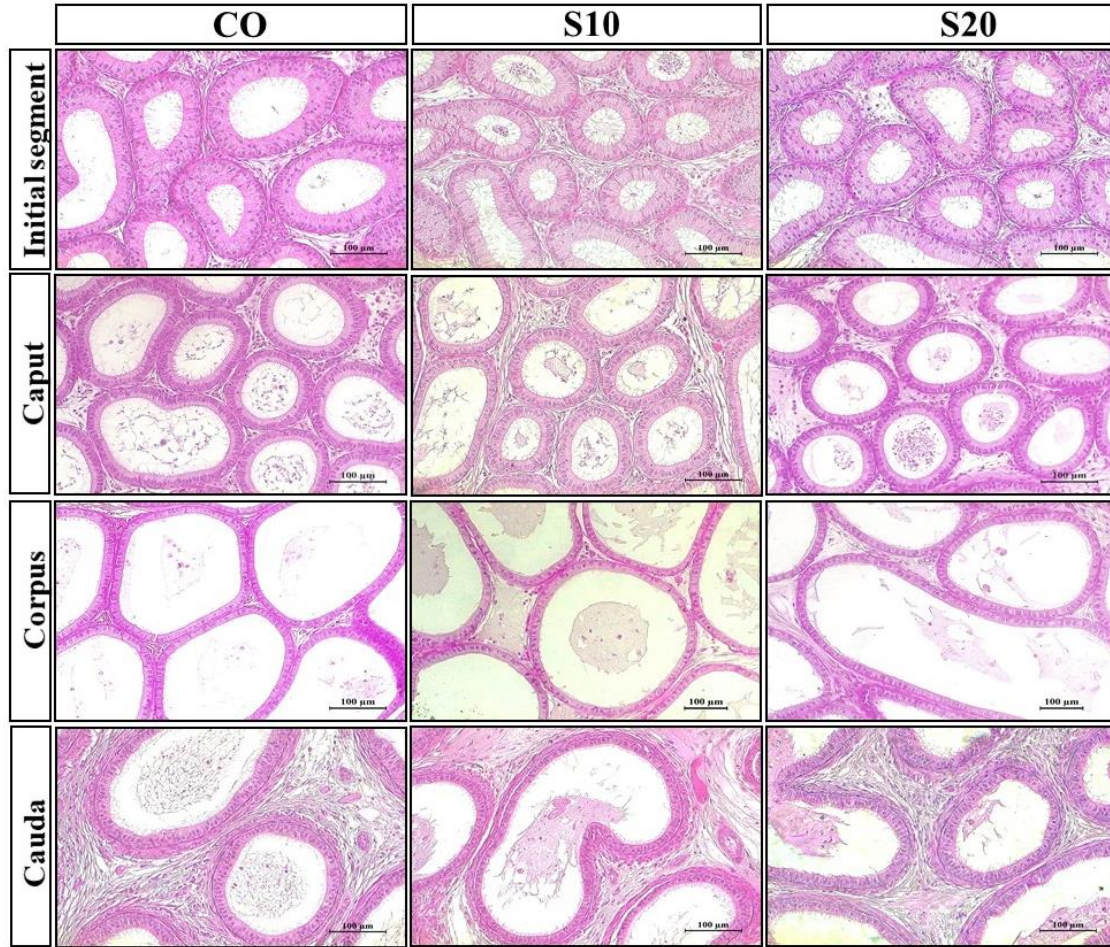


Figure 9. Epididymis on PND 45 stained with HE. (1) Initial segment, (2) caput, (3) corpus and (4) cauda. Final magnification: 200x. CO -received filtered water; S10 and S20- received 10 and 20 mg/kg of sertraline.

Histological analysis of the epididymis on PND 100, showed no difference between the experimental groups (Figure 10).

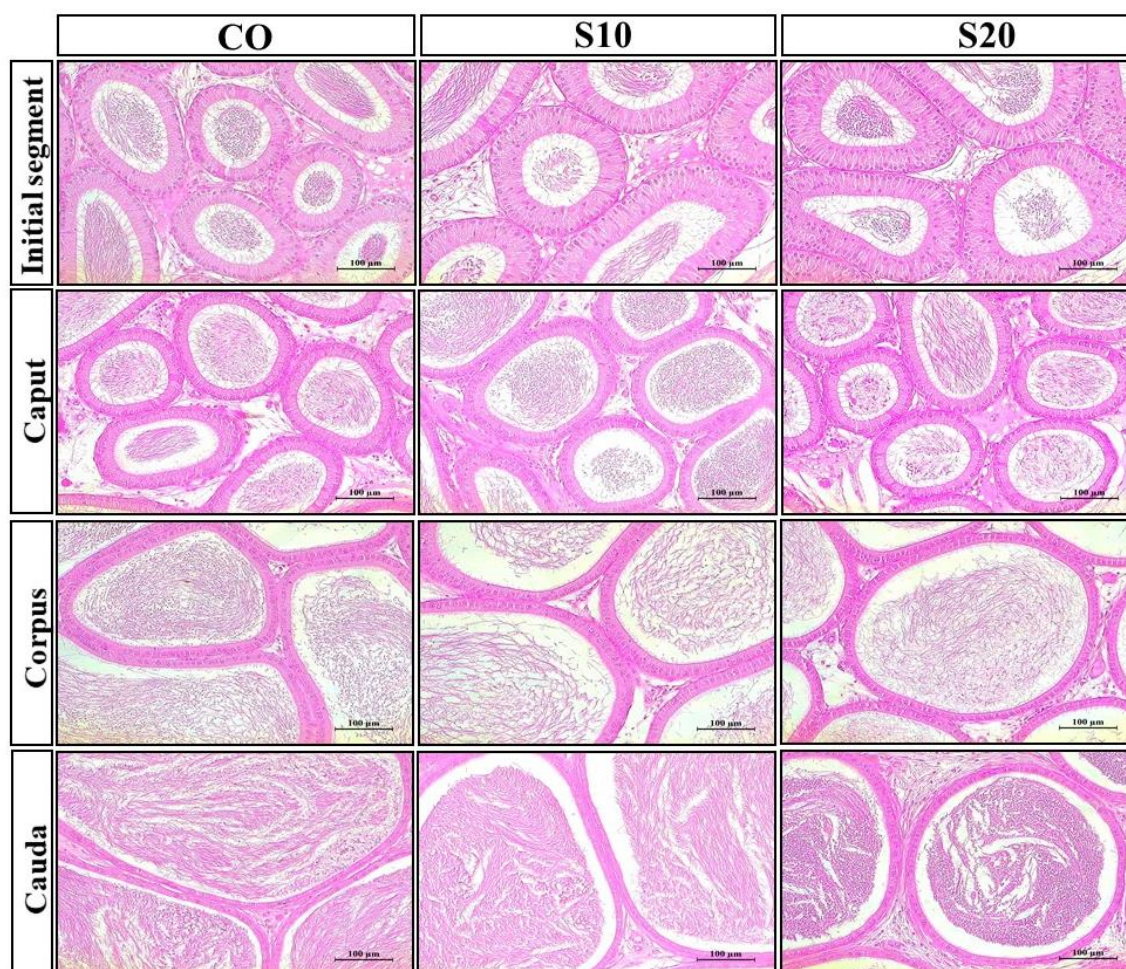


Figure 10. Epididymis on PND 100 stained with HE. (1) Initial segment, (2) caput, (3) corpus and (4) cauda. Final magnification: 200x. CO -received filtered water; S10 and S20- received 10 and 20 mg/kg of sertraline

Sperm motility and morphology

There was an increase in the number of immobile sperm (type C) in the S10 group compared to the CO group ($p=0.02$). The results obtained in the analysis of sperm morphology showed a higher prevalence of cytoplasmic droplets in the groups treated with sertraline ($p=0.005$) (Table 6).

Table 6- Sperm motility and morphology

Parameters (%)	CO (n=10)	S10 (n=10)	S20 (n=9)
<i>Motility</i>			
Type A	72.7±3.0	55.7±5.8	57.0±5.2
Type B	14.4±3.0	22.7±3.4	17.5±2.7
Type C	11.6±1.0 ^a	21.6±2.8 ^b	19.0±2.8 ^{ab}
<i>Morphology</i>			
Normally shaped sperm	96.56±0.4	96.10±0.4	96.33±0.4
Isolated head	0.25±0.1	0.60±0.2	0.22±0.1
Abnormalities of the sperm head	0.12±0.1	0.70±0.1	0.55±0.2
Curled tail	0.44±0.2	0.60±0.2	0.77±0.3
Broken tail	2.2±0.3	2.1±0.2	2.1±0.5
Sperm with CD	55.78±2.6 ^a	70.60±2.2 ^b	68.25±3.6 ^b
Sperm without CD	44.22±2.6 ^a	29.40±2.2 ^b	31.75±3.6 ^b

Values expressed as mean ± S.E.M. Kruskal-Wallis test followed by Dunn's test. Different letters expressed $p \leq 0.05$. Type A: sperm with the progressive movement, Type B: sperm without progressive movement, and Type C: immobile sperm; CD: cytoplasmic droplet. CO -received filtered water; S10 and S20- received 10 and 20 mg/kg of sertraline.

Sperm count

There was no difference between the experimental groups in daily sperm production and sperm transit time ($p > 0.05$) (Figure 11).

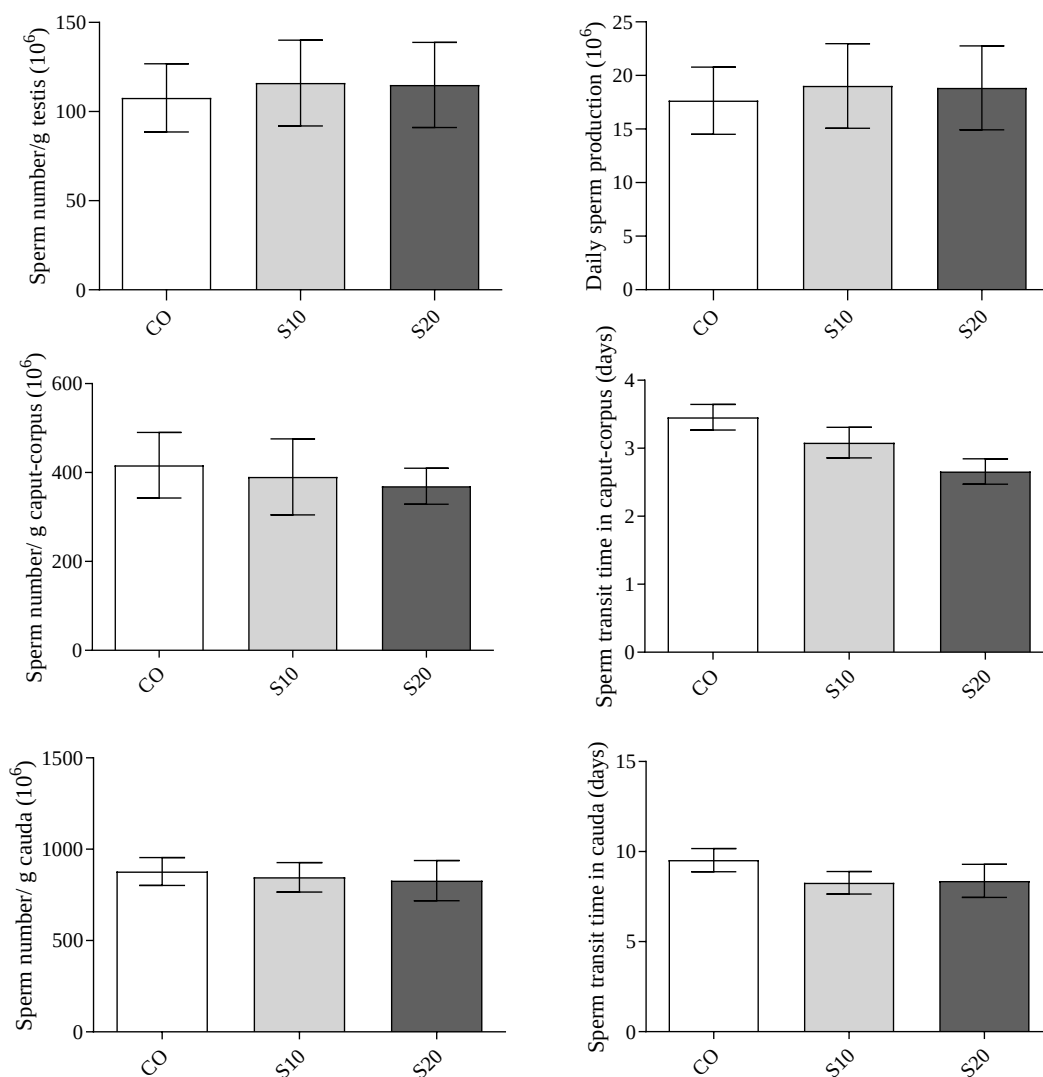


Figure 11. Sperm concentration, daily sperm production, and sperm transit of animals from CO, S10, and S20 groups (n= 1 male from each litter; 10 litters/group). Values expressed as mean $\pm$ S.E.M. ANOVA followed by Tukey's test. CO -received filtered water; S10 and S20- received 10 and 20 mg/kg of sertraline.

Sexual behavior natural fertility

The S20 group presented a higher frequency of mounts ($p < 0.0001$), as well as a lower frequency of intromissions when compared to the CO group ($p < 0.0001$).

Natural fertility showed no difference between the experimental groups ($p > 0.05$). (Table 7).

Table 7- Sexual behavior and natural fertility

Parameters	CO (n = 10)	S10 (n = 10)	S20 (n = 10)
<i>Sexual behavior</i>			
¹ Frequency of mounts (%)	88.9 ^a	80 ^a	100 ^b
² Latency of first mount (s)	118.1±18.2	132±33.2	222.4±68.2
¹ Frequency of intromissions (%)	77.8 ^a	80 ^a	55.6 ^b
² Latency of first intromission(s)	183±46.2	432.6±78.3	225.8±67.5
<i>Fertility</i>			
¹ Fertility potential (%)	98.4±1.0	97.2±1.3	94.6±1.4
¹ Pre-implantation loss (%)	1.7±1.1	2.7±1.3	5.3±2.6
¹ Pos-implantation loss (%)	6.0±1.8	0.6±0.6	8.5±4.1
² Number of live fetuses	11.8±0.7	12.7±0.6	12.1±0.5
² Sex ratio (M:F)	1.4±0.3	1.8±0.5	1.7±0.6
² Male fetal weight	4.5±0.1	4.4±0.05	4.5±0.07
² Female fetal weight	4.3±0.1	4.3±0.09	4.1±0.1
² Male placental weight	0.6±0.02	0.5±0.01	0.6±0.02
² Female placental weight	0.5±0.01	0.5±0.01	0.6±0.02

¹Value expressed as a percentage (Chi-square test). ²Values expressed as mean ± S.E.M.

Different letters express $p \leq 0.05$ (ANOVA followed by Tukey's test). CO -received filtered water; S10 and S20- received 10 and 20 mg/kg of sertraline.

4- DISCUSSION

Lactation is a period of extreme importance for the nutrition and growth of the newborn and is considered a vulnerable period for development. Not only nutrition but also the quality of maternal care has a profound impact on the child's cognitive, emotional, and social development [27]. It is known that maternal care during the puerperium can modulate the development of neural systems positively or negatively, and may induce different psychiatric disorders in the newborn, such as anxiety and depression [28].

In rodents, maternal behavior is a useful model for studying neurobiological mechanisms [29][30]. The lactating rat presents a repertoire of care during the period of 2 to 3 weeks after the birth of the pup, and the intensity and quality of these actions reflect on the development of the offspring [31]. The evaluation of maternal behavior in rodents is usually done in the first week after birth, as newborns need their mothers exclusively to survive [32].

Postpartum depression impacts a woman's physical and mental well-being, as well as the baby's health and the relationship between them. Studies have shown that women with depression are at greater risk of early cessation of breastfeeding and less positive

interaction with their children. As a consequence, behavioral and cognitive changes can impact the child's development [33][34].

Several antidepressants, including sertraline, paroxetine, and nortriptyline, were identified as having the best evidence base for use in breastfeeding women [34]. A study conducted by Logsdon, Wisner, and Hanusa [35], demonstrated that both sertraline and nortriptyline were equally effective in decreasing women's depressive symptoms and improving overall functioning and gratification in the maternal role. Leedy and Alexander [36] also observed that sertraline was able to improve the maternal behavior of stressed ewes during the postpartum period. In contrast, Zaccarelli-Magalhães et al. [37] observed, in rats, that fluoxetine caused dysfunctions in maternal behavior, without causing alterations and/or deficiencies in the offspring. In conditions of bipolar disorder, for example, postpartum pharmacotherapy should also be conducted with caution, as it may deteriorate maternal behavior [30].

In the present study, we did not observe behavioral changes in lactating rats exposed to sertraline, however, the number of grooming was higher in the S20 group compared to the CO group. It is known that an excessive increase in this behavior can reflect, in rodents, conflicting situations in the face of stressful stimuli [38].

Although in the present study maternal behavior was not impaired, male offspring exposed to sertraline revealed delayed palmar grasp, cliff avoidance, and negative geotaxis. We also showed that changes in these parameters were independent of antidepressant concentration. Neonatal reflexes are one of the first assessments used to verify the functional integrity of the nervous system [39]. It is known that serotonin is a neuromodulator that plays an important role in brain development, as it regulates critical processes such as cell division, differentiation, migration, myelination, and synaptogenesis. During the first few weeks of lactation, there is intense arborization of serotonin axonal terminals in the cerebral/ cerebellar cortex, hippocampus, and striatum [40].

Campos et al. [41] reported that chronic administration of fluoxetine in the neonatal period caused a delay in reflex ontogeny and somatic development in rats. In previous studies [42][16], we found that gestational exposure to sertraline compromised the somatic and neurobehavioral development of rat offspring. Vasconcelos et al. [43] also observed that treatment with sertraline during pregnancy causes damage to the somatic and neuromotor development of the offspring. Thus, compared with the present

study, we demonstrate that the window of exposure is crucial for the integrity of the nervous system.

Abnormal neurobehavioral developments have been reported in different studies with SSRIs, many associating them with psychiatric disorders such as autism spectrum disorder, attention deficit hyperactivity disorder, anxiety, and depression [44][45][46][42]. Although these changes have been reported in the literature, the present study did not detect neurobehavioral abnormalities in male offspring exposed to sertraline during lactation. Some human studies suggest that prenatal SSRI exposure does not increase the risk of behavioral changes in children when maternal mood is taken into account [47].

During treatment, a reduction in body weight was observed in the mothers and their offspring, as observed by other studies [48][49]. The significant weight reduction of offspring in the S20 group occurred precisely during a period when the only food source was breast milk. Also, other changes such as a smaller anogenital distance and histopathological changes were limited to this period.

It is known that low concentrations of sertraline can be transferred from breast milk to the plasma of infants [50][11]. Studies show that the relative infant dose (mg/kg/day) ingested by the infant through breast milk is < 10% for all SSRIs which is much lower than the in utero exposure [8]. Evidence suggests that an increase in serotonin neurotransmission can cause hypophagia, leading to low food intake, as it regulates neuropeptide Y, pro-opiomelanocortin and melanocortinergeric pathways [49][51].

In addition to the changes in the mother's body weight, the thyroid weight was increased in the S20 group, which may have been caused by the direct action of sertraline on the organ. Although measurements of thyroid hormones were not performed in the present study, it is known that other types of SSRIs can influence the production of these hormones [52][53][54][55].

Unlike other studies with fluoxetine exposures during lactation [56][57], in the present study a lower AGD was detected in animals from the S20 group. AGD has been used as a marker of fetal exposure to androgens, as it develops during the "masculinization programming window" that occurs at the same time as all male reproductive organs [58]. Although on PND 21 AGD had normalized, a smaller volume of Leydig cells was observed, reflecting a possible reduction in testosterone production [59]. This reduction possibly caused the atrophy of the seminiferous tubules and an increase in acidophilic cells, which represent dying cells [60][15].

The non-observation of histological alterations in the testes and epididymis on PND 45 and 100 reflect a normalization of these parameters in animals exposed to sertraline. However, a decrease in sperm motility and a large amount of cytoplasmic droplets were observed. The cytoplasmic droplets is produced during spermiation and is allocated to the distal position of the tail during sperm transport through the epididymis [61]. In this organ, spermatozoa acquire their potential for progressive motility and fertilization capacity [62].

Moreira et al. [63] also observed that lactational exposure to venlafaxine (SSRI) caused a reduction in sperm motility. Karimipour et al. [64] found that lactational exposure to fluoxetine negatively affects sperm parameters such as motility, morphology, and DNA integrity in mice. Kumar et al. [65], showed that antidepressants such as sertraline, fluoxetine, fluvoxamine, and citalopram have spermicidal activity, possibly by impairing ATP synthesis and by interactions with sulfhydryl groups present along the spermatogenic membrane. Rahban et al. [66] demonstrated that sertraline inhibits the Ca^{2+} channel (CatSper) and affects human sperm function *in vitro*.

Finally, although our results have demonstrated the negative impact that sertraline has on the development of the reproductive and nervous systems, we reinforce the importance of new studies to clarify the molecular processes involved in these changes. In addition, further analyzes are needed to verify the mechanism of action of sertraline on the structure and functionality of the thyroid.

5- CONCLUSION

Our results demonstrated that lactational exposure of rats to sertraline compromised maternal health, leading to decreased body weight and increased thyroid weight. The male offspring, during childhood, showed delay in the development of reflex and also testicular histological changes. In adulthood, although sperm quality was impaired, it was not able to impact fertility. Thus, our results alert the medical and scientific community about the drug's side effects.

6- DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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O experimento IV, intitulado como “Reproductive and neurobehavioral evaluation of mice exposed to sertraline and probiotic”, será submetido a um periódico internacional.

REPRODUCTIVE AND NEUROBEHAVIORAL EVALUATION OF MICE EXPOSED TO SERTRALINE AND PROBIOTIC

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ABSTRACT

Sertraline (SE) is a selective serotonin reuptake inhibitor widely used in clinical medicine. However, there are still few studies that have investigated its impact on male fertility, as well as adjuvant treatments that alleviate its side effects, such as sexual dysfunctions. It is known that some types of probiotics have a protective effect on reproductive parameters, and can bring benefits to mental health. Thus, the present study aimed to investigate the effects of SE on reproductive parameters in mice, as well as to verify whether the probiotic *Lactobacillus rhamnosus* alleviates the side effects of this antidepressant. Initially, a dose study (Experiment I) was carried out with 24 C57BL/6xBALB/c mice (n=6/group): CO group received filtered water (vehicle); and groups S10, S20, and S40 received respectively 10, 20 and 40 mg/Kg of SE diluted in the vehicle. The treatment occurred by gavage for 30 days and during this period the evolution of body weight was observed. At the end of the treatment, the animals were euthanized and the organs were weighed. Sperm quality was analyzed as well as testicular histology. After choosing the SE dose capable of causing reproductive alterations, which was 20 mg, experiment II was carried out. For that, 32 mice were distributed in 4 experimental groups (n=8/group): CO- received filtered water (vehicle); S – received 20 mg/kg of SE, diluted in the vehicle; P- received the probiotic *Lactobacillus rhamnosus* (1×10^9 CFU) diluted in the vehicle; and SP group that received both probiotic and SE. At the end of treatment, behavioral aspects were analyzed using the open field and elevated plus maze. After euthanasia, the organs were weighed, the histology of the testes and epididymis was analyzed, and the sperm quality and also natural fertility were verified. Complementarily, *in vitro* assays were carried out to verify whether sertraline could affect sperm capacitation and embryonic development. The statistical test used was ANOVA (followed by Tukey) or Kruskal-Wallis ($p \leq 0.05$). The results of Experiment I demonstrated that the animals in the S10 and S20 groups presented a decrease in the weight of the seminal glands compared to the CO group ($p < 0.05$). The other results showed no difference between the experimental groups ($p > 0.05$). In experiment II, it was observed that the probiotic reduced the loss of body weight in the animals exposed to SE. There was also no reduction in seminal gland weight in the SP group. Although a delay in sperm transit time was observed in the SP group, natural fertility was not compromised. In addition, this same group had a higher number of fetuses and a lower number of resorptions compared to the S group ($p < 0.05$). In the neurobehavioral tests, it was

observed that the profiles of the animals in groups P and S were different from each other ($p < 0.05$), the latter being less exploratory and more anxious. Thus, the present study demonstrated that SE has an impact on the reproductive system and the neurobehavior of adult mice. And for the first time, we demonstrate that the probiotic can alleviate these alterations.

Keywords: Sertraline, probiotic, reproductive system, neurobehavior, mice.

INTRODUCTION

Depression is a common disease worldwide, affecting about 280 million individuals, and is considered a public health problem that can lead to suicide. According to the World Health Organization (2021), more than 700.000 people die due to suicide every year, being the fourth leading cause of death in young people aged 15 to 29 years. The increase in psychiatric disorders since the beginning of the pandemic has been significant and in the United States, for example, the incidence of depression was 3 times higher during this pandemic period (Renaud-Charest *et al.*, 2021).

To improve people's quality of life and reduce the impacts of depression, anxiety, and stress on physical and mental health, the use of antidepressants and anxiolytics is recommended (Pratt *et al.*, 2017; Hanley and Oberlander, 2014). Since 1980, selective serotonin reuptake inhibitors (SSRIs) have been the most used and recommended drugs in medical clinics (Simon *et al.*, 2002).

Sertraline (SE) is one of the most used SSRIs, and in the United States, it is the third most indicated psychiatric drug, as it offers advantages in terms of efficacy and safety compared to others of its class. Its administration covers different types of people, such as adults, children and lactating women (Bałkowiec-Iskra *et al.*, 2017).

Gestational exposure to this drug can have an impact on fetal development, impairing the structure and functioning of different systems, such as the cardiovascular and nervous systems (Paulzen *et al.*, 2017). Previous studies carried out in our laboratory have shown that intrauterine exposure can compromise the neurobehavioral development of rat offspring (Lozano *et al.*, 2021; Moura *et al.*, 2023) as well as the development of the reproductive system (Quiarato Lozano *et al.*, 2022). In adult rats, sertraline has been shown to affect sperm motility and morphology, possibly by causing testicular oxidative stress and hormonal changes (Atli *et al.*, 2017).

Thus, it is noted that SE can affect the functionality of the reproductive system. However, it is still necessary to further studies to clarify the effect of this drug on male fertility, as well as to propose associated treatments that alleviate its side effects.

Probiotics are live microorganisms used as a supplement because they improve intestinal functionality and cause beneficial effects on human health (Williams, 2010). They can modulate the intestinal environment by inhibiting pathogens and improving epithelial barrier function through signaling pathways such as NF- κ B, Akt, and mitogen-activated protein kinase-dependent pathways (Segers and Lebeer, 2014).

Studies indicate that probiotics reduce anxiety and depressive symptoms by directly regulating the microbiota-gut-brain axis (Pinto-Sanchez *et al.*, 2017; Li *et al.*, 2019). The antioxidant capacity and reproductive protective effect have also been evidenced. Supplementation with *Lactobacillus rhamnosus* was shown to increase the percentage of progressively motile sperm, in addition to modulating serum testosterone, LH, and FSH levels in normal and obese mice (Dardmeh *et al.*, 2017). The probiotics *Lactobacillus spp.* and *Bacillus spp.* had antioxidant activity and protective effects against sperm damage induced by a high-fat diet (Chen *et al.*, 2013).

Therefore, the present study aimed to investigate the effects of SE on reproductive and neurobehavioral parameters. In addition, we verified whether the probiotic *Lactobacillus rhamnosus* mitigates the reproductive impacts caused by this antidepressant.

METHODOLOGY

Animals and treatment

Hybrid C57BL/6xBALB/c mice were housed under controlled conditions (22°C, 30% air humidity, 12/12h light/dark cycle), with food and water *ad libitum*. The animals were kept in accordance with the Ethical Principles for Animal Experimentation, adopted by the Brazilian College of Animal Experimentation. The project was filed under protocol number CEUA n° 3597170322.

Initially, a dose-response study (Experiment I) was carried out to identify the dose of SE capable of impacting reproductive parameters. Therefore, at 80 days of age, 24 animals were distributed into four experimental groups (n=6/group): Group CO that received filtered water by gavage; groups S10, S20, and S40 that received respectively 10, 20, and 40 mg/Kg of SE (diluted in the vehicle) by gavage. The concentrations of the antidepressant are considered, respectively, low, moderate, and high for rodents (Atli *et al.*, 2017; Kokras *et al.*, 2015).

The treatment took place for 30 days and the evolution of body weight was verified. In the end, the animals were euthanized by cervical dislocation to weigh organs

and verify sperm quality (motility and morphology). Daily sperm production, sperm transit time through the epididymis, and histology of the testes were also analyzed.

After identifying the SE concentration capable of impacting reproductive parameters, Experiment II was performed. For this purpose, 32 mice were divided into 4 experimental groups (n=8/group): CO- received filtered water (vehicle) by gavage; S – received 20 mg/Kg of SE, diluted in vehicle; P- received the probiotic *Lactobacillus rhamnosus* (1×10^9 CFU) diluted in vehicle; and SP group that received both probiotic and SE.

The probiotic dose chosen is based on a previous study that demonstrated the protective action of this compound on the reproductive system (Dardmeh *et al.*, 2017).

The treatment occurred for 30 days. At the end, the animals were euthanized by cervical dislocation to collect organs. Testicular and epididymis histology, sperm quality (motility and morphology), sperm production, and transit time through the epididymis were analyzed. Natural fertility was also investigated. It is important to highlight that all analyzes were carefully carried out blindly.

In vitro assay verified the acrosomal reaction of sperm exposed to different concentrations of SE (0.5, 5, 50, 500 $\times 10^{-5}$ mg/mL) during the capacitation process. The lowest doses chosen were found in the plasma of humans and rodents (DeVane *et al.*, 2002). The concentration of 500 $\times 10^{-5}$ mg/mL of SE was chosen for conducting the second stage. At this stage, embryonic development was verified after exposure to SE in different periods, such as capacitation, fertilization, and embryonic development. Thus, the following experimental groups were formed (Figure 1): SSS: Exposed during all stages to SE; CCC: Not exposed to SE; SCC: Exposed to SE during capacitation; CSC: Exposed to SE only during fertilization; SSC: Exposed to SE during capacitation and fertilization; CCS: Exposed to SE during embryonic development.

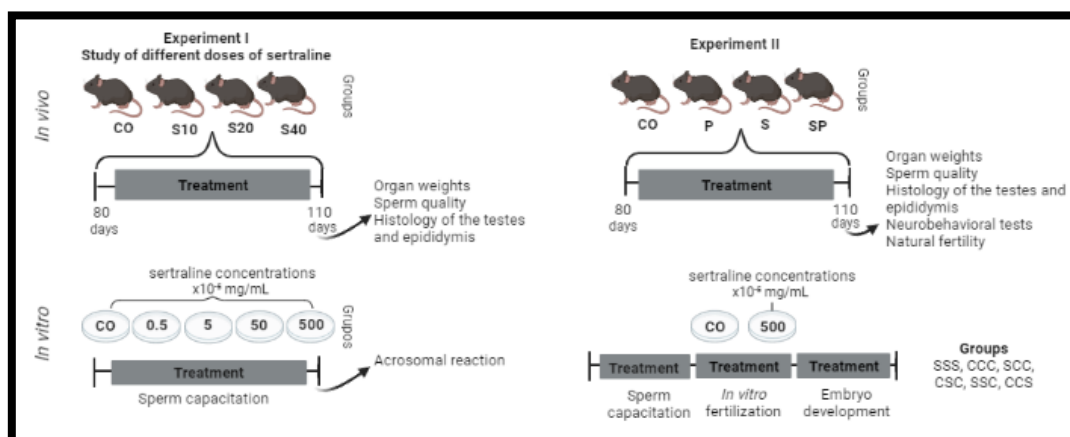


Figure 1. Experimental design.

Histology of the testes and epididymis

The testicles and epididymis were immersed in the mDF fixative solution and later were histologically processed for inclusion in Paraplast and preparation of histological sections with a thickness of 5µm. Sections were subsequently stained with hematoxylin and eosin.

Testicular histopathology was performed following the criteria established by Creasy (2002). For this purpose, 100 sections of the seminiferous tubules were analyzed along with the interstitial aspect.

Qualitative analysis of the epididymis occurred along the entire length of the organ, using the Leica Q-win Version 3 software for Windows TM (De Grava Kempinas and Klinefelter, 2015).

Sperm motility

Spermatozoa collected from the cauda were transferred to a PBS + 1% BSA solution and kept for 5 minutes at 37° C. Subsequently, a 10 µl aliquot of the suspension was placed in a Makler chamber. Using a phase contrast microscope (400x magnification), 100 sperm were counted and classified as progressive or non-progressive (Missassi *et al.*, 2017).

Sperm morphology

To perform the sperm morphology, 100µl of the solution containing spermatozoa was transferred to 900 µl of 10% formalin-saline solution. Then, smears were made on histological slides for analysis under a phase contrast microscope (400x magnification). Two hundred spermatozoa were analyzed per animal (Borges *et al.*, 2020).

Calculation of daily sperm production and transit time in the epididymis

The left testes and epididymis were processed according to methodologies used in other studies with some modifications (Borges *et al.*, 2020; Carvalho *et al.*, 2020). The testicular parenchyma was homogenized in 2 ml of a mixture of NaCl and Triton x100 (Sigma Chemical Co., St. Louis, Mo.), then processed in a sonicator for 30 seconds. After a 2-fold dilution, a sample was transferred to Neubauer chambers, and mature spermatids were counted to calculate sperm production per day (DSP). DSP was calculated by dividing the total number of spermatids resistant to homogenization found per testis by

4.84 (the number of days spermatids are present in the seminiferous epithelium of mice)(Carvalho *et al.*, 2020).

In the same way, the caput/corpus and cauda of the epididymis were cut into small fragments and homogenized in 1 mL of a mixture of NaCl and Triton x100. After a 3-fold dilution, a sample was transferred to Neubauer chambers. The transit time of spermatozoa through the epididymis was determined by dividing the number of spermatozoa by the DSP.

Sperm capacitation

Spermatozoa were recovered by incising the cauda epididymis in 300 μ L capacitation medium (Fraser and Drury, 1975) supplemented with 0.3% bovine serum albumin. After allowing motile sperm to swim out, aliquots of the suspension were added to 300 μ L fresh medium previously placed in tissue culture dishes containing different concentrations of sertraline (0.5, 5, 50, 500 $\times 10^{-5}$ mg/mL) to give a final concentration of $1-10 \times 10^6$ cells/mL. Incubation lasted 90 min at 37°C in 5% CO₂. Subsequently, the acrosomal reaction was verified (Gómez-Elías *et al.*, 2016).

Acrosomal reaction assays

Acrosome reaction of capacitated spermatozoa was evaluated by staining with Coomassie Brilliant Blue following the methodology described by Gómez-Elías *et al.* (2016). Thus, 200 spermatozoa were analyzed under a light microscope (400x) and the percentage of spermatozoa with acrosome in reaction was calculated.

In vitro fertilization

Mice were superovulated by an injection (i.p.) of equine chorionic gonadotropin (eCG; 5 IU), followed by an injection (i.p.) of human chorionic gonadotropin (hCG; 5 IU) 48 h later. Egg–cumulus complexes were collected 12–13 h after hCG administration and inseminated with capacitated spermatozoa (final concentration: $5-6 \times 10^5$ spermatozoa/mL). Subsequently, gametes were co-incubated for 3 h. Then, the oocytes were transferred into the KSOM medium. After 15h the percentage of two cell embryos was recorded. Four days later, the percentage of blastocysts was checked (Gómez-Elías *et al.*, 2016).

Natural fertility

Seven days before the end of treatment, a sexually receptive female (in proestrus/or estrus) was placed in the box with a male. After verifying the first mount, the animals remained together throughout the dark period. The following day they were separated and 16 days later, laparotomy was performed on the pregnant females. After collecting the uterus, the numbers of implants, resorptions, and live fetuses were determined. From these results, the post-implantation loss rate was calculated: $(\text{number of implants} - \text{number of live fetuses}) / \text{number of implants} \times 100$.

Results Analysis

For the experiment I, the one-way ANOVA test was used (followed by the Tukey test). The two-way ANOVA test (followed by the Tukey test) was used in experiment II. For non-parametric data, the Kruskal-Wallis test (followed by Dunn) was performed. Differences were considered significant when $p \leq 0.05$. Statistical analyzes were realized using the GraphPad Prism program.

RESULTS

Evolution of body weight

It was observed that in experiment II on day 30, the S group had a lower body weight compared to the SP group ($p=0.04$) (Figure 2). In other periods, no significant differences were observed ($p>0.05$)

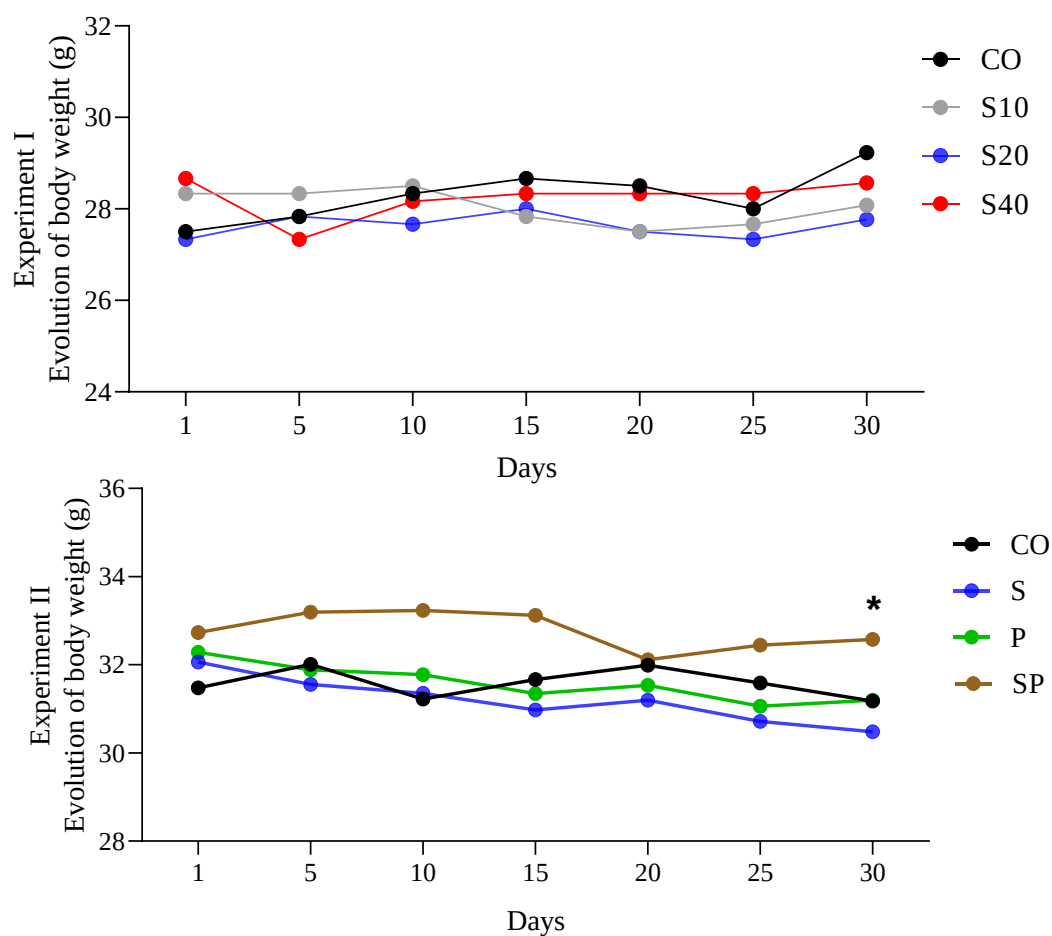


Figure 2. Evolution of body weight. Values expressed as mean $\pm$ S.E.M (ANOVA followed by Tukey's test). * $p=0.04$

Organ weights

In experiment I, the empty and full seminal gland weight of the S20 group was lower compared to the CO group ($p<0.05$). The empty seminal gland was also smaller in the S10 group compared to the CO group ($p<0.05$).

In experiment II, the S group also had a smaller empty seminal vesicle compared to CO and SP groups ($p=0.02$ and 0.002) (Table 1).

Table 1. Organ weights

Organ (mg)	Experiment I				Experiment II			
	CO (n=6)	S10 (n=6)	S20 (n=6)	S40 (n=6)	CO (n=8)	P (n=8)	S (n=8)	SP (n=8)
Testis	104.6±1.8	103.1±2.4	105.9±1.8	110.4±1.3	100.7±2.2	105.5±1.6	104.3±2.1	106.6±1.3
Epididymis	39.9±1.4	36.8±1.0	40.9±1.0	42.7±2.9	40.0±0.9	42.2±1.0	39.3±0.7	41.7±1.2
Seminal vesicle	210.8±7.2 ^a	175.9±8.7 ^{ab}	175.2±9.0 ^b	185.8±8.9 ^{ab}	184.2±10	196.0±13	200.9±11	223.3±10
Empty seminal vesicle	139.6±7.4 ^a	103.0±4.1 ^b	109.6±4.0 ^b	127.6±8.5 ^{ab}	72.9±2 ^a	68.2±1 ^{ab}	62.0±2 ^b	76.3±2 ^a
Liver	1420±18.2	1351±59.3	1331±61.6	1374±53.6	1529±53	1483±77	1401±27	1560±33
Brain	449.5±4.0	443.7±6.4	433.9±5.5	447.6±4.3	466.3±4.7	455.2±8.1	447.5±7.2	464.3±5.9
Lungs	172.6±7.8	163.3±1.8	171.4±12.0	187.1±10.4	218.2±15	211.3±10	199.7±7	210.2±7
Heart	188.1±5.8	187.4±5.9	188.9±17.0	190.3±7.0	239.3±6.8	215.1±13.1	225.1±6.9	241.9±11.8
Adrenals	5.9±0.7	3.4±0.6	5.9±0.7	4.7±0.4	4.8±0.2	4.8±0.5	4.8±0.4	3.8±0.5
Pituitary	1.4±0.1	1.0±0.1	1.2±0.1	1.3±0.1	1.4±0.1	1.0±0.1	1.2±0.1	1.3±0.1

Values expressed as mean ± S.E.M (ANOVA followed by Tukey's test). Different letters indicate $p < 0.05$.

Histology of testis and epididymis

There were no differences in testicular histology between the experimental groups ($p > 0.05$) (Table 2).

Table 2. Testicular histopathology

Parameters (%)	Experiment I				Experiment II			
	CO (n=6)	S10 (n=6)	S20 (n=6)	S40 (n=6)	CO (n=8)	P (n=8)	S (n=8)	SP (n=8)
Normal tubules	91.6±1.1	94.0±1.1	94.2±0.5	91.2±1.5	85.0±1.0	82.2±2.5	82.8±1.1	84.5±1.3
Vacuolization	0.5±0.2	0.0±0.0	0.0±0.0	0.0±0.0	0.7±0.2	0.7±0.2	0.8±0.2	2.3±0.5
Acidophilic cells	4.4±0.6	2.0±0.5	4.0±0.9	5.4±0.9	12.4±1.3	11.6±0.8	14.7±1.2	11.8±1.3
Exfoliation	0.3±0.3	2.0±2.0	1.4±0.9	2.6±0.8	0.2±0.1	0.2±0.1	0.0±0.0	0.0±0.0
Degeneration	0.83±0.6	1.0±1.0	0.0±0.0	0.0±0.0	0.1±0.1	0.2±0.1	0.2±0.2	0.3±0.1
Multinucleated cells	0.8±0.6	1.0±1.0	0.0±0.0	0.0±0.0	0.1±0.1	0.5±0.3	0.3±0.1	0.1±0.1

Values expressed as mean ± S.E.M (Kruskal-Wallis test).

In Figure 3, it is possible to observe the testicular histology of the animals from Experiment I. No histological differences were observed between the experimental groups ($p>0.05$).

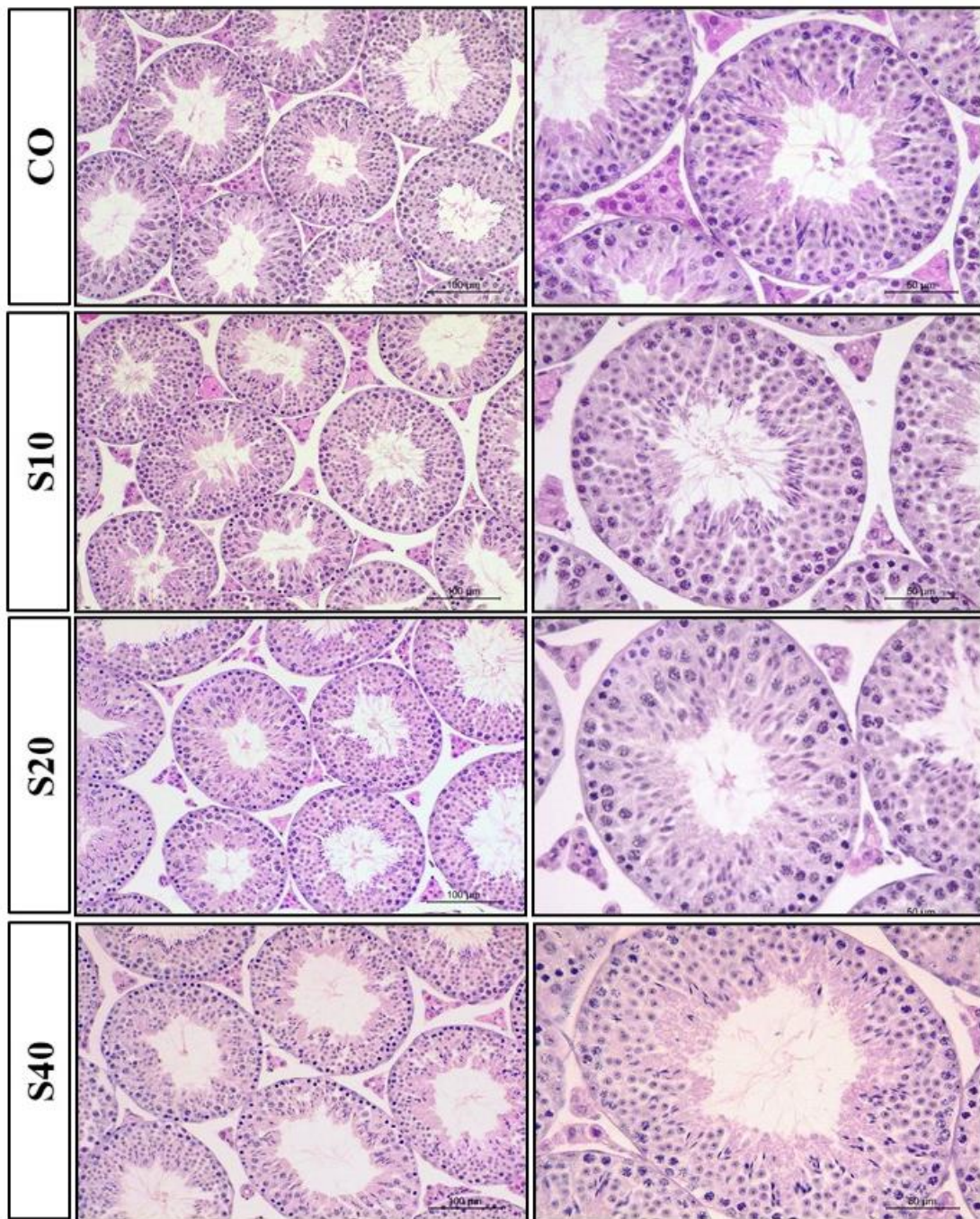


Figure 3. Testicular histology of the experimental groups of Experiment I.

In Figure 4, it is possible to observe the testicular histology of the animals from Experiment II. No histological differences were observed between the experimental groups ($p>0.05$).

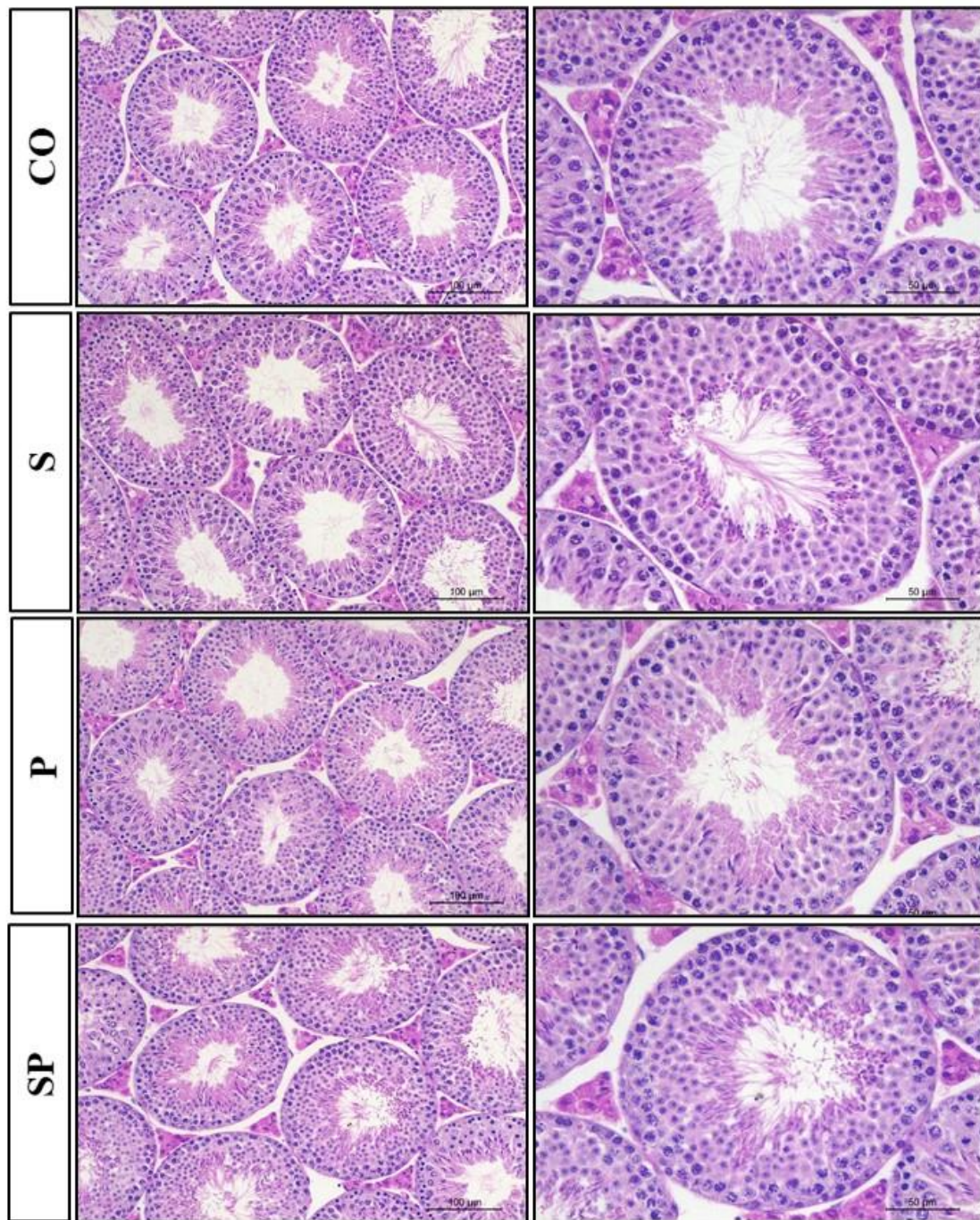


Figure 4. Testicular histology of the experimental groups of Experiment II.

There was no difference in the histological analysis of the epididymis in Experiment I (Figure 5).

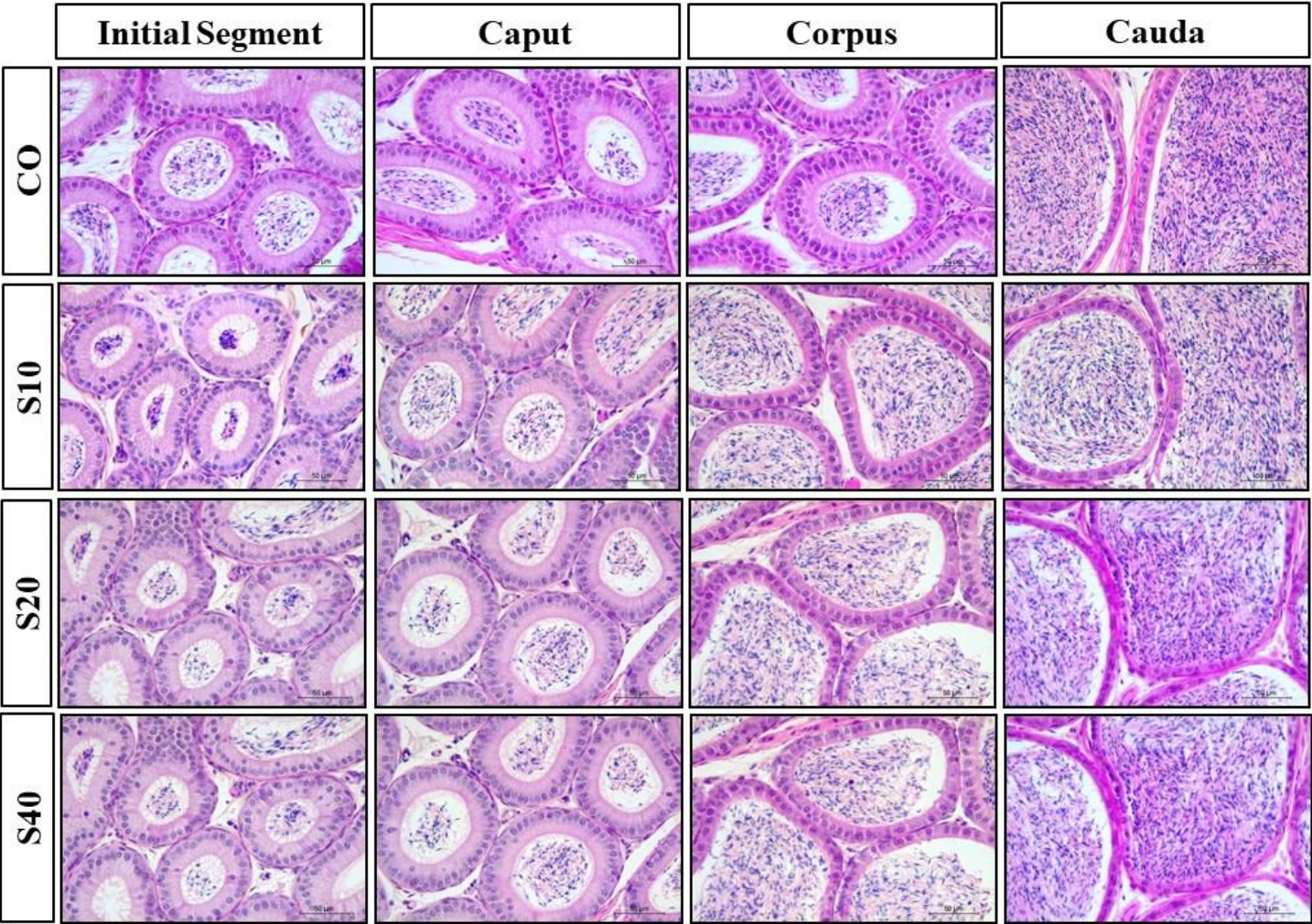


Figure 5. Histology of the epididymis of the different experimental groups.

In Experiment II, no histological alterations were observed either in epididymis (Figure 6).

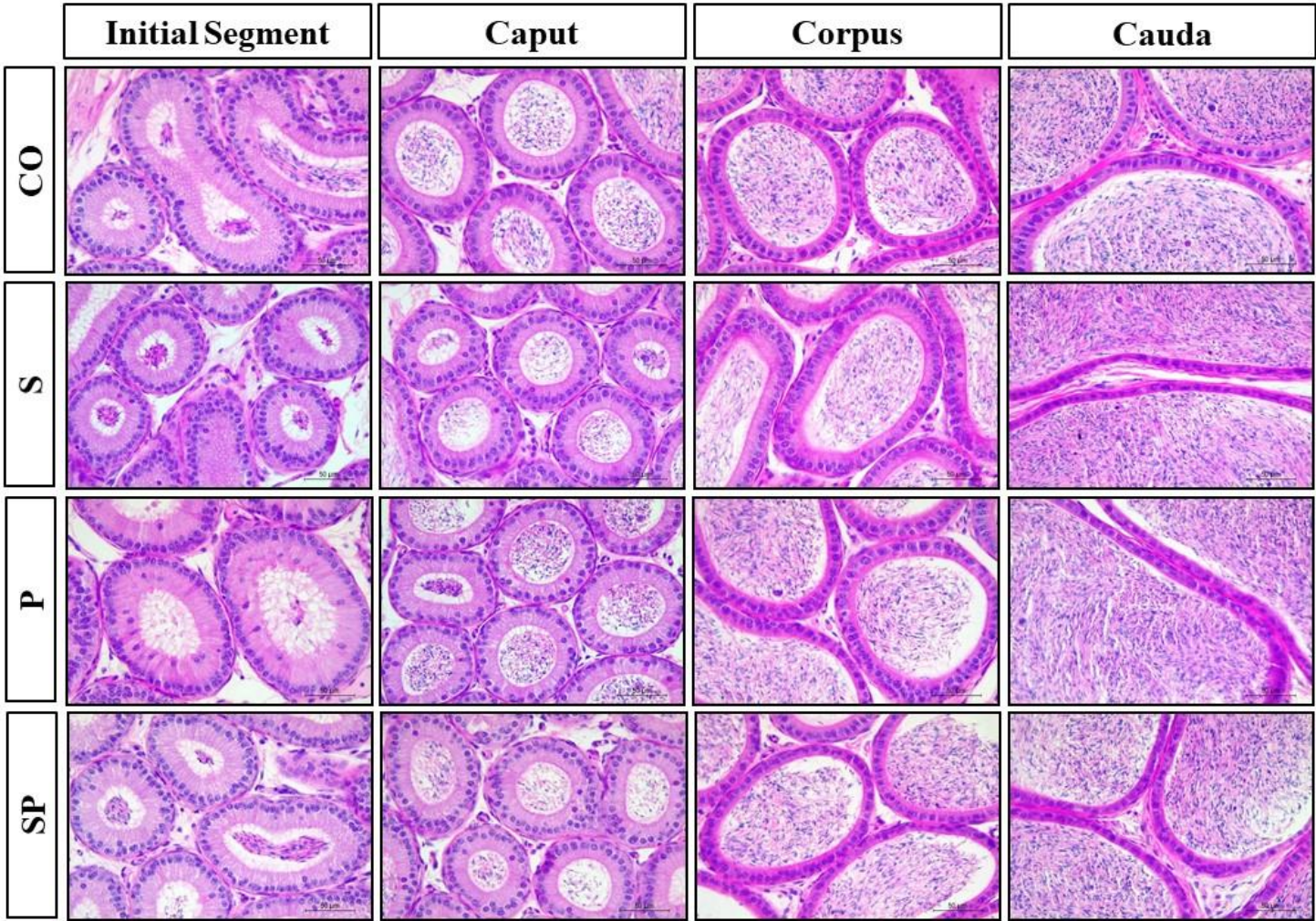


Figure 6. Histology of the epididymis of the different experimental groups.

Motility

No differences were observed in sperm motility between groups ($p>0.05$) (Figure 7).

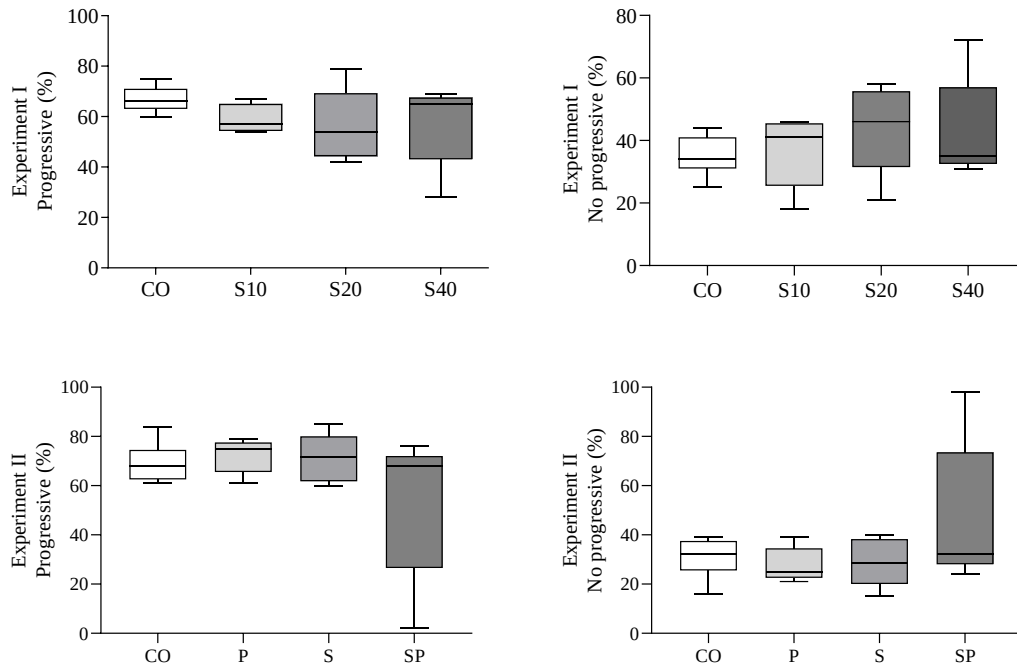


Figure 7. Sperm motility. Values expressed as mean $\pm$ S.E.M (Kruskal-Wallis test).

Sperm count and morphology

A delay in sperm transit time through the epididymis was observed in the SP group compared to the other groups ($p>0.05$) (Table 3).

Table 3. Sperm count and morphology

Parameters	Experiment I				Experiment II			
	CO (n=6)	S10 (n=6)	S20 (n=6)	S40 (n=6)	CO (n=8)	P (n=8)	S (n=8)	SP (n=8)
¹ <i>Sperm morphology (%)</i>								
Normal shaped sperm	79.5±1.5	81.2±2.5	80.5±2.8	80.5±3.2	78.6±2.5	74.4±3.2	77.6±2.4	79.2±2.2
Curly tail	0.5±0.3	0.8±0.3	0.7±0.2	1.6±0.6	3.8±1.2	1.4±0.6	3.5±1.2	1.0±0.2
Broken tail	12.6±1.3	8.2±1.2	12.0±3.3	7.1±2.4	11.4±1.8	15.2±2.1	10.6±1.3	11.3±2.3
Isolated head	1.5±0.4	2.4±0.8	2.0±0.4	3.3±0.7	1.7±0.4	2.2±0.8	1.8±0.6	2.7±0.6
Head without curvature	5.8±0.6	7.4±1.2	4.7±1.6	7.3±0.9	4.6±1.1	4.8±1.3	7.0±1.3	5.0±1.1
Sperm with cytoplasmic droplet	55.0±2.5	52.0±3.9	48.5±4.2	49.5±2.9	46.3±2.0	41.8±2.7	47.1±2.5	42.6±3.9
² <i>Sperm count</i>								
Sperm number (10 ⁶)	15.2±1.5	13.5±1.5	16.1±1.2	14.0±1.4	10.1±0.7	10.9±0.8	11.0±0.7	9.1±1.3
Daily sperm production (10 ⁶)	3.1±0.3	2.7±0.3	3.3±0.2	2.8±0.3	2.1±0.1	2.2±0.1	2.2±0.1	1.8±0.2
Sperm number (10 ⁶)- CC	6.6±0.7	7.1±0.4	6.9±0.4	6.5±1.0	5.7±0.4	6.9±0.5	6.3±0.4	6.7±0.5
Sperm transit time (days)- CC	2.4±0.4	2.6±0.3	2.1±0.2	2.3±0.3	2.8±0.2 ^a	3.1±0.3 ^a	2.7±0.2 ^a	4.6±0.5 ^b
Sperm number (10 ⁶)- CA	9.5±1.1	7.5±1.6	8.6±1.3	9.7±1.8	9.8±0.7	10.2±1.1	9.4±0.7	11.8±0.5
Sperm transit time (days)- CA	3.1±0.6	2.2±0.42	2.6±0.4	3.6±0.9	4.9±0.6 ^{ab}	4.6±0.4 ^a	4.1±0.3 ^a	7.1±0.8 ^b

Values expressed as mean ± SEM. ¹Kruskal-Wallis test; ²ANOVA followed the Tukey test. Different letters indicate p<0.05. CC- caput/corpus; CA- cauda

Natural fertility

In experiment II, an increase in resorption was observed in the S group compared to the SP group ($p<0.05$), also an increase in the number of live fetuses in the SP group compared to the S and P groups ($p<0.05$) (Table 4).

Table 4. Natural fertility- Experiment II

Parameters	CO (n=8)	P (n=8)	S (n=8)	SP (n=8)
¹ Implantation number	9.6±0.4	9.3±0.7	9.6±0.4	10.3±0.2
¹ Resorption number	1.1±0.4 ^{ab}	1.2±0.3 ^{ab}	2.3±0.6 ^a	0.4±0.1 ^b
¹ Live fetuses' number	8.5±0.2 ^{ab}	8.1±0.5 ^a	7.1±0.7 ^a	9.7±0.2 ^b
² Sex ratio (M: F)	0.8±0.08	1.8±0.5	1.0±0.2	1.2±0.2
² Pos-implantation loss (%)	10.9±3.2	12.2±2.4	25.8±6.9	5.2±1.6

Values expressed as mean ± SEM. ¹ANOVA followed the Tukey test. ²Kruskal-Wallis test. Different letters indicate $p<0.05$.

In vitro assay

The percentage of spermatozoa without acrosome in the acrosome reaction analysis was not different between groups. As well as the percentage of 2 cells and blastocysts ($p>0.05$) (Figure 6).

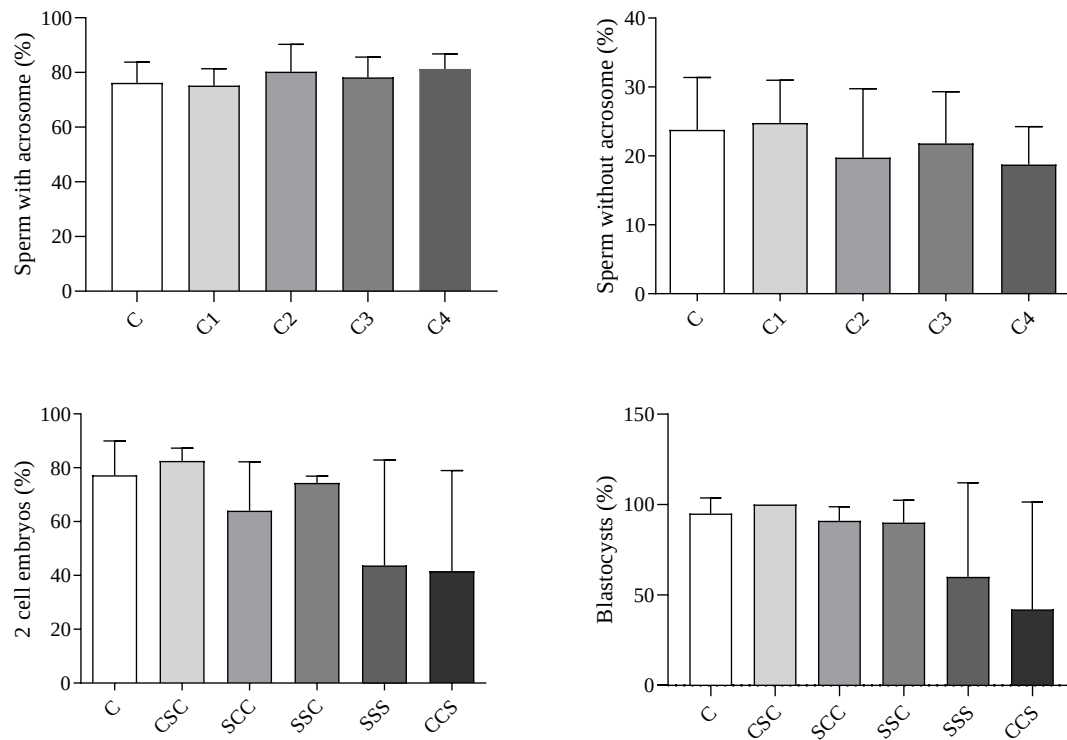


Figure 6. *In vitro* assay. Values expressed as mean + SEM. Kruskal-Wallis test. C= control; C1, C2, C3, C4 were exposed to 0.5; 5; 50; 500 $\times 10^{-5}$ mg/ml SE, respectively. SSS: Exposed during all stages to SE; CCC: Not exposed to SE; SCC: Exposed to SE during capacitation; CSC: Exposed to SE only during fertilization; SSC: Exposed to SE during capacitation and fertilization; CCS: Exposed to SE during embryonic development.

Neurobehavior

In experiment II, neurobehavioral tests showed that S group entered less in open and closed arms compared to P group ($p=0.02$). The SP and S groups entered the closed arms fewer times compared to the P group ($p<0.05$). The number of crossings through the open field was also lower in the S group compared to the P and CO groups ($p<0.05$) (Table 5).

Table 5. Neurobehavior- Experiment II

Parameters	CO (n=8)	P (n=8)	S (n=8)	SP (n=8)
<i>Elevated plus maze</i>				
Time in open arms (s)	101.0±24.7	97.1±14.5	34.5±15.0	85.3±16.8
Open arms entries (count)	8.3±1.5 ^{ab}	9.8±1.5 ^a	3.3±1.0 ^b	7.8±1.4 ^{ab}
Closed arms entries (count)	9.8±0.7 ^{ab}	12.0±1.1 ^a	7.7±0.8 ^b	8.3±1.0 ^b
Rearing (count)	8.4±1.7	12.0±1.5	6.8±1.2	8.3±1.7
Stretched (count)	23.8±3.3	21.5±1.1	24.7±1.6	22.0±2.3
Head dipping (count)	15.8±3.4	17.7±3.2	8.5±1.3	18.0±4.0
<i>Open field</i>				
Ambulation near the wall	55.6±5.0	61.7±10.0	50.6±7.1	44.1±6.0
Time near the wall (s)	94.0±8.4	92.3±8.6	119.8±7.3	97.6±11.2
Total crosses (count)	4.5±0.5 ^a	4.7±0.4 ^a	2.1±0.5 ^b	3.6±0.6 ^{ab}

Values expressed as mean ± SEM. ANOVA followed the Tukey test. Different letters indicate p<0.05

DISCUSSION

SSRIs are widely prescribed medications for depression and anxiety-related disorders. Although they are selective for serotonin transport, they can also act on other targets, which gives rise to some side effects. It is believed that over 80% of individuals who begin therapy with these antidepressants experience at least one side effect (Joshi, 2018; Silva *et al.*, 1999; Gregorian *et al.*, 2002).

As an unwanted effect, SSRIs can promote effects contrary to what was expected. For example, fluoxetine may lead to an exacerbation of anxiety due to increased serotonergic transmission (Silva *et al.*, 1999). Perinatal exposures are reported to induce children and experimental animals to have increased traits of anxiety-like or depression-like behavior (Glover and Clinton, 2016).

In the present study, the animals in the S group showed less exploration in the elevated plus maze and open field, reflecting an anxious profile compared to the P group. Interestingly, not all the altered parameters in the S group were observed in the SP group, which showed similar results to the CO group. A study carried out by Bravo *et al.* (2011) observed greater exploitation of animals treated with *Lactobacillus rhamnosus*, reflecting an anxiolytic action. In addition, the authors reported that the microorganism was able to modulate the mRNA expression of GABAA α 2, GABAA α 1, and GABAB1 receptors that are implicated in anxiety behavior.

A clinical trial conducted by Akkasheh *et al.* (2016) investigated the effects of probiotic supplementation on patients with major depression. The probiotic capsule contained three strains of *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium bifidum*. Results indicated that patients who received the probiotic intervention had significantly lower Beck Depression Inventory scores compared to the placebo group.

The human intestine has about 100 trillion bacteria essential to intestinal functioning, and also to other systems such as the immune and nervous systems. Studies indicate that rodent behavioral responses are affected when the gut bacterial status is manipulated (Foster and McVey Neufeld, 2013). Since probiotics can modulate the microbiota-gut-brain axis, a new category of drugs called "psychobiotic" has emerged. The exact mechanisms by which the brain and gut interact are not fully understood, but bidirectional communication through the autonomic nervous system, the immune system, and the hypothalamic-pituitary-adrenal axis is well documented (Ansari *et al.*, 2020.; Pinto-Sanchez *et al.*, 2017; Neufeld *et al.*, 2011).

The interaction of drugs with the intestinal microbiota can also cause important changes. For example, the antipsychotic Olanzapine is capable of inducing severe weight gain and

metabolic disturbances by interacting with the gut microbiome (Morgan *et al.*, 2014). Little is known about the direct effects of antidepressants on the intestinal mucosa. A study carried out by Eyzaguirre-Velásquez *et al* (2021) demonstrated that both citalopram and SE can affect intestinal mucosal function. This mechanism is involved with changes in intestinal epithelial barrier properties triggered by tight junction modifications and post-translational changes in signaling proteins.

It is already well established that SE is capable of promoting body weight reduction since serotonin plays a role in controlling food intake and in the body's energy homeostasis (Nielsen *et al.*, 1992; Lozano *et al.*, 2023; Rachdi *et al.*, 2019). In the present study, it was demonstrated that the animals in the SP group had a significant increase in body weight when compared to the S group. Interestingly, the group isolated to the probiotic did not show differences in this aspect, suggesting that the weight gain is a result of the action combined with the SSRI. Thus, it is possible that the intestinal changes caused by SE were reversed by probiotics. However, more studies should be conducted to clarify this mechanism.

It was observed that the weight of seminal vesicles was lower in S10 and S20 groups. This reproductive organ is involved in the ejaculatory process and any alteration in its functioning results in ejaculatory alterations. Premature ejaculation is one of the most common sexual disorders, affecting 20 to 40% of sexually active men. It is known that SSRIs emerged as a new treatment modality, since serotonin is one of the most important neurotransmitters in ejaculation, as it is involved in the control of contraction and also in smooth muscle relaxation (Kim *et al.*, 2004).

Previous studies have shown that patients with premature ejaculation usually have larger seminal vesicles, and SE can mitigate this effect (Hong *et al.*, 2017; Longping *et al.*, 2018). It is known that SE inhibits the increase in seminal vesicle pressure and the contractile responses induced by stimulation of the hypogastric nerve (Kim *et al.*, 2004). Interestingly, in the present study, the SP group did not present alterations in the weight of the vesicles, and the probiotic may have interfered with the SE mechanism of action. However, we reinforce the need for further studies to clarify this hypothesis.

The SP group also showed a delay in sperm transit time. During this event, many morphological and physiological characteristics of the spermatozoa are modified to enhance motility and fertilizing capacity. It is known that the acceleration in sperm transit causes a reduction in the time required for maturation (Fernandez *et al.*, 2008). Although in the present study sperm transit was slow in the SP group, this group did not show changes in motility or natural

fertility. In addition, a greater number of fetuses and a smaller number of resorptions were observed in relation to the S group.

Finally, as sertraline is used by patients with psychiatric disorders, we warn that our study has a limitation, as the animals used did not present these disorders before treatment.

CONCLUSION

We verified that SE was able to decrease the weight of the seminal vesicle and body weight, in addition to causing a less exploratory profile in adult mice. These changes were mitigated when this antidepressant was associated with the probiotic *Lactobacillus rhamnosus*. These results provide an important contribution to understanding the effects of probiotics. However, we reinforce the need for further studies to be carried out to clarify the molecular mechanisms involved.

DECLARATION OF COMPETING INTEREST

The authors declare that there are no conflicts of interest

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A depressão, o estresse e a ansiedade são comumente observados na população, e como consequência o consumo de antidepressivos tem aumentado exponencialmente nos últimos anos. A SE, classificada como ISRS, é um antidepressivo/ansiolítico utilizado por pessoas com distintos transtornos psiquiátricos e diferentes faixas etárias. Os ISRS podem provocar graves efeitos colaterais, no entanto existem poucos relatos sobre os impactos da SE em aspectos reprodutivos e comportamentais, tampouco há tratamentos adjuvantes que amenizem seus efeitos indesejáveis. Sabendo-se dos benefícios dos probióticos no eixo microbiota-intestino-cérebro e sua ação benéfica na saúde reprodutiva, o presente estudo realizou quatro experimentos com SE associada ou não ao estresse/ ou probiótico, em diferentes períodos do desenvolvimento de roedores.

No Experimento I, investigou-se o impacto da exposição gestacional ao estresse associado ou não à SE no desenvolvimento neurocomportamental e reflexo de filhotes machos de ratas Wistar. O comprometimento da saúde materna também foi verificado.

Neste estudo, verificou-se que o antidepressivo causou atraso no desenvolvimento somático e no reflexo da prole masculina. No DPN 25, o grupo SE (tratado apenas com sertralina) apresentou menor grau de exploração em relação ao grupo CO, e principalmente ao grupo ST (exposto ao estresse). Ratos com perfil encontrado no grupo SE são caracterizados como ansiosos, pois são menos exploradores principalmente em regiões potencialmente ameaçadoras. Estudos anteriores, com ratos, também evidenciaram que a exposição aos ISRS *in útero* induz a uma menor exploração na prole (Olivier *et al.*, 2011; Sadegzadeh *et al.*, 2020). Sabe-se que crianças expostas durante o período pré-natal correm risco de desenvolver psicopatologias, incluindo aumento do comportamento de internalização, autismo e depressão durante a adolescência (Glover e Clinton, 2016).

A saúde materna também foi comprometida nos animais expostos à SE, pois, apresentaram redução do peso corporal e sangramento vaginal. Sabe-se que os ISRSs podem, em algumas condições clínicas raras, causar disfunções plaquetárias e provocar sangramento intenso nas pacientes (Calhoun e Calhoun, 1996; Smith e Robinson, 2002).

No experimento II, verificou-se o impacto que o estresse, associado ou não à SE, causa no desenvolvimento reprodutivo de ratos machos. Observou-se que a prole exposta *in útero* à SE apresentou menor peso corporal, possivelmente pela alteração no fluxo sanguíneo placentário desencadeada pelo aumento dos níveis de serotonina (Vorhees *et al.*, 1994; Wen *et al.*, 2006). No DPN 45, os órgãos reprodutores, como o epidídimo e os testículos, mostraram-se menores no

grupo SS (expostos ao estresse e à SE). Sabe-se que a SE, quando administrada desde a juventude até a puberdade, diminui as concentrações de LH, FSH, testosterona e 17 β -hidroxiesteróide desidrogenase (ElMazoudy *et al.*, 2020). Os animais do grupo SS apresentaram uma menor DAG no DPN 21 e atraso no início da puberdade. Alterações desses marcadores reprodutivos demonstram a ocorrência de distúrbios androgênicos (Schwartz *et al.*, 2019).

Alterações androgênicas também podem ser causadas por desequilíbrios na expressão do WT1. Este fator de transcrição, que está envolvido na expressão de enzimas esteroidogênicas e no desenvolvimento de células de Leydig fetais (Chen *et al.*, 2014), foi fracamente corado nas células de Leydig e Sertoli no grupo SS no DPN 45. Nesse mesmo período, os animais expostos à SE apresentaram baixa marcação de PCNA e menor quantidade de espermatogônias, reforçando a hipótese de que esse antidepressivo possui ação antiproliferativa (Kuwahara *et al.*, 2015; Amit *et al.*, 2009).

Na fase adulta, os animais não apresentaram alterações histológicas significativas no epidídimo e nos testículos. No entanto, a qualidade espermática nos grupos expostos à SE foi comprometida, possivelmente por alterações em canais CatSper, um importante canal de cálcio envolvido na função espermática (Rahban *et al.*, 2021).

O experimento III foi conduzido de forma a investigar as repercussões reprodutivas e comportamentais de ratos machos expostos durante a lactação à SE. Observou-se que a prole das mães expostas ao antidepressivo apresentaram atrasos no desenvolvimento de reflexos. Sabe-se que nas primeiras semanas de lactação, há intensa arborização dos terminais axonais de serotonina no córtex cerebral/cerebelar, hipocampo e corpo estriado (Gaspar *et al.*, 2003). Entretanto não foram detectadas anormalidades neurocomportamentais nos testes de labirinto em cruz elevado e campo aberto.

Durante o tratamento com SE, observou-se uma redução do peso corporal das mães e da prole. Essa significativa redução de peso dos lactentes do grupo S20 ocorreu justamente no período em que a única fonte de alimento era o leite materno. Sabe-se que baixas concentrações de SE podem ser transferidas ao leite (De Vane *et al.*, 2002). Além disso, outras alterações como menor distância anogenital e alterações histopatológicas foram limitadas a esse período.

Na idade adulta a análise da qualidade espermática revelou diminuição da motilidade nos grupos expostos à SE. Karimipour *et al.* (2020) descobriram que a exposição lactacional à fluoxetina afeta negativamente os parâmetros espermáticos de camundongos, como motilidade e morfologia. Rahban *et al.*, (2021) demonstraram que a SE inibe o canal de Ca²⁺ (CatSper) e afeta a função de espermatozoides humanos *in vitro*.

Verificou-se que o peso da tireoide das mães aumentou no grupo S20, o que pode ter sido causado pela ação direta do antidepressivo no órgão. Embora as dosagens dos hormônios tireoidianos não tenham sido realizadas no presente estudo, sabe-se que outros tipos de ISRS podem influenciar a produção desses hormônios (Escudero *et al.*, 2013).

O experimento IV investigou os efeitos da SE nos parâmetros reprodutivos de camundongos, bem como verificou se o probiótico *Lactobacillus rhamnosus* ameniza os efeitos colaterais desse antidepressivo. Os resultados mostraram que os animais do grupo S (administração isolada à SE) apresentaram menor exploração no labirinto em cruz elevado e campo aberto, demonstrando um perfil ansioso em relação ao grupo P (administração isolada ao probiótico). Curiosamente, nem todos os parâmetros alterados no grupo S foram observados no grupo SP (receberam antidepressivo e probiótico), que apresentou resultados semelhantes ao grupo CO. Um estudo realizado por Bravo *et al.*, (2011) relatou que o *Lactobacillus rhamnosus* quando administrado por animais ocasiona ação ansiolítica. Além disso, os autores verificaram que o microrganismo foi capaz de modular a expressão do mRNA dos receptores GABAA α 2, GABAA α 1 e GABAB1, que estão implicados no comportamento de ansiedade.

Foi demonstrado que o grupo SP teve um aumento significativo no peso corporal quando comparados ao grupo S. Já o grupo P não apresentou diferenças nesse aspecto, sugerindo que o ganho de peso seja resultado da ação combinada com o ISRS. Observou-se também que o peso das glândulas seminais foi menor nos grupos S10 e S20. Estudos anteriores mostraram que pacientes com ejaculação precoce geralmente têm glândulas seminais maiores e a SE pode atenuar esse efeito (Hong *et al.*, 2017; Longping *et al.*, 2018). Sabe-se que este medicamento diminui a pressão da glândula seminal e as respostas contráteis induzidas pela estimulação do nervo hipogástrico (Kim *et al.*, 2004). No entanto, o grupo SP não apresentou modificações no peso deste órgão, demonstrando que o probiótico possivelmente consegue interferir no mecanismo de ação da SE.

O grupo SP também apresentou atraso no tempo de trânsito espermático. Durante este evento, muitas características morfológicas e fisiológicas dos espermatozoides são modificadas para aumentar a motilidade e a capacidade fertilizante. Embora no presente estudo o trânsito espermático tenha sido lento no grupo SP, este não apresentou alterações na motilidade ou fertilidade natural.

Diante dos estudos aqui realizados, demonstrou-se que a gravidade dos impactos reprodutivos provocados pela SE está relacionada com o período de exposição. Sendo a fase gestacional mais crítica ao desenvolvimento reprodutivo e neurocomportamental. Elucidou-se

também, que os efeitos colaterais observados em camundongos adultos foram atenuados quando esse antidepressivo foi associado ao probiótico *Lactobacillus rhamnosus*.

Conclusão

Os resultados demonstraram que o tratamento com SE, em diferentes períodos da vida de roedores, tem repercussões negativas em parâmetros reprodutivos e neurocomportamentais. Por sua vez, o *Lactobacillus rhamnosus* mostrou-se efetivo em atenuar estes efeitos colaterais desenvolvidos em camundongos adultos.

Assim, sob o aspecto translacional, esclarecemos à comunidade científica e médica sobre os impactos desencadeados pela SE. E também fornecemos informações importantes que podem contribuir com a compreensão dos efeitos de probióticos.

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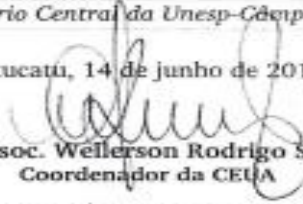
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Certificado

Certificamos que o projeto intitulado "Exposição gestacional ao estresse e ao antidepressivo Sertralina: impacto sobre o desenvolvimento sexual e trato genital da prole masculina de ratos, com ênfase à diferenciação das células de Leydig", Protocolo nº **1169-CEUA**, sob a responsabilidade de **Wilma De Grava Kempinas**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 9 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela **COMISSÃO DE ÉTICA NO USO DE ANIMAIS** (CEUA), nesta data.

Finalidade:	☐ Ensino	☒ Pesquisa Científica
Vigência do Projeto:	Início: 1/8/2019	Término: 30/6/2022
Espécie/linhagem:	Rato Wistar	
Nº de animais:	280	
Peso:	300-500g	Idade: 90 dias
Sexo:	Macho e fêmea	
Origem	Biotério Central da Unesp-Campus de Botucatu/SP.	

Botucatu, 14 de junho de 2019.


Prof. Assoc. Wellerson Rodrigo Scarano
Coordenador da CEUA



Comissão de Ética no Uso de Animais - CEUA
Instituto de Biociências da Unesp - Campus de Botucatu
Rua Prof. Dr. Antonio Carlos Wagner Zanin, 258 - Distrito de Rubião Júnior - CEP 13618-689 Botucatu/SP
Tel 14-3892-6851 - e-mail: unespceua@fzib.unesp.br

CERTIFICADO

Certificamos que a proposta intitulada "Desenvolvimento pós-natal e performance reprodutiva das proles masculina e feminina de ratas Wistar expostas à Sertralina durante a lactação", protocolada sob o CEUA nº 3548071220 (00 000158), sob a responsabilidade de **Wilma de Grava Kempinas** e equipe; *Mayara Silva Moura; Ana Flávia Quiarato Lozano* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **APROVADA** pela Comissão de Ética no Uso de Animais da Universidade Estadual Paulista/Instituto de Biociências (IBB/UNESP) na reunião de 21/12/2020.

We certify that the proposal "Postnatal development and reproductive performance of male and female offspring of Wistar rats exposed to Sertraline during lactation", utilizing 320 Heterogenics rats (males and females), protocol number CEUA 3548071220 (00 000158), under the responsibility of **Wilma de Grava Kempinas** and team; *Mayara Silva Moura; Ana Flávia Quiarato Lozano* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **APPROVED** by the Ethic Committee on Animal Use of the São Paulo State University/Biosciences Institute (IBB/UNESP) in the meeting of 12/21/2020.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 01/2021 a 06/2021 Área: Ciências Biológicas

Origem: Biotério Central da UNESP (Biotério Central da UNESP de Botucatu)

Espécie: Ratos heterogênicos

sexo: Machos e Fêmeas

idade: 1 a 120 dias

N: 320

Linhagem: Wistar

Peso: 3 a 450 g

Botucatu, 07 de janeiro de 2023

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"



Profa. Dra. Ana Carolina Inhasz Kiss
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Estadual Paulista/Instituto de Biociências

Prof. Assoc. Luis Fernando Barbisan
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Estadual Paulista/Instituto de Biociências

CERTIFICADO

Certificamos que a proposta intitulada "Exposição ao antidepressivo sertralina associada ou não ao probiótico *Lactobacillus rhamnosus*: Uma abordagem sobre os aspectos reprodutivos e neurocomportamentais de camundongos", protocolada sob o CEUA nº 3597170322 (ID 000351), sob a responsabilidade de **Wilma de Grava Kempinas e equipe; Ana Flávia Quirato Lozano** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **APROVADA** pela Comissão de Ética no Uso de Animais da Universidade Estadual Paulista/Instituto de Biociências (IBB/UNESP) na reunião de 29/04/2022.

We certify that the proposal "Exposure to the antidepressant sertraline associated or not with the probiotic *Lactobacillus rhamnosus*: An approach to the reproductive and neurobehavioral aspects of mice", utilizing 40 isogenics mice (males and females), protocol number CEUA 3597170322 (ID 000351), under the responsibility of **Wilma de Grava Kempinas and team; Ana Flávia Quirato Lozano** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **APPROVED** by the Ethic Committee on Animal Use of the São Paulo State University/Biosciences Institute (IBB/UNESP) in the meeting of 04/29/2022.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 05/2022 a 07/2022 Área: Ciências Biológicas

Origem:	CPPA - IBTEC/UNESP (CPPA-IBTEC/UNESP)		
Espécie:	Camundongos isogênicos	sexo:	Machos e Fêmeas
		idade:	45 a 120 dias
		N:	40
Linhagem:	C57BL/6 (fêmeas) e BALB/c (machos)	Peso:	20 a 40 g

Botucatu, 07 de janeiro de 2023

UNIVERSIDADE ESTADUAL PAULISTA
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