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Investigação de parâmetros reprodutivos em ratos machos expostos ao psicoestimulante Lisdexanfetamina do período juvenil a peripuberdade

JULIA STEIN

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Orientadora: Profa. Dra. Arielle Cristina Arena

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Palavras-chave: Hepatotoxicidade; Lisdexanfetamina; Psicoestimulantes; Toxicidade reprodutiva ; Toxicidade sistêmica.

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LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SÍMBOLOS

5-HT: Serotonina

ANVISA: Agência Brasileira de Vigilância Sanitária

APA: Associação Americana de Psiquiatria

ASC: Área sob a curva

CID10: classificação Estatística Internacional de Doenças e problemas Relacionados com a Saúde, décima edição

$C_{máx}$: Concentração plasmática máxima

DA: Dopamina

D-AMF: Dextroanfetamina

DAT: Transportador de dopamina

DHT: Di-hidrotestosterona

DL50: dose-letal para 50% dos invidíduos

DPN: Dia pós-natal

DSM-5: Manual diagnóstico e estatístico de transtornos mentais, Quinta edição

FDA: food and Drug Administration

FSH: Hormônio Folículo Estimulante

GABA: Ácido gama-aminobutírico

GH: Hormônio do crescimento

GnRH: Hormônio liberador de gonadotrofina

HPG: Eixo hipotálamo-pituitária-gonadal

LDX: Dimesilato de lisdexanfetamina

LH: Hormônio Luteinizante

MOA: Monoamina oxidase

MPH: Metilfenidato

NA: Noradrenalina

NAT: Transportador de noradrenalina

NOAEL: nível de efeitos adversos não observáveis

NOEL: nível de efeitos não observáveis

PEPT1: Transportador de peptídeos 1

SERT: Transportador de serotonina

SNC: Sistema nervoso central

$T_{1/2}$: tempo de meia-vida

TAAR1: Receptor associado a traços de aminas do tipo 1

TDAH: Transtorno de déficit de atenção e hiperatividade

$T_{máx}$: Tempo para se atingir a Cmáx

VMAT2: Transportador de monoamina vesicular 2

RESUMO

O transtorno de déficit de atenção e hiperatividade (TDAH) é um distúrbio do neurodesenvolvimento comum em crianças e adolescentes, com maior incidência de diagnósticos no sexo masculino. Desatenção, hiperatividade e impulsividade em níveis acima do esperado são os principais sintomas do TDAH, afetando negativamente a vida diária dos pacientes. O tratamento adequado é essencial e baseia-se numa estratégia multimodal, com intervenções cognitivo-comportamentais e tratamento farmacológico, geralmente com medicações estimulantes. Um dos principais medicamentos utilizado é o Dimesilato de Lisdexanfetamina (LDX), uma formulação de liberação prolongada, pois é uma pró-droga da dextroanfetamina, hidrolisada no interior dos eritrócitos. Sua ação é mediada pelo aumento dos níveis de catecolaminas, principalmente dopamina e noradrenalina, nas fendas sinápticas. Como os neurotransmissores influenciam o controle hormonal e atuam diretamente nas gônadas, a LDX poderia interferir no desempenho reprodutivo. O período juvenil e a peripuberdade são janelas críticas do desenvolvimento, mais susceptíveis ao efeito de substâncias exógenas. Assim, o objetivo deste estudo foi avaliar os efeitos da exposição ao LDX do período juvenil até a peripuberdade sobre os parâmetros de toxicidade sistêmica e reprodutiva de ratos machos. Ratos Wistar machos (23 dias de idade) foram divididos em um grupo controle (água deionizada) e três grupos expostos a LDX em doses terapêuticas (convertidas para dose animais): 5,2; 8,6 e 12,1 mg/kg/dia. O tratamento ocorreu do dia pós-natal 23 (DPN) ao 53, via gavage. Durante o tratamento, foram avaliados sinais clínicos de toxicidade, peso corporal, ingestão de água e ração, comportamento social de brincar e estabelecimento da puberdade. No dia pós-natal (PND) 54, metade dos animais foi morta para peso dos órgãos, análise hematológica e bioquímica, histologia testicular e estresse oxidativo do testículo. Na vida adulta (PND 90), os animais foram avaliados quanto ao comportamento sexual masculino e parâmetros de fertilidade. No DPN 120, os animais foram mortos para registro do peso dos órgãos, parâmetros hematológicos e espermáticos, contratilidade do ducto deferente, histologia testicular e estresse oxidativo do testículo. Observou-se redução no consumo diário de ração e água durante o período de tratamento. Na avaliação do comportamento social de brincar, os animais apresentaram redução nos comportamentos sociais totais. Nenhum grupo experimental teve o dia da separação prepucial alterado em relação ao controle, mas o peso dos animais do grupo de maior dose foi reduzido neste dia. No DPN 54, vários parâmetros foram alterados, principalmente relacionados à toxicidade sistêmica: redução do ganho de peso corporal, aumento do peso relativo do fígado, baço e glândula seminal, redução da contagem de eritrócitos e leucócitos, redução dos níveis de proteínas totais e

alterações em parâmetros oxidativos. Na idade adulta, o comportamento sexual masculino e a fertilidade não foram alterados. Já no DPN 120, houve aumento de espermatozoides do tipo C (imóvel), diminuição da contagem nos testículos e epidídimos, alterações histológicas nas células de Leydig e nos túbulos seminíferos e alteração dos parâmetros oxidativos. Com base nos dados apresentados, o LDX foi capaz de induzir distúrbios nos parâmetros toxicológicos sistêmicos (principalmente durante o tratamento) e na função reprodutiva (principalmente na vida adulta), indicando que o uso deste medicamento deve considerar esses aspectos.

Palavras-chave: Lisdexanfetamina, psicoestimulantes, toxicidade sistêmica, hepatotoxicidade, toxicidade reprodutiva

ABSTRACT

Attention deficit hyperactivity disorder (ADHD) is a common neurodevelopmental disorder in children and adolescents, with a higher incidence of diagnoses in male sex. Inattention, hyperactivity and impulsivity in levels above expected are the main symptoms in ADHD, negatively affecting patients' daily life. Proper treatment is essential and it is based on a multimodal strategy, with cognitive-behavioral interventions and pharmacological treatment, usually with stimulants medications. One of the main drugs is Lisdexamfetamine Dimesylate (LDX), an extended-release formulation since it is a prodrug of dextroamphetamine, hydrolyzed inside erythrocytes. Its action is mediated by increased levels of catecholamines, mainly dopamine and noradrenaline, in synaptic clefts. Neurotransmitters influence hormonal control and act directly in gonads, so LDX could interfere in reproductive performance. Juvenile and peripubertal period are critical windows of development, more susceptible to the effect of exogenous substances. The aim was to evaluate the effects of exposure to Lisdexamfetamine during the juvenile to peripubertal period on parameters of systemic and reproductive toxicity of male rats. Male Wistar rats (23 days old) were divided into a control group (deionized water) and three groups exposed to LDX in therapeutic doses (converted to animal doses): 5.2; 8.6 and 12.1 mg/kg/day. The treatment occurred from post-natal day 23 (PND) to 53, by gavage. During the treatment, clinical signs of toxicity, body weight, water, and food intake, play behavior and puberty onset were evaluated. On postnatal day (PND) 54, half of the animals were killed for organ weight, hematological and biochemical analysis, testis histology and oxidative stress of the testis. In adult life (PND 90), the animals were evaluated in relation to male sexual behavior and fertility parameters. On PND 120, the animals were killed to record organ weight, hematological parameters, sperm parameters, contractile of vas deferens, testis histology and oxidative stress. There was a reduction in the daily food and water consumption during the treatment period. On play behavior evaluation, the animals presented a reduction in total social behaviors. No experimental group had the day of preputial separation altered compared to control, but the weight of the animals in the highest dose group were reduced on this day. At PND 54, several parameters were altered, mainly related to systemic toxicity: reduction in body weight gain, increase in the relative weight of the liver, the spleen and the seminal gland, reduction in the erythrocyte and leukocyte count, reduced total protein levels and a disturbance in oxidative parameters. At adulthood, the male sexual behavior and fertility were not altered. But at PND 120, there was an increase in type C sperm (immobile), reduced sperm count in testis and epididymis, histological alterations in Leydig cells and seminiferous tubules, and oxidative parameters altered. Based on the data presented, LDX was

able to induce disturbances in systemic toxicological parameters (mainly during treatment) and in reproductive function (mainly in adult life), indicating that the use of this medication should consider these aspects.

Keywords: Lisdexamfetamine, psychostimulants, systemic toxicity, hepatotoxicity, reproductive toxicity

SUMÁRIO

1. REVISÃO DA LITERATURA

1.1. Transtorno de déficit de atenção e hiperatividade (TDAH):

O transtorno de déficit de atenção e hiperatividade (TDAH) é a desordem neurocomportamental mais comum em crianças e adolescentes (WOLRAICH et al., 2019), sendo que cerca de dois terços dos casos persistem na vida adulta destes indivíduos (BARKLEY et al., 2002; FARAONE; BIEDERMAN; MICK, 2006; WEISS et al., 1985). A prevalência deste transtorno apresenta dados com grande heterogeneidade devido a diferenças metodológicas, variações de idade descritas e mudanças em critérios diagnósticos (THOMAS et al., 2015), além de um número considerável de casos não diagnosticados (POLANCZYK et al., 2007; WOLRAICH et al., 2019). Um estudo de meta-análise calculou uma prevalência mundial de 7,2% entre as crianças (THOMAS et al., 2015), sendo que outros estudos apontam para uma taxa entre 8,7 a 15,5% (ROWLAND et al., 2015; WOLRAICH et al., 2014). No Brasil, a estimativa varia de 5,8 a 19,9% em toda a população (ANSELM et al., 2010; PASTURA; MATTOS; ARAÚJO, 2007; POLANCZYK et al., 2010; ROHDE et al., 1999), sendo estimado 7,6% para crianças e adolescentes entre 6 e 17 anos, 5,2% entre os 18 e 44 anos e 6,1% acima dos 44 anos (ARRUDA et al., 2015; FAYYAD et al., 2017; JERNELÖV et al., 2019; SIMON et al., 2009).

Os comportamentos hiperativos, que são mais facilmente observáveis e disruptivos, são mais frequentes no sexo masculino, e por esta razão, há uma probabilidade duas vezes maior de ocorrer o diagnóstico (DANIELSON et al., 2018; PASTOR et al., 2011; WOLRAICH et al., 2014), aumentando assim, os dados de prevalência de TDAH em meninos. Ainda sobre características dimórficas, os meninos apresentam uma maior propensão a transtornos desafiadores de oposição e transtornos de conduta (CUFFE et al., 2020; ELIA; AMBROSINI; BERRETTINI, 2008; GAUB; CARLSON, 1997), enquanto as meninas tendem a ter como comorbidade do TDAH, transtornos de ansiedade e depressão (TUNG et al., 2016).

Em muitos dos casos, o TDAH se apresenta como parte de um complexo conjunto de manifestações emocionais, físicas e condições sociais (MATTINGLY et al., 2020). Desatenção, hiperatividade, dificuldades no aprendizado e impulsividade em níveis acima do esperado estão entre os principais sintomas do TDAH, e, com o passar do tempo, o perfil de sintomas tende a mudar, de forma que, ao entrar na adolescência, comportamentos hiperativos e impulsivos da infância tendem a reduzir, fazendo com que a desatenção permaneça nesta nova fase (MOLINA et al., 2009). Além disso, a manifestação dos sintomas tende a mudar de acordo com o contexto ambiental e comorbidades envolvidas (MATTINGLY et al., 2020).

O diagnóstico e tratamento do TDAH, bem como de algumas comorbidades envolvidas, pode ser orientado pelo Manual diagnóstico e estatístico de transtornos mentais, Quinta edição (DSM-5), da Associação Americana de Psiquiatria (APA) (AMERICAN PSYCHIATRIC ASSOCIATION, 2013; WOLRAICH et al., 2019). De acordo com o DSM-5 estabelece-se 3 classificações do TDAH: o tipo predominante hiperativo, o tipo desatento, e o tipo combinado (SHIRE, [s.d.]; WOLRAICH et al., 2019). No Brasil, outra classificação também utilizada é classificação Estatística Internacional de Doenças e problemas Relacionados com a Saúde, décima edição (CID-10), que considera: Distúrbios de atividade e da atenção [F90.0], transtorno hipercinético de conduta [F90.1], outros transtornos hipercinéticos [F90.8] e transtorno hipercinético não especificado [F90.9] (BOTELHO; BARROS, 2022).

A presença deste transtorno impacta as atividades diárias destes pacientes, incluindo as relações sociais, relacionamentos, atividades escolares, bem como suas performances operacionais e financeiras (AMERICAN PSYCHIATRIC ASSOCIATION, 2013), o que resulta em significativa redução da qualidade de vida (DANCKAERTS et al., 2010), impacto econômico (devido aos altos gastos médicos, farmacêuticos e redução da produtividade dos pacientes e familiares) (WU et al., 2012) e baixo desempenho no contexto familiar, social e acadêmico, principalmente quando não-diagnosticado e/ou tratado de forma ineficaz (BARRY; LYMAN; KLINGER, 2002; GREENE et al., 2001; HARPIN, 2005).

Pacientes com TDAH têm um maior risco de morte precoce, suicídio e comorbidades psiquiátricas, principalmente abuso de substâncias (BIEDERMAN et al., 2009; CHANG et al., 2016b), além de um desempenho educacional mais baixo (BARBARESI et al., 2007; SCHEFFLER et al., 2009) e maiores índices de encarceramento (BARBARESI et al., 2013). Em um estudo com a população da Dinamarca, a taxa de mortalidade aumentou 86% em pré-escolares, 58% em crianças e 325% em adultos em relação àqueles sem TDAH (DALSGAARD et al., 2015). A falta ou descontinuação do tratamento aumenta o risco de resultados catastróficos, como acidentes automobilísticos (CHANG et al., 2017; WOLRAICH et al., 2019), criminalidade (CHANG et al., 2016a; LICHTENSTEIN et al., 2012), depressão (CHANG et al., 2016b), questões interpessoais (HARSTAD et al., 2014), entre outros.

O tratamento adequado pode diminuir estes riscos, melhorando a função executiva, reduzindo comportamentos de risco e diminuindo as taxas de criminalidade (LICHTENSTEIN et al., 2012; MOHR-JENSEN et al., 2019; WILENS et al., 2008). Iniciando este tratamento na infância propicia efeitos protetivos para comportamentos disruptivos, ansiedade, comportamentos de adicção, falha acadêmica e acidentes de carro (BIEDERMAN et al., 2019),

ou seja, o tratamento precoce e adequado é essencial para minimizar os riscos e aumentar a qualidade de vida dos pacientes (MATTINGLY et al., 2020).

1.2. Etiologia do TDAH:

A complexa etiologia do TDAH ainda não foi completamente elucidada, mas evidências sugerem que fatores genéticos e não genéticos, tais como fatores neurológicos, biológicos e ambientais estão implicados neste processo (ADESMAN, 2001; CURATOLO; D'AGATI; MOAVERO, 2010; FARAONE; MICK, 2010). Neste sentido, estudos apontam que a neurotransmissão de catecolaminas está prejudicada no cérebro desses pacientes (ARNSTEN, 2009a, 2009b; BRENNAN; ARNSTEN, 2008). Estudos sugerem que diversos neurotransmissores e estruturas cerebrais estejam envolvidos no TDAH, principalmente os sistemas dopaminérgicos e noradrenérgicos, que são críticos para a função cortical e para a função do corpo estriado adequada, e por isso seriam os prováveis responsáveis por parte da fisiopatologia do TDAH (ARNSTEN, 2009a, 2009b; CORTESE, 2012; FARAONE et al., 2015). Entretanto, disfunções de outros sistemas de neurotransmissores, como serotonina (5-HT), acetilcolina, opioides e glutamato também já foram relacionados (BLUM et al., 2008; CORTESE, 2012; ELIA; AMBROSINI; BERRETTINI, 2008; MALTEZOS et al., 2014; POTTER; SCHAUBHUT; SHIPMAN, 2014).

Estudos de meta-análise demonstraram alterações em estruturas cerebrais que incluem integridade alterada na matéria branca em diversas regiões (VAN EWIJK et al., 2012), volume reduzido da matéria cinzenta (NAKAO et al., 2011) e redução do volume dos núcleos accumbens, amígdala, caudado, hipocampo e putâmen (HOOGMAN et al., 2017). Além disso, o TDAH já foi relacionado a diversas alterações funcionais no cérebro, principalmente relacionadas a hipoativação e conectividade alterada em diversas regiões cerebrais (FARAONE, 2018).

O TDAH é frequentemente associado a transtornos psiquiátricos comórbidos, como ansiedade, desordens de humor, abuso de substâncias, distúrbios do sono e desordens de personalidade antissocial (KONOFAL; LECENDREUX; CORTESE, 2010; KOOIJ et al., 2012; MAO; FINDLING, 2014), visto que compartilham as regiões cerebrais e sistemas de neurotransmissores em suas fisiopatologias (FARB; RATNER, 2014). Fatores de risco genéticos também podem ser compartilhados por estes transtornos psiquiátricos e o TDAH (CAREY et al., 2016; SHARP et al., 2014).

1.3. Tratamento:

Na maioria dos casos, considera-se ideal o tratamento baseado em uma estratégia multimodal, que inclui estratégias não-farmacológicas, como intervenções cognitivas e comportamentais, psicoeducacionais e psicoterapia (CADDRA, 2018; MATTOS, 2014; PLISZKA, 2007; WOLRAICH et al., 2019); além de tratamentos farmacológicos, em que os psicoestimulantes são os medicamentos mais eficazes e comumente prescritos (FARAONE; MICK, 2010; LUAN et al., 2017; RIERA et al., 2017; SHIER et al., 2013), visto que a terapia com estimulantes promove um efeito imediato mais forte, enquanto a terapia comportamental tende a ser preferida pelos pais e mantém seus efeitos positivos (WOLRAICH et al., 2019). Desta forma, esta estratégia multimodal é recomendada para melhora dos sintomas e da qualidade de vida (MATTINGLY et al., 2020).

Medicamentos estimulantes, por terem alta eficácia, são considerados a terapia farmacológica de primeira linha no tratamento do TDAH, tanto em crianças, adolescentes ou adultos (FARAONE, 2018). Estimulantes à base de metilfenidato (MPH) e anfetamina são considerados os pilares da farmacoterapia do TDAH, e visam aumentar os níveis dos neurotransmissores com o objetivo de melhorar tanto os sintomas centrais como as comorbidades envolvidas (CADDRA, 2018; PLISZKA, 2007; WOLRAICH et al., 2019). Em contrapartida, devido a este mecanismo, há uma preocupação a respeito do uso de forma não prescrita ou como substância de abuso destas substâncias (JASINSKI; KRISHNAN, 2009).

Para idade pré-escolar (4 a 6 anos), o tratamento de primeira linha inclui intervenções comportamentais, e quando necessário, a utilização do MPH (WOLRAICH et al., 2019). A partir dos 6 anos, há a aprovação do uso de outras medicações (as quais têm a anfetamina como princípio ativo) como tratamento de primeira linha, também acompanhadas de intervenções comportamentais (WOLRAICH et al., 2019).

Alguns estudos apontam a anfetamina e o MPH como tendo eficácia comparada (CATALÁ-LÓPEZ et al., 2017; FARAONE; GLATT, 2010), enquanto outros apontam efeitos moderadamente maiores com o uso de anfetaminas (FARAONE; BUITELAAR, 2010; JOSEPH et al., 2017; STUHEC et al., 2015). Além disso, há uma grande variabilidade entre pacientes na resposta ao tratamento, com efeitos idiossincráticos, em que 40% dos pacientes respondem a ambos os medicamentos, e 40% a apenas um destes (WOLRAICH et al., 2019). Essa variabilidade faz com que a dose tenha que ser ajustada ao longo do tempo, iniciando com uma menor dosagem até que se atinja os efeitos máximos e ideias para controle dos sintomas, sem efeitos adversos observáveis, visto que não há uma relação entre a dose e o peso ou altura dos pacientes (WOLRAICH et al., 2019).

Os psicoestimulantes podem ser de ação curta (como a Ritalina®, cujo princípio ativo é o MPH) ou prolongada (como a Ritalina LA® e o Venvanse®, sendo os princípios ativos o MPH e a dextroanfetamina - D-AMF, respectivamente), diferindo farmacologicamente na duração do efeito terapêutico. Na prática clínica, múltiplas doses diárias podem diminuir a adesão devido a alguns problemas, como: políticas escolares que proíbam a administração de substâncias controladas, estigmatização e ridicularização dos pacientes, falta de administração no horário prescrito (GREENHILL; PLISZKA; DULCAN, 2002; PELHAM et al., 2001). Além disso, as formulações de liberação imediata são associadas a uso indevido e com fins recreativos, bem como com risco aumentado de abuso posterior a substâncias. Neste sentido, por ter uma cobertura mais consistente e sem a necessidade de administrações repetidas ao longo do dia, as formulações com liberação prolongada são preferidas pela maioria dos pacientes e melhoram a adesão e a eficácia do tratamento (BANASCHEWSKI et al., 2006; ERMER et al., 2010; FEGERT et al., 2011; MAY; KRATOCHVIL, 2010).

Assim como outros medicamentos para TDAH, houve um aumento no uso de anfetaminas nos últimos anos (CHERMÁ et al., 2020). Na Suécia, as vendas aumentaram 16 vezes entre os anos de 2012 e 2017, principalmente impulsionados pelo pró-fármaco Dimesilato de Lisdexanfetamina (LDX) que representou cerca de 84% destas vendas (CHERMÁ et al., 2020).

Além dos estimulantes, existem algumas medicações não estimulantes também prescritas para o tratamento do TDAH, como o inibidor da recaptação seletiva de noradrenalina (atomoxetina) (CHENG et al., 2007; MICHELSON et al., 2002) e agonistas seletivos do receptor α2-adrenergico (guanfacina e clonidine) (BIEDERMAN et al., 2008; SALLEE et al., 2009), que demonstraram eficácia em reduzir os sintomas, mas, apesar disto, seus efeitos são menores e menos robustos, e ainda possuem pouca evidência que suporte sua utilização (WOLRAICH et al., 2019).

1.4. Lisdexanfetamina:

Dentre os medicamentos mais utilizados para o tratamento de TDAH, destaca-se o LDX, comercialmente vendido no Brasil como Venvanse® e Juneve®, sendo o mais novo fármaco psicoestimulante de ação prolongada, e o primeiro e único pró-fármaco da D-AMF. Nos Estados Unidos, o LDX foi aprovado pela "*Food and Drug Administration (FDA*)" em 2007 (SCHNEIDER et al., 2021), e no Brasil foi aprovado em 2011 pela Agência Brasileira de Vigilância Sanitária (ANVISA) como tratamento de primeira linha de crianças, adolescentes e

adultos, na apresentação farmacêutica de cápsulas nas doses de 30, 50 e 70 mg (ABDA, 2017; CADDRA, 2018; WHO, 2014).

Esta droga libera sua substância ativa por um processo de hidrólise da ligação covalente entre a D-AMF e a l-lisina (um aminoácido essencial) (COGHILL et al., 2014; WEBER; ASIF; SIDDIQUI, 2009), ocorrendo de forma lenta e, diminuindo assim, a probabilidade de abuso (potencialmente ao evitar os aumentos grandes/rápidos da dopamina que estão associados aos efeitos de reforço do consumo de drogas) (ERMER et al., 2011; HUTSON; PENNICK; SECKER, 2014; MATTOS, 2014) e prolongando o efeito deste fármaco em até 13 horas após a administração, sugerindo que seja a formulação psicoestimulante de ação mais longa (DEW; KOLLINS, 2010; GOODMAN, 2007). A LDX é um fármaco solúvel em água, que deve ser administrado por via oral uma vez ao dia, preferencialmente pela manhã, em indivíduos a partir dos 6 anos de idade (GOODMAN, 2007; STEER et al., 2012; WEBER; ASIF; SIDDIQUI, 2009).

Estudos apontam que 100 mg de LDX tem peso molar equivalente a 40 mg de D-AMF (JASINSKI; KRISHNAN, 2009). Outro estudo aponta que 100 mg de LDX equivale a 29,5 mg de D-AMF, visto que 30, 50 e 70 mg de LDX equivalem respectivamente a 8,9; 14,8 e 20,8 mg de D-AMF (COMIRAN et al., 2021). Outra fonte aponta ainda como 100 mg de LDX sendo equivalente a 50 mg/kg de D-AMF (HUTSON; PENNICK; SECKER, 2014; SHIRE, [s.d.]).

A LDX também é aprovada em alguns países (Estados Unidos, Canadá, Brasil, Porto Rico, México e Israel) para o tratamento do transtorno de compulsão alimentar, somente em indivíduos adultos (HIMMERICH et al., 2020).

1.5. Farmacocinética:

Diferente de outros medicamentos estimulantes com ação prolongada, a LDX não é liberada no trato gastrointestinal após administração oral (ERMER; PENNICK; FRICK, 2016). A absorção intestinal da LDX ocorre por transporte mediado por transportadores (possivelmente o transportador de peptídeos 1 - PEPT1), visto que apresenta baixa difusão passiva por membranas biológicas considerando que apresenta propriedades físico-químicas de alta solubilidade aquosa (maior que 0,85 g/ml em pH entre 1 e 8) e baixa lipofilicidade (logP - 1,75) (COMIRAN et al., 2021; PENNICK, 2010).

Após ser absorvida e chegar ao sangue, a LDX é metabolizada em anfetamina farmacologicamente ativa no citosol dos eritrócitos (ERMER; PENNICK; FRICK, 2016; HIMMERICH et al., 2020), através de um processo de hidrólise enzimática por uma peptidase eritrocitária (figura 1) (JIAN-MIN et al., 2022). Por esta ser uma etapa limitante, os efeitos

farmacológicos são independentes da via de administração (HUTSON; PENNICK; SECKER, 2014). Essa conversão não é afetada pelo pH gastrointestinal e nem por variações no tempo de trânsito gastrintestinal (ERMER et al., 2010; KRISHNAN; ZHANG, 2008). Com base nos estudos presentes até o momento, não há saturação enzimática na conversão de LDX para D-AMP nas doses testadas (de 50 a 250 mg) (ERMER et al., 2010). Além disso, o processo de hidrólise não é afetado por variações clinicamente relevantes tanto na variabilidade quanto nos níveis dos eritrócitos (PENNICK, 2010, 2013).

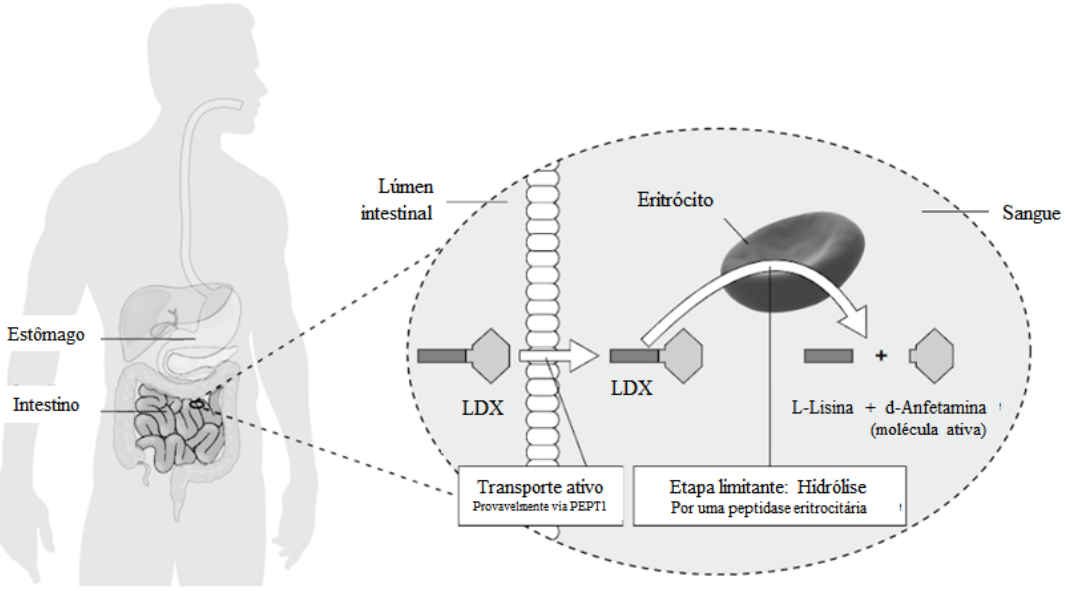

Figura 1 - Etapas de absorção e hidrólise das moléculas de LDX (adaptado de ERMER; PENNICK; FRICK, 2016).

O LDX apresentou uma área sob a curva (ASC) no plasma idêntica às formulações de D-AMF de liberação imediata, entretanto, uma concentração plasmática máxima ($C_{máx}$) menor e um tempo para se atingir a $C_{máx}$ ($T_{máx}$) prolongado em doses equimolares dos medicamentos (JIAN-MIN et al., 2022; ROWLEY et al., 2012). Essa questão farmacocinética que propicia uma liberação sustentada com menor magnitude contribui para que a LDX tenha uma janela terapêutica mais prolongada do que a D-AMF na administração dos sintomas, além de uma menor variabilidade intra-individual (JIAN-MIN et al., 2022) e interindividual (BIEDERMAN et al., 2007b; ERMER et al., 2010). Além disso, faz com que apresente uma menor propriedade de recompensa em comparação a D-AMF de liberação imediata (ROWLEY et al., 2012; THIELE; BELLGROVE, 2018). Estes aspectos, juntamente com o fato que a LXD não pode ser manipulada mecanicamente para se extrair a D-AMF, faz com que este medicamento tenha um risco reduzido de adulteração e de abuso indevido (BIEDERMAN et al., 2007a; JASINSKI;

KRISHNAN, 2009). Comiran et al. (2021) observou que 70 mg de LDX atingiu uma $C_{máx}$ de 45,8 μg/L após 1,2 horas ($T_{máx}$), com a D-AMF iniciando a aparecer entre 0,5 e 1 horas e atingindo sua $C_{máx}$ de 62.2 μg/L com $T_{máx}$ de 3,8 horas (COMIRAN et al., 2021). Estes dados corroboram com estudos anteriores, em que o $T_{máx}$ para LDX foi entre 1 e 2,1 horas e para D-AMF variou de 3 a 4,7 horas (ERMER; PENNICK; FRICK, 2016) e 2,5 a 6 horas (COMIRAN et al., 2016).

Após atingir o $C_{máx}$, os níveis de LDX caem rapidamente, sendo que seu tempo de meia vida ($t_{1/2}$) varia de 0,4 a 0,9 horas, enquanto a D-AMF decai mais lentamente, com $t_{1/2}$ entre 8,6 e 15 horas (ERMER; PENNICK; FRICK, 2016). Em relação a D-AMF, a ASC aumenta quase linearmente e a $C_{máx}$, aumenta linearmente, proporcional às doses crescentes de LDX (ERMER et al., 2010). A D-AMF apresenta característica de base fraca, e por isso, acumula-se na saliva devido à diferença de pH plasma-saliva (COMIRAN et al., 2021), e característica lipofílica, que possibilita sua entrada no sistema nervoso central (SNC) (SANCHEZ, 2017).

Posteriormente, o metabolismo da D-AMF é feito no fígado, por mecanismos de hidroxilação, N-desalquilação e desaminação ("Amphetamine|DrugBank", 2023; SANCHEZ, 2017). A principal enzima envolvida é a CYP2D6, a qual gera metabólitos ativos (como a norefedrina e 4-hidroxianfetamina) ("Amphetamine|DrugBank", 2023; SANCHEZ, 2017). Outras vias metabólicas geram metabólitos inativos, como a fenilcetona, que é transformada em ácido benzóico, e posteriormente, em ácido hipúrico ("Amphetamine|DrugBank", 2023; SANCHEZ, 2017). A principal rota de eliminação é a urina, com eliminação entre 1,3 e 2,2% de LDX, 41,5% a 48,5% de seu metabólito direto (D-AMF) (COMIRAN et al., 2021; KRISHNAN; ZHANG, 2008; SHIRE, [s.d.]), e ainda, 35% de ácido hipúrico (SHIRE, [s.d.]).

1.6. Mecanismo de ação:

As anfetaminas, de uma forma geral, trata-se de uma mistura racêmica, ou seja, sua molécula contém um centro quiral e apresenta-se como dois enantiômeros (duas formas opticamente ativas): a dextroanfetamina (d-anfetamina - D-AMP) e a levoanfetamina (l-anfetamina). Nesse sentido, a D-AMF é a forma mais potente e clinicamente eficaz, com efeitos estimulantes no SNC, sendo assim usada em maior porcentagem ou exclusivamente nas medicações à base de anfetaminas (HEAL et al., 2013). Nas formulações de LDX, há somente a presença da D-AMF, sendo que não ocorre a formação de misturas racêmicas durante seu metabolismo, ou seja, nenhum resíduo de l-anfetamina é encontrado em sua formulação e no organismo dos pacientes que fazem uso deste medicamento (FITZGERALD et al., 1988; KRAEMER; MAURER, 2002).

A anfetamina é um estimulante do SNC, cuja ação ocorre através de diversos mecanismos, tais como aumentando os níveis de dopamina (DA), noradrenalina (NA) e 5-HT nas fendas sinápticas (figura 2). A molécula de anfetamina possui estrutura muito similar às catecolaminas, com uma longa conformação planar, presença de um anel aromático e de nitrogênio na cadeia lateral aril ("Amphetamine|DrugBank", 2023), o que permite sua entrada nos terminais axônicos pré-sinápticos, por difusão ou sendo captadas pelos transportadores de dopamina (DAT), noradrenalina (NET) e serotonina (SERT) ("Amphetamine|DrugBank", 2023; MARTIN; LE, 2022). Assim, a anfetamina exógena atua como um ligante competitivo com as monoaminas endógenas pela ligação ao transportador, e quanto maior a concentração de anfetaminas, menos moléculas de neurotransmissores serão captadas (permanecendo na fenda) e mais moléculas de D-AMF serão internalizadas (HEAL et al., 2013), sendo assim, um inibidor fraco da recaptação da DA, moderado para NA e muito fraco para 5- HT ("Amphetamine|DrugBank", 2023).

Dentro dos terminais axônicos, a D-AMF é capaz de bloquear o transportador de monoamina vesicular 2 (VMAT2) e alterar o gradiente eletroquímico necessário para o funcionamento do transportador vesicular, liberando as monoaminas das vesículas de armazenamento e impedindo que sejam novamente armazenadas, o que aumenta sua quantidade livre no citosol (MARTIN; LE, 2022), e consequentemente, aumenta o efluxo destes neurotransmissores para a fenda sináptica.

A anfetamina também inibe a enzima monoamina oxidase (MOA), responsável pela degradação do excesso de monoaminas. Além disso, estimula o receptor associado a traços de aminas do tipo 1 (TAAR1), intracelular, que induz a internalização ou reversão do transportador DAT, NET e/ou SERT. Desta forma, o efluxo de neurotransmissores é aumentado, juntamente com a diminuição de sua recaptação (MILLER, 2011; NICKELL et al., 2014).

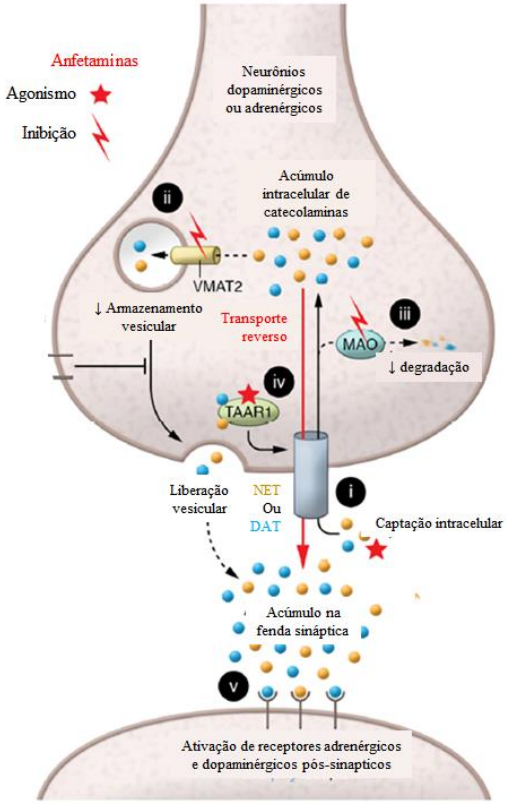

Figura 2 - Mecanismo de ação das anfetaminas. (I) anfetaminas agem como agonistas competitivos pelos transportadores (DAT, NET e SERT); (II) AMF se ligam ao VMAT2, inibindo a translocação das monoaminas do pool citosólico para as vesículas de armazenamento; (III) D-AMF inibe fracamente a enzima MAO; (IV) D-AMF ativam o receptor TAAR1, promovendo o efluxo das monoaminas via transporte reverso pelo NET ou DAT; e (V) A liberação elevada de monoaminas ativam os receptores adrenérgicos e dopaminérgicos pós-sinápticos para exercer seus efeitos terapêuticos (adaptado de STEMMER et al., 2019).

O mecanismo pelo qual exerce a ação terapêutica nos indivíduos com TDAH não é completamente elucidado, mas sabe-se que está relacionado à habilidade de aumentar as concentrações sinápticas destes neurotransmissores. Há estudos que demonstram que o aumento das concentrações sinápticas de DA e NA afetam o sistema corticoestrial cerebral, principalmente de NA no córtex pré-frontal e DA no corpo estriado, de maneira dose e tempo dependente (“Amphetamine|DrugBank”, 2023), resultando em mudanças comportamentais (“Lisdexamfetamine|DrugBank”, 2022; MHRA, 2015), visto que estas regiões relacionam-se com a cognição e função executiva (MOELLER et al., 2014; SCHLÖSSER et al., 2009; TOMASI et al., 2011), tomada de decisão arriscada (OSWALD et al., 2015), responsividade emocional (HARIRI et al., 2002) e regulação dos processos de recompensa (HABER, 2016). É importante ressaltar que estas regiões cerebrais onde a D-AMF altera a atividade dopaminérgica

e noradrenérgica têm sido regiões com alterações estruturais e funcionais associadas ao TDAH (FARAONE, 2018).

1.7. Efeitos adversos:

O perfil de segurança e tolerabilidade da LDX é semelhante a outros psicoestimulantes (MATTOS, 2014), incluindo efeitos colaterais leves e moderados de diminuição do apetite, dor de cabeça, boca seca, taquicardia, tontura, dor abdominal, redução de peso, insônia, irritabilidade e inquietação, entre outros (BIEDERMAN et al., 2007a; FINDLING, 2008; FRAMPTON, 2016; MARTIN; LE, 2022).

Um dos principais efeitos reportados, apesar de alguns dados ainda conflitantes, referem-se a um atraso na taxa de crescimento pediátrico e redução na altura de pacientes adultos que utilizam medicações estimulantes durante a infância, possivelmente como uma consequência da redução no apetite, redução na ingestão calórica associada a medicações estimulantes (BERMAN et al., 2009; HIMMERICH et al., 2020; POULTON, 2005; RICHARDSON; SEIBERT; ULI, 2017; SAFER; ALIEN, 1973; TROKSA et al., 2019) e perda de peso (COGHILL et al., 2014; MATTOS, 2014). Este efeito no processo de crescimento também pode estar associado a uma diminuição na liberação do hormônio do crescimento (GH) através de mecanismos desencadeados pelo aumento de DA e NA nas sinapses (NEGRAO; VILJOEN, 2011).

A associação com eventos cardiovasculares é controversa, e apesar de algumas observações neste sentido, a maioria dos estudos de acompanhamento a longo-prazo não relataram aumento de eventos cardiovasculares severos em pacientes tratados com medicações estimulantes (COOPER et al., 2011; SCHELLEMAN et al., 2011). Érmer et al. (2010) observou aumento da pressão arterial e pulsação, mas especialmente em doses acima das recomendadas na terapêutica, e com os sinais voltando à normalidade após o término da exposição (ERMER et al., 2010). Ainda assim, os pacientes sob tratamento com medicamentos estimulantes, como o LDX, devem ter avaliações individuais de riscos e benefícios da terapia, bem como um monitoramento ao longo do tratamento (FARAONE, 2018).

1.8. Dados toxicológicos:

A LDX foi considerada segura em estudos de toxicidade aguda (estudos de dose oral única), apesar de ter desencadeado aumento da atividade motora, considerando que a DL50 (dose capaz de causar mortalidade em 50% dos animais) foi maior que 1.000 mg/kg (KRISHNAN; MONTCRIEF, 2007), definida em outro estudo como 7.060 mg/kg para ratos

(CAYMAN, 2013). Em camundongos, o valor definido para DL50 foi 3450 mg/kg, que apesar de mais baixo, ainda é considerado seguro (CAYMAN, 2013).

Krishnan e Montcrief (2007) também realizaram um estudo de dose-repetidas (de 7 e 28 dias de tratamento). Com 7 dias de exposição, alguns animais foram mortos por estarem moribundos e um animal foi encontrado morto, todos devido a automutilação, possivelmente resultado de efeitos comportamentais da LDX (KRISHNAN; MONTCRIEF, 2007). Os animais também apresentaram algumas observações clínicas de toxicidade, redução no peso corporal e consumo, e alteração de parâmetros bioquímicos, bem como redução no peso do fígado e do baço. No entanto, as doses que apresentaram estes resultados foram altas (100 e 300 mg), e o estudo indicou níveis de efeitos não observáveis (*no observed effect level* - NOEL) como menor que 30 mg/kg/dia. No estudo de 28 dias foram utilizadas doses menores (40 e 80 mg/kg), o que resultou em menos impactos negativos nos animais, mas ainda assim, exibiram sinais clínicos de toxicidade, consumo reduzido e parâmetros bioquímicos alterados, definindo-se a NOEL como menor que 40 mg/kg/dia (KRISHNAN; MONTCRIEF, 2007).

Quando o medicamento foi administrado durante o período juvenil por 8 semanas consecutivas (a partir do DPN 7), também houve redução no peso corporal e consumo de ração, bem como redução no crescimento, sendo que essa redução no peso e crescimento continuou até o DPN 91, mesmo após o término da exposição, nos ratos machos (HUTSON; PENNICK; SECKER, 2014; SHIRE, [s.d.]). A função reprodutiva não apresentou nenhum efeito observado. Nestes estudos, o nível para efeitos adversos não observável (NOAEL) foi considerado 4 mg/kg/dia (HUTSON; PENNICK; SECKER, 2014; SHIRE, [s.d.]). Em ratos e coelhos, não há evidências de teratogenicidade (HUTSON; PENNICK; SECKER, 2014; SHIRE, [s.d.]). No entanto, já é bem estabelecido que a dependência de anfetaminas pela gestante aumenta o risco de parto prematuro e baixo peso ao nascer e, neste sentido, não há estudos adequados e bem controlados em mulheres grávidas com o uso do LDX, sendo assim considerado categoria C na gravidez (HUTSON; PENNICK; SECKER, 2014; SHIRE, [s.d.]).

No entanto, estas informações contidas na bula do medicamento não apresentam detalhes metodológicos envolvidos nos estudos para se obter estas conclusões, bem como carecem de informar todos os parâmetros avaliados. Outros estudos que abordem estas questões ainda são pouco explorados. Assim, um estudo toxicológico do LDX que investigue dados de toxicidade sistêmica e reprodutiva mostra-se de extrema importância, principalmente considerando que outras substâncias estimulantes são capazes de desencadear efeitos endócrinos e reprodutivos negativos no organismo exposto.

1.9. Efeitos endócrino e reprodutivos de estimulantes:

Substâncias estimulantes, por terem seus efeitos decorrentes de alteração nos níveis de neurotransmissores (DA e NA), podem comprometer a regulação de várias vias hormonais, afetando a liberação de hormônios, tais como prolactina, hormônios tireoidianos, andrógenos e estrógenos (NEGRAO; VILJOEN, 2011). Desta forma, a administração de drogas psicoterapêuticas pode interferir com a regulação neuroendócrina (GREELEY; KIZER, 1980), e consequentemente, afetar o sistema genital destes indivíduos. Visto que a LDX é um fármaco desta classe, é de se esperar que apresente efeitos similares. Neste sentido, evidências demonstram que a DA exerce um efeito inibitório sobre a secreção do Hormônio Luteinizante (LH) (MARTIN et al., 1981), o qual pode ser mediado pela supressão da secreção de hormônio liberador de gonadotrofina (GnRH), ou por um controle negativo direto sobre a secreção de LH (que anula os efeitos do GnRH), possivelmente devido a um controle inibitório produzido por neurônios dopaminérgicos originários da região pré-óptica antero-ventral e projetado para a *pars distalis* proximal da hipófise (PETER et al., 1986; VIDAL et al., 2004).

Além da regulação hormonal, as gônadas estão sujeitas a influências regulatórias direta de neurotransmissores, sejam eles derivados da inervação sináptica local ou trazidos pela circulação (GNESSI; FABBRI; SPERA, 1997; MAYERHOFER et al., 1996; SKINNERF, 1991). Evidências sugerem a existência de um eixo cérebro-testicular direto, principalmente relacionado a inervação hipotalâmica, o qual estaria envolvido nesse controle das funções gonadais (JAMES; RIVIER; LEE, 2008). Em ratos machos, as catecolaminas podem estar envolvidas na regulação espermatogênica (OTTH et al., 2007), já que receptores para DA (D2) e NA estão presentes nas células testiculares (ADEOYA-OSIGUWA; FRASER, 2007; OTTH et al., 2007).

A NA pode influenciar o desenvolvimento de células de Leydig, observado através da expressão de 3b-hidroxiesteróide-desidrogenase, um marcador de proliferação (HUO et al., 2012), e pode ativar a capacitação espermática (WAY; KILLIAN, 2002). Além disso, um estudo observou que ao se ligar a receptores adrenérgicos testiculares, a NA é capaz de estimular a produção de testosterona e de regular o número e a responsividade dos receptores para LH nas células de Leydig (LEE; MISELIS; RIVIER, 2002; MAYERHOFER et al., 1992). Entretanto, outros estudos apresentaram informações discrepantes, onde substâncias estimulantes levaram a uma redução nos níveis de testosterona (CHIN et al., 2002; LIN et al., 2014). Estes dados apontam para uma regulação esteroidogênica testicular através de catecolaminas e independente da ação das gonadotrofinas hipofisárias (LEE; MISELIS;

RIVIER, 2002). Dessa forma, as implicações da administração deste medicamento no sistema reprodutor devem ser consideradas.

Substâncias com mecanismos de ação similares a LDX já foram investigadas quanto ao seu impacto no sistema genital masculino. Estudos *in vivo* e *in vitro* demonstraram que a anfetamina (DUCA et al., 2019) diminui a produção de testosterona, através da diminuição da atividade de enzimas esteroidogênicas, do aumento da geração de AMP cíclico testicular e pela atenuação do influxo de cálcio (TSAI et al., 1996, 1997). A metanfetamina altera os níveis de NA e seus metabólitos no testículo, mecanismo pelo qual induz disfunções reprodutivas´ (JANPHET; NUDMAMUD-THANOI; THANOI, 2017). A metanfetamina diminuiu os níveis de testosterona em ratos (LIN et al., 2014), além de induzir apoptose em células germinativas testiculares, diminuir a proliferação celular, causar alterações histológicas (ALAVI; TAGHAVI; MOALLEM, 2008; YAMAMOTO et al., 2002), reduzir a contagem espermática (NUDMAMUD-THANOI; THANOI, 2011), bem como provocar estresse oxidativo em altas doses (LIN et al., 2014). Outro efeito observado deste fármaco foi o aumento na concentração testicular de Ácido gama-aminobutírico (GABA) (KAEWMAN; NUDMAMUD-THANOI; THANOI, 2018), o qual está envolvido na proliferação das células de Leydig, produção de testosterona e na supressão da proliferação de espermatogônias (KAEWMAN; NUDMAMUD-THANOI; THANOI, 2018). Alterações nos níveis de GABA após administração de psicoestimulantes podem impactar negativamente o processo de maturação reprodutiva. Sabe-se que no final da infância e início do período juvenil, o GABA tem efeito inibitório sobre os hormônios gonadotróficos, até que no período peripubertal, esta ação seja perdida e leve ao aumento dos níveis de LH e Hormônio Folículo Estimulante (FSH), o que contribui para maturação do sistema reprodutor e do eixo hipotálamo-pituitária-gonadal (HPG) (PICUT et al., 2015).

MONTAGNINI et al. (2014) avaliou os efeitos do MPH em parâmetros reprodutivos tardio (dia pós-natal - DPN 100) após a exposição durante a peripuberdade (DPN 21-60), e observou uma alteração na função testicular destes animais na idade adulta, evidenciado pelo aumento no número de anormalidades na cauda dos espermatozoides, aumento do volume no tecido intersticial do testículo, e alteração na população das células germinativas, indicando um prejuízo reprodutivo desencadeado por esta classe de medicamentos quando administrados neste período (MONTAGNINI et al., 2014).

1.10. Infância e puberdade:

A infância é um período de rápido crescimento e desenvolvimento, que envolve mudanças funcionais, metabólicas, físicas e comportamentais, sendo que as taxas de desenvolvimento de diferentes sistemas variam entre si (ATSDR, 2014). Conforme a idade da criança vai avançando, novas mudanças vão ocorrendo, e isso impacta diretamente em aspectos farmacocinéticos das substâncias às quais ela é exposta (ATSDR, 2014). Na puberdade ocorrem eventos de maturação, no sistema genital masculino acontecem a maturação final das células de Leydig, mudança no perfil hormonal e amadurecimento do eixo HPG desses indivíduos (PICUT et al., 2015). A maioria das divisões celulares, tanto mitóticas como meióticas, para a produção diária de espermatozoides são desencadeadas somente com o estabelecimento da puberdade e persistem pelo resto da vida adulta (PRYOR et al., 2000).

O desenvolvimento, maturação e diferenciação do sistema genital masculino é orquestrado pela ação de hormônios esteroidais, iniciando-se no período pré-natal e continuando até o final da puberdade (HO et al., 2017). No período pré-natal, apesar da presença de receptores hormonais no testículo, a ação hormonal não é um fator crítico, visto que o desenvolvimento gonadal embrionário, crescimento celular e morfogênese testicular ocorrem independentes de hormônios (tanto gonadotrofinas como hormônios esteroidais) (DIERICH et al., 1998; KUMAR et al., 1997; OJEDA; SKINNER, 2006; ZHANG et al., 2001). Nesta fase, a ação hormonal é essencial para que ocorra a diferenciação sexual hipotalâmica e a diferenciação e desenvolvimento da genitália externa.

Por outro lado, durante o desenvolvimento puberal, os hormônios androgênicos são os mais críticos para indução deste processo e manutenção das funções testiculares (OJEDA; SKINNER, 2006). Na pré-puberdade, os principais hormônios são a androstenediona, 5α-androstenediol e esteroides 5α-reduzidos (como a di-hidrotestosterona - DHT), e com a redução da atividade da 5α-redutase após o DPN 40, a testosterona torna-se o hormônio mais importante (GUPTA; ZARZYCKI; RAGER, 1975; MOGER, 1977; PIACSEK; GOODSPEED, 1978; PODESTÁ; RIVAROLA; JYUJO, 1974). Desta forma, qualquer substância administrada nesta faixa etária que altere o perfil hormonal destes indivíduos pode comprometer a maturação do sistema genital e levar a prejuízos na função reprodutiva imediatamente após a exposição ou na vida adulta. Esse aspecto tem relação direta com as chamadas “Janelas de vulnerabilidade”, que são fases específicas do desenvolvimento no qual determinado sistema do indivíduo apresenta uma maior susceptibilidade devido a um conjunto de eventos organizacionais dinâmicos, principalmente no que se refere à exposição a substâncias potencialmente tóxicas (ATSDR, 2014; PRYOR et al., 2000). O período da infância e puberdade pode ser considerado um período

crítico para o desenvolvimento reprodutivo (CHAPIN et al., 1997), com uma maior susceptibilidade a ação de insultos externos, pois compreende um período de mudanças morfológicas e endocrinológicas dinâmicas (PICUT et al., 2015). O momento da maturação puberal é um dos importantes alvos para ação tóxica de diversas substâncias neste sistema (PRYOR et al., 2000).

Isso significa, que mesmo com uma exposição mais curta, o indivíduo durante sua infância ou peripuberdade, pode apresentar determinados prejuízos espermáticos e reprodutivos de acordo com a fase em que ela se encontra (ATSDR, 2014). Caso a exposição ocorra antes da maturação final de determinado sistema, os danos causados podem ser irreversíveis (ATSDR, 2014). Neste sentido, a exposição a químicos durante a infância e peripuberdade pode impactar a saúde de maneira diferente do que em um indivíduo adulto (ATSDR, 2014), e esse impacto pode diminuir ou suprimir a competência reprodutiva do indivíduo exposto (PRYOR et al., 2000). A ação tóxica das substâncias pode ocorrer em diferentes níveis do organismo, isto é, pode afetar os tecidos reprodutivos via ação no eixo HPA, ou atingir diretamente as células testiculares, sejam elas as células germinativas, células de Sertoli ou Leydig (PRYOR et al., 2000).

O uso do rato como modelo experimental é conveniente para estudo do desenvolvimento sexual (OJEDA; SKINNER, 2006), considerando as semelhanças na arquitetura tecidual, mecanismos homeostáticos e expressão gênica (PRYOR et al., 2000). Entretanto, existem algumas diferenças que valem ser observadas. Uma delas é o fato que os humanos apresentam uma qualidade espermática relativamente baixa e um alto nível de perda fetal (PRYOR et al., 2000), o que faz com que os resultados encontrados no modelo experimental possam se manifestar de forma ainda mais intensa no organismo humano. Outra diferença entre as espécies é que os ratos não possuem o período de quiescência testicular pós-natal, onde não há secreção de gonadotrofina ou hormônios esteroides, presente nos humanos (OJEDA; SKINNER, 2006; PICUT et al., 2015), o que ressalta a importância de se atentar, em estudos do desenvolvimento reprodutivo nesta fase, que substâncias que reduzem a secreção de gonadotrofinas nos ratos neste período podem ter uma relevância menor em humanos (PICUT et al., 2015).

Além disso, isso faz com que as definições dos períodos em ratos e em humanos tenham certa diferença (figura 3). Em ratos, o período juvenil engloba do DPN 21 ao 32, o qual corresponde a uma criança de 2 a 12 anos. O período peripuberal, do DPN 33 ao 55 em ratos, equivale a adolescência humana dos 12 aos 16 anos. Nos ratos, há ainda uma última divisão, a chamada puberdade tardia, que engloba do DPN 56 ao 70 (OJEDA; SKINNER, 2006; PICUT

et al., 2015), visto que só são considerados sexualmente maduros entre 9 e 10 semanas (DPN 63 - 70) (LANNING et al., 2002).

Nome dos estágios Ratos/humanos	Ratos (DPN) (a)	Humanos (b)
Neonatal/Recém-nascido	0 a 7	0 a 28 dias
Infantil/Infância	8 a 20	1 a 23 meses
Juvenil/Criança	21 a 32	2 a 12 anos
Peripuberal/Adolescencia	33 a 55	12 a 16
Puberdade tardia/Adolescência	56 a 70	

Figura 3 - Comparação dos períodos do desenvolvimento em ratos machos e humanos. (a) OJEDA; ADVIS; ANDREWS (1980) e (b) BARROW; BARBELLION; STADLER (2011) (adaptado de PICUT et al., 2015).

1.11. Psicoestimulantes na puberdade:

O estudo da administração de psicoestimulantes durante o período da puberdade mostra-se ainda mais necessário visto que trabalhos prévios demonstraram que adultos e adolescentes respondem de maneira diferentes quando expostos a estes fármacos (BOLANOS; GLATT; JACKSON, 1998; LAVIOLA et al., 2002). Apesar dos mecanismos para estas diferenças ainda não serem completamente elucidados, supõem-se que haja uma maturação diferencial dos receptores dopaminérgicos mesolímbicos, uma diminuição no número de transportadores de DA (responsáveis pela recaptação deste neurotransmissor) ou da afinidade da DA por estes locais, o que resultaria em um acúmulo de DA durante a peripuberdade, amplificando sua transmissão, e acarretando uma alta atividade dopaminérgica basal em ratos desta faixa etária (BOLANOS; GLATT; JACKSON, 1998; LAVIOLA et al., 2002).

A maioria dos efeitos reprodutivos de substâncias psicoestimulantes (como a anfetamina e metanfetamina citadas anteriormente) foram observadas *in vitro* ou *in vivo* em animais adultos, demonstrando a escassez de estudos abrangendo a fase juvenil e puberdade. Corroborando com este fato, não há informações a respeito da influência da LDX na reprodução, em especial após sua administração durante o período juvenil e puberdade.

2. HIPÓTESE

O uso contínuo da Lisdexanfetamina durante a infância e puberdade provoca alterações nos níveis de dopamina e noradrenalina, catecolaminas relacionadas ao controle do metabolismo corporal, controle hormonal e com atuação direta nas gônadas. Assim, seus níveis alterados pela LDX levam a um desequilíbrio metabólico, indicativo de toxicidade sistêmica, bem como a impactos negativos no sistema genital masculino por ação hormonal indireta ou ação direta na inervação local.

3. JUSTIFICATIVA

Considerando que o TDAH, a desordem neurocomportamental mais comum em crianças e adolescentes, têm grande impacto na qualidade de vida dos pacientes, seu tratamento adequado é de extrema importância. Os psicoestimulantes, como a LDX, vem sendo amplamente prescritos, mas os efeitos a respeito de sua toxicidade sistêmica e reprodutiva, tanto concomitante ao tratamento como na vida adulta, ainda são escassos. Desta forma, estudos adicionais que investiguem possíveis desfechos de seu uso contínuo, em especial nos indivíduos do sexo masculino, devem ser realizados.

4. OBJETIVOS:

4.1. Objetivo geral

Avaliar os efeitos da exposição a Lisdexanfetamina durante o período juvenil à peripuberdade sobre parâmetros de toxicidade sistêmica e reprodutiva imediatamente após a exposição e na vida adulta de ratos machos.

4.2. Objetivos específicos

- Avaliar a toxicidade clínica durante o período de tratamento (DPN 23 ao 53);
- Avaliar parâmetros de toxicidade sistêmica imediatamente após o término da exposição (DPN 54) e após um período sem exposição (DPN 120);
- Investigar os impactos reprodutivos imediatamente após o término da exposição (DPN 54) e na vida adulta, após a maturidade sexual completa (DPN 120);

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6. APÊNDICES

6.1. Artigo: Toxicological and reproductive impairments in peripubertal and adult male rats treated with lisdexamfetamine dimesylate during juvenile and peripubertal period.

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TOXICOLOGICAL AND REPRODUCTIVE IMPAIRMENTS IN PERIBUBERTAL AND ADULT MALE RATS TREATED WITH LISDEXAMFETAMINE DIMESYLATE DURING JUVENILE AND PERIPUBERTAL PERIOD

Julia Stein[a], Bárbara Campos Jorge[a], Lívia Trippe Nagaoka[a], Ana Carolina Casali Reis[a], Beatriz de Matos Manoel[a], Letícia Cardoso Valente[b], Alana Rezende Godoi[c], Vanessa Caroline Fioravante[c], Francisco Eduardo Martinez[c], Patrícia Fernanda Felipe Pinheiro[c], André Sampaio Pupo[d], Arielle Cristina Arena[a,e*]

[a]Department of Structural and Functional Biology, Morphology sector, Institute of Biosciences of Botucatu – São Paulo State University (UNESP) - Botucatu, São Paulo, Brazil;

[b]Faculty of Health Sciences - Federal University of Grande Dourados (UFGD) - Dourados, Mato Grosso do Sul, Brazil;

[c]Department of Structural and Functional Biology, Anatomy sector, Institute of Biosciences of Botucatu - São Paulo State University (UNESP) - Botucatu, São Paulo, Brazil;

[d]Department of Biophysics and Pharmacology, Institute of Biosciences of Botucatu - São Paulo State University (UNESP) - Botucatu, São Paulo, Brazil;

[e]Toxicological Assistance Center (CEATOX), Institute of Biosciences of Botucatu – São Paulo State University (UNESP) - Botucatu, São Paulo, Brazil.

*Author for correspondence: Arielle Cristina Arena

Address: Department of Structural and Functional Biology, Institute of Biosciences of Botucatu, São Paulo State University, UNESP, Distrito de Rubião Junior, s/n, Caixa Postal – 510; CEP: 18618970; Botucatu – SP. Tel: + 55 14 38800495

E-mail address: arielle.arena@unesp.br (A. C. Arena)

Running Title: Negative health impacts of Lisdexamfetamine

ABSTRACT

Lisdexamfetamine (LDX) is a d-amphetamine (d-AMP) pro-drug used to treat Attention Deficit and Hyperactivity Disorder (ADHD), a common neurodevelopmental disorder in children and adolescents. Since its action is mediated by increased levels of catecholamines, mainly dopamine and noradrenaline, which influence hormonal control and have direct action in gonads, this drug may interfere with reproductive performance. Thus, this study evaluated the effects of exposure to LDX from the juvenile to peripubertal period (critical windows of development) on parameters of systemic and reproductive toxicity of male rats. Male Wistar rats (23 days old) were treated with 0; 5.2; 8.6 or 12.1 mg/kg/day of LDX from post-natal day (PND) 23 to 53, by gavage. The treatment with LDX provoked a reduction in the daily food and water consumption, as well as a reduction in total social behaviors. The day of preputial separation was unaltered, but the weight of the treated animals was reduced on this day. At PND 54, the treated animals presented signs of systemic toxicity, evidenced by a reduction in body weight gain, increase in the relative weight of the liver, spleen and seminal gland, reduction in the erythrocyte and leukocyte count, reduced total protein levels and disturbances in oxidative parameters. In adulthood, there was an increase in immobile sperm, reduced sperm count, histological changes in the testes, and altered oxidative parameters, without compromising male sexual behavior and fertility of the animals. These results showed that juvenile and peripubertal LDX-treatment induced systemic toxicity immediately after treatment and adversely influenced the reproductive function in adult life, indicating that caution in prescribing this drug on peripubertal period is necessary.

Keywords: Lisdexamfetamine, stimulants, systemic toxicity, hepatotoxicity, reproductive toxicity, sperm disturbances

Abbreviations

ADHD: Attention deficit hyperactivity disorder

ALP: Alkaline phosphatase

ALT: Alanine aminotransferase

ANVISA: Brazilian Health Surveillance Agency

AST: Aspartate aminotransferase

CAT: Catalase

CEUA: Commission for Ethics in the Use of Animals

CONCEA: National Council for the Control of Animal Experimentation

D-AMP: Dextroamphetamine

DAT: Dopamine transportes

DSP: Daily sperm production

$E_{máx}$: Maximal contractions

GEH: Germinal epithelium height

GGT: Gamma-glutamyl transferase

GSH: Glutathione

LC: Leydig cell

LDX: Lisdexamfetamine Dimesylate

LH: Luteinizing hormone

MAO: Monoamine oxidase

MCH: Mean corpuscular hemoglobin

MCHC: Mean corpuscular hemoglobin concentration

MCV: Mean corpuscular volume

MDA: Malondialdehyde

METH: Methamphetamine

NA: Noradrenaline

NET: Noradrenaline transporters

PD: Pregnancy Day

pEC50: -log of the effective concentration at 50%

PND: Post-natal day

SEM: Standard error of the mean

SERT: Serotonin transporters

SOD: Superoxide dismutase

STsD: Seminiferous tubules diameter

TAAR1: Trace amine-associated receptor 1

TBA: Thiobarbituric acid

UNESP: São Paulo State University

VMAT2: Monoamine vesicular transporter 2

Highlights:

- LDX induced systemic toxicity concomitant to treatment period.
- Systemic toxicity alterations were restored to normal levels at adulthood.
- LDX negatively impacted reproductive parameters at adulthood.
- LDX interfered in maturation of male genital system.

1. INTRODUCTION

Attention Deficit Hyperactivity Disorder (ADHD) is the most common neurodevelopmental disorder in children and adolescents [1], with two thirds of cases persisting in adulthood [2–4]. Despite the high heterogeneity of data, a worldwide prevalence of 7.2% is estimated [5] and in Brazil the prevalence is between 5.8 to 19.9% [6–9]. This disorder has a higher rate of diagnosis in males since they have a high prevalence of more disruptive and easily observed hyperactive behaviors [10–12]. The main symptoms in ADHD include inattention, hyperactivity, and impulsiveness above expected levels [1,13], negatively affecting patients' life, mainly when untreated [14–16].

The treatment is based on non-pharmacological methods associated with the use of stimulant medications [17–20], such as methylphenidate and amphetamines [1,21,22]. Among the most used drugs is Lisdexamfetamine Dimesylate (LDX), the first and only prodrug of dextroamphetamine (D-AMP), approved by Brazilian Health Regulatory Agency (ANVISA) in 2011 [21,23,24]. LDX is rapidly absorbed in the gastrointestinal tract after oral administration [25] and, once in the blood circulation, undergoes a hydrolysis process in the covalent ligation of D-AMP and l-lysine (an essential amino acid) [26,27]. It occurs slowly, decreasing the potential for abuse and prolonging its action for about 13 hours [28–31].

The amphetamine molecule has a very similar structure of endogenous catecholamines, which permits their entrance in presynaptic axon terminals by dopamine (DAT), noradrenaline (NET) and serotonin (SERT) transporters [32,33], reducing reuptake of the endogenous molecules [34]. Once inside, D-AMP can block monoamine vesicular transporter 2 (VMAT2), blocking neurotransmitters storage, and subsequently, increasing their efflux to synaptic clef [33]. Besides that, D-AMP also inhibits monoamine oxidase (MAO), leading to a reduction in monoamines degradation, and stimulates trace amine-associated receptor 1 (TAAR1) to internalize or reverse DAT, NET and SERT, increasing even more the neurotransmitters efflux

[35,36]. All these mechanisms contribute to elevating the levels of monoamines in the synaptic cleft, which seems to be related to the improvement of ADHD symptoms [1,21,22].

In addition to the expected adverse effects of stimulant medications, such as reduced appetite, headache, dry mouth, dizziness, tachycardia, insomnia, and others [33,37–39], an important effect of this class of drugs is the delayed growth rate and reduced height of patients treated for a long period [40–44]. Two repeated-dose toxicology studies (7 and 28 days) observed alterations in parameters related to general toxicology, such as clinical signs, hematological and biochemical parameters and organ weights in adult animals treated with doses above the therapeutic level [45]. However, more detailed toxicological information after treatment during important developmental windows is scarce.

During puberty, a significant maturation of the genital system occurs [46], which is orchestrated by androgens [47]. Thus, any substance that can compromise hormonal action at this stage can negatively impact an individual's reproductive performance [46]. It is known that when stimulants alter neurotransmitter levels, they can compromise the regulation of hormonal pathways [48], resulting in adverse effects on the genital system. A study of Mayerhofer et al. (1992) showed that catecholamines stimulates testosterone production by interaction with adrenergic receptors, and besides that, noradrenaline (NA) regulates the number of luteinizing hormone (LH) receptors in the testis altering its function and responsiveness [49]. In addition, neurotransmitters, originating from local innervation or coming from the blood circulation [50–52], are directly responsible for gonadal regulation, contributing to spermatogenic regulation [53] and Leydig cells (LC) function [53,54], through action in adrenergic and dopaminergic receptors present in the testis [53,54]. In this sense, other stimulants substances, like methamphetamine (METH), alter NA and its metabolites levels in testis, inducing reproductive dysfunction [55], which emphasizes the importance to study LDX effects.

Considering that catecholamines are related to hormonal control and have direct action on the gonads, their altered levels due to the action of continuous treatment with LDX could bring negative impacts to the general health and reproductive system of the exposed individual. Since LDX is mainly prescribed during childhood and adolescence, this study evaluated the effects of LDX treatment during this critical developmental window (juvenile and peripubertal) on systemic and reproductive toxicity parameters immediately after treatment and in adulthood in male rats.

2. MATERIALS E METHODS

2.1. Animals

Wistar rats from the Central *Bioterium* of São Paulo State University (UNESP), 24 females and 24 males (postnatal day - PND 90) were naturally mated to obtain the animals that have been used in this study. The pups were reduced to 4 males and 4 females per litter on PND 1, and the male pups were subsequently (after weaning in PND 21) randomly distributed among the experimental groups, avoiding littermates within the same group [56]. The animals were kept in collective cages (2 animals/cage) under standardized conditions (22ºC, 12/12h light/dark cycle), with water and commercial food (commercial chow phytoestrogen-free; Nuvilab CR1-Nuvital-PR, Brazil) *ad libitum*. The experimental procedures are in accordance with the Ethical Principles of the National Council for the Control of Animal Experimentation (CONCEA) and were approved by the Commission for Ethics in the Use of Animals (CEUA nº 3148130421) of the Biosciences Institute of UNESP in Botucatu.

2.2. Experimental design

After weaning (PND 21), male rats (n=20/group, 1/litter) were distributed into four experimental groups: a control group that received only the vehicle (deionized water) and three

groups exposed to different doses of LDX: 5.2; 8.6 and 12.1 mg/kg/day. Doses were based on therapeutic doses of 30, 50 and 70 mg, and converted to animal dose considering an animal weight of 0.150 kg and a human weight of 35 kg, corresponding to the average weight during the treatment period [57,58], and the following formula was used [59,60]: Human dose (mg/kg) = animal dose (mg/kg) x (animal weight kg/human weight kg)$^{0.33}$.

The exposure was orally (by gavage), for 31 consecutive days, from PND 23 to 53, following the protocol for evaluation of pubertal male rats [56], corresponding to the juvenile (PND 23 to 37 in rats and 2 to 12 years in children) and peripubertal period (PND 38 to 53 in rats and 12 to 16 years in adolescents) [46].

During the exposure period, clinical signs of toxicity, the onset of puberty and social play behavior of the animals were evaluated. At the end of this period (PND 54), half of the animals (n=10/group) were killed for immediate evaluation of toxicological and reproductive parameters. The other half (n=10/group) were maintained until sexual maturation when they were evaluated in relation to their sexual behavior (PND 90), fertility test and, on PND 120, they were killed to evaluation of toxicological and reproductive parameters after this period of recuperation.

2.3. Toxicological parameters

During the treatment, clinical signs of toxicity including body weight, water and food intake, diarrhea, piloerection, bleeding, abnormal breathing, tremors, seizures, changes in gait, posture and reaction to manipulation were evaluated (n=20/group).

On PND 54 (one day after the end of exposure) and 120 (30 days after sexual behavior), the animals (n=10/group/age) were killed by cardiac puncture after anesthesia with sodium Thiopental (50 mg/kg) to collection of blood in dry falcon tube (for serum collection to biochemical analyses) and EDTA tube (for hematological analyses). Furthermore, organs (liver,

kidney, spleen, thyroid, adrenal, testis, epididymis, seminal gland, ventral prostate, and vas deferens) were collected and weighted in analytical balance and storage for analyses (described below). The relative weights of these organs were calculated by dividing their absolute weight by their body weight and multiplying the result by 100.

2.3.1. Biochemical parameters: Serum biochemical parameters were analyzed on PND 54 (n=10/group) for determination of the levels of albumin, total protein, cholesterol, urea, alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), calcium and uric acid, using Bioclin analysis kits and semi-automatic BIOPLUS 200 devices.

2.3.2. Hematological parameters: on PND 54 and 120 (n=10/group/age), erythrocytes, total leukocytes and platelet counts were performed manually using a Neubauer chamber on a Nikon E-200 light microscope. For differential leukocytes count, blood smears were stained with the Rapid Panotic kit (Laborclin, Pinhais, PR, Brazil) and 200 cells were classified in monocytes, eosinophils, basophils, neutrophils, and lymphocytes. Hemoglobin was measured by a semi-automatic BIOPLUS 200 device. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated.

2.3.3. Social play behavior: the social play behavior, an important marker of neurodevelopment, influenced by the action of neurotransmitters [61,62], were evaluated, once, between PND 40 and 45 (n=20/group), with animals from the same cage (same experimental group, 2 animals/cage), being observed for 15 minutes in the dark period of the cycle, with a red light, where social (sniff, on back, boxing/bite, chase/flee, pinning/pounce, allogrooming,

jumping and running, and genital sniffing) and non-social (dig, chew substrate, explore and self-grooming) behavior were classified [63].

2.4. Reproductive evaluation

2.4.1. Assessment of puberty onset*:* preputial separation (n=20/group) was assessed from PND 30 by manually detaching the foreskin and weighing the animals on that day [56].

2.4.2. Male copulatory behavior: On PND 90, male rats from each group (n=10/group) were placed individually in polycarbonate boxes, 5 minutes before the introduction of an untreated sexually receptive female (PND 80). The animals were observed in the dark period of the cycle, 2-4 hours after the beginning of this period, in a separate room and under dark red light. For 40 minutes, the animals were observed regarding the following parameters: latency for the first mount, intromission, and ejaculation; number of intromissions and mounts until the first ejaculation; Interval between first intromission and ejaculation; latency to resume behavior after ejaculation and total number of ejaculations [64,65]. Males that do not mount within the first 10 minutes have been considered sexually inactive. After completing the evaluation of sexual behavior, the animals were maintained in the same cage overnight, next morning, they were separated and the vaginal smear of the females were collected to confirm pregnancy (presence of sperm and estrus cells), and this was considered pregnancy day 0 (PD0) and then, these females were used for the fertility test (n=10/group).

2.4.3. Fertility test: The sperm-positive females were killed on the PD 20, by decapitation, to evaluation of maternal weight gain, uterus plus fetuses weight, fetuses weight and calculation of the fertility potential (implantation sites/corpora lutea x 100); pre-implantation loss rate (Nº of corpora lutea – Nº of implantations/ nº of corpora lutea X 100);

post-implantation loss rate (Nº of implantations –Nº of live fetuses/Nº of implantations x 100); and sex-ratio (Nº male/nº female fetuses).

2.4.4. Sperm parameters on PND 120:

2.4.4.1. Sperm motility: It was assessed in the right cauda of the epididymis (n=6/group), as described by Perobelli et al. (2010) [66]. A 10 ml aliquot of the sperm suspension in HTF (human tubal fluid - Irvine) was transferred to the Makler chamber (34°C). Under a phase-contrast microscope, 100 sperm were counted and classified into: type A (progressive), type B (non-progressive mobile) and type C (immobile) [66].

2.4.4.2. Sperm count: Spermatids resistant to homogenization (stage 19) and sperm in caput/corpus and cauda of the left epididymis were counted (n=8/group), as previously described by (Robb et al., 1978), with adaptations adopted by Fernandes et al. (2007) [67,68]. Briefly, testis was decapsulated, weighed and homogenized in 5 mL of 0.9% NaCl containing 0.5% TritonX100. The sample was diluted 10 times and transferred to Neubauer chambers, and mature spermatids were counted in 4 different sections. To calculate the daily sperm production (DSP), the total number of mature spermatids was divided by 6.1 (number of days required to complete the spermatogenic cycle in rats). Similarly, portions of the epididymal caput/corpus and cauda were cut into fragments and homogenized, and sperm count proceeded as described for the testis, with 20-fold dilution of the samples. Sperm transit time through the epididymis was determined by dividing the total number of sperm in each portion by DSP [67,68].

2.4.4.3. Sperm morphology: To perform the morphology (n=10/group), the left vas deferens of the animals was sectioned at the ends and washed with a needle and syringe with 1mL of 10% formalin. 10 µl of this lavage were deposited on histological slides, and 200 sperm per animal were evaluated under light microscopy (400 X magnification). The morphological

abnormalities found in the sperm were classified into head and tail abnormalities and cytoplasmatic drops were counted [69].

2.4.5. Contractile activity of isolated epididymal duct: right vas deferens (epididymal portion) were carefully excised, cleaned of adherent tissues and mounted in organ baths according to Nojimoto et al. (2009), under 9.8 mN tension in a nutrient solution with the following composition (mM): NaCl 138; KCl 5.7, CaCl2 1.8, NaH2PO4 0.36, NaHCO3 15, dextrose 5.5, prepared in glass-distilled, deionized water, maintained at 30 °C, pH 7.4, and continuously bubbled with 95%O2/5%CO2 [70]. This nutrient solution is a modified Tyrode's solution optimized to minimize erratic "spontaneous" contractions and along with the respective temperatures, improves the contractions of these two smooth muscles (Zuleika P. Picarelli, personal communication). Changes in tension were recorded using FORT10 isometric force transducers (WPI, USA) connected to Transbridge 4M Transducer Amplifier (WPI, USA) connected to a PC based MP100 System and analysed off-line using AcqKnowledge version 3.5.7 software (Biopac Systems Inc., U.S.A.). After a 30 min stabilization period, tissues were challenged with 80 mM KCl. This procedure was repeated until reproducible contractions were obtained. Then, a concentration–response curve to phenylephrine was constructed by adding cumulative concentrations of the drug (10^{-8}M - 10^{-4}M). The maximal contractions ($E_{máx}$) and potency (pEC50: -log of the effective concentration at 50%) were evaluated.

2.4.6. Histological evaluation of the testis: right testis of the male rats on PND 54 and 120 were fixed in Bouin solution (n=5/group/age) and, subsequently processed as described by Balin et al. (2020) [71].

2.4.6.1. Leydig cell nuclei Count, area, and volume: In each histological section of the testis, LC nuclei were counted in 10 random fields. Mean LC diameter was measured to

calculate volume and area, and for this, 50 random nuclei per animal were analyzed [72]. Nuclei diameter values were obtained using a Nikon E-200 (X400) microscope coupled with a digital camera and a computer with NisElements software (version 4.20 for Windows). From these values, the radii of the nucleus were obtained, and thus the area, calculated using the formula $A = 3.14 \times r^2$, and the volume, in turn, calculated using the formula $V = 4 \times 3.14 \times r^3 / 3$.

2.4.6.2. Germinal epithelium height (GEH) and seminiferous tubules diameter (STsD): in each rat, 200 cross sections (one hundred per testis), round or nearly round, of the seminiferous tubules were randomly analyzed. From this, two perpendicular diameters of each cross section of the seminiferous tubules were measured using a NisElements (version 4.20 for Windows) light microscopy ocular micrometer and their means were calculated. For the measurement of the GEH, four equidistance of each cross section of the seminiferous tubules were considered [73], so their means were calculated.

2.4.6.3. Staging of seminiferous tubules: to evaluate the spermatogenic dynamics of the animals, the relative frequency of the stages of spermatogenesis was measured, so that it was classified as I-VI (two generations of spermatids - round and elongated), VII-VIII (spermiation), IX-XIII (one generation of elongated spermatids) and XIV (presence of secondary spermatocytes) in 100 sections of seminiferous tubules per animal.

2.4.7. Oxidative stress of the testis: for antioxidant enzymes determination, testis samples were homogenized in RIPA extraction buffer (Pierce, Rockford, IL, USA), incubated for 2h at 4 ◦C and centrifuged at 4000 g at 4 ◦C for 30 min to remove insoluble material. The supernatants were collected, and the total protein concentration was determined using the Bradford method (Bradford, 1976). The samples were stored at -80ºC until the day of analyses, when they were diluted in distilled water in an adequate ratio for each enzyme. The enzymatic activity of catalase (CAT), superoxide dismutase (SOD), and glutathione (GSH) levels were

quantified by the colorimetric method and the results were analyzed according to the methodology of Ohara et a. (2020) and Portela et al. (2021) [74,75]. For oxidative enzyme determination, testis samples were homogenized with 1.15% potassium chloride (KCl; 1:5, w/v). The samples were centrifuged for 10 min at 4 °C and 7690 g. Lipid peroxidation was determined by measuring the thiobarbituric acid (TBA) reaction according to Ohara et al. 2020. The results were expressed as nmol of malondialdehyde (MDA)/g of tissue.

2.5. Statistical analysis

Values were expressed as mean ± standard error of the mean (SEM) or median with interquartile ranges (Q1-Q3). Normality of the data were analyzed using D'Agostino & Pearson omnibus and Shapiro-Wilk normality test, and further compared using Analysis of variance – One-Way ANOVA with Dunett's posterior test for parametric data or Kruskal-Wallis analysis with Dunn's posterior test for non-parametric data. Differences were considered significant with $p<0,05$.

3. RESULTS

3.1. Toxicological parameters

Clinical signs of toxicity were not observed in the animals (data not shown), however all doses of LDX reduced the food intake during the treatment period (Figure 1A), as well as the water intake in the two highest doses (Figure 1B). In addition, the body weight gain of the animals exposed to the highest dose of LDX were reduced in relation to the control group (Figure 1C). The social play behavior analysis was negatively affected in the animals exposed to LDX. The two highest doses had a reduction in total social behavior (Figure 1E), and each of the social parameters analyzed (on back, pinning/pounce, boxing/bite, chase/flee, jumping and running; and genital sniffing) were also affected (SM - Figure 2).

On PND 54, an increase in the relative weight of the liver and spleen in the highest dose group (Table 1) was observed, however, these alterations were not permanent in the adult life of these animals (SM - Table 1). After biochemical analyzes, there was a reduction in total protein serum level in the two highest dose groups (Table 2). The erythrocytes count was reduced in all experimental groups compared to control, and there was also a reduction in leukocytes count only in the highest dose group on PND 54 (Table 2). However, on PND 120, these hematological parameters were unaltered (SM - Table 2).

3.2. Reproductive parameters

In relation to puberty onset, the day of preputial separation were not altered among the experimental groups (SM - Figure 1B), however, the body weight on this day was reduced in the highest dose (Figure 1D). On PND 54, an increase in the relative weight of seminal gland in the two highest dose groups (Table 1) was observed.

At adult life (PND 90), the male rats from the lowest dose presented an increased latency to the first intromission, but no other parameters were altered in the male sexual behavior (Table 3). There were also no significant differences in the fertility of the treated animals (SM - Table 3). In relation to sperm parameters, the animals exposed to 5.2 and 8.6 mg/kg of LDX had an increase in type C sperm (immobile) and a reduction in the relative number of mature spermatids in the testis (Table 4). Besides that, the animals exposed to the highest dose had a reduction in the relative number of sperm count in the cauda of epididymis compared to control (Table 4). The sperm morphology (Table 4) and the contractile activity (pEC50 and Emax) of vas deferens (SM - Figure 3 and Table 4) were not altered among groups.

On PND 54, there were no alterations in the number, area and volume of LCs (Figure 2A-C). In relation to the staging of the seminiferous tubules, we had an increase in stage XIV (Figure 3A) in the animals exposed to 8.6. The GEH was unaltered (Figure 3B), but there was

a reduction in the STsD in the lowest dose group of LDX (Figure 3C). On PND 120, the rats from all groups exposed to LDX had a higher number of LC count (Figure 2D) and the area and volume of these cells were increased in the animals exposed to 5.2 and 12.1 mg/kg of LDX (Figure 2E-F) (SM - Figure 4). The staging of the seminiferous tubules was normalized in adult rats (Figure 3D). However, on PND 120, the GEH was increased in the two highest doses (Figure 3E) and the STsD was increased in groups exposed to 5.2 and 12.1 mg/kg (Figure 3F).

Immediately after treatment, there were higher levels of CAT (5.2 and 8.6 mg/kg) and SOD (5.2 and 12.1 mg/kg), simultaneously with higher levels of MDA in all groups (Figure 4A-D). In adult life, this oxidative profile was altered, with a reduction in SOD and MDA levels only in the intermediated dose group (Figure 4G-H).

4. DISCUSSÃO

LDX is a medication approved in several countries for use in people aged > 6 years with ADHD. Although studies evaluating the adverse effects of LDX in children and adolescents are found in the literature [76], possible late effects of this drug on reproductive function are not reported. In order to address this gap, this study simulated the LDX treatment on pediatric patients and showed that this drug causes a systemic toxicity immediately after treatment and compromises the reproductive function in adult life.

This drug was developed with the aim of providing a prolonged duration of effect that is consistent throughout the day, with reduced potential for abuse and overdose toxicity [30]. In acute toxicity tests, LDX was considered safe, despite triggering increased motor activity [45]. However, after prolonged use (7 and 28 days) of doses above the therapeutic level, hemato-biochemical and organ weight changes were observed in treated animals [45]. In our study, male rats exposed to equivalent therapeutic doses of LDX during the juvenile to peripubertal period showed some signs of systemic toxicity during and immediately after (PND 54)

391 treatment, evidenced by alterations in hemato-biochemical parameters, weight gain, and liver
392 and spleen relative weight. The alterations found had a classic dose-response profile, with the
393 prevalence of negative impacts increasing with LDX dose. However, those parameters returned
394 to normal levels at adulthood (PND 120), demonstrating that the alterations were not permanent
395 in the conditions tested.

396 The reduction in body weight found in this study can be considered as a first indicator
397 of metabolic changes induced by exogenous substances [77]. Our results corroborate with
398 previous studies, where evidence demonstrated that pediatric patients with prolonged use of
399 amphetamines, including LDX, had a delayed growth index [42,78], with reduced height in
400 adulthood [33]. This consequence would be mainly associated with weight loss [26,31], reduced
401 appetite [44] and/or reduced caloric intake associated with stimulant medications [40,43],
402 which was also observed in our results as a reduction in food and water intake. These findings
403 corroborate with previous repeated dose studies, where adult rats also showed a reduction in
404 body weight and food intake after treatment with LDX [45]. This may occur since these
405 monoamine neurotransmitters, elevated by LDX, are involved in appetite control, increased
406 satiety [79], food recompense processes [80–83], food palatability [84], and regulation of
407 hormonal pathways (such as the growth hormone pathway) [48]. The reduced appetite and food
408 intake could also be a consequence of other common side effects, like nausea, constipation, and
409 diarrhea [85,86].

410 Play behavior, expressed in juvenile period [61], is modulated by DA and NA [87],
411 neurotransmissions systems that maturates in this phase, and thus, it is vulnerable to the effect
412 of drugs that act on their levels [88–90], which can lead to altered patterns of social behavior
413 later in life, such as low social interaction and aggressivity [91,92], as well as altered
414 reproductive performance [93]. Stimulation of dopamine receptors results in increased
415 motivation for play behavior, while stimulation of α2-adrenergic receptors by their agonists

suppresses the expression of this type of behavior [87], which could be responsible for the reduction in social play behavior in the animals of this study. These data, resulting from stimulation of α2-adrenoceptors, have already been demonstrated in previous studies as an effect of other psychostimulants, such as amphetamine and methylphenidate [94–97]. Despite these points, it is important to highlight that neurodevelopmental disorders, such as ADHD, can also lead to inadequate social behavior [89], thus, since the results of this study were obtained in healthy rats, this behavioral alteration triggered by LDX could occur differently in individuals with ADHD.

Treatment-related effects can often appear as changes in organ weights [98], which emphasizes the importance of this assessment in toxicological studies. Liver plays a central role in detoxification [99], which means that hepatotoxicity can be triggered by several substances[100] and lead to an increase in its weight and, in some cases, to changes in histology and serum biomarkers of toxicity [101]. In our study, LDX-treated animals had an increase in relative liver weight on PND 54, which may indicate a possible hepatotoxic process produced by the continuous use of this drug. The reduced total protein levels found support this hypothesis, since this biomarker indicates liver dysfunction [102].

In addition, since the spleen has a close anatomical and functional relationship with the liver, the increase in the weight of this organ found may be a direct consequence of hepatotoxicity [103–105]. Once increased, its function is compromised and it can capture and store an excessive number of erythrocytes, even leading to anemia, and destroy leukocytes and platelets, reducing their count and increasing the risk of infection [106], which could explain the change in hematological parameters after LDX treatment. The hematopoietic system is very sensitive to toxic compounds [107], and can also alter as a direct response of xenobiotics in synthesis or degradation of erythroid elements [77] and leukocytes [108]. In contrast to our findings, Krishnan and Montcrief (2007) observed a reduction in liver and spleen weights after

441 treatment with LDX, also indicating a perturbation. However, this study was conducted in adult
442 animals and higher doses, which may have contributed to these differences.

443 Despite the altered total protein levels discussed above, the doses of LDX used in this
444 study did not alter the other biochemical parameters analyzed. Similarly, Ermer et al. (2010)
445 also did not report significant alterations related to blood tests, urinalysis, biochemistry, or
446 physical examinations in healthy adults exposed to a single dose of LDX [109]. However, other
447 studies showed that high doses of LDX was capable of altering several biochemical parameters,
448 including an increase in albumin levels in rats [45].

449 Exposure to different substances at a pre-conceptional stage (such as peripubertal period
450 or early adulthood) can affect male germ cell development, affecting reproductive health and
451 performance in adulthood [110]. In this study, males were treated during peripuberty, an
452 extremely androgen-dependent period, when substances with endocrine disruption potential can
453 negatively affect the reproductive health of the animal in adulthood, compromising its fertility
454 [111]. In this period, neurotransmitters act altering hormonal levels, indirectly regulating
455 reproductive function, and can also regulate this function by a direct action in testis receptors
456 [49,54].

457 Although the treatment with LDX did not interfere with the process of puberty
458 installation, it adversely influenced the reproductive health of exposed animals at the two life
459 stages evaluated (on PND 54 and 120). Impairments of sperm function correspond to the most
460 prevalent cause of male infertility, which is a major medical and social problem and a difficult
461 condition to treat [112]. To this regard, the evaluation of sperm parameters is essential for
462 determining the safety of LDX use. In present study, treatment with LDX during peripubertal
463 period compromised the sperm motility of animals in adulthood, as well as caused a reduction
464 in the sperm count in the testis and epididymis cauda. Since dopaminergic and adrenergic
465 receptors are expressed in testes and sperm [53], changes in catecholamine levels in the testes

caused by LDX may have impacted spermatogenic process. Similar results were found in other studies, where exposure of male rats to other stimulants, such as methamphetamine (METH) and p-chloroamphetamine (PCA), also led to reduced sperm motility, in addition to other reproductive function-related damage, such as alteration in normal morphology, sperm count, and increased apoptotic cells in the seminiferous tubules [113–115], suggesting a negative impact of these stimulants on male fertility.

Male sexual behavior, especially sexual motivation and copulatory performance are regulated by steroid hormones, which influence the production and release of neurotransmitters from different areas of the brain [116], indicating that changes in hormone or catecholamine levels can have a negative impact on reproductive performance. LDX-treated rats had only increased latency to first intromission but did not represent a significant negative contribution to sexual performance, considering other parameters including latency and number of ejaculations were not altered. The absence of changes in fertility indicates that the behavioral and sperm changes found were not able to negatively affect gamete maturation, efficiency in copulatory performance, fertilization, implantation, zygote, and fetus development [117]. Similarly, the reactivity of the vas deferens *in vitro* demonstrated that the maximum sensitivity and effectiveness to the action of an agonist drug was unaltered.

The prepubertal LDX-treatment increased the frequency of the stage XIV in the testis of males on PND 54, demonstrating an imbalance in the spermatogenic cycle [118]. These animals also exhibited a reduction in the seminiferous tubules diameter, which may indicate that a degeneration of the testis, with loss of the spermatogenic epithelium, has occurred [119]. However, in adulthood, possibly due to a compensatory mechanism or over-stimulation by hormonal factor, there was a recovery of the spermatogenic cycle, and we found an increase in STsD and in GEH.

Leydig cell (LC) is under gonadotropin control [47] and catecholamine influence [53,54]. Our results demonstrated that the LDX-treatment induced a hypertrophy and hyperplasia in LC. Similar results have already been seen. Besides DA being considered an inhibitor of LH secretion, a study of Mendis-Handagama (1997) demonstrated that exposure for 57 weeks with a dopamine agonist resulted in an increased number of LC, since the medication induced a sustained increase in LH levels, which results in increased stimulation of LC volume and number [120]. Another hypothesis is that this increased number of LC could suggest a compensatory mechanism to low testosterone levels [121]. Thus, a hormonal dosage would be important to confirm the hormonal profile of the rats exposed to LDX. LC hypertrophy was also seen after newborn rats were treated for 49 days with an adrenoceptor agonist [122], possibility because noradrenaline promotes proliferation and differentiation of LC, that is evidenced by increased expression of 3b-hydroxysteroid-dehydrogenase (3β-HSD - a differentiation marker of precursor cells) and proliferating cell nuclear antigen (PCNA - a marker of proliferation) [54]. So, elevated levels of catecholamines at pubertal period, resulted from LDX use, may influence the establishment of adult LC by promoting their development and proliferation.

The presence of abundant oxidative stress or reactive oxygen species (ROS) has growing evidence of playing a key role in the etiology of sperm dysfunction and male infertility [112], either by lipid peroxidation of sperm [112,123] or LC membrane [124,125], compromising their function. Free radicals, related to oxidative activity, can be originated from catecholamines [126], during their degradation by MAO that result in H_2O_2 or their autoxidize process that form quinones (which induce damages) [126]. Considering this, the toxic effect of LDX in reproductive parameters may be mediated by increased oxidative stress associated with elevated levels of catecholamines. The increased levels of Malondialdehyde (MDA) on PND 54 indicate this oxidative damage to lipids [127], resulted of lipid peroxidation (LPO) induced

by LDX, which induced an increase in the levels of enzymes of antioxidant system to inhibit this lipid peroxidation and combat oxidative insults to the testis [112,127]. The induction of antioxidant enzymes is a strategy to develop tolerance to a subsequent larger insult [128,129], which combined with the absence of an insult in adult life, lead to an altered oxidative balance with reduced levels of MDA and SOD.

The response profile of the reproductive data was different, with most of the impacts being seen only in adult life, a time after the end of the treatment, which can represent that impacts during genital system maturation led to future injuries. Besides that, they did not present a classical dose-response, but caused results mainly in the lowest and highest dose group, in other words, showing a non-monotonic dose-response. Similar response profiles have been found as a dopamine action resulting in an inverted U-shaped dose-response pattern in cognitive performance [130], locomotion [131] and working memory [132,133].

5. CONCLUSION

Considering our results, the prescription and use of LDX should be done with caution, considering risks and benefits, since this medication seems to induce systemic toxicological alteration during its use and was able to impact developmental points of reproductive maturation and result in important injuries in genital system at adulthood. Besides that, more long-term clinical studies should be done to better understand these actions and mechanisms.

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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TABLES AND FIGURES

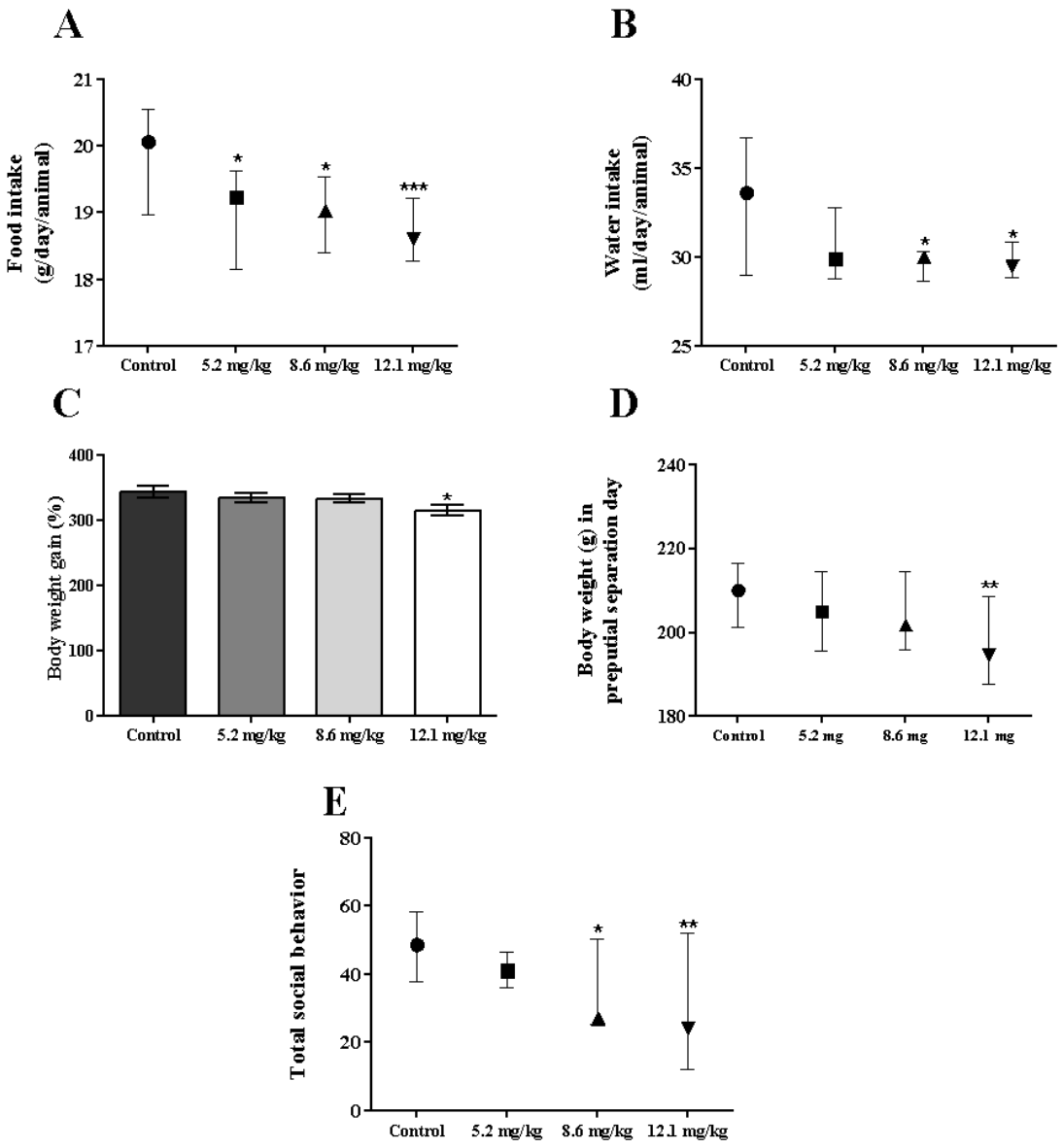

Figure 1. Parameters evaluated during the treatment period (juvenile to peripubertal period) to LDX. **A**. Food and **B**. water intake per rat per day; **C**. Body weight gain at PND 54; **D**. Body weight on the day of puberty onset; **E**. Total social behavior.

A, B, D and E: Values expressed as median - (Q1-Q3) of 20 animals per group. Kruskal-Wallis analysis with Dunn's posterior test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to the control group.

C: Values expressed as mean ± standard error of the mean (SEM) of 20 animals per group. Analysis of variance – One-Way ANOVA with Dunett's posterior test. *$p < 0.05$ compared to the control group.

Table 1. Relative organ weight (mg/100g of body weight) on PND 54 of male rats exposed to LDX from juvenile to peripubertal period.

Parameters	Experimental groups			
	Control	**5.2 mg/kg**	**8.6 mg/kg**	**12.1 mg/kg**
Liver	4662.00 ± 97.08	4784.00 ± 67.62	4937.00 ± 77.64	4994.00 ± 100.30*
Kidney	480.80 ± 11,88	466.70 ± 7.24	482.20 ± 7.19	503.20 ± 9.96
[1]Adrenal	10.54 (9.72 – 12.49)	10.33 (8.67 – 11.72)	10.61 (9.88 – 11.46)	10.08 (9.50 – 11.64)
Spleen	244.60 ± 11.16	267.10 ± 11.53	259.50 ± 8.87	294.40 ± 15.47*
Thyroid	6.57 ± 0.62	5.86 ± 0.39	5.94 ± 0.54	6.22 ± 0.40
Testis	512.40 ± 14.52	490.40 ± 14.19	524.00 ± 16.84	520.40 ± 14.88
[1]Epididymis	81.65 (71.11 – 88.54)	73.45 (70.99 – 79.48)	85.64 (68.40 – 92.38)	79.51 (70.32 – 84.08)
Vas deferens	24.05 ± 2.41	21.81 ± 0.99	23.43 ± 1.12	22.44 ± 1.62
Prostate	47.09 ± 2.85	49.82 ± 3.40	59.55 ± 5.59	52.81 ± 4.84
Seminal gland	98.45 ± 9.93	125.30 ± 9.06	150.50 ± 13.99*	143.80 ± 15.09*

Values expressed as mean ± SEM of 10 animals per group. Analysis of variance – One-way ANOVA with Dunett's posterior test. *$p < 0.05$ compared to the control group.

[1]Values expressed as median - (Q1-Q3) of 10 animals per group. Kruskal-Wallis analysis with Dunn's posterior test. $p > 0.05$ compared to control group.

Table 2. Biochemical and hematological analyzes on PND 54 of male rats exposed to LDX from juvenile to peripubertal period.

Parameters	Experimental groups			
	Control	**5.2 mg/kg**	**8.6 mg/kg**	**12.1 mg/kg**
[1]Total protein (g/dL)	6.10 (5.73 – 6.43)	5.45 (5.27 – 5.80)	4.90 (4.80 – 5.05)***	4.25 (3.88 – 4.88)***
[1]Albumin (g/dL)	2.80 (2.75 – 2.90)	2.60 (2.50 – 2.72)	2.65 (2.38 – 2.83)	2.70 (2.48 – 2.70)
[1]Creatinine (mg/dL)	0.50 (0.40 – 0.50)	0.50 (0.40 – 0.60)	0.55 (0.50 – 0.60)	0.40 (0.40 – 0.43)
Urea (mg/dL)	48.20 ± 1.70	47.80 ± 1.79	50.60 ± 1.47	52.20 ± 2.00
[1]AST (U/dL)	181.00 (169.00 – 194.30)	165.00 (141.00 – 181.00)	170.50 (137.50 – 179.80)	172.50 (146.50 – 183.80)
ALT (U/dL)	82.75 ± 2.57	86.40 ± 4.79	83.30 ± 5.59	77.50 ± 3.82
[1]GGT (U/L)	0 (0 – 1.50)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)
[1]ALP (U/L)	416.00 (352.50 – 436.50)	378.50 (303.80 – 444.30)	402.00 (362.50 – 451.00)	360.50 (330.30 – 463.00)
Cholesterol (mg/dL)	86.78 ± 6.02	82.11 ± 6.82	85.20 ± 4.13	84.22 ± 6.95
Calcium (mg/dL)	94.91 ± 21.06	77.78 ± 18.47	49.69 ± 10.10	114.8 ± 28.54
Uric acid (mg/dL)	13.62 ± 1.26	19.94 ± 1.14	10.92 ± 1.11	15.08 ± 1.67
Hematocrit (%)	37.80 ± 1.04	36.20 ± 0.93	34.20 ± 1.18	35.89 ± 1.14
[1]RBC (10^6/µL)	6.24 ± 0.20	5.57 ± 0.15*	5.21 ± 0.14**	5.34 ± 0.22**
[1]Hemoglobin (g/dL)	17.70 (17.63 – 18.08)	17.18 (17.18 – 17.93)	16.90 (16.58 – 18.05)	17.40 (16.73 – 17.90
Platelets (10^3/µL)	14.33 ± 0.37	15.38 ± 0.73	14.56 ± 1.26	15.70 ± 0.90
Leukocyte (10^3/µL)	10.30 ± 0.59	9.87 ± 0.66	9.89 ± 0.66	8.08 ± 0.40*
Lymphocyte	80.90 ± 1.55	78.60 ±1.35	82.60 ± 1.92	82.60 ± 2.17
Neutrophil	15.50 ± 1.64	17.80 ± 1.41	14.60 ± 1.64	14.00 ±1.90
[1]Monocyte	2.00 (1.00 – 4.25)	3.00 (2.75 – 3.00)	1.50 (1.00 – 2.50)	2.00 (0.75 – 4.00)
[1]Eosinophil	0 (0 – 1.25)	0 (0 – 1.00)	1.00 (0 – 1.00)	0 (0 – 1.25)
[1]Basophil	0 (0 – 0.50)	0 (0 – 1.00)	0 (0 – 1.00)	0 (0 – 1.00)
MCV (fL)	61.60 ± 3.48	65.28 ± 2.10	63.52 ± 3.53	65.83 ± 4.81
MCH (pg)	29.74 ± 0.96	31.63 ± 0.74	32.36 ± 1.52	32.84 ± 1.23
MCHC (%)	49.05 ± 1.62	48.68 ± 1.13	50.98 ± 1.34	48.39 ± 1.35

Values expressed as mean ± SEM of 10 animals per group. Analysis of variance – One-way ANOVA with Dunett's posterior test. ***p < 0.001 compared to control group. ALP - alkaline phosphatase; GGT – gamma-glutamyl transferase; ALT - alanine aminotransferase; and AST - aspartate aminotransferase.

[1]Values expressed as median - (Q1-Q3) of 10 animals per group. Kruskal-Wallis analysis with Dunn's posterior test. *p < 0.05 and **p < 0.01 compared to control group. MCV -Mean corpuscular volume; MCH - mean corpuscular hemoglobin; MCHC - mean corpuscular hemoglobin concentration.

Table 3. Male sexual behavior on PND 90 of male rats exposed to LDX from juvenile to peripubertal period.

Parameters	**Experimental groups**			
	Control	**5.2 mg/kg**	**8.6 mg/kg**	**12.1 mg/kg**
[1]Latency to the first mount	38.00 (17.00 - 77.75)	121.00 (32.50 - 283.00)	52.00 (25.50 - 195.50)	149.00 (16.00 - 164.00)
Number of mounts	6.62 ± 1.94	7.11 ± 1.81	3.75 ± 0.84	5.43 ± 1.73
Latency to the first intromission	56.14 ± 15.51	200.90 ± 39.94*	145.60 ± 49.11	122.40 ± 36.09
Number of intromissions	23.25 ± 4.30	20.44 ± 2.16	19.11 ± 2.65	21.71 ± 0.87
Latency to the first ejaculation	1118.00 ± 223.40	1173.00 ± 250.90	1114.00 ± 108.40	1076.00 ± 173.60
Interval between first intromission and ejaculation	1032.00 ± 219.80	966.00 ± 227.00	956.80 ± 130.10	938.80 ± 152.80
Latency to resume behavior after ejaculation	363.50 ± 45.58	308.00 ± 44.58	352.20 ± 22.83	289.20 ± 52.82
[1]Number of ejaculations	1.00 (0.25 – 2.00)	1.00 (0 - 2.00)	1.00 (0 - 1.00)	1.00 (0 - 2.00)
Sexually inactive animals	2	1	1	3

Values expressed as mean ± SEM of 10 animals per group. Analysis of variance – One-Way ANOVA with Dunett's posterior test. *$p < 0.05$ compared to the control group.

[1]Values expressed as median - (Q1-Q3) of 10 animals per group. Kruskal-Wallis analysis with Dunn's posterior test. $P > 0,05$ compared to the control group.

Table 4. Sperm parameters on PND 120 of male rats exposed to LDX from juvenile to peripubertal period.

Parameters	Experimental groups			
	Control	**5.2 mg/kg**	**8.6 mg/kg**	**12.1 mg/kg**
Sperm motility (n=6)				
Type A	70.96 ± 3.05	60.61 ± 2.60	60.27 ± 3.33	63.34 ± 6.05
Type B	19.84 ± 3.07	24.16 ± 1.99	22.40 ± 1.98	24.00 ± 4.07
Type C	8.24 ± 0.72	15.23 ± 1.08*	17.33 ± 2.83**	10.29 ± 1.16
Sperm Count (n=8)				
Testis				
Number of mature spermatids ($x10^6$)	220.10 ± 10.99	217.20 ± 8.48	212.10 ± 12.31	215.50 ± 15.06
Relative number of mature spermatids ($x10^6$/g)	163.30 ± 5.64	139.00 ± 4.40*	140.70 ± 5.20*	146.70 ± 7.97
Daily sperm production	36.09 ± 1.80	36.61 ± 1.39	34.76 ± 2.02	35.32 ± 2.47
Epididymis - Caput/corpus				
Number of sperm ($x10^6$)	117.70 ± 6.65	132.90 ± 6.80	129.70 ± 7.76	129.40 ± 12.08
Relative number of sperm ($x10^6$/g)	458.90 ± 23.19	456.10 ± 26.78	438.00 ± 18.90	436.60 ± 16.36
Transit (days)	3.39 ± 0.37	3.78 ± 0.27	3.64 ± 0.23	3.71 ± 0.32
Epididymis - Cauda				
Number of sperm ($x10^6$)	222.90 ± 15.10	233.10 ± 10.39	227.60 ± 8.70	233.20 ± 13.64
Relative number of sperm ($x10^6$/g)	1105.00 ± 45.12	1060.00 ± 39.30	1035.00 ± 36.84	923.20 ± 19.80**
Transit (days)	6.24 ± 0.47	6.62 ± 0.38	6.63 ± 0.28	6.80 ± 0.52
Total transit (days)	9.62 ± 0.67	10.39 ± 0.57	10.27 ± 0.44	10.50 ± 0.76
Sperm morphology (n=10)				
[1]Normal	96.75 (89.88 – 98.63)	97.25 (95.63 – 98.00)	96.50 (95.50 – 96.50)	97.00 (92.00 – 98.25)
[1]Abnormal	3.25 (1.38 – 10.13)	2.75 (2.00 – 4.38)	3.50 (3.50 – 4.50)	3.00 (1.75 – 8.00)
[1]Head abnormalities	1.25 (0.88 – 4.63)	1.00 (0.50 – 1.75)	1.50 (0.50 – 2.63)	1.00 (0.50 – 2.38)
[1]Tail abnormalities	2.50 (0 – 5.13)	1.50(0.88 -2.50)	2.50 (1.25 – 3.50)	1.25 (0.50 – 4.50)
[1]Cytoplasmatic drops	17.40 ± 2.91	15.20 ± 3.08	18.38 ± 3.24	16.50 ± 2.30

Values expressed as mean ± SEM of 6-10 animals per group. Analysis of variance – One-Way ANOVA with Dunett's posterior test. *$p < 0.05$ and ** $p < 0,01$ compared to control group. Type A: Motile and progressive sperm; Type B: non progressive motile sperm; Type C: immobile sperm.

[1]Values expressed as median - (Q1-Q3) of 5-8 animals per group. Kruskal-Wallis analysis with Dunn's posterior test. $p > 0.05$ compared to the control group.

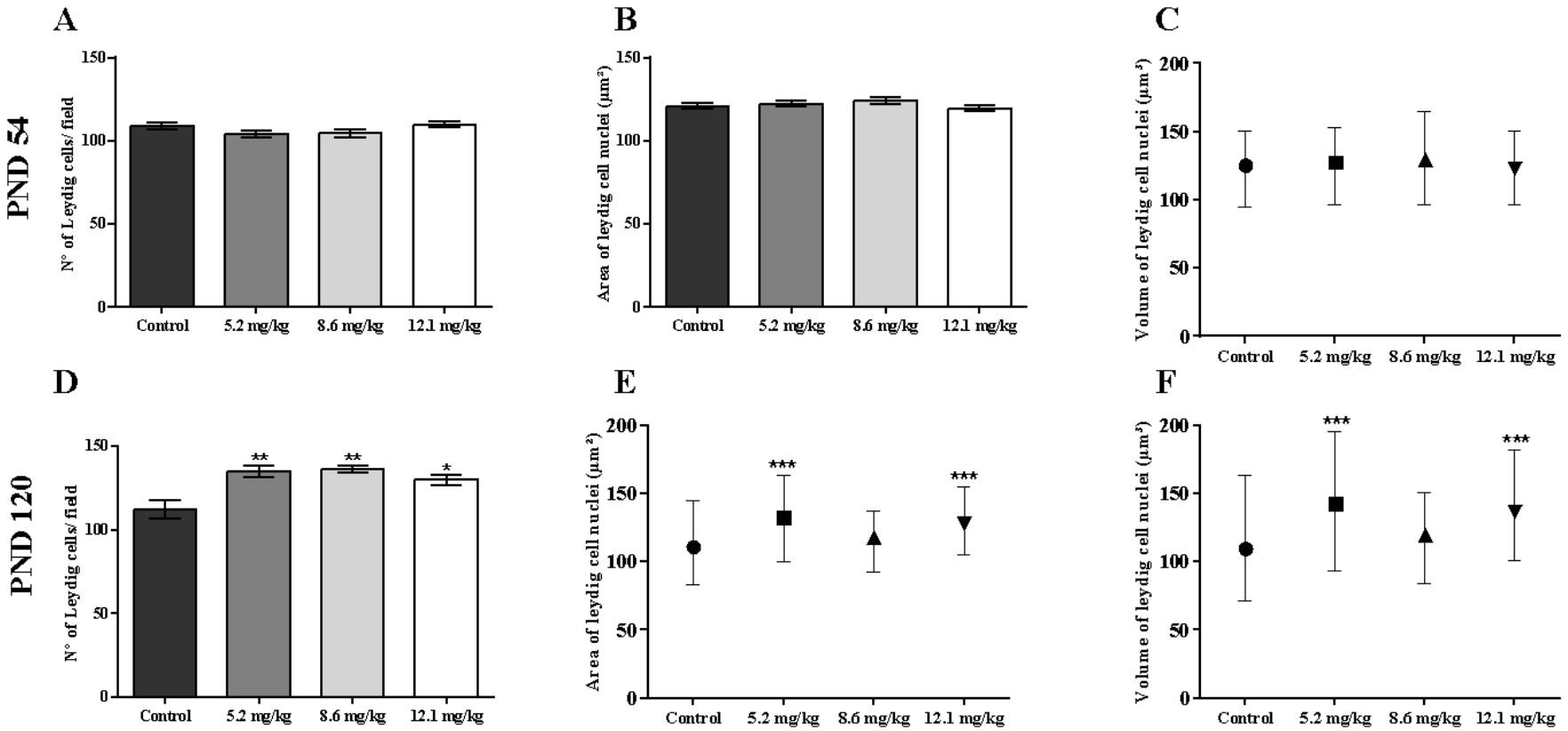

Figure 2. Histological analysis of Leydig cells on PND 54 (Fig A - C) and PND 120 (D - F) of male rats exposed to LDX from the juvenile to peripubertal period. Number of Leydig cells in 10 fields (A and D), area (B and E) and volume (C and F) of Leydig cell nuclei.

A, B and D: Values expressed as mean ± SEM of 5-7 animals per group. Analysis of variance – One-way ANOVA with Dunett's posterior test. *$p < 0.05$ and **$p < 0.01$ compared to the control group.

C, E and F: Values expressed as median - (Q1-Q3) of 5-7 animals per group. Kruskal-Wallis analysis with Dunn's posterior test. ***$p < 0.001$ compared to the control group.

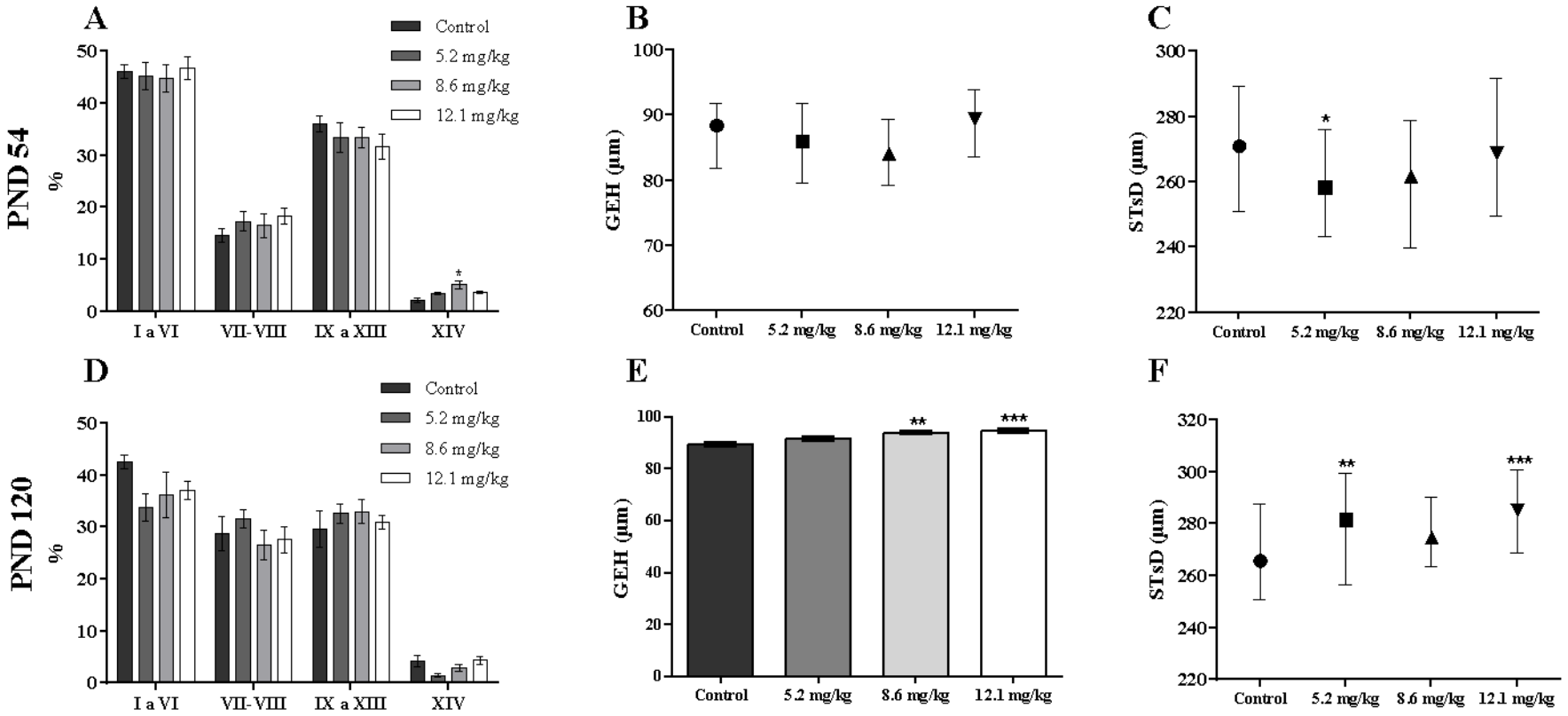

Figure 3. Histological analysis of seminiferous tubules on PND 54 (Fig A - C) and PND 120 (D - F) of male rats exposed to LDX from the juvenile to peripubertal period. Stages of seminiferous tubules (A and D), GEH (B and E) and STsD (mm) (C and F).

A, D and E: Values expressed as mean ± SEM of 5-7 animals per group. Analysis of variance – One-way ANOVA with Dunett's posterior test. *<0.05, **p < 0.01 and ***p < 0.001 compared to the control group.

B, C and F: Values expressed as median - (Q1-Q3) of 5-7 animals per group. Kruskal-Wallis analysis with Dunn's posterior test. *p < 0.05, **p < 0.01 and ***p < 0.001 compared to the control group.

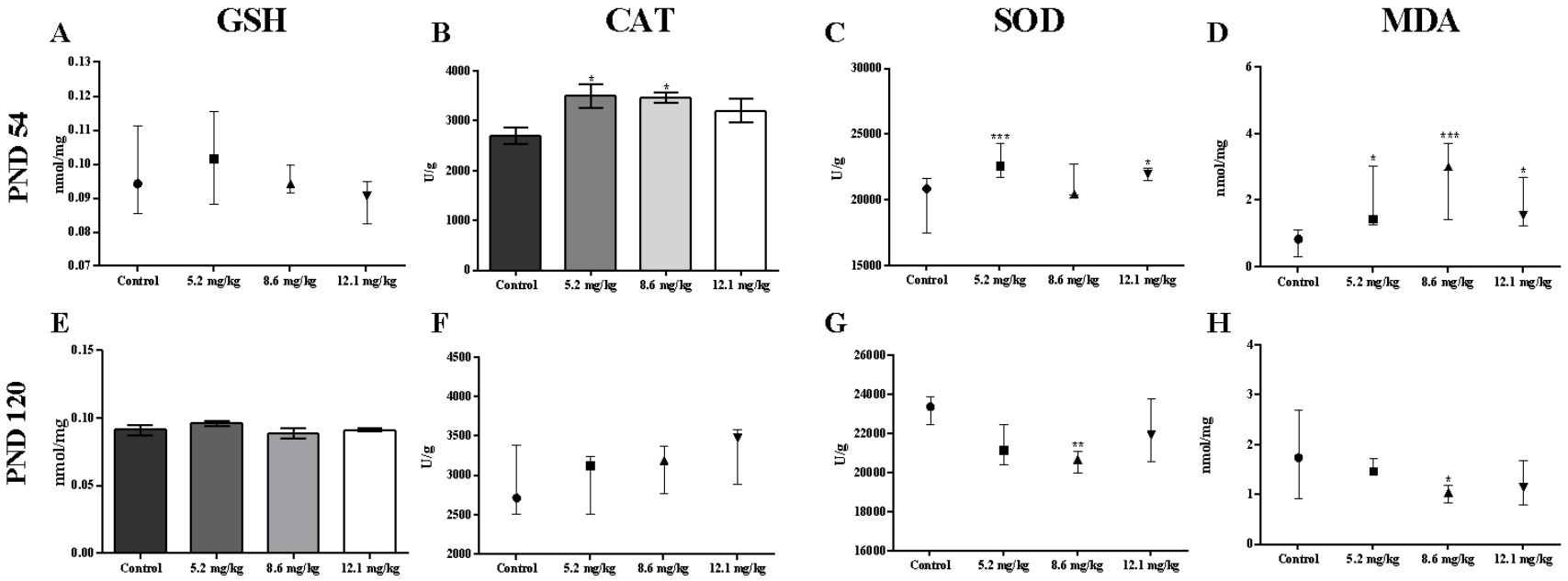

Figure 4. Oxidative stress biomarkers on PND 54 (Fig A - D) and PND 120 (E - H) of male rats exposed to LDX from the juvenile to peripubertal period. CAT: catalase; SOD: superoxide dismutase; GSH: glutathione; and MDA: malondialdehyde

A, C, D, F, G and H: Values expressed as median - (Q1-Q3) of 5 animals per group. Kruskal-Wallis analysis with Dunn's posterior test. *p<0.05, **p < 0.01 and ***p < 0.001 compared to the control group.

B and E: Values expressed as mean ± SEM of 5 animals per group. Analysis of variance – One-way ANOVA with Dunett's posterior test. *p<0.05 compared to the control group.

SUPPLEMENTARY MATERIAL

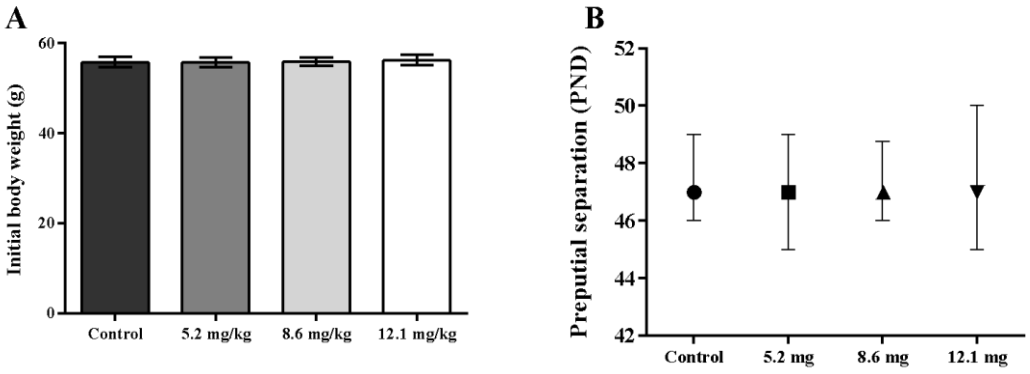

Figure 1. A. Initial body weight (PND 23); **B**. postnatal day (PND) of preputial separation.

A: Values expressed as mean ± standard error of the mean (SEM) of 20 animals per group. Analysis of variance – One-Way ANOVA with Dunett's posterior test. $p > 0.05$ compared to the control group.

B: Values expressed as median - (Q1-Q3) of 20 animals per group. Kruskal-Wallis analysis with Dunn's posterior test. $p > 0.05$ compared to the control group.

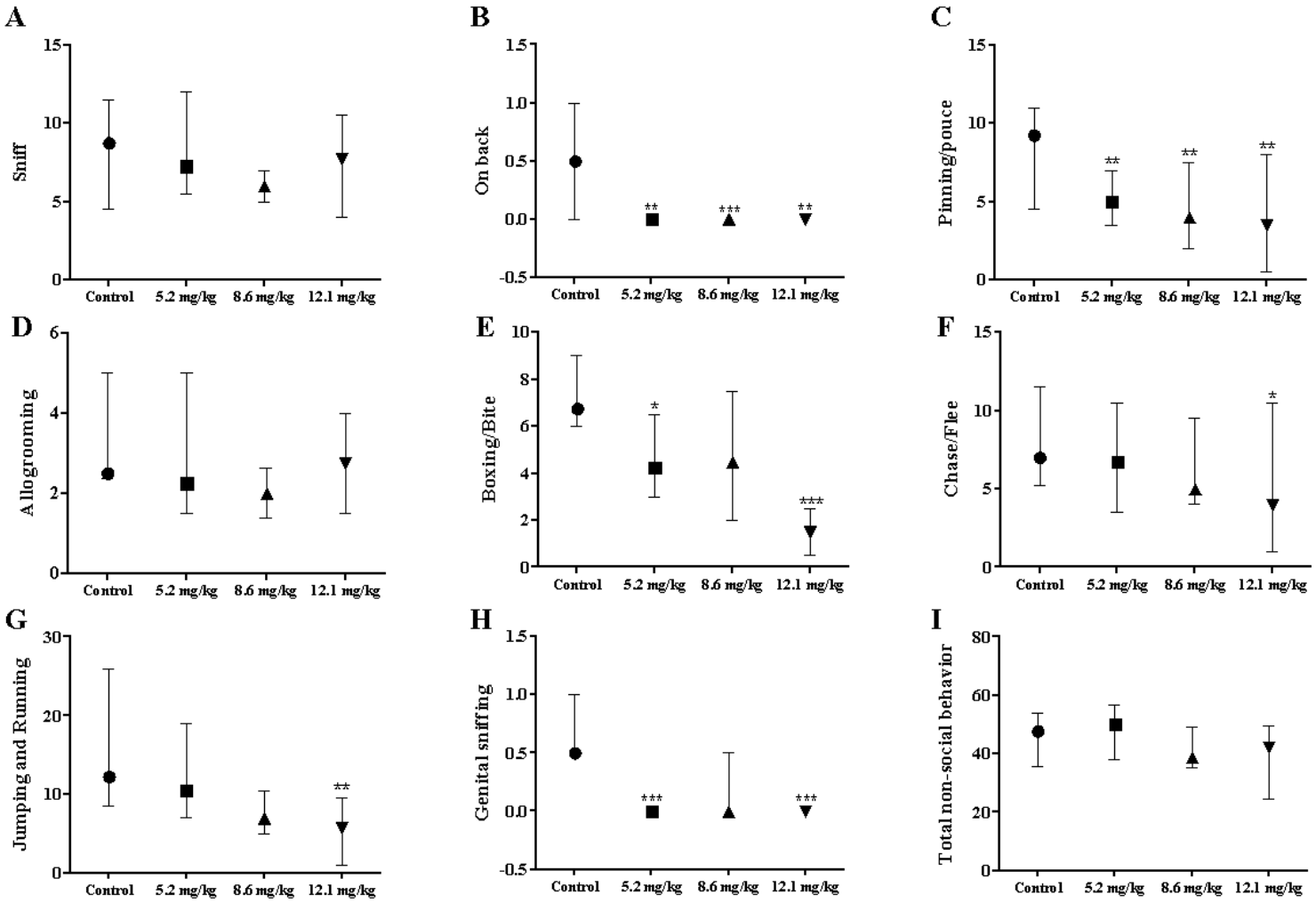

Figure 2. Social parameters evaluated: **A**. Sniff; **B**. On back; **C**. Pinning/pounce; **D**. Allogrooming; **E**. Boxing/Bite; **F**. Chase/Flee; **G**. Jumping and running; and **H**. Genital sniffing. **I.** Total non-social behavior. Values expressed as median - (Q1-Q3) of 20 animals per group. Kruskal-Wallis analysis with Dunn's posterior test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to the control group.

Table 1. Relative organ weight (mg/100g of body weight) on PND 120 of male rats exposed to LDX from juvenile to peripubertal period.

Parameters	**Experimental groups**			
	Control	**5.2 mg/kg**	**8.6 mg/kg**	**12.1 mg/kg**
Body weight (g)	444.60 ± 7.51	470.30 ± 11.24	471.40 ± 8.66	450.70 ± 10.18
liver	3353.00 ± 71.19	3474.00 ± 111.40	3479.00 ± 61.46	3487.00 ± 61.78
Kidney	393.40 ± 9.14	400.70 ± 15.41	390.20 ± 9.00	400.90 ± 4.45
Adrenal	7.68 ± 0.51	7.99 ± 0.84	7.60 ± 0.36	7.01 ± 0.37
Spleen	171.20 ± 11.46	148.30 ± 4.95	162.20 ± 10.86	158.00 ± 11.84
Thyroid	4.83 ± 0.42	4.22 ± 0.40	4.94 ± 0.23	4.66 ± 0.34
[1]Testis	381.00 (351.20 – 421.60)	391.20 (374.70 – 394.60)	378.10 (341.60 – 394.60)	394.80 (349.90 – 439.70)
Epididymis	144.00 ± 4.67	145.30 ± 3.13	140.90 ± 3.63	147.50 ± 5.44
Vas deferens	22.10 ± 1.33	22.91 ± 0.85	22.14 ± 0.87	23.91 ± 1.43
[1]Prostate	114.90 (99.31 – 137.30)	110.70 (91.09 – 157.50)	107.90 (91.13 – 130.50)	121.00 (91.84 – 137.70)
Seminal gland (full)	439.50 ± 26.32	452.70 ± 21.38	461.20 ± 16.89	443.10 ± 25.45
[1]Seminal gland (empty)	270.20 (218.30 – 375.50)	325.30 (283.60 – 353.70)	333.80 (240.50 – 374.80)	254.50 (230.50 – 346.70)

Values expressed as mean ± SEM of 10 animals per group. Analysis of variance – One-way ANOVA with Dunett's posterior test. $p > 0.05$ compared to the control group.

[1]Values expressed as median - (Q1-Q3) of 10 animals per group. Kruskal-Wallis analysis with Dunn's posterior test. $p > 0.05$ compared to control group.

Table 2. Hematological parameters on PND 120 of male rats exposed to LDX from juvenile to peripubertal period.

Parameters	Experimental groups			
	Control	**5.2 mg/kg**	**8.6 mg/kg**	**12.1 mg/kg**
[1]Hematocrit (%)	56.00 (53.75 – 63.50)	57.50 (50.00 – 61.25)	57.50 (50.00 – 61.25)	60.00 (49.50 – 65.00)
RBC (10^6/µL)	8.22 ± 0.41	7.47 ± 0.29	7.18 ± 0.13	7.64 ± 0.31
Hemoglobin (g/dL)	21.15 ± 0.31	21.17 ± 0.24	21.01 ± 0.27	21.06 ± 0.60
[1]Platelets (10^3/µL)	11.50 (8.00 – 13.75)	12.00 (8.50 – 14.50)	10.00 (9.00 – 13.50)	13.00 (9.00 – 18.00)
Leukocyte (10^3/µL)	12.83 ± 0.96	15.46 ± 0.67	12.77 ± 1.06	15.13 ± 1.38
Lynphocyte	73.63 ± 1.68	76.63 ± 2.25	76.25 ± 2.23	75.25 ± 2.72
Neutrophil	20.50 ± 1.61	17.75 ± 1.85	19.75 ± 1.90	20.00 ± 2.49
[1]Monocyte	3.12 ± 0.58	3.00 ± 0.78	2.00 ± 0.49	2.38 ± 0.68
[1]Eosinophil	1.00 (0.25 – 2.00)	1.00 (0.25 – 2.50)	0 (0 – 1.75)	1.00 (0.25 – 1.00)
[1]Basophil	2.00 (0.25 – 2.75)	1.00 (1.00 – 1.75)	1.00 (0 – 1.00)	1.50 (0.25 – 2.75)
MCV (fL)	71.11 ± 4.37	76.98 ± 4.44	79.71 ± 3.24	77.54 ± 5.18
MCH (pg)	26.28 ± 1.23	26.68 ± 0.95	29.34 ± 0.59	27.89 ± 1.12
MCHC (%)	37.42 ± 1.05	37.85 ± 1.29	37.24 ± 1.40	36.85 ± 1.82

Values expressed as mean ± SEM of 10 animals per group. Analysis of variance – One-Way ANOVA with Dunett's posterior test. $p > 0.05$ compared to the control group.

[1]Values expressed as median - (Q1-Q3) of 10 animals per group. Kruskal-Wallis analysis with Dunn's posterior test. $p > 0.05$ compared to the control group.

RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; and MCHC: mean corpuscular hemoglobin concentration.

Table 3. Fertility test of male rats exposed to LDX from juvenile to peripubertal period mated with untreated females on PND 90.

Parâmetros	**Grupos experimentais**			
	Control	**5.2 mg/kg**	**8.6 mg/kg**	**12.1 mg/kg**
[1]Fertility potential (%)	89.29 (79.12 - 100)	100 (93.73 - 100)	93.10 (92.86 - 100)	94.44 (90.00 - 100)
Maternal weight gain (g)	139.30 ± 3.79	139.40 ± 6.29	131.50 ± 5.81	141.10 ± 9.34
Uterus + fetus (g)	80.81 ± 2.66	81.54 ± 3.27	79.33 ± 4.23	90.07 ± 3.62
Fetus weight (g)	4.28 ± 0.07	4.28 ± 0.07	4.37 ± 0.09	4.07 ± 0.08
[1]Pre-implantation loss (%)	10.71 (0 – 20.88)	0 (0 – 6.28)	6.91 (0 – 7.14)	5.56 (0 - 10.00)
[1]Post-implantation loss (%)	0 (0 – 7.92)	0 (0 – 7.44)	0 (0 - 13.57)	5.56 (0 - 6.67)
Sex-ratio (M/F)	1.12 ± 0.26	1.23 ± 0.20	0.93 ± 0.12	1.25 ± 0.13

Values expressed as mean ± SEM of 8 animals per group. Analysis of variance – One-Way ANOVA with Dunett's posterior test.* $p < 0.05$ and ** $p < 0,01$ compared to the control group.

1Values expressed as median - (Q1-Q3) of 8 animals per group. Kruskal-Wallis analysis with Dunn's posterior test. $p > 0.05$ compared to the control group.

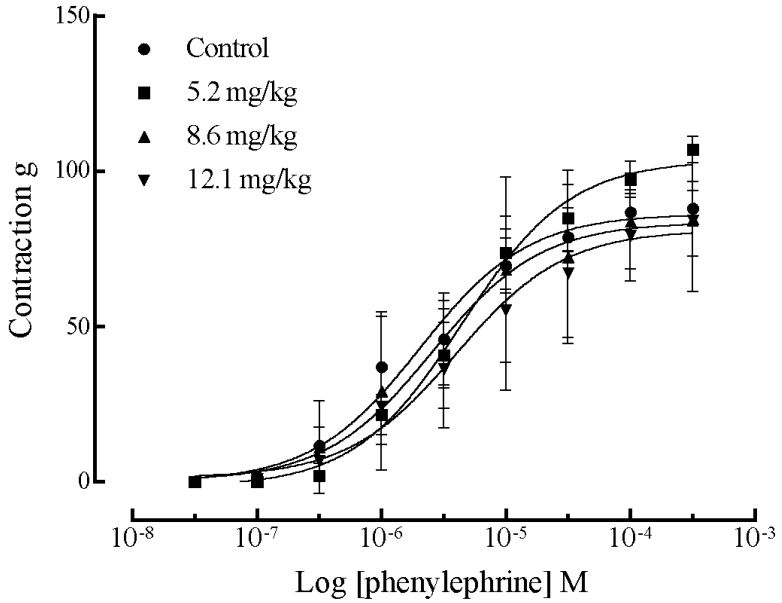

Figure 3. Concentration-response curve of phenylephrine in vas deferens.

Table 4. Parameters of agonism (pEC50: -log of the effective concentration at 50%; and $E_{máx}$: maximal contractions) in the vas deferens for norepinephrine on PND 120 of male rats exposed to LDX from juvenile to peripubertal period.

Parâmetros	**Grupos experimentais**			
	Control	**5.2 mg/kg**	**8.6 mg/kg**	**12.1 mg/kg**
pEC50	-5.64 ± 0.25	-5.44 ± 0.13	-5.70 ± 1.17	-5.27 ± 0.27
$E_{máx}$	90.64 ± 6.34	111.10 ± 5.28	89.61 ± 5.34	89.72 ± 10.88

Values expressed as mean ± SEM of 6 animals per group. Analysis of variance – One-Way ANOVA with Dunett's posterior test. $p > 0.05$ compared to the control group.

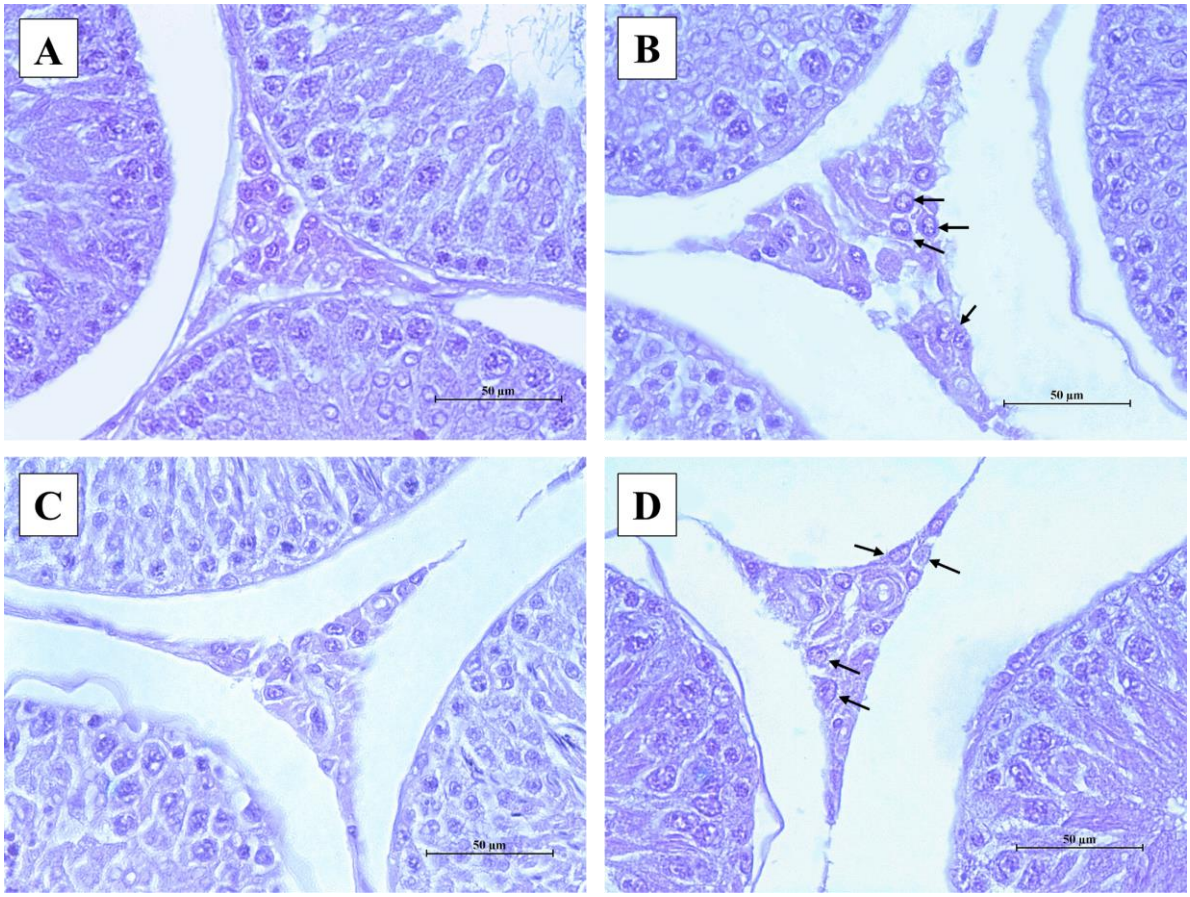

Figure 4. Histological images of interstitial tissue with Leydig cells at PND 120 of male rats exposed to LDX from juvenile to peripubertal period in control group (A) and experimental groups: 5.2 mg/kg (B), 8.6 mg/kg (C) and 12.1 mg/kg (D). The arrows indicate increased volume of Leydig cell nuclei.

7. CONSIDERAÇÕES FINAIS

Considerando a importância do tratamento adequado do TDAH para melhora na qualidade de vida dos pacientes e que o LDX apresenta boa adesão e melhora dos sintomas envolvidos, um estudo mais aprofundado a respeito de sua segurança, principalmente durante o período juvenil e puberdade mostrou-se de grande necessidade.

O tratamento de ratos machos Wistar durante esta janela crítica do desenvolvimento, que representa o principal período alvo para diagnóstico e tratamento do TDAH em humanos, indicou o potencial do LDX em induzir alterações toxicológicas sistêmicas, principalmente relacionada a hepatotoxicidade, durante e imediatamente ao final do tratamento, com efeitos apresentando um padrão de dose-resposta clássico, ou seja, maiores efeitos conforme o aumento da dose. No entanto, um tempo de recuperação após o término da exposição foi suficiente para restaurar os níveis fisiológicos similares ao controle na vida adulta.

Por outro lado, no que se refere aos parâmetros reprodutivos, algumas alterações foram encontradas logo após o tratamento (DPN 54), mas além disso, os resultados demonstraram que o LDX foi capaz de interferir na maturação do sistema genital masculino durante a puberdade e impactar negativamente o desenvolvimento reprodutivo, levando a danos no sistema genital masculino na vida adulta, mesmo após o término da exposição. Estas alterações apresentaram um perfil diferencial de resposta dependendo do órgão-alvo, levando a um padrão dose-resposta não monotônico, característico de substâncias com potencial de desregulação endócrina. Esta questão reforça que análises hormonais adicionais serão de extrema importância para melhor compreensão dos resultados encontrados e dos mecanismos envolvidos.

Considerando nossas observações, o uso do LDX deve ser feito com cautela, considerando os prós e contras envolvidos, e com melhor entendimento sobre seus possíveis efeitos adversos após uso prolongado durante uma fase crítica de desenvolvimento, o que ressalta a importância de mais estudos clínicos a longo prazo nestes pacientes.

8. ANEXOS

8.1. Parecer de aprovação do comitê de ética

unesp
UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

Comissão de Ética no Uso de Animais
Instituto de Biociências de Botucatu

CERTIFICADO

Certificamos que a proposta intitulada "Avaliação da toxicidade imediata e repercussões reprodutivas na vida adulta de ratos machos expostos ao psicoestimulante lisdexanfetamina do período juvenil a peripuberdade", protocolada sob o CEUA nº 3148130421 (ID 000210), sob a responsabilidade de **Arielle Cristina Arena** *e equipe; Julia Stein* - 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 do Universidade Estadual Paulista (IBB/UNESP) na reunião de 28/05/2021.

We certify that the proposal "Evaluation of immediate toxicity and reproductive repercussions in adult life of male rats exposed to the psychostimulant lysdexamphetamine from the juvenile period to peripuberty", utilizing 156 Heterogenics rats (males and females), protocol number CEUA 3148130421 (ID 000210), under the responsibility of **Arielle Cristina Arena** *and team; Julia Stein* - 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 (IBB/UNESP) in the meeting of 05/28/2021.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 05/2021 a 11/2021 Área: Ciências Biomédicas

Origem:	Biotério Central da UNESP						
Espécie:	Ratos heterogênicos	sexo:	Machos e Fêmeas	idade:	0 a 4 meses	N:	156
Linhagem:	Wistar			Peso:	4 a 500 g		

Local do experimento: A experimentação animal ocorrerá tanto no biotério de mamíferos quanto no laboratório do professor responsável pela pesquisa no departamento de biologia estrutural e funcional.

Botucatu, 28 de maio de 2021

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

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

8.2. Resultados sumarizados

Systemic toxicological parameters					
DPN	**Type of Analysis**	**Parameters**	**5.2**	**8.6**	**12.1**
23 – 53	Treatment	Body weight gain	=	=	↓
		Food intake	↓	↓	↓
		Water intake	=	↓	↓
40	Play behavior	Total social behavior	=	↓	↓
54	Organ weight	Liver	=	=	↑
		Spleen	=	=	↑
	Biochemical parameters	Total protein	=	↓	↓
	Hematological parameters	RBC	↓	↓	↓
		Leukocytes	=	=	↓

Reproductive parameters					
DPN	**Type of Analysis**	**Parameters**	**5.2**	**8.6**	**12.1**
47	Puberty onset	Body weight	=	=	↓
54	Organ weight	Seminal gland	=	↑	↑
	Seminiferous tubules	Staging - XIV	=	↑	=
		STsD	↓	=	=
	Oxidative stress	CAT	↑	↑	=
		SOD	↑	=	↑
		MDA	↑	↑	↑
120	Male sexual behavior	Latency to 1ª intromission	↑	=	=
	Sperm motility	Type C	↑	↑	=
	Sperm count	Relative nº spermatids	↓	↓	=
		Relative nº of sperm (C)	=	=	↓
	Leydig analysis	Count	↑	↑	↑
		Area	↑	=	↑
		Volume	↑	=	↑
	Seminiferous tubules	GEH	=	↑	↑
		STsD	↑	=	↑
	Oxidative stress	SOD	=	↓	=
		MDA	=	↓	=