Universidade Estadual de Londrina

DAYANE PRISCILA DOS SANTOS

CONSEQUÊNCIAS DA ADMINISTRAÇÃO DE DIMESILATO DE LISDEXANFETAMINA SOBRE O DESENVOLVIMENTO PÓS NATAL DO SISTEMA GENITAL MASCULINO DE RATOS WISTAR

Londrina
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Tese apresentada ao Programa de pós-graduação *Stricto Sensu* em Patologia Experimental da Universidade Estadual de Londrina como requisito parcial para a obtenção do título de Doutora.

Orientador: Profa. Dra. Glaura Scantamburlo Alves Fernandes.

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Londrina, 16 de fevereiro de 2024.

Dedico este trabalho,

A minha amada família e meus grandes amigos, que me apoiaram e seguraram a minha mão em todos os momentos dessa intesa jornada.

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"Gratidão é a memória do coração"

“Deus não escolhe os capacitados,
capacita os escolhidos. Fazer ou não fazer algo
só depende de nossa vontade e perseverança”.

Albert Einstein.

RESUMO

SANTOS, Dayane Priscila. **Consequências da administração de dimesilato de lisdexanfetamina sobre o desenvolvimento pós natal do sistema genital masculino de ratos wistar**. 2024. 115 p. Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2024.

O transtorno do Déficit de Atenção/Hiperatividade (TDAH) é caracterizado por excessiva desatenção, desorganização ou hiperatividade/impulsividade. Esses sintomas iniciam-se na infância produzindo prejuízos pessoais, sociais e acadêmicos. Para melhorar a qualidade de vida dos pacientes, medicamentos neuroestimulantes são recomendados. Dentre eles, encontra-se o dimesilato de lisdexanfetamina (LDX), um inibidor da recaptação de noradrenalina e dopamina prescrito para pacientes a partir dos 6 anos de idade. Nessa fase o sistema genital masculino não está completamente desenvolvido e o aumento da estimulação catecolaminérgica nos órgãos genitais poderia prejudicar seu desenvolvimento e funcionalidade. Esse estudo objetivou avaliar, em modelo animal, os efeitos da administração de LDX durante o período de desenvolvimento pós-natal do testículo e epidídimo e verificar se esses efeitos perduram até a vida adulta. Oitenta ratos Wistar foram divididos nos grupos experimentais controle imediato (C-I), LDX imediato (LDX-I), controle tardio (C-T) e LDX tardio (LDX-T). Os grupos LDX-I e LDX-T receberam 11,3 mg/kg/dia de LDX diluído em 500 µL de salina a 0,9% diariamente via gavagem do dia pós-natal (DPN)25 ao DPN65. Após o período de tratamento os grupos C-I e LDX-I foram eutanasiados no DPN66 e os grupos C-T e LDX-T permaneceram no biotério por um tempo de recuperação de 65 dias recebendo ração e água *ad libidum* até a data de sua eutanásia no DPN131. Os testículos foram utilizados para determinação da produção diária de espermatozoides (PDE), análises histopatológicas, morfométricas e de estresse oxidativo. Os epidídimos foram utilizados para análise de estresse oxidativo, estereologia, morfologia, histopatologia e determinação do tempo de trânsito espermático (TTE). Espermatozoides foram avaliados quanto a morfologia, integridade do acrossôma, atividade mitocondrial e motilidade. A testosterona foi determinada no plasma. Nos testículos, o LDX induziu hipoplasia de células de Leydig e consequente redução dos níveis plasmáticos de testosterona no grupo LDX-I. A baixa disponibilidade de testosterona neste grupo favoreceu o desprendimento de células imaturas do epitélio germinativo, culminando em aumento do percentual de túbulos seminíferos contendo vacúolos no epitélio e células imaturas na luz. Esses fatores contribuíram para redução da altura do epitélio germinativo, do diâmetro dos túbulos seminíferos e da PDE. O grupo LDX-T evidenciou que a hipoplasia de células de Leydig permanece após a interrupção do tratamento, causando atrofia gonadal e persistência da testosterona plasmática reduzida. Consequentemente, houve persistência do desprendimento de células imaturas do epitélio germinativo e da redução da altura do epitélio germinativo e da PDE nos animais adultos. No epidídimo, a administração de LDX gerou hipoplasia de células basais e retardou a expansão do ducto epididimário na região de cauda dos grupos LDX-I e LDX-T. O grupo LDX-I apresentou aceleração do TTE pelo epidídimo e redução na atividade de glutationa-S-transferase na região de cauda resultando em redução da atividade mitocondrial e motilidade espermática. Após a interrupção do tratamento o TTE foi reestabelecido no grupo LDX-T, mas a atividade da glutationa S-transferase na cauda se manteve reduzida levando ao aumento da peroxidação lipídica nessa região e persistência da redução da atividade mitocondrial e motilidade nos espermatozoides dos animais adultos. Em conclusão, esses resultados demonstram que a administração de LDX retarda o desenvolvimento pós-natal dos testículos e da cauda do epidídimo, levando a alterações irreversíveis na morfologia e funcionalidade desses órgãos.

Palavras-chave: Dextroanfetamina, Venvanse, Peripuberdade, Testículo, Epidídimo.

ABSTRACT

SANTOS, Dayane Priscila. **Consequences of lisdexamfetamine dimesylate administration on the postnatal development of the male genital system of Wistar rats.** 2024. 115 p. Thesis (Doctoral in Experimental Pathology) – State University of Londrina, Londrina, 2024.

Attention Deficit/Hyperactivity Disorder (ADHD) is characterized by excessive inattention, disorganization or hyperactivity/impulsivity. These symptoms begin in childhood, causing personal, social and academic harm. To improve the quality of life of patients, neurostimulating medications are recommended. Among them is lisdexamfetamine dimesylate (LDX), a norepinephrine and dopamine reuptake inhibitor prescribed for patients from 6 years of age. At this stage, the male genital system is not completely developed and increased catecholaminergic stimulation in the genital organs could harm their development and functionality. This study aimed to evaluate, in an animal model, the effects of LDX administration during the period of postnatal development of the testis and epididymis and to verify whether these effects last into adulthood. Eighty Wistar rats were divided into immediate control (C-I), immediate LDX (LDX-I), tardy control (C-T) and tardy LDX (LDX-T) experimental groups. The LDX-I and LDX-T groups received 11.3 mg/kg/day of LDX diluted in 500 µL of 0.9% saline daily via gavage from postnatal day (PND)25 to PND65. After the treatment period, groups C-I and LDX-I were euthanized on DPN66 and groups C-T and LDX-T remained in the biotherium for a recovery time of 65 days receiving food and water *ad libidum* until the date of their euthanasia on DPN131. The testes were used to determine daily sperm production (DSP), histopathological, morphometric and oxidative stress analyses. The epididymis were used for analysis of oxidative stress, stereology, morphology, histopathology and determination of sperm transit time (STT). Spermatozoa were evaluated for morphology, acrosome integrity, mitochondrial activity and motility. Testosterone was determined in plasma. In the testicles, LDX induced Leydig cell hypoplasia and a consequent reduction in plasma testosterone levels in the LDX-I group. The low availability of testosterone favored the detachment of immature cells from the germinal epithelium, culminating in an increase in the percentage of seminiferous tubules containing vacuoles in the epithelium and immature cells in the lumen. These factors contributed to reducing the height of the germinal epithelium, the diameter of the seminiferous tubules and DSP. The LDX-T group showed that Leydig cell hypoplasia persists after treatment interruption, causing gonadal atrophy and persistence of reduced plasma testosterone. Consequently, there was a persistence of detachment of immature cells from the germinal epithelium and a reduction in the height of the germinal epithelium and DSP in adult animals. In the epididymis, the administration of LDX irreversibly generated basal cell hypoplasia and delayed the expansion of the epididymal duct in the cauda region of groups LDX-I and LDX-T. The LDX-I group showed acceleration of TTE through the epididymis and a reduction in glutathione-S-transferase activity in the cauda region, resulting in a reduction in mitochondrial activity and sperm motility. After interrupting treatment, STT was reestablished in the LDX-T group, but glutathione S-transferase activity in the cauda remained reduced, leading to increased lipid peroxidation in this region and persistent reduction in mitochondrial activity and motility in sperm from adult animals. In conclusion, these results demonstrate that LDX administration delays the postnatal development of the testes and cauda epididymis, leading to irreversible changes in the morphology and functionality of these organs.

Key-words: Dextroamphetamine, Venvanse, Peripuberty, Testicle, Epididymis.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

ADRA2C	Gene adrenoceptor alfa 2C
ATX	Atomoxetina
CLO	Clonidina
DAT	Transportador de dopamina
DAT1	Gene Transportador de Dopamina 1
DPN	Dia Pós-Natal
DRD4	Gene receptor de dopamina 4
DRD5	Gene receptor de dopamina 5
GFC	Guanfacina
LD_{50}	Dose letal mediana
LDX	Dimesilato de Lisdexanfetamina
MAO	Monoamina oxidase
NOAEL	Nível de efeito adverso não observado
NET	Transportador de Noradrenalina
PEPT1	Proteína Transportadora de Peptídeos 1
SERT	Transportador de serotonina
SNC	Sistema Nervoso Central
TDAH	Transtorno do Déficit de Atenção/Hiperatividade
VMAT2	Transportador de Monoamina Vesicular 2

SUMÁRIO

1. INTRODUÇÃO

1.1 Transtorno do Déficit de Atenção/Hiperatividade: Considerações Gerais

O Transtorno do Déficit de Atenção/Hiperatividade (TDAH) é o transtorno neurocomportamental mais comum diagnosticado em crianças. O TDAH pertence ao grupo de transtornos do neurodesenvolvimento, caracterizado por níveis inapropriados de desatenção, desorganização e/ou hiperatividade/impulsividade que se manifestam na infância e, frequentemente, persistem pela adolescência e vida adulta. Consequentemente, o TDAH é responsável por produzir prejuízos pessoais, sociais, acadêmicos e ocupacionais ao indivíduo (AMERICAN PSYCHIATRIC ASSOCIATION, 2013; WILENS et al., 2018; TOURJMAN et al., 2018). O Manual Diagnóstico e Estatístico de Transtornos Mentais 5 estima que a taxa de prevalência mundial do TDAH seja cerca de 5% em crianças e 2,5% em adultos. Aproximadamente 50% das crianças diagnosticadas com TDAH continuarão a manifestar sintomas desse transtorno durante a vida adulta (AMERICAN PSYCHIATRIC ASSOCIATION, 2013; LOOBY, 2008).

Os problemas de comportamento presentes no TDAH afetam profundamente não só o desempenho acadêmico de crianças, mas também seu bem-estar e suas interações sociais. A desatenção e desorganização incapacitam a criança de realizar atividades que lhe são propostas, uma vez que implicam em dificuldade excessiva e involuntária de manter a atenção, permanecer na tarefa e frequentemente levam o indivíduo a perder objetos pessoais. Já a hiperatividade/impulsividade implicam em excessiva inquietação, incapacidade de permanecer parado, incapacidade de esperar e constante intrusão em atividades de outras pessoas (AMERICAN PSYCHIATRIC ASSOCIATION, 2013; AMERICAN ACADEMY OF PEDIATRICS, 2011). Em adultos, geralmente os sintomas de hiperatividade/impulsividade diminuem com a idade, enquanto as dificuldades cognitivas como desatenção e desorganização persistem ou se intensificam (WILENS et al., 2018; TOURJMAN et al., 2018).

Apesar de diversos estudos tentarem explicar as possíveis relações entre fatores genéticos, neurobiológicos, ambientais e psicossociais com as manifestações do TDAH, a etiologia e fisiopatologia desse transtorno ainda não estão claras (TRIPP e WICKENS, 2009). Diversas pesquisas evidenciam anormalidades no sistema nervoso central (SNC) fundamentando a base neurobiológica do TDAH. Achados neuropsicológicos sugerem que pacientes com TDAH apresentam comprometimento na função executiva, ou seja, comprometimento nos processos de controle mental que permitem o autocontrole, no caso do TDAH isso ocorre principalmente na inibição a respostas impulsivas e fluência verbal (PENNINGTON e OZONOFF, 1996; GEURTS et al., 2004). Exames de imagem indicam que o comprometimento da função executiva nesses pacientes está relacionado com redução volumétrica do lobo frontal (Figura 1) e hipoativação do lobo frontal direito. Também é visualizada disfunção nos circuitos fronto-estriatais do cérebro, um sistema que une diversas regiões do lobo frontal aos gânglios da base e, juntamente com outras áreas cerebrais, participa do controle do movimento, cognição e comportamento (NOORDERMEER et al., 2017; RUBIA et al.,2001; COUTO; MELO-JUNIOR; GOMES, 2010).

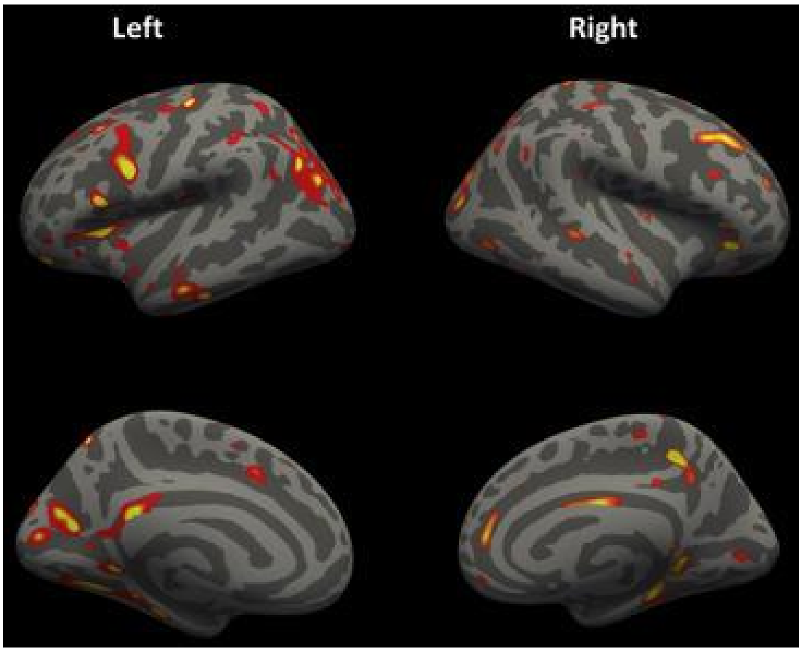

Figura 1. Áreas que apresentam redução volumétrica em pacientes com TDAH com base em análises de imagens obtidas por ressonância magnética. Vista lateral (superior) e sagital (inferior) dos hemisférios esquerdo e direito. As áreas coloridas indicam agrupamentos que exibem diferenças gerais no volume cortical. Cinza escuro indica sulcos; cinza claro indica giros. Fonte: NOORDERMEER et al., 2017.

Outro ponto considerado importante na etiologia do TDAH são os fatores genéticos. Pesquisadores tem evidenciado que o TDAH provavelmente envolve a participação de múltiplos genes e investigações genéticas e moleculares têm demonstrado que a redução na atividade dos sistemas dopaminérgico e noradrenérgico desempenha um papel crucial na patogênese do TDAH (COUTO; MELO-JUNIOR; GOMES, 2010; BUDZISZEWSKA et al., 2010). Os genes mais estudados neste contexto são o gene transportador de dopamina 1 (DAT1), os genes receptores de dopamina 4 e 5 (DRD4 e DRD5) e o gene adrenoceptor alfa 2C (ADRA2C). Observou-se que alterações nos genes relacionados a dopamina estão implicados a diversos comportamentos impulsivos, compulsivos e aditivos, enquanto alterações nos genes relacionados a noradrenalina estão implicados com mal funcionamento do lobo frontal que resultam em maior distração, falta de atenção, hiperatividade, baixa tolerância à frustração e ansiedade (COMINGS et al., 2000; LI et al., 2006; PURPER-OUAKIL et al., 2011).

1.2 Abordagens terapêuticas para o TDAH

O tratamento atualmente recomendado para crianças e adolescentes com TDAH é multimodal, portanto, engloba a combinação de abordagens não farmacológicas com tratamentos farmacológicos. As abordagens não farmacológicas são base primordial para o tratamento do TDAH, envolvendo terapias comportamentais, acompanhamentos psicológicos e psicopedagógicos. Por outro lado, os tratamentos farmacológicos envolvem a administração de medicamentes estimulantes do sistema nervoso (AMERICAN ACADEMY OF PEDIATRICS, 2011).

Os neuroestimulantes de primeira linha mais eficazes e comumente utilizados no tratamento do TDAH são o metilfenidato (MF) e a anfetamina (DRECHSLER et al., 2020). Esses fármacos atuam em vários pontos das vias da dopamina e noradrenalina inibindo sua recaptação e degradação enzimática (Figura 2). Dessa forma, o aumento da disponibilidade desses neurotransmissores favorece o controle da função executiva e demonstram grande efetividade na redução dos sintomas gerais do TDAH em crianças e adolescentes (FRAMPTON, 2018; ADESMAN, 2001).

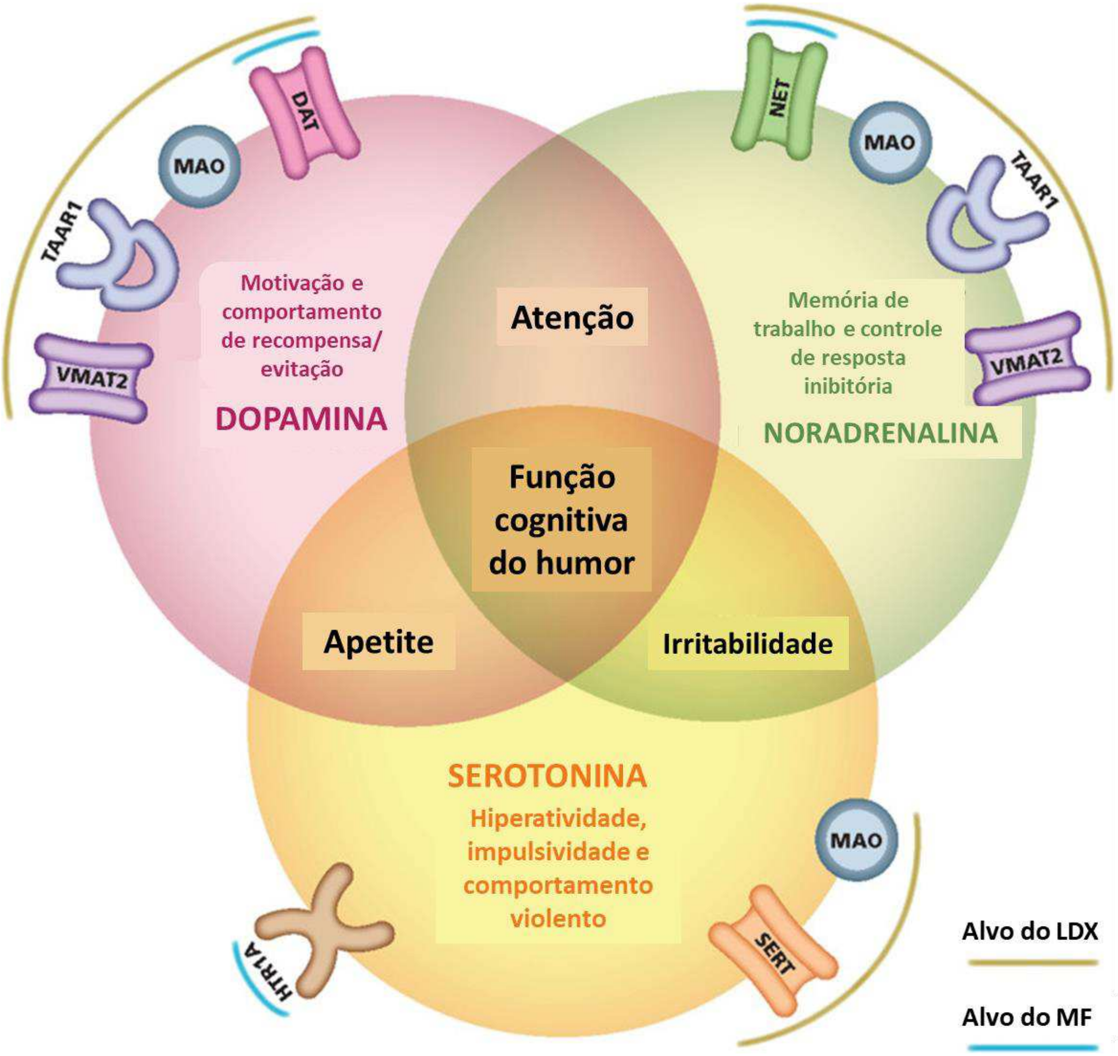

Figura 2. Alvos do metilfenidato e dimesilato de lisdexanfetamina e seus efeitos comportamentais. DAT= transportador de dopamina; MAO= monoamina oxidase; VMAT2= transportador de monoamina vesicular 2; HTR1A= receptor 1A de 5-hidroxitriptamina, SERT= transportador de serotonina; TAAR1= receptor 1 associado a traços de amina; NET= transportador de norepinefrina. MF= metilfenidato, LDX= dimesilato de lisdexanfetamina. Adaptado de QUINTERO; GUTIÉRREZ-CASARES e ÁLAMO, 2022.

São considerados medicamentos de segunda linha para o tratamento do TDAH a atomoxetina (ATX), guanfacina (GFC) e clonidina (CLO), indicados em casos de ausência de resposta, alergias ou outras contraindicações ao MF e anfetamina (CAYE et al., 2018). A ATX inibe o transportador de noradrenalina (NET) 1 reduzindo a recaptação e aumentando a disponibilidade de noradrenalina em todas as regiões do cérebro (CAYE et al., 2018). Já a GFC e

CLO são agonistas dos receptores de noradrenalina alfa-2 aumentando a estimulação noradrenérgica no córtex pré-frontal (ARNSTEN, 2010).

Embora os medicamentos de primeira e segunda linha apresentem mecanismos de ação diferentes, ambos atuam aumentando a disponibilidade de dopamina e/ou noradrenalina na fenda sináptica. Essa estimulação culmina na modulação da neurotransmissão entre os circuitos do núcleo accumbens e córtex pré-frontal, córtex parietal e córtex pré-frontal além do cerebelo e córtex pré-frontal que controlam uma série de funções cognitivas, incluindo funcionamento executivo, resposta à recompensa, memória e tempo (CAYE et al., 2018).

1.3 Dimesilato de Lisdexanfetamina: Estrutura química, farmacocinética e toxicidade

Dentre os medicamentos de primeira linha atualmente disponíveis no mercado para o tratamento do TDHA encontra-se o dimesilato de lisdexanfetamina (LDX), comercializado como Venvanse® e amplamente prescrito para pacientes com idade a partir de 6 anos em que o tratamento prévio com metilfenidato não foi efetivo. Trata-se de um pró-fármaco da dextroanfetamina de administração oral, em uma única dose diária, que apresenta menor risco de abuso devido a seu efeito de longa duração (AMERICAN ACADEMY OF PEDIATRICS, 2011; FRAMPTON, 2018; HUTSON; PENNICK; SECKER, 2014; MATTOS, 2014). Os efeitos terapêuticos desse medicamento são observados até 13 horas após sua administração em crianças e até 14 horas em adultos (WIGAL et al., 2009; WIGAL et al.,2010).

A estrutura química do LDX é representada por uma dextroanfetamina ligada covalentemente ao aminoácido essencial L-lisina (Figura 3). Após sua ingestão, o LDX é absorvido rapidamente no trato gastrointestinal, de forma intacta. A absorção ocorre através de transporte ativo desse fármaco por meio de sua interação com uma proteína altamente expressa no intestino delgado, a proteína transportadora de peptídeos 1 (PEPT1). Após absorção, o LDX é direcionado à circulação portal e rapidamente metabolizado a dextroanfetamina e L-lisina pelos eritrócitos na circulação sanguínea (Figura 4) (HUTSON; PENNICK; SECKER, 2014; PENNICK, 2010).

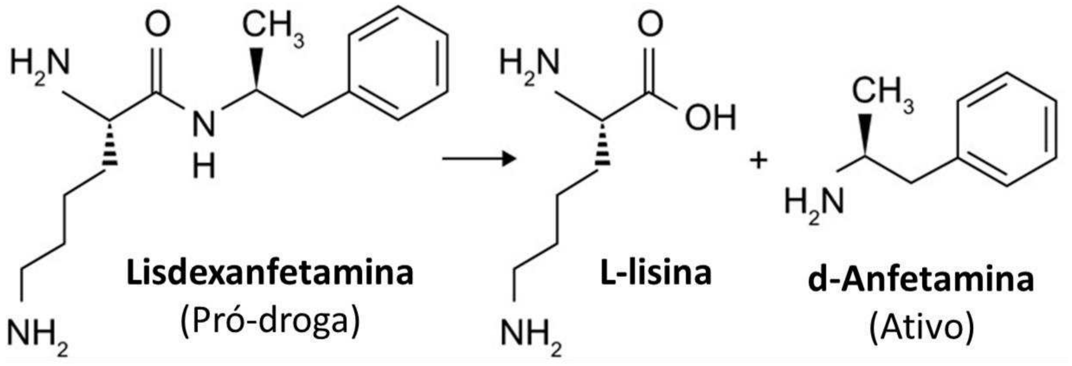

Figura 3- Estrutura química e conversão enzimática de dimesilato de lisdexanfetamina em d-anfetamina e L-lisina nos eritrócitos. Adaptado de: HUTSON; PENNICK; SECKER, 2014.

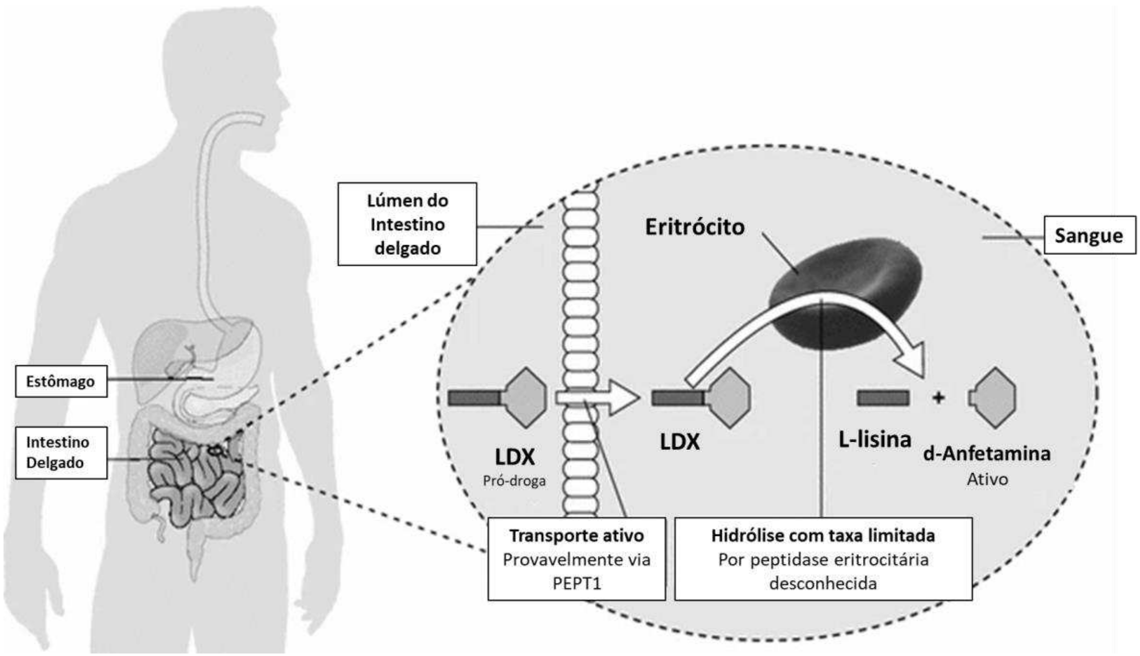

Figura 4- Absorção e distribuição sistêmica de d-Anfetamina por hidrólise de dimesilato de lisdexanfetamina. LDX= Dimesilato de lisdexanfetamina, PEPT1= transportador de peptídeo 1. Adaptado de: ERMER; PENNICK; FRICK, 2016.

A dextroanfetamina é o composto farmacologicamente ativo desse medicamento e sua concentração se eleva no plasma após a hidrólise do pró-fármaco. Essa substância atua no sistema simpático autônomo como um inibidor moderadamente potente do transportador de dopamina (DAT), do NET e do transportador de monoamina vesicular 2 (VMAT2). Além disso, apresenta afinidade fraca para inibição do transportador de serotonina (SERT) e é um inibidor fraco da monoamina oxidase (MAO). Deste modo a dextroanfetamina atua inibindo principalmente a recaptação neural e vesicular de noradrenalina e dopamina na fenda sináptica, além de inibir de maneira mais fraca a degradação dessas monoaminas pela MAO na fenda sináptica (HEAL et al., 2013; HEAL et al., 2008).

Como a dextroanfetamina apresenta capacidade de atravessar a barreira hematoencefálica alguns pesquisadores têm evidenciado que a inibição realizada pela dextroanfetamina em animais resultou no aumento da concentração de noradrenalina no córtex pré-frontal e de dopamina no córtex estriado, provocando aumento da estimulação catecolaminérgica nessas áreas e melhora dos sintomas de desatenção e hiperatividade/impulsividade observados no TDAH (HUTSON; PENNICK; SECKER, 2014; MATTOS, 2014). Além disso, devido a seu efeito de longa duração, o LDX gera um padrão de liberação constante e duradouro de dopamina no córtex pré-frontal medial de ratos Sprague-Dawley o que o torna mais eficiente na melhoria do desempenho cognitivo espacial do que a dextroanfetamina de liberação imediata (JIAN-MIN et al., 2022).

Embora a efetividade do LDX na melhoria dos sintomas do TDAH seja bem descrita na literatura, estudos que avaliem a toxicidade e efeitos adversos da administração de LDX em humanos e modelo animal são escassos na literatura (BIEDERMAN et al., 2007; KRISHNAN e MONTCRIEF, 2007; WOOD e KRASOWSKI, 2016). Os efeitos adversos relatados por crianças de 6 a 12 anos que receberam LDX foram semelhantes aos efeitos observados durante o uso de outras anfetaminas, ocorrendo predominantemente diminuição do apetite, insônia, dor abdominal superior, dor de cabeça, irritabilidade, vômitos, diminuição de peso e náusea (BIEDERMAN et al., 2007). Krishnan e Montcrief (2007) foram os únicos pesquisadores a avaliar a toxicidade da administração de LDX a ratos. Esses autores revelaram que a dose letal mediana (LD_{50}) de LDX em ratos Sprague-Dawley é maior do que 1000 mg/kg, equivalente a 548 mg/kg de sulfato de dextroanfetamina. O LDX apresenta uma LD_{50} extremamente maior que a do sulfato de dextroanfetamina (LD_{50} 32 mg/Kg), possivelmente por sua hidrólise taxa de hidrólise limitada nos eritrócitos. O nível de efeito adverso não observado (NOAEL) não foi determinado nesse estudo.

1.4 Sistema Genital Masculino: Considerações Gerais

O sistema genital masculino consiste em um conjunto de órgãos responsáveis pela produção do gameta masculino (espermatozoide), dos componentes seminais e de testosterona (DANGELO e FANTTINI, 2011). A união do gameta masculino com o gameta feminino resulta em fertilização e desenvolvimento de um novo indivíduo com um conjunto cromossômico completo e único. Portanto, o sistema genital tem como finalidade produzir componentes necessários para a reprodução e, dessa forma, garantir a preservação e perpetuação da espécie (SHEERWOOD, 2016).

Esse sistema esteroide-dependente é composto, na maioria dos mamíferos, por testículos (gônadas), epidídimos, ductos deferentes e glândulas sexuais acessórias: próstata e vesícula seminal (Figura 5) (TORTORA; NIELSEN, 2013). Além disso, o sistema genital masculino é inervado por fibras adrenérgicas do sistema nervoso simpático, as quais variam em quantidade de acordo com sua localização e exercem funções distintas através da ativação de receptores específicos de cada órgão ou glândula (BAUMGARTEN, 1968).

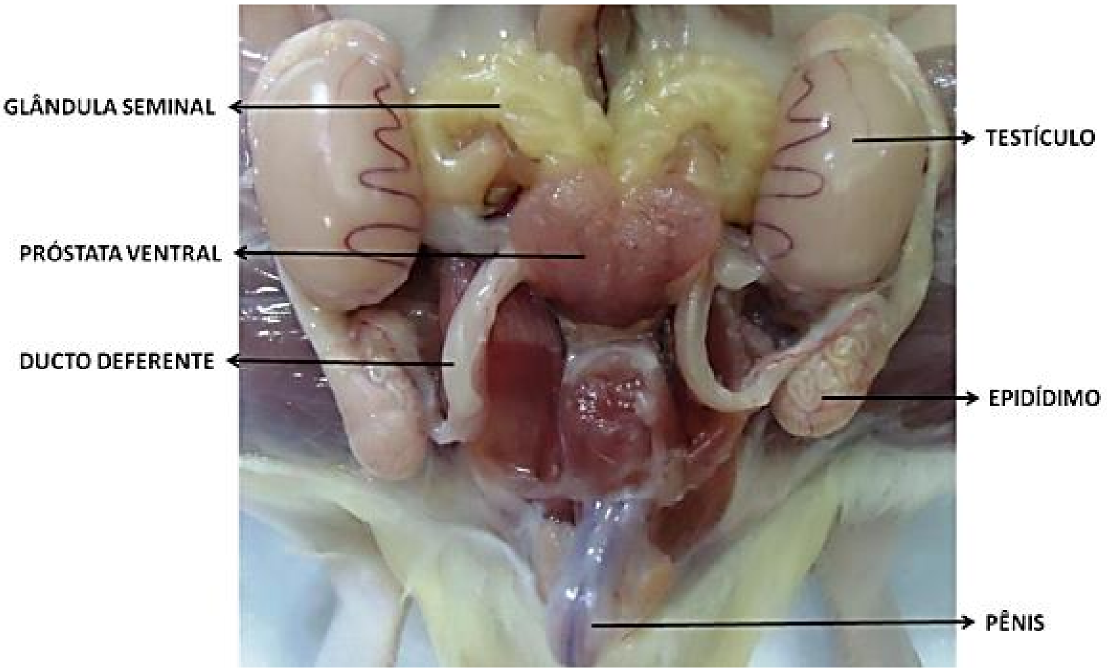

Figura 5. Órgãos do sistema genital de rato Wistar. A imagem mostra a visualização macroscópica dos componentes do sistema genital. Fonte: BORGES, 2013.

Os testículos estão localizados no interior do escroto e são recobertos por duas cápsulas: a túnica albugínea e a túnica vaginal (TORTORA e NIELSEN, 2013). Internamente, apresentam o compartimento tubular e intersticial responsáveis, respectivamente, pela espermatogênese e esteroidogênese (Figura 6). O compartimento tubular corresponde a um sistema de túbulos seminíferos constituídos por células germinativas (espermatogônias, espermatócitos e espermátides) e células somáticas (células mioides peritubulares e células de Sertoli) (JOHNSON et al., 2010). O compartimento intersticial, localizado entre os túbulos seminíferos, é composto por células de Leydig, células imunológicas (macrófagos e mastócitos), vasos sanguíneos e linfáticos, fibras nervosas, fibroblastos e tecido conjuntivo frouxo (JUNQUEIRA; CARNEIRO, 2013; JOHNSON et al., 2010).

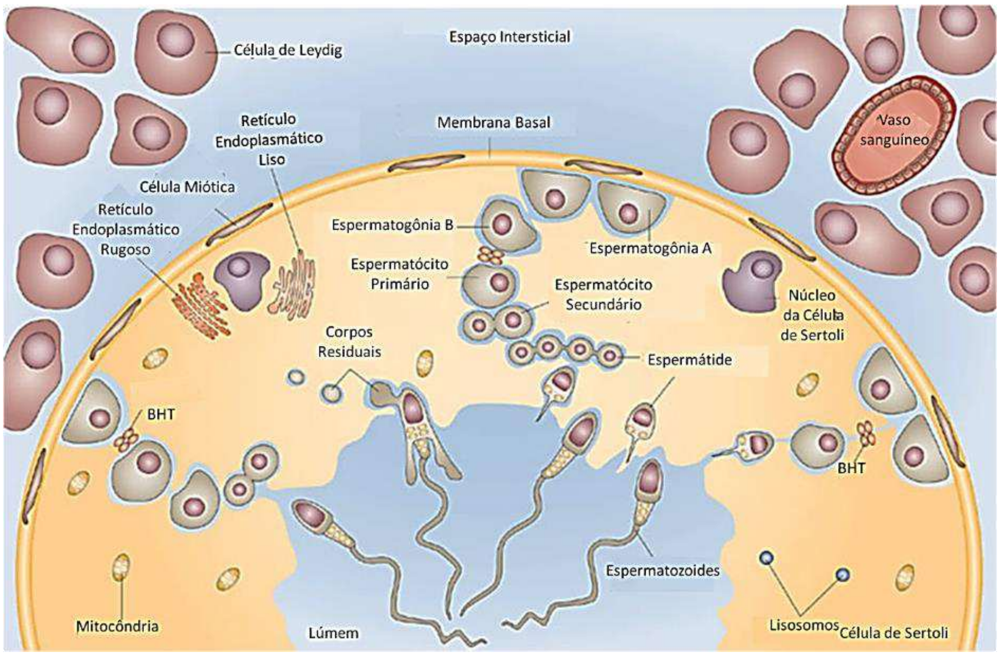

Figura 6. Representação de um túbulo seminífero e compartimento intersticial. A figura demonstra a localização das células somáticas e células de linhagem germinativa presentes no túbulo seminífero. Também podem ser observadas células somáticas do tecido intersticial e a diferenciação das células germinativas até a formação do espermatozoide no tecido tubular. Adaptado de RATO et al., 2012.

No compartimento tubular o epitélio germinativo realiza o processo denominado espermatogênese no qual uma espermatogônia dá origem a espermatozoides (HESS; FRANCA, 2009; MRUK; CHENG, 2004). Esse processo ocorre, respectivamente, em três fases denominadas mitótica, meiótica e espermiogênese (CLERMONT, 1972). Na fase mitótica as espermatogônias, células diplóides (2n) localizadas no compartimento basal dos túbulos seminíferos, sofrem divisões mitóticas periodicamente resultando no desenvolvimento de duas células filhas idênticas (2n). Uma célula filha (espermatogônia A) permanece como célula reserva, promovendo a auto renovação das espermatogônias. Já a outra (espermatogônia B) é destinada à diferenciação para originar os espermatócitos primários (JAN et al., 2012; KOLASA et al., 2012). Ao fim da fase mitótica é iniciada a fase meiótica, onde os espermatócitos primários (2n) sofrem a primeira divisão meiótica originando os espermatócitos secundários (1n). Rapidamente, os espermatócitos secundários sofrem a segunda divisão meiótica originando espermátides imaturas (1n) de formato arredondado. Na sequência inicia-se a espermiogênese, processo de maturação das espermátides, caracterizada por condensação do material genético, formação do acrossoma, reposicionamento das mitocôndrias, perda de citoplasma e formação da cauda. Por fim, as espermátides tardias são liberadas para o lúmen dos túbulos seminíferos (espermiação) e passam a ser denominadas espermatozoides (JAN et al., 2012; O´DONNELL, 2011; CLERMONT, 1972).

As células de Sertoli encontram-se apoiadas na membrana basal dos túbulos seminíferos e se projetam em direção ao lúmen dando suporte estrutural para o epitélio germinativo (WEINBAUER et al., 2010). Essas células se conectam entre si através de junções oclusivas formando a barreira hemato-testicular e dividindo o epitélio germinativo em dois compartimentos: basal e adluminal (PASCHALIA et al., 2015; JOHNSON et al., 2010). As espermatogônias ficam posicionadas no compartimento basal, enquanto os espermatócitos e espermátides estão localizadas no compartimento adluminal. Essa barreira impede o reconhecimento de células haploides como antigênicas pelo sistema imunológico, além de proporcionar um ambiente adequado para o processo meiótico e desenvolvimento espermático (PASCHALIA et al., 2015; JOHNSON et al., 2010; WEINBAUER et al., 2010). As células de Sertoli também são responsáveis por secretar nutrientes (carboidratos, lipídios, aminoácidos, vitaminas e íons metálicos), fatores de crescimento (fator de crescimento de fibroblastos, fator de células-tronco, fatores de crescimento transformadores alfa e beta, fator de crescimento semelhante à insulina-I e fator de crescimento epidérmico), bem como, fatores necessários para o metabolismo das células germinativas (lactato, transferrina e proteína de ligação a andrógenos) criando um microambiente favorável para a espermatogênese (SKINNER; ANWAY, 2005; MRUK; CHENG, 2004).

No interstício as células de Leydig são responsáveis pela síntese e secreção de testosterona, um hormônio que atua como estimulador da espermatogênese e do desenvolvimento das características sexuais masculinas secundárias (SHEERWOOD, 2016; WEINBAUER et al., 2010). Esse processo é regulado pelo hormônio luteinizante, que ao se ligar a seus receptores na membrana plasmática das células de Leydig ativa a enzima adenilato ciclase gerando aumento da concentração de adenosina monofosfato cíclico intracelular (AMPc). A AMPc estimula a síntese da proteína reguladora esteroidogênica e translocação de colesterol para a mitocôndria que é então associado a enzima de clivagem de cadeia lateral P450 (P450scc) resultando em conversão de colesterol em pregnenolona. Na sequência, a pregnenolona é translocada da mitocôndria para o retículo endoplasmático liso onde é convertida em testosterona (Figura 7) (ZIRKIN; CHEN, 2000).

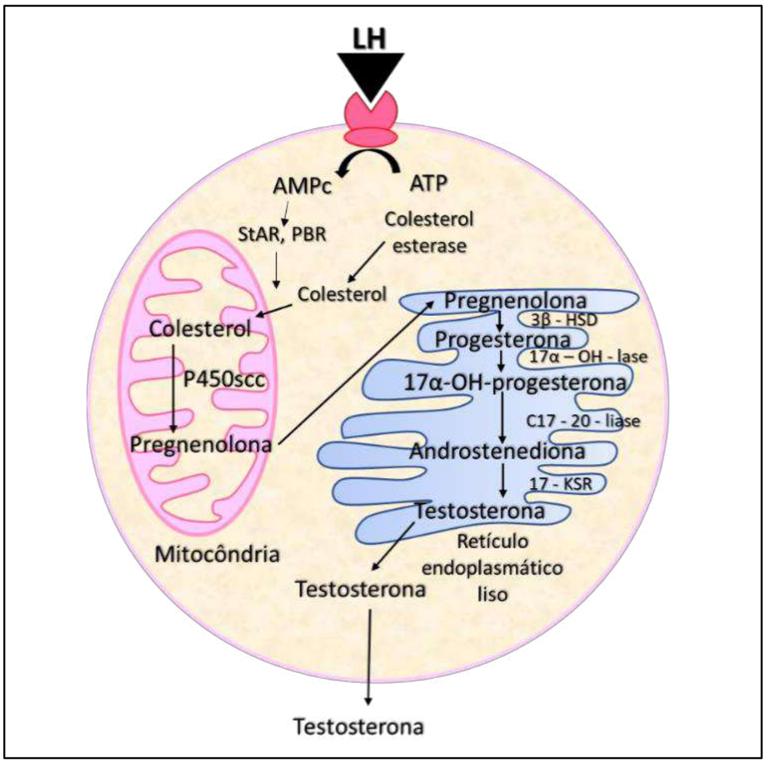

Figura 7. Eventos moleculares envolvidos na produção de testosterona pelas células de Leydig. AMPc: adenosina monofosfato cíclico; ATP: adenosina

trifosfato; StAR: proteína reguladora esteroidogênica; PBR: Receptor de benzodiazepina periférico; P450scc: enzima de clivagem de cadeia lateral P450; 3β-HSD: 3β hidroxiesteróide desidrogenase; 17α-OH-lase: 17α hidroxilase, 17-KSR: 17β-hidroxiesteróide desidrogenase. Fonte: Dayane Priscila dos Santos.

Os túbulos seminíferos do testículo se convergem dando origem à rede testicular que por sua vez tem continuidade com os ductos eferentes. Os ductos eferentes formam um ducto único e altamente enovelado denominado epidídimo (ROBAIRE et al., 2006). O epidídimo é responsável pela maturação, proteção, transporte e armazenamento dos espermatozoides oriundos dos testículos. Esse órgão é dividido em três regiões distintas histologicamente e funcionalmente: cabeça, corpo e cauda. A cabeça e corpo do epidídimo estão envolvidos principalmente no processo de maturação dos espermatozoides onde, através do trânsito por essas regiões, os espermatozoides obtêm a capacidade de movimento progressivo e fertilização do oócito secundário (HERMO e ROBAIRE, 2002; CORNWALL, 2009). Esse processo é caracterizado pelo remodelamento da membrana plasmática do espermatozoide, onde proteínas de origem testicular são removidas ou modificadas através da ação de enzimas epididimárias e proteínas epididimárias são secretadas e adsorvidas à membrana espermática para que sua capacidade funcional seja adquirida (BARRIOS, 2005; DA ROS et al., 2004; CUASNICÚ et al., 2002). Já a região da cauda está envolvida principalmente no processo de armazenamento e proteção desses espermatozoides (HERMO e ROBAIRE, 2002; CORNWALL, 2009). Células musculares lisas estão dispostas ao redor de toda extensão da camada epitelial do ducto epididimário e a contração muscular rítmica realizada por elas auxilia no transporte dos espermatozoides através do ducto no tempo adequado para a maturação espermática (JUNQUEIRA e CARNEIRO, 2013).

O ducto deferente é responsável pelo transporte dos espermatozoides armazenados na cauda do epidídimo para o ducto ejaculatório e auxilia no término do processo de maturação espermática. Este ducto é caracterizado por um lúmen estreito e uma camada de células musculares lisas bastante espessa. O epitélio secretor mantém os espermatozoides banhados enquanto estão no lúmen do ducto, já a camada muscular realiza fortes contrações que são responsáveis pela impulsão dos espermatozoides para o ejaculado durante o momento da ejaculação (DIXON; JEN; GOSLING, 1998; JUNQUEIRA e CARNEIRO, 2013).

A vesícula seminal é um ducto altamente enovelado que apresenta uma mucosa pregueada com epitélio cuboide ou pseudoestratificado colunar envolvida por uma espessa camada de músculo liso. Essa vesícula não armazena espermatozoides, mas produz uma secreção viscosa de pH ácido que contêm substâncias fundamentais para viabilidade dos espermatozoides após a ejaculação como frutose, prostaglandinas e proteínas coagulantes (JUNQUEIRA e CARNEIRO, 2013; RISBRIDGER, 2006).

A próstata, considerada a maior glândula sexual assessória, é constituída por um conjunto de glândulas túbulo-alveolares ramificadas formadas por um epitélio cuboide ou pseudoestratificado que envolvem a uretra prostática e desembocam na mesma. Além disso, a próstata ainda apresenta um estroma fibromuscular ao redor das glândulas e é revestida por uma cápsula rica em células musculares lisas (JUNQUEIRA e CARNEIRO, 2013). As glândulas prostáticas produzem uma secreção que contém fribrinolisina, uma substância que auxilia a dissolver o plug ejaculatório produzido pelos fluidos da glândula seminal tornando o sêmen liquefeito. A secreção prostática também contém citrato, fosfatase ácida e amilase que servem como meio para a otimização da motilidade e viabilidade espermática (JOHNSON et al., 2010; PENNEFATHER et al., 2000).

1.5 Inervação do Sistema Genital Masculino

A inervação dos testículos de ratos ocorre através de duas vias: nervo espermático superior e nervo espermático inferior. O nervo espermático superior tem origem no gânglio mesentérico superior e adentra o testículo através da artéria testicular, enquanto o nervo espermático inferior se origina nos gânglios mesentéricos inferiores, acompanha o ducto deferente e epidídimo até alcançar o testículo (SOSA et al., 2008; MOTOC; RUSU; JIANU, 2010). A maior parte das fibras testiculares são adrenérgicas e receptores adrenérgicos estão presentes em diversas células tanto do parênquima quanto do interstício testicular, como células de Leydig, células de Sertoli, espermatócitos primários, e células mióides (SOSA et al., 2008; ROSSI et al., 2018). Devido à grande disponibilidade destes receptores em diferentes tipos celulares, a inervação simpática pode influenciar diversos parâmetros testiculares, como a espermatogênese (GERENDAI; BANCZEROWSKI; HALÁSZ, 2005), sobrevivência das células de Leydig (GONG et al., 2009), secreção de testosterona (HUO et al., 2010) e a atividade contrátil das células peritubulares e células musculares lisas constituintes da túnica albugínea, responsáveis pelo transporte dos espermatozoides dos túbulos seminíferos ao epidídimo (ELLIS et al., 1981; HUO et al., 2010).

O epidídimo recebe inervação do sistema simpático originada do gânglio mesentérico inferior, gânglio pélvico maior e gânglio pélvico acessório que adentram o epidídimo através do nervo hipogástrico, nervo espermático inferior e nervo espermático mediano. Fibras adrenérgicas percorrem todo o interstício epididimal, variando em concentração de acordo com a região do epidídimo, uma vez que a região da cabeça e corpo do epidídimo apresenta inervação escassa, enquanto a cauda do epidídimo é ricamente inervada (RICKER, 1998). Essas fibras adrenérgicas apresentam como principal função mediar os eventos neuromusculares necessários para o transporte dos espermatozoides através do ducto epididimário. No entanto, neurotransmissores relacionados com as vias adrenérgicas, como a noradrenalina, também podem mediar funções de células epiteliais como transporte eletrolítico entre os compartimentos celular e luminal e processamento proteico (RICKER, 1998; RICKER et al., 1996; CHAN et al., 1994).

A contratilidade muscular do ducto deferente, rico em fibras adrenérgicas, é comandada pelo sistema simpático autônomo. As fibras adrenérgicas se originam no gânglio mesentérico inferior e chegam ao ducto através do nervo hipogástrico que juntamente com fibras simpáticas formam o plexo pélvico. Portanto, as fibras adrenérgicas apresentam como principal função mediar os eventos neuromusculares necessários para a impulsão dos espermatozoides pelo do ducto deferente (ALM,1982; DIXON; JEN; GOSLING, 1998).

A vesícula seminal apresenta inervação do sistema simpático autônomo através de fibras pós-ganglionares que se estendem de corpos celulares nervosos presentes na extremidade periférica do nervo hipogástrico. As fibras adrenérgicas da vesícula seminal localizam-se na camada muscular dessa glândula e estimulam a contração das células musculares lisas, auxiliando o processo de secreção do conteúdo do ducto para a uretra (SETCHELL e BREED, 2006; ÜCKERT, 2009; LAMANO-CARVALHO et al. 1990).

Assim como a vesícula seminal, a próstata apresenta inervação do sistema simpático autônomo através de fibras pós-ganglionares que se estendem de corpos celulares nervosos presentes na extremidade periférica do nervo hipogástrico. As fibras adrenérgicas estimulam a contração das células musculares lisas, auxiliando o processo de secreção do conteúdo glandular para a uretra prostática. Além disso, os estímulos nervosos auxiliam no crescimento e maturação da próstata (SETCHELL e BREED, 2006; WANG et al., 1991).

Como a inervação simpática dos órgãos do sistema genital masculino exercem funções distintas, desde a estimulação da espermatogênese, sobrevivência das células de Leydig, até o controle do tempo de trânsito dos espermatozoides, é possível inferir que o desequilíbrio dessa comunicação, seja por inibição ou estimulação, poderia acarretar em prejuízos na funcionalidade do sistema genital. Desta forma, substâncias que aumentem a disponibilidade de neurotransmissores catecolaminérgicos na fenda sináptica podem interferir sobre o controle nervoso que há no sistema genital masculino (BORGES, 2013).

1.6 Desenvolvimento Pós-Natal dos Testículos e Epidídimos de Ratos

O desenvolvimento pós-natal dos testículos de ratos é dividido em período neonatal, infantil, juvenil, peripuberal e adulto (OJEDA e SKINNER 2006). O período neonatal corresponde ao intervalo de tempo que vai do momento do nascimento ao dia pós-natal (DPN) 7. Essa fase é caracterizada por três eventos: diferenciação dos gonócitos fetais em espermatogônias mitoticamente ativas, proliferação e maturação de células de Sertoli e, substituição das células de Leydig fetais por células de Leydig progenitoras (Figura 8). Portanto, nessa fase os túbulos seminíferos são estreitos e não possuem espermatócitos e espermátides (PICUT et al., 2014).

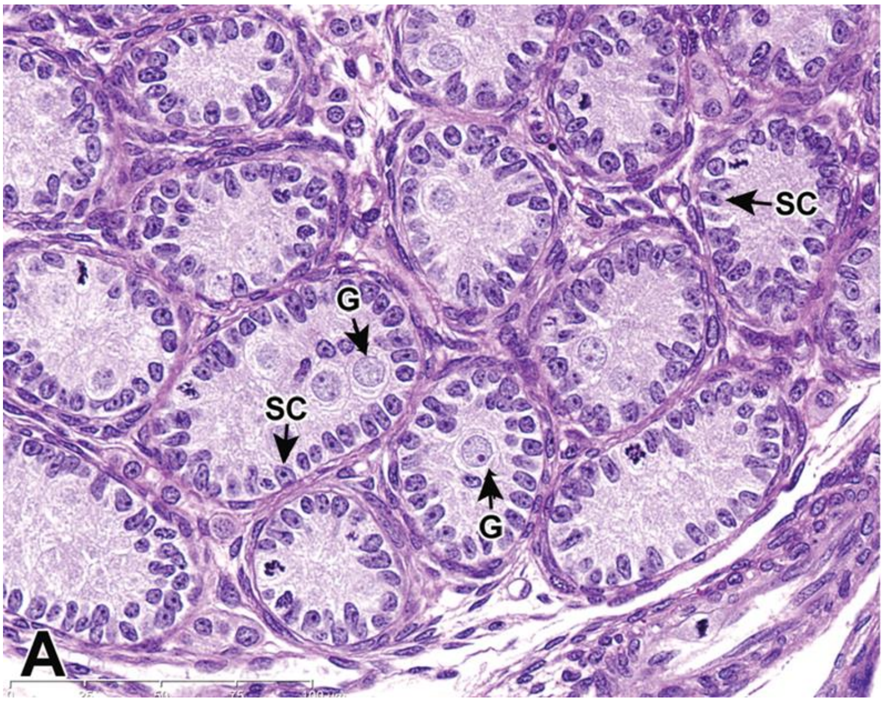

Figura 8. Testículo no DPN 3 (período neonatal). Túbulos seminíferos revestidos por numerosas células de Sertoli mitoticamente ativas (SC) posicionadas na membrana basal. Os gonócitos (G) posicionam-se centralmente nos túbulos, são mitoticamente inativos e possuem um grande núcleo redondo. Coloração hematoxilina e eosina. Fonte: PICUT et al., 2014.

O período infantil se inicia no DPN 8 e se estende até o DPN 20. Nessa fase todos os três tipos celulares apresentam alta taxa proliferativa alcançando a densidade celular máxima de cada túbulo seminífero (PICUT; ZIEJEWSKI; STANISLAUS, 2017). Durante esse período as espermatogônias se diferenciam em tipo A e tipo B podendo ser identificadas pelas características nucleares. As espermatogônias do tipo A apresentam núcleo redondo, volumoso com cromatina finamente pontilhada e um nucléolo evidente. As espermatogônias do tipo B revelam um núcleo redondo, pequeno com cromatina condensada na periferia e pontilhado grosseiro (Figura 9) (PICUT et al., 2014). Por volta do DPN 18 as células de Sertoli amadurecem e sua atividade mitótica é interrompida. Concomitantemente, essas células desenvolvem junções de oclusão entre si e secretam liquido luminal, originando a barreira hemato-testicular e o lúmen tubular (WANG; WREFORD; DE KRETSER, 1989).

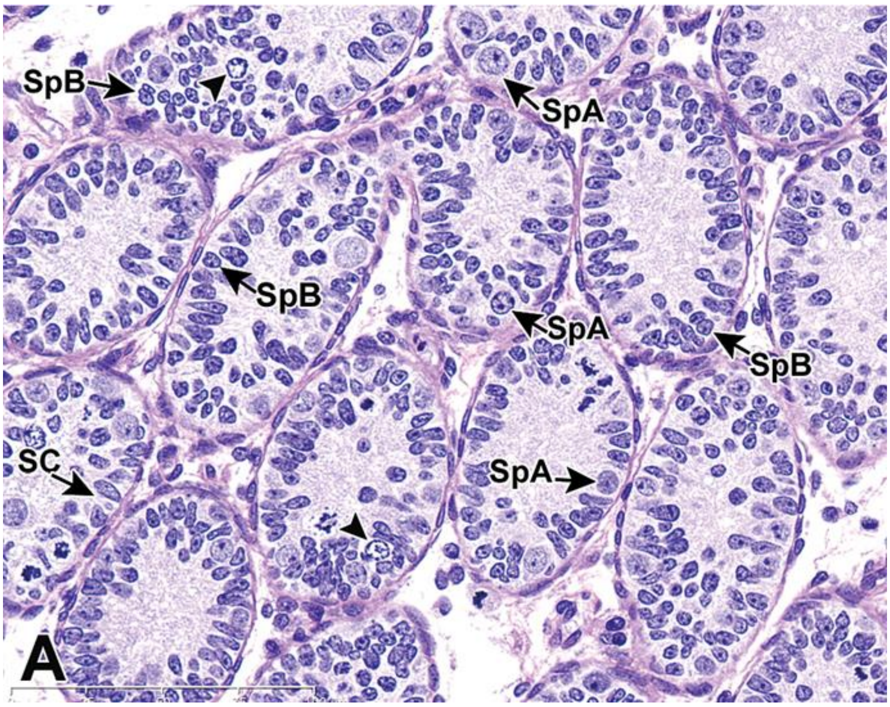

Figura 9. Testículo no DPN 9 (período infantil). Túbulos seminíferos revestidos por numerosas células de Sertoli (SC) e espermatogônias, formando uma camada pseudoestratificada. As células de Sertoli possuem núcleo alongado a poligonal com cromatina frouxa e um nucléolo evidente. SpA= espermatogônias tipo A; SpB= espermatogônias tipo B; ponta de seta= célula em prófase. Coloração hematoxilina e eosina. Fonte: PICUT et al., 2014.

Ainda na fase infantil, as células de Sertoli maduras secretam inibina em quantidade suficiente para induzir um feedback negativo resultando em redução dos níveis de hormônio folículo estimulante (FSH). No interstício, as células de Leydig progenitoras dão origem a uma pequena população de células de Leydig imaturas, resultando em níveis bastante baixos de testosterona nessa fase (LEE et al., 1979).

O período juvenil vai do DPN 21 ao DPN 32. Essa fase é marcada pelo início da espermatogênese, ocorrendo o desenvolvimento de espermatócitos e espermátides arredondadas. As células de Sertoli e espermatogônias se posicionam no compartimento basal dos túbulos, enquanto no compartimento adluminal predominam espermatócitos pré-leptótenos, leptótenos e zigótenos (Figura 10) (PICUT e REMICK, 2016). No interstício, a população de células de Leydig imaturas aumenta, gerando uma elevação discreto nos níveis de testosterona, porém suficiente para induzir um feedback negativo na produção de hormônio luteinizante (LH). Isso ocorre porque durante o período juvenil o eixo hipotálamo-hipófise-gonodas (HHG) imaturo é extremamente sensível a testosterona (PICUT et al., 2014).

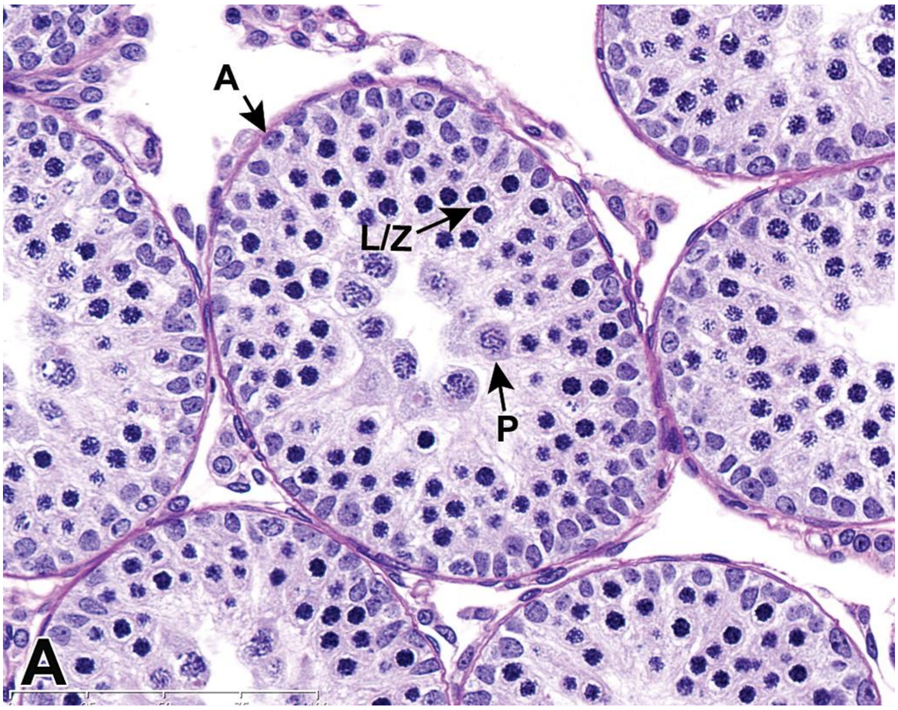

Figura 10. Testículo no DPN 25 (juvenil). Espermatogônias e células de Sertoli recuam para o compartimento basal à medida que o diâmetro do túbulo se expande. A= Espermatogônias tipo A; L/Z= espermatócitos leptóteno/zigóteno; P= espermatócitos paquítenos. Coloração hematoxilina e eosina. Fonte: PICUT et al., 2014.

A partir do DPN 28, as células de Leydig imaturas se diferenciam a células maduras resultando no aumento rápido dos níveis de testosterona (WALKER et al. 2012). Paralelamente ocorre maturação do eixo HHG, em parte devido à perda dos efeitos inibitórios do ácido gama aminobutírico (GABA) permitindo a secreção de níveis mais elevados de gonadotrofinas. Como resultado, ocorre o aumento dos níveis de LH e FSH que são críticos para a maturação final das células de Leydig (PICUT E REMICK, 2016).

A fase peripuberal se inicia no DPN 33 e se estende até o DPN 55/60. Essa fase compreende desde o início da puberdade até a maturidade sexual. A puberdade é determinada pela presença de espermátides em etapa 19 no epitélio de túbulos no estágio VII da espermatogênese (Figura 11) (PICUT et al.

2014). Essas espermátides são maduras e alongadas, apresentam cabeça altamente condensada e representam os 6 dias finais do desenvolvimento da espermátide antes da sua liberação (CLERMONT, 1972). A maturidade sexual por sua vez, é adquirida quando todos os túbulos seminíferos têm espermiogênese completa e os espermatozoides maduros são facilmente encontrados no epidídimo (PICUT; ZIEJEWSKI; STANISLAUS, 2017). Como a espermatogênese e a esteroidogênese ainda não estão totalmente estabelecidas durante esse período, esta pode ser uma fase crítica do desenvolvimento reprodutivo (JOHNSON; WELSH; WILKER, 1997).

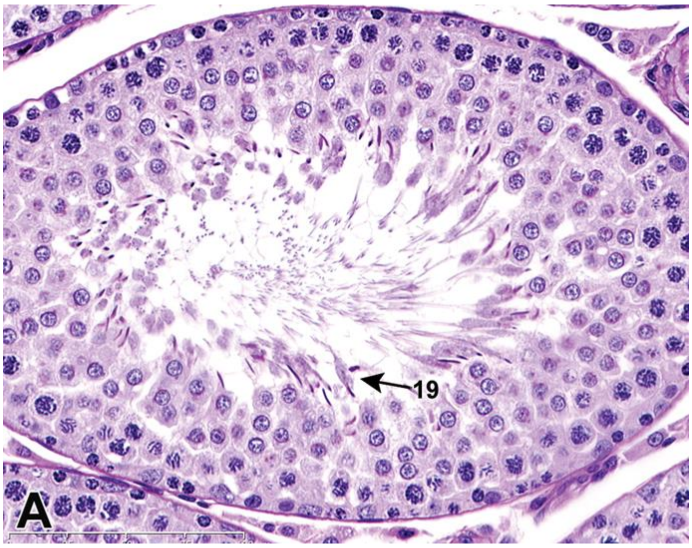

Figura 11. Testículo no PND 46 (período peripuberal). Observe a presença de espermátides em etapa 19 (19) presentes no túbulo do estágio VII. Coloração hematoxilina e eosina. Fonte: PICUT et al., 2014.

O desenvolvimento pós-natal do epidídimo é dividido em períodos de indiferenciação (nascimento até DPN 15), período de diferenciação (DPN 16–44) e período de expansão (>DPN 44) (SUN e FLICKINGER, 1979). No período de indiferenciação, o epidídimo é considerado menos susceptível a insultos, uma vez que as células epiteliais são indiferenciadas e permanecem quiescentes durante toda essa fase (PICUT E REMICK, 2016).

O período de diferenciação é marcado por rápida proliferação das células epiteliais e sua diferenciação em células basais, principais, apicais, estreitas, claras e halo. Essa diferenciação é controlada por testosterona e, portanto, agentes que reduzam a disponibilidade de testosterona ao epidídimo nessa fase podem retardar a maturação do epidídimo. Durante esta fase, também ocorre proliferação de tecido fibromuscular ao redor do ducto epididimário e células germinativas esfoliadas dos testículos são encontradas no lúmen a partir do DPN 20 (PICUT; ZIEJEWSKI; STANISLAUS, 2017).

O período de expansão é caracterizado pelo aumento do diâmetro do ducto para abrigar os espermatozoides oriundos dos testículos. A expansão do ducto epididimário também é dependente de testosterona. Nessa fase os níveis de testosterona circulantes devem ser altos, uma vez que, a maturação de células de Leydig já ocorreu no testículo (SCHEER e ROBAIRE, 1980).

1.7 Dimesilato de Lisdexanfetamina e o Sistema Genital Masculino

Atualmente, não existem estudos clínicos e em modelos animais que avaliem o efeito do LDX sobre desenvolvimento pós-natal dos órgãos do sistema genital masculino. Disfunção erétil e perda de libido em homens adultos são apontadas como efeitos adversos do uso dessa medicação (LYONS; LENTZ; COWARD 2017). Porém, considerando a faixa etária de administração do fármaco, efeitos adversos, disponibilidade de fibras adrenérgicas no sistema reprodutor masculino e os eventos que ocorrem durante o período juvenil e peripuberal, fazem-se necessárias investigações sobre o assunto.

2 JUSTIFICATIVA

O TDAH é o transtorno neurocomportamental mais comum diagnosticado em crianças, com alta prevalência na população mundial. Além disso, aproximadamente 50% das crianças diagnosticadas com TDAH manifestam os sintomas desse transtorno durante a vida adulta. Os sintomas de desatenção, desorganização e hiperatividade/impulsividade manifestados por crianças com TDAH prejudicam seu bem-estar, desempenho acadêmico e interação social. Visando amenizar esses sintomas e melhorar a qualidade de vida dessas crianças a prescrição de tratamentos farmacológicos tem aumentado, assim como a busca por medicamentos com maior tempo de ação farmacológica. Dentre os medicamentos disponíveis no mercado, o dimesilato de lisdexanfetamina é o fármaco com maior tempo de ação farmacológica observada e, devido a essa propriedade, sua prescrição para crianças com idade a partir de 6 anos tem aumentado nos últimos anos. Esse medicamento é metabolizado na corrente sanguínea em dextroanfetamina e atua inibindo a recaptação neuronal e vesicular de noradrenalina e dopamina, aumentando a disponibilidade desses neurotransmissores. Os órgãos do sistema genital masculino apresentam inervação pelo sistema nervoso simpático, principalmente fibras adrenérgicas que variam em quantidade de acordo com sua localização e exercem funções distintas como, por exemplo, a sobrevivência das células de Leydig. Neste contexto é possível inferir que o desequilíbrio dessa comunicação, seja por inibição ou estimulação, poderia acarretar em prejuízos no desenvolvimento e funcionalidade do sistema genital.

Portanto, este estudo tem grande aplicabilidade ao avaliar os efeitos da administração de LDX durante a juventude e peripuberdade sobre o sistema genital masculino em dois momentos distintos: na peripuberdade imediatamente após a administração do fármaco e na vida adulta após um período de 65 dias da interrupção do tratamento. Os resultados aqui obtidos contribuem para o maior entendimento dos riscos do uso de LDX durante o período de desenvolvimento pós-natal do sistema genital masculino.

3 OBJETIVOS

3.1 Geral:

Avaliar a resposta celular dos testículos, epidídimo e espermatozoides de ratos Wistar após a administração de LDX durante o período de desenvolvimento pós-natal (DPN25 ao DPN65) imediatamente após ao término de sua administração (peripuberdade - DPN66) e tardiamente, após um período 65 dias para recuperação da administração do fármaco (vida adulta - DPN131).

3.2 Específicos:

- Avaliar se a administração de LDX gera alterações morfológicas, funcionais e perda de viabilidade nos espermatozoides imediatamente após ao término de sua administração e após 65 dias de recuperação da administração;
- Investigar se esse tratamento promove alterações estruturais e funcionais nos testículos e epidídimos imediatamente após ao término de sua administração ou após 65 dias de recuperação da administração;
- Investigar se o LDX altera os níveis de testosterona plasmática;
- Contribuir com dados sobre os efeitos imediatos e tardios da administração de LDX, sobre o desenvolvimento pós-natal de órgãos do sistema genital masculino de ratos e entendimento de sua ação sobre estes órgãos.

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ARTIGO 1

LISDEXAMPHETAMINE DIMESYLATE ADMINISTRATION TO RATS DURING THE JUVENILE AND PERIPUBERTAL PERIODS GENERATES IRREVERSIBLE TESTICULAR DAMAGE IN PUBERTAL AND ADULT AGES

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LISDEXAMPHETAMINE DIMESYLATE ADMINISTRATION TO RATS DURING THE JUVENILE AND PERIPUBERTAL PERIODS GENERATES IRREVERSIBLE TESTICULAR DAMAGE IN PUBERTAL AND ADULT AGES

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Abstract

Lisdexamfetamine dimesylate (LDX) is a neurostimulant medication, prescribed from the age of 6, to treat attention deficit/hyperactivity disorder. At this age, important reproductive development events, such as spermatogenesis and steroidogenesis, are not fully established. This study aimed to evaluate the effects of LDX administration on the postnatal development of the testicles in an animal model and verify whether these effects last into adulthood after a drug-free period. For this, Wistar rats were distributed into the immediate control (C-I), immediate LDX (LDX-I), tardy control (C-T) and tardy LDX (LDX-T) groups. The LDX-I and LDX-T groups received 11.3 mg/kg/day of LDX daily via gavage from postnatal day (PND)25 to PND65. On DPN66, groups C-I and LDX-I were euthanized and groups C-T and LDX-T were kept in the bioterium until their euthanasia on DPN131. Histopathological, morphometric and testicular oxidative stress analyses, sperm morphology, acrosome integrity, determination of daily sperm production (DSP) and serum testosterone were realized. LDX administration generated Leydig cell hypoplasia and reduction in serum testosterone in the LDX-I group. This favored the exfoliation of immature cells of the germinal epithelium (GE), culminating in a reduction in the diameter of the seminiferous tubules, the GE height and DSP. Leydig cell hypoplasia persisted in the LDX-T group, causing gonadal atrophy, reduced serum testosterone, persistent detachment of cells from the GE, reduced GE height and DSP. In conclusion, administration of LDX during the juvenile and peribuperal periods disrupted rat gonadal development by inducing irreversible Leydig cell hypoplasia, impairing steroidogenesis and spermatogenesis.

Keywords: dextroamphetamine, Vyvanse, postnatal development, testosterone

1. Introduction

Lisdexamfetamine dimesylate (LDX) is part of the group of first-line medications used in the treatment of attention deficit/hyperactivity disorder (ADHD) and is currently approved for use in several countries around the world (such as the United States of America, Canada, Australia and Brazil) [1,2]. It is a prodrug of dextroamphetamine, with a long-lasting effect, prescribed in a single daily dose for patients over 6 years of age in whom previous treatment with methylphenidate for at least 6 weeks was not effective [3].

Structurally, LDX corresponds to a dextroamphetamine covalently linked to an essential amino acid L-lysine [2]. It is absorbed in the gastrointestinal tract by active transport, through the peptide transport protein 1 and, upon reaching the portal circulation, it is hydrolyzed to dextroamphetamine and L-lysine in the cytosol of red blood cells. [4,5].

Dextroamphetamine acts as an important neurostimulant, as it overcomes the blood-brain barrier and inhibits the transporters of dopamine (DAT), noradrenaline (NET), vesicular monoamine 2 (VMAT2) and serotonin (SERT), in addition to inhibiting monoamine oxidase (MAO) [6,7]. Evaluation of LDX administration in rats revealed that dextroamphetamine increases the concentration of norepinephrine and dopamine in the prefrontal cortex and dopamine in the striate cortex, causing an increase in catecholaminergic stimulation in these areas and improving symptoms of inattention and hyperactivity/impulsivity [8].

LDX treatment can be initiated in the child period of postnatal development of the human male reproductive system and lasts throughout adolescence [9]. In rats, these developmental periods correspond to the juvenile and peripubertal periods, respectively [10]. In these phases, important events of male postnatal reproductive maturation occur, such as: development of germ cells, increase in testosterone levels and proliferation of Sertoli cells. [11]. As spermatogenesis and steroidogenesis are not fully established during this period, this is considered a critical phase of reproductive development [10].

Furthermore, the testicles of rats receive innervation from the autonomic nervous system through two routes: superior spermatic nerve and inferior spermatic nerve. [12]. Due to the large availability of adrenergic receptors in different cell types, sympathetic innervation can influence several testicular parameters, such as spermatogenesis [13], Leydig cell survival [14], testosterone secretion [15] and the contractile activity of peritubular cells [16].

Understanding the importance of the juvenile and peripubertal period for male fertility, this study aimed to evaluate in an animal model the effects of LDX administration during the period of postnatal testicular development, as well as to verify whether these effects last into adulthood.

2. Methodology

2.1 Experimental design

The experiment evaluated the effects of LDX administration on the male genital system immediately after the treatment period and 65 days after treatment interruption. 80 male Wistar rats were used, with an initial age of 22 days, from the Central Biotherium of the State University of Londrina – UEL. The animals were acclimatized to the biotherium of the Toxicology and Metabolic Disorders of Reproduction Laboratory for three days. After acclimatization, the animals were randomly distributed into four experimental groups (n=20 animals/group) named:

immediate control group (C-I), immediate LDX group (LDX-I), tardy control group (C-T) and tardy LDX group (LDX-T). The animals were housed in cages containing a maximum of 3 animals per cage and received water and food *ad libidum*. Water and food consumption and body weight were measured every three days throughout the experimental period. The biotherium was maintained under constant temperature conditions (23±2°C) and under a 12-hour light-dark cycle, with the light phase beginning at 7 a.m. Animals in groups LDX-I and LDX-T received LDX at a dose of 11.3 mg/kg/day diluted in 0.9% saline solution in a volume of 500 µl, via gavage, for 40 consecutive days. This dose is equivalent to the administration of a 70mg capsule of the drug and corresponds to 1.13% of the oral LD50 for rats (oral LD50 for rats >1000 mg/kg) [17,18]. Groups C-I and C-T received only the vehicle (0.9% saline solution) in an equal volume. The treatment period occurred between postnatal day (PND)25 and PND65, which correspond to the juvenile and peripubertal phase of Wistar rats [19,20].

The experimental protocol was approved by the Animal Use Ethics Committee of the State University of Londrina. (CEUA-UEL/ OF. CIRC. CEUA nº 82/2019).

2.2 Materials collection

After the treatment period, the C-T and LDX-T groups were kept in the biotherium for a period free-drug of 65 days, receiving only water and food *ad libidum*. Euthanasia of rats in groups C-I and LDX-I occurred on PND66, while euthanasia of groups C-T and LDX-T occurred on PND131. On their respective days, the animals were anesthetized by intraperitoneal injection of Ketamine (90 mg/kg of weight) and Xylazine (10 mg/kg of weight) and euthanized by cardiac puncture to collect blood in a heparinized tube (sodium heparin).

The right testicles were collected, weighed on a precision analytical balance and used to determine the number of mature spermatids, histopathological and morphometric analyzes (n=10/group). The left testes were collected, weighed and used for oxidative stress analyzes (n=10/group). Spermatozoa were collected from the vas deferens and used to evaluate sperm morphology and acrosome integrity. Blood was used to determine plasma testosterone concentration.

2.3 Histological processing

Testicles were collected and fixed in Methacarn for 4 hours. After fixation, the testicles were embedded in Paraplast® and semi-serial sections were obtained at 5µm (50µm intervals) [21]. The sections were stained with hematoxylin and eosin (HE) and examined under light microscopy for morphometric and histopathological analysis, spermatogenesis dynamics, Sertoli cell number count and Leydig cell number count.

2.3.1 Seminiferous tubule diameter and germinal epithelium height

Ten random seminiferous tubules, per animal, in cross-section at stage IX of the germinal epithelial cycle were photodocumented and examined. Images were obtained using an Opton photomicroscope under 100x magnification and BELview software version 6.2.3.0 (BEL Engineering) was used to analyze the images. The same tubules described above were used to analyze the seminiferous tubules diameter and germinal epithelium height. In each seminiferous tubule, the mean of four measurements for the diameter and height of the epithelium was calculated and the means were used in the statistical analysis [21].

2.3.2 Spermatogenic process evaluation

To evaluate the spermatogenic process, 100 random seminiferous tubules per animal were classified into four categories: Stages I-VI, VII-VIII, IX-XIII and XIV of the germinal epithelium cycle, according to Leblond and Clermont, 1952, under a light microscope (Opton) at 100x magnification.

2.3.3 Histopathological analysis of the testicles

The histopathological evaluation was carried out quantitatively using transverse semi-serial sections of the organ. For each animal, 100 seminiferous tubules were analyzed, classified as normal or abnormal. The tubules were considered abnormal in the presence of acidophilic cells, multinucleated cells, retained spermatids, degeneration of cell types, vacuolation of the epithelium or the presence of immature cells in the tubular lumen [21].

2.3.4 Leydig cell count

The number of Leydig cell nuclei was counted in 10 random fields of interstitial tissue in each testis section per rat under light microscopy at 400x magnification [23].

2.3.5 Sertoli cell count

The number of Sertoli cell nuclei was determined in 20 cross-sections of the seminiferous tubules per testis in each rat, under a light microscope at 400x magnification [24].

2.4 Plasma testosterone dosage

Blood plasma was obtained by centrifugation at 3000 rpm for 15 min at 4°C and stored at -20°C until analyzed by automated immunoassay. Plasma total testosterone was determined by chemiluminescence in microparticles using the Alinity ABBOTT equipment and specific kit (2nd Generation Testosterone Architect System, Abbott, Wiesbaden, Germany), according to the manufacturer's recommendations. The intra-assay coefficient of variation and minimum assay sensitivity were 4.6% and 0.15 nmol/l, respectively.

2.5 Testicular Oxidative Stress Biomarkers

2.5.1 Tissue preparation

The testes were homogenized in 1 mL of phosphate buffer (pH 7.4) and centrifuged at 9500 rpm for 10 minutes at 4°C. Protein quantification of samples was determined by the Bradford method, using bovine serum albumin as standard [25]. Samples were then normalized to 1 mg protein/mL and used for subsequent analyses.

2.5.2 Lipid peroxidation

Lipid peroxidation was measured indirectly by quantifying the peroxides produced. The result reflects the intensity of lipid peroxidation [26]. Dosages were performed by the method of thiobarbituric acid reactive substances (TBARS) with absorbance of 535 nm and 572 nm in relation to the standard curve for malondialdehyde (MDA), the main byproduct of cellular lipid peroxidation [27]. To prepare the test, 50 µl of each normalized sample was pipetted in duplicate into a microplate, followed by the addition of FeCl3 (1M), ascorbic acid, TCA (2.8%) and TBA (1.0%). The microplate was shaken and placed in a water bath at 90 °C for 15 min. It was then cooled to stop the reaction, and readings were taken at 535 nm and 572 nm. Lipid peroxidation was estimated by correcting the amount of protein, and the results were expressed as nmol of MDA per mg of protein.

2.5.3 Reduced glutathione

Reduced glutathione (GSH) levels were determined as proposed by Rahman et al., (2007) , with some modifications. For this, the sulfhydryl reagent 5,5'-dithio-bis (2-nitrobenzoic acid) DTNB was added to the supernatant of the testis homogenate to oxidize GSH. The result of this reaction is the formation of 5′-thio-2-nitrobenzoic acid evidenced by the formation of yellow color. GSH levels were measured at 412 nm and results expressed as micromoles/mg of protein.

2.5.4 Glutathione S-transferase activity

The enzymatic activity of glutathione S-transferase (GST) in the testis was determined through the formation of a thioether from the interaction of GSH with CDNB. The increase in absorbance through thioether formation was monitored at 340 nm (RS: 100 mM potassium phosphate buffer pH 6.5; 1.5 mM GSH; 2 mM CDNB) for 5 min at 40 s intervals [29]. Values were expressed as µM Thioether formed/min/mg of protein.

2.5.5 Total glutathione

Total glutathione was quantified through the oxidation of glutathione by the sulfhydryl reagent 5,5'-dithio-bis (2-nitrobenzoic acid) DTNB to form the yellow derivative 5'-thio-2-nitrobenzoic acid. The glutathione disulfide formed was recycled into glutathione by glutathione reductase in the presence of NADPH. Total glutathione content in the samples was determined by comparison with a predetermined glutathione standard curve [28].

2.5.6 Superoxide dismutase activity

Assessment of superoxide dismutase (SOD) enzyme activity was performed as described by Senthilkumar et al. (2021), with some modifications. The enzyme comes from homogenates normalized to 1 mg/mL. A reaction mixture was prepared containing sodium carbonate buffer (50 mM, pH 10.2), nitroblue tetrazoilium (NBT) (96 uM) and Triton X-100 (0.6%), which was incubated for 2 min with hydroxylamine hydrochloride (20 mM $NH_2OH \cdot HCl$, pH 6.0). The final volume was adjusted to 200 µl. The reaction consists of quantifying the complexes formed by superoxide anions with the addition of NBT and NH2OH·HCl of a yellowish color with the reduction of NBT, forming a bluish color read at 560 nm for 2 min at intervals of 15 s.

2.5.7 Catalase activity

The enzymatic activity of catalase (CAT) was determined by the degradation of hydrogen peroxide into oxygen and water. After determining the protein concentration (normalized to 1.0 mg/mL in PBS), the sample was added to 297 µl of reaction medium in a UV4 microplate (in triplicate) and reading was performed at 240 nm for 60 s. [31].

2.6 Daily sperm production

To evaluate daily sperm production (DSP), the testes were weighed and homogenized, as described by Fernandes et al. (2007). After dilution of the homogenate, a small volume of sample was transferred to a Neubauer chamber (four fields per animal) for counting spermatids resistant to homogenization (stage 19 of spermatogenesis). To calculate DSP, the concentration of spermatids per testis was divided by 6.1, which refers to the number of days for which mature spermatids are present in the seminiferous epithelium.

2.7 Sperm morphology

This analysis was performed as described by Fernandes et al. (2007). The contents of the vas deferens were removed via internal rinsing with 1.0 mL of 10% formol saline. Histological slides were prepared from this solution and observed using an Opton photomicroscope (400x magnification). One hundred spermatozoa were analyzed per animal. Morphological analysis was classified into three general categories: normal morphology, head abnormalities (without characteristic curvature or isolated form, i.e., no tail attached) and tail abnormalities (broken, rolled into a spiral and isolated, i.e., no head attached).

2.8 Analysis of sperm acrosome integrity

To assess the integrity of the acrosome, a method based on the PNA-FITC reaction with components of the external acrosomal membrane of the sperm according to Cheng et al. (1996). Sperm were collected in modified human tubal fluid (HTF) and a smear was made from this solution. The slide was permeabilized with absolute methanol and then stained with the 40 µg/mL PNA-FITC solution in PBS buffer. One hundred sperm were analyzed under a fluorescence microscope (Axio Zeiss photonic microscope) and the cells were classified into spermatozoa with integral acrosome or spermatozoa with acrosome damage.

2.9 Statistical analysis

Data distribution was assessed using the Shapiro-Wilk normality test and homogeneity of variances was assessed using Levene's test. Parametric data were evaluated by Student's t test for independent samples and data were expressed as mean (±SEM). Non-parametric data were evaluated using the Mann-Whitney test and data were expressed as median (Q1-Q3). Values were considered significantly different when $p<0.05$.

3. Results

3.1 Body weight and testicular weight

Throughout the experimental period, body weight gain was similar between animals in groups C-I and LDX-I (data not shown). Likewise, there was no significant difference in body weight gain between the C-T and LDX-T groups (data not shown), showing that treatment with LDX does not interfere with the weight gain of Wistar rats during the period of drug administration, nor does it have any effects on body weight gain after treatment interruption.

Both the weights of the right testes and left testes were similar between the C-I and LDX-I groups (Figure 1A and 1B). However, the weight of the right and left testicles of the LDX-T group was reduced compared to the C-T group (Figure 1A and 1B).

3.2 Testicular morphometric analysis and spermatogenesis dynamics

Testicular morphometric analysis revealed that LDX administration during the juvenile and peripubertal periods caused a reduction in the diameter of the seminiferous tubules and in the height of the germinal epithelium of rats in the LDX-I group (Table 1). After 65 days of interrupting treatment, the diameter of the seminiferous tubules was reestablished in the LDX-T group, however, the height of the germinal epithelium remained reduced in this group (Table 1).

The analysis of the dynamics of spermatogenesis showed a reduction in the number of seminiferous tubules in stages I-VI and a consequent increase in the number of seminiferous tubules in stages VII-VIII in the LDX-I group when compared to the C-I group (Table 1). However, 65 days after interrupt LDX administration the number of seminiferous tubules in stages I-VI; VII-VIII; IX-XIII and XIV showed no significant differences between the LDX-T and C-T experimental groups (Table 1).

3.3 Histopathological analysis, Sertoli cells number, Leydig cells number and plasma testosterone

Histopathological analysis of the testis showed that LDX administration increased the number of abnormal seminiferous tubules in the LDX-I group compared to C-I (Figure 2A). The abnormalities found correspond to the presence of vacuoles in the germinal epithelium and immature cells in the tubular lumen (Figure 2C and 2D). After treatment interruption, the number of abnormal seminiferous tubules remained increased in the LDX-T group, indicating that the histopathological changes induced by LDX are irreversible up to this stage of life (Figures 2E, 2G and 2H).

The number of Sertoli cells remained similar between the experimental groups (Figure 3A). However, the LDX administration was responsible for reducing the number of Leydig cells in the interstitium and reducing plasma testosterone levels in rats in the LDX-I group (Figure 3B and 3C). After treatment interruption, the Leydig cell population was not reestablished in the LDX-T group and serum testosterone levels remained reduced in this group (Figure 3B and 3C).

3.4 Oxidative Stress Biomarkers

Substances reactive to thiobarbituric acid concentrations were similar between the experimental groups (Figures 4A).

Similarly, there was no significant difference between catalase activity and superoxide dismutase activity between the experimental groups (Figures 4B and 4C). However, the LDX-I group showed an increase in glutathione S-transferase activity compared to the C-I group (Figure 4D). After 65 days of treatment interruption, glutathione S-transferase activity returned to normal (Figure 4D).

The quantification of total glutathione and reduced glutathione was similar between the experimental groups (Figures 4E and 4F).

3.5 Sperm parameters

LDX administration during the juvenile and peripubertal periods was responsible for reducing the number of mature spermatids resistant to homogenization and the daily sperm production by the testes of rats in the LDX-I group (Table 2). Even after treatment has been stopped, the reduced number of mature spermatids resistant to homogenization and the low daily sperm production remained in the animals in the LDX-T group (Table 2). Furthermore, the administration of this medication was responsible for increasing the percentage of morphologically abnormal spermatozoa in the LDX-I and LDX-T groups (Table 2). Among the abnormalities observed, there was a predominance of spermatozoa with isolated heads and spermatozoa with isolated tail.

Although increased morphological abnormalities were observed in sperm from the LDX-I group, drug treatment did not cause damage to the acrosome of these sperm (Table 2).

4 Discussion

This study provides information not yet available in the literature, which is relevant to understanding the effects of LDX administration during the juvenile and peripubertal periods on the development of male gonads in an animal model. Our main findings show that the administration of LDX delays the postnatal development of the testicles of Wistar rats, leading to irreversible changes responsible for impairing the functioning of this organ.

Postnatal development of the rat testis is divided into neonatal (DPN 0 – 7), infantile (DPN 8 – 20), juvenile (DPN 21 – 32), peripubertal (DPN 33 – 60) and adult (after PND 60) periods. During these periods, testicular maturation occurs as Leydig cells and Sertoli cells proliferate, undergo differentiation and acquire functional maturity associated with the development and differentiation of spermatogonia that enable the first spermatogenesis cicly [34].

One of the most important events of the juvenile and peripubertal period is the differentiation of progenitor Leydig cells (PLC), specialized in the synthesis of dihydrotestoterone (DHT), into immature Leydig cells (ICL) and later mature Leydig cells (MLC), responsible by testosterone synthesis [35]. During the juvenile phase, PLC predominate in the testicular interstitium, leading to increased levels of DHT, which induces meiosis and directs the emergence of spermatocytes in the germinal epithelium [36]. These authors further demonstrate that this phase is also marked by apoptosis and detachment of pachytene spermatocytes from the germinal epithelium, since the survival of this cell type is dependent on testosterone. As ICL and MLC develop, testosterone levels gradually increase so that, during the peripubertal period, the first spermatogenic cycle is completed. [35].

Our data revealed that the development of Leydig cells during the juvenile and peripubertal period is delayed by the administration of LDX, resulting in hypoplasia of this cell population and consequently a reduction in plasma testosterone levels. Although studies show that amphetamine-derived drugs reduce the population of Leydig cells by increasing lipid peroxidation and reducing the activity of antioxidant enzymes [37], our results showed that LDX-induced damage occurs independently of lipid peroxidation and antioxidant activity.

Testosterone is essential for successful spermatogenesis [38]. Gendt et al. (2004) , evidenced that knockout mice for the androgen receptor (AR) present a reduction in the diameter of the seminiferous tubules and height of the germinal epithelium associated with the absence of spermatids. These data proved that, although meiosis is initiated independently of androgenic stimulation, in the absence of testosterone signaling, spermatogenesis does not progress beyond the pachytene stages of meiosis. Furthermore, testosterone signaling in AR in Sertoli cells is necessary for round spermatids to remain adhered to the germinal epithelium and spermiogenesis to be completed [40]. Thus, in the present study, the reduction in serum testosterone levels induced by the administration of LDX contributed not only to spermatogenesis failure, evidenced by the reduction in daily sperm production and decrease in sperm quality, but also for the early detachment of spermatocytes and immature spermatids from the germinal epithelium, culminating in a reduction in the diameter of the tubules, a reduction in the height of the germinal epithelium, as well as an increase in the percentage of seminiferous tubules containing vacuoles in the epithelium and immature cells in the lumen.

During stages I-VII, AR gradually increases in Sertoli cells, reaching a peak in stage VII, which is considered the most sensitive stage to testosterone [41]. The reduction in serum testosterone levels during the juvenile and peripubertal periods is responsible for impairing the dynamics of spermatogenesis, reducing the number of tubules in stages VII-VIII, culminating in delayed spermiation [42]. Although the reduction in testosterone impairs the dynamics of spermatogenesis, substances that inhibit the reuptake of norepinephrine and dopamine also interfere with this process. Thanoi et al. (2020) revealed that administration of methamphetamine, a sympathomimetic drug like LDX, to rats reduces the number of seminiferous tubules in the early stages of spermatogenesis. These stages are marked by high mitotic rates of type A spermatogonia and their differentiation to type B, so that a reduction in the number of tubules in these stages contributes to reduced daily sperm production in the testes [44]. Thus, the reduction in the number of seminiferous tubules in stages I-VI of spermatogenesis observed in the LDX-I group occurred due to the action of the drug and not as a consequence of the reduction in testosterone, with the dynamics of spermatogenesis being reestablished after treatment interruption.

ILCs have restricted mitotic activity and can enter the cell cycle up to twice. During the peripubertal period, ILC maturation is completed and the interstitium predominantly presents MLC incapable of proliferating [35,45]. For this reason, even after the interruption of LDX administration, the population of Leydig cells remains reduced in the gonads of adult Wistar rats, resulting in gonad atrophy and maintenance of reduced testosterone levels throughout the lives of these animals. Since testosterone plays a key role in directing the completion of meiosis [39], maintain the integrity of the blood-testicular barrier [46], maintain spermatids adhered to the germinal epithelium [47] and direct the spermiation [48] the testicles of adult animals that received LDX during the juvenile and peripubertal period continue to show early detachment of cells from the germinal epithelium, culminating in a persistent reduction in the height of the germinal epithelium and a reduction in daily sperm production. Together, these parameters characterize hypogonadism [49].

5 Conclusion

In conclusion, the administration of LDX to rats during the juvenile and peribuberal periods proved to be harmful to the postnatal development of the testicles, revealed by Leydig cell hypoplasia, reduced steroidogenesis and consequent impairment in spermatogenesis. As the Leydig cell population is unable to proliferate after the peripubertal period, Leydig cell hypoplasia is not reversed after treatment interruption, culminating in hypogonadism and functional impairment in the testicles of rats in adult life. These results describe for the first time the effects of LDX administration on postnatal testicular development in an animal model.

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Interest conflicts

The authors declare there are no conflicts of interest.

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Table 1- Seminiferous tubule diameter, germinal epithelium height and spermatogenesis dynamics

	Experimental groups	
Parameters	**C-I (n=10)**	**LDX-I (n=10)**
[1]Seminiferous tubule diameter (µm)	160.76 [147.6 – 177.8]	152.03** [143.8 – 166.0]
[2]Germinal epithelium height (µm)	49.87 ±0.7	46.40 ±0.6***
Spermatogenesis dynamics (nº)		
[2]I – VI	49.44 ±1.2	44.11 ±1.7*
[2]VII – VIII	25.78 ±0.8	29.11 ±0.9*
[2]IX – XIII	22.00 ±1.2	24.89 ±1.6
[2]XIV	2.78 ±0.6	1.89 ±0.6
	C-T (n=10)	**LDX-T (n=10)**
[2]Seminiferous tubule diameter (µm)	267.5 ±4.1	259.2 ±4.0
[2]Germinal epithelium height (µm)	83.73 ±1.3	70.77 ±1.2***
Spermatogenesis dynamics (nº)		
[2]I – VI	45.86 ±2.8	46.38 ±1.7
[2]VII – VIII	25.14 ±2.3	27.88 ±1.5
[2]IX – XIII	26.43 ±1.3	23.88 ±1.2
[2]XIV	2.57 ±0.6	1.88 ±0.4

[1]Values expressed as median [Q1-Q3]. Mann-Whitney test. [2]Values expressed as mean ±S.E.M. Student's t test. *$p<0.05$; **$p<0.01$; ***$p<0.001$. C-I= immediate control group; LDX-I= immediate lisdexamfetamine dimesylate group; C-T= tardy control group; LDX-T= tardy lisdexamfetamine dimesylate group.

Table 2- Sperm parameters

	Experimental groups	
Parameters	**C-I (n=10)**	**LDX-I (n=10)**
Number of mature spermatids resistant to homogenization ($x10^6$/testicle)	72.63 ±3.3	60.27 ±3.8*
Daily sperm production ($x10^6$/testicle/day)	11.90 ±0.5	9.88 ±0.6*
Abnormal sperm (%)	25 ±1.6	36.93 ±2.7**
Spermatozoa with damage to the acrosome (nº)	20 ±2.0	32 ±7.0
	C-T (n=10)	**LDX-T (n=10)**
Number of mature spermatids resistant to homogenization ($x10^6$/testicle)	121.60 ±7.4	96.83 ±6.8*
Daily sperm production ($x10^6$/testicle/day)	19.93 ±1.2	15.87 ±1.1*
Abnormal sperm (%)	5.10 ±1.0	10.40 ±1.6*

Values expressed as mean ±S.E.M. Student's t test. *p<0.05; **p<0.01. C-I= immediate control group; LDX-I= immediate lisdexamfetamine dimesylate group; C-T= tardy control group; LDX-T= tardy lisdexamfetamine dimesylate group.

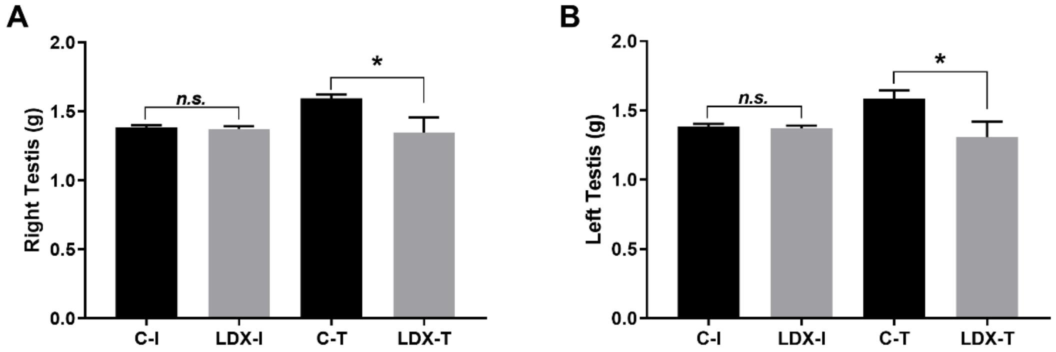

Figure 1 – Weight of the testicles. (A) Weight of the right testicles (B) Weight of the left testicles. Values expressed as mean ±S.E.M. Student's t test. *n.s.*= not significant; *p<0.05. C-I= immediate control group; LDX-I= immediate lisdexamfetamine dimesylate group; C-T= tardy control group; LDX-T= tardy lisdexamfetamine dimesylate group.

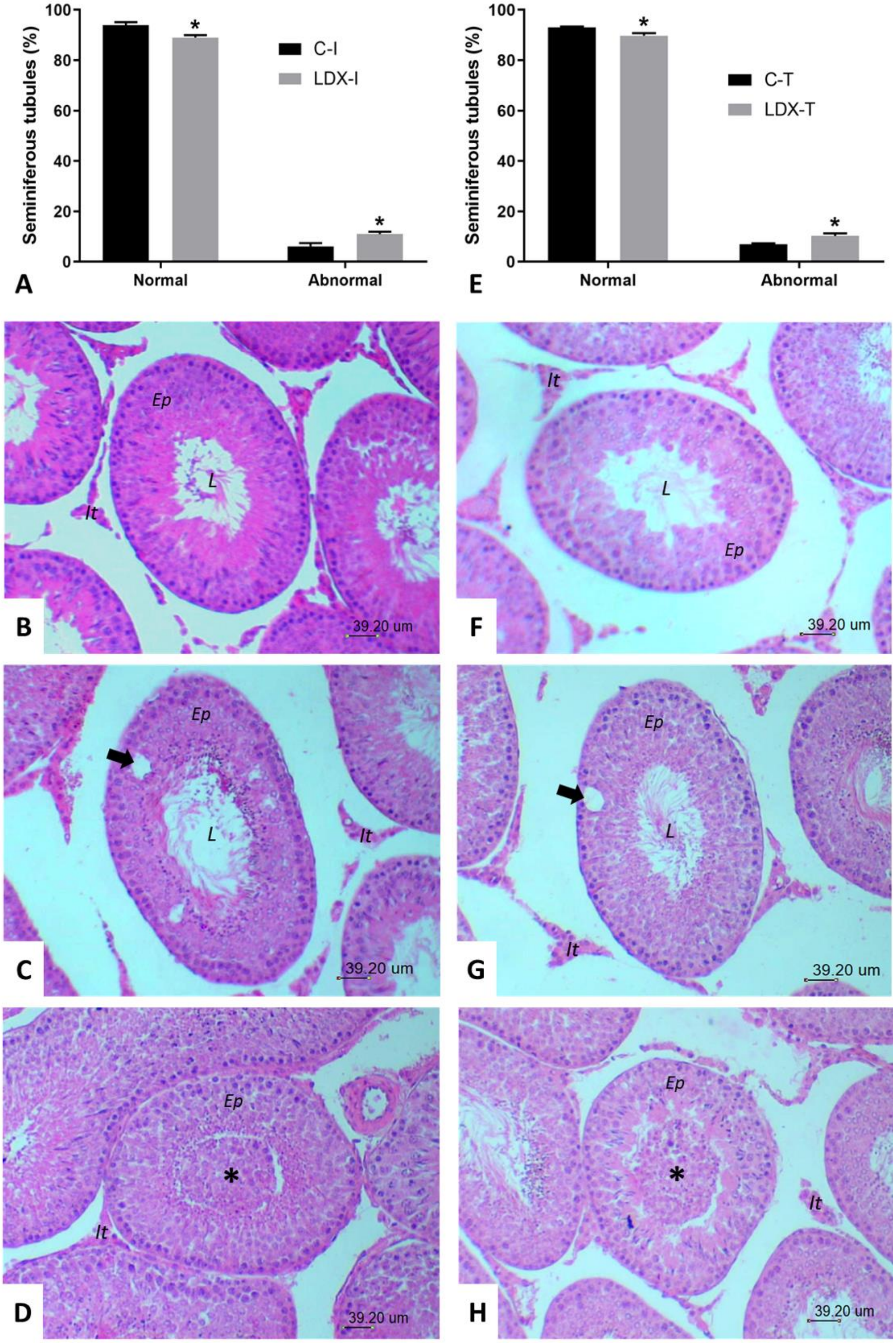

Figure 2- Histopathological analysis of the testicles. (A e E) Percentage of normal and abnormal seminiferous tubules. (B) Photomicrograph of a testis

section from group C-I. (C e D) Photomicrographs of testis sections from LDX-I group. (F) Photomicrograph of a testis section from group C-T. (G e H) Photomicrographs of testis sections from the LDX-T. (B e F) Normal appearing germinal epithelium is observed. (C e G) Note the presence of a vacuole in the germinal epithelium (arrow). (D e H) Note the presence of immature germ cells in the tubular lumen (asterisk). C-I= immediate control group; LDX-I= immediate lisdexamfetamine dimesylate group; C-T= tardy control group; LDX-T= tardy lisdexamfetamine dimesylate group. Hematoxylin and eosin stain. Ep, epithelium; It, interstice, L, lumen. Values expressed as mean ±S.E.M. Student's t test. *$p<0.05$.

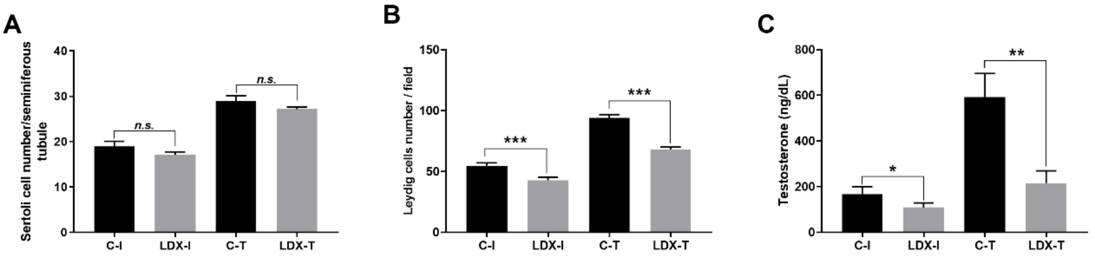

Figure 3- Sertoli cell number, Leydig cell number and plasma testosterone levels. (A) Number of Sertoli cells per seminiferous tubule of Wistar rat testis. (B) Number of Leydig cells per field in the interstitium of the testicle of Wistar rats. (C) Plasma testosterone levels. Values expressed as mean ±S.E.M. Student's t test. *n.s.*= not significant; *p<0.05; **p<0.005; ***p<0.001. C-I= immediate control group; LDX-I= immediate lisdexamfetamine dimesylate group; C-T= tardy control group; LDX-T= tardy lisdexamfetamine dimesylate group.

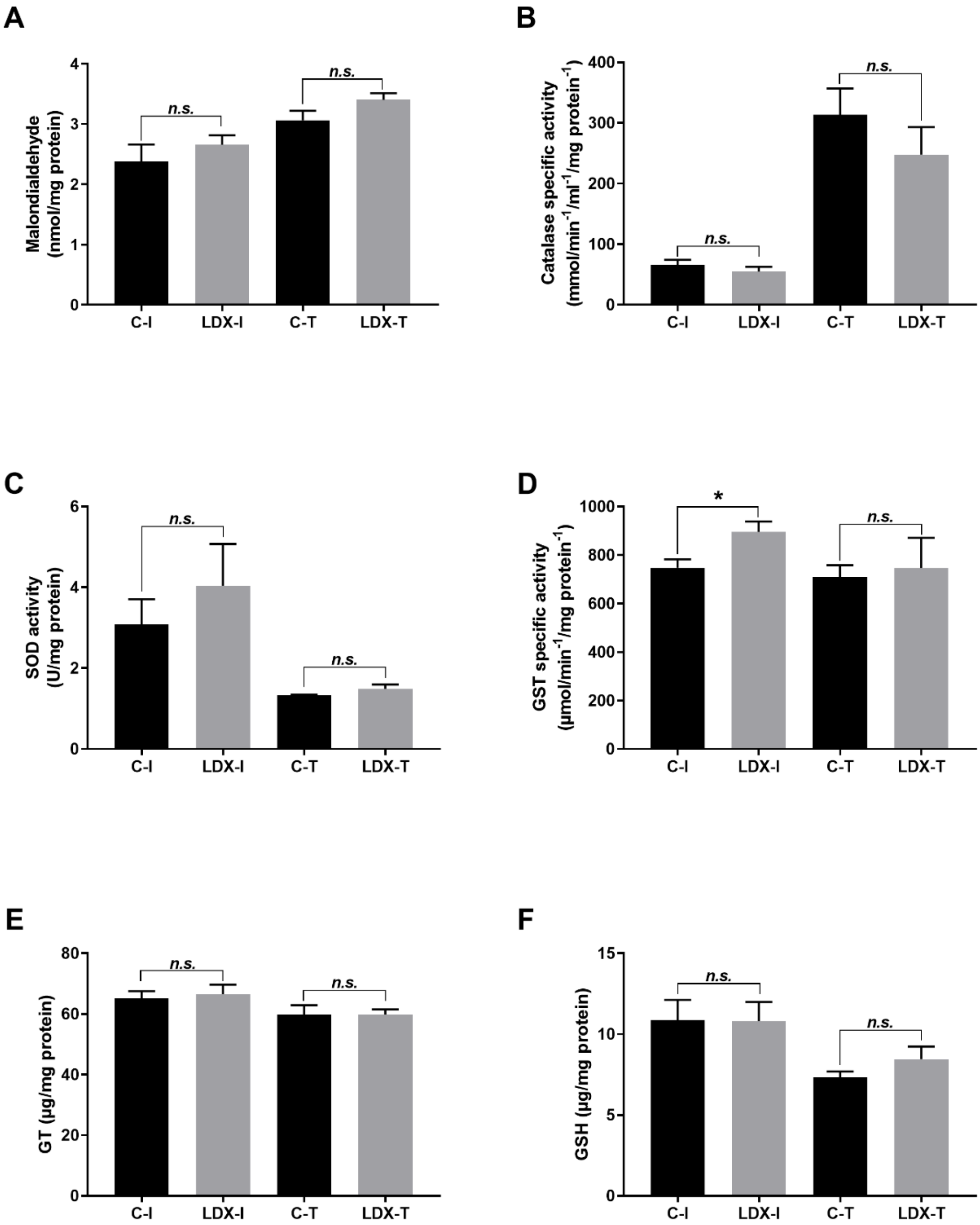

Figure 4- Biomarkers of oxidative stress. (A) Malondialdehyde concentration in the testicle. (B) Catalase activity in the testicle. (C) Superoxide dismutase activity in the testicle. (D) Glutathione S-transferase activity in the testicle. (E) Total glutathione concentration in the testicle. (F) Reduced glutathione concentration in the testicle. Values expressed as mean ±S.E.M. Student's t test. *n.s.*= not significant; *p<0.05. C-I= immediate control group; LDX-I= immediate

lisdexamfetamine dimesylate group; C-T= tardy control group; LDX-T= tardy lisdexamfetamine dimesylate group.

5 ARTIGO 2

IRREVERSIBLE DAMAGE TO POSTNATAL DEVELOPMENT AND FUNCTIONALITY OF THE EPIDIDYMIS DUE TO THE LISDEXAMPHETAMINE DIMESYLATE ADMINISTRATION TO WISTAR RATS

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IRREVERSIBLE DAMAGE TO POSTNATAL DEVELOPMENT AND FUNCTIONALITY OF THE EPIDIDYMIS DUE TO THE LISDEXAMPHETAMINE DIMESYLATE ADMINISTRATION TO WISTAR RATS

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Abstract

Lisdexamfetamine dimesylate (LDX) is a dextroamphetamine prodrug widely prescribed for the treatment of ADHD from 6 years of age. During this period, the epididymis is not completely developed and certain medications can interfere with the maturation of the organ. This study aimed to evaluate the effects of LDX on the postnatal development of the epididymis and verify whether these effects persist into adult life. Wistar rats were divided into 4 groups: immediate control (C-I), immediate LDX (LDX-I), tardy control (C-T) and tardy LDX (LDX-T). The LDX-I and LDX-T groups received daily LDX via gavage from postnatal day (PND)25 to PND65. On DPN66, groups C-I and LDX-I were euthanized and groups C-T and LDX-T remained in the bioterium until euthanized (DPN131). Histopathological, morphological, stereological analyses, oxidative stress biomarkers and determination of sperm transit time were performed on the epididymis. Spermatozoa were used for motility and mitochondrial activity analyses. LDX administration generated basal cell hypoplasia and damage to the expansion of the epididymal duct in the cauda region irreversibly. Sperm showed reduced mitochondrial activity and sperm motility, due to acceleration of sperm transit time throughout the epididymis and reduced glutathione S-transferase activity in the cauda. Although transit time was reestablished after interruption treatment, reduced mitochondrial activity and decrease in sperm motility was maintained due to the persistent of reduced glutathione S-transferase activity and increased lipid peroxidation. In conclusion, the administration of LDX during the period of postnatal development impairs the maturation of the epididymal cauda and decrease in sperm quality in rats.

Keywords: dextroamphetamine, epididymis, Vyvanse, sperm quality, basal cells, glutathione S-transferase.

1 Introduction

Attention Deficit/Hyperactivity Disorder (ADHD) is a global health problem belongs to the group of neurodevelopmental disorders and is characterized by inappropriate levels of inattention, disorganization and/or hyperactivity/impulsivity that manifest in childhood and often persist through adolescence and adulthood [1,2]. The recommended treatment for children and adolescents with ADHD includes a combination of behavioral therapies, psychological and psychopedagogical support with medication therapy [3]. The most commonly prescribed medications for the treatment of ADHD are neurostimulant substances that act on the dopamine and noradrenaline pathways. These medications stimulate the prefrontal cortex and, in this way, favor the control of executive function, demonstrating great effectiveness in reducing the general symptoms of ADHD [4,5].

In recent years, lisdexamfetamine dimesylate (LDX) has gained prominence in the medical scene, being widely prescribed for patients from 6 years of age [4]. LDX corresponds to a prodrug of dextroamphetamine, whose metabolism occurs directly in the bloodstream through its hydrolysis in the cytosol of red blood cells [6]. This medication acts as a moderately potent inhibitor of the dopamine transporter (DAT), norepinephrine transporter (NET), and vesicular monoamine transporter 2 (VMAT2) [7]. Furthermore, LDX is described as a drug with a lower risk of abuse due to its long-lasting effect, since the therapeutic effects of this medication are observed up to 13 hours after its administration in children [8] and up to 14 hours in adults. [9].

LDX treatment can be started in the child period of postnatal development of the human male reproductive system and last throughout adolescence [10]. In rats, these developmental periods correspond to the juvenile and peripubertal periods, respectively [11]. Important events in male postnatal reproductive maturation occur in these phases, such as differentiation of epithelial cells and expansion of the diameter of the epididymal duct [12].

The epididymis of rats, the organ responsible for the maturation, storage and transport of sperm, receives innervation from the sympathetic nervous system [13]. The caput and corpus regions of the epididymis have scarce innervation, while the cauda of the epididymis is richly innervated [14]. Adrenergic fibers have the main function of mediating the neuromuscular events necessary for the transport of sperm through the epididymal duct [13]. However, norepinephrine can also mediate epithelial cell functions such as electrolyte transport between cellular and luminal compartments and protein processing [15]. Therefore, substances that increase the availability of catecholaminergic neurotransmitters in the synaptic cleft could interfere with the physiology of this organ.

Since drug therapy with LDX can be initiated during the juvenile period and last throughout the peripubertal period of postnatal development of the male genital system, this study aimed to evaluate the effects of LDX administration during the period of postnatal development of the epididymis, as well as verifying whether these effects last into adulthood in an animal model.

2. Methodology

2.1 Experimental design

The experiment evaluated the effects of LDX administration on the male genital system immediately after the treatment period and 65 days after treatment interruption. Eighty male Wistar rats were used, with an initial age of 22 days, from the Central Biotherium of the State University of Londrina – UEL. The animals were acclimatized to the biotherium of the Toxicology and Metabolic Disorders of Reproduction Laboratory for three days. After acclimatization, the animals were randomly distributed into four experimental groups (n=20 animals/group) named: immediate control group (C-I), immediate LDX group (LDX-I), tardy control group (C-T) and tardy LDX group (LDX-T). The animals were housed in cages containing a maximum of 3 animals per cage and received water and food *ad libidum*. Water and food consumption and body weight were measured every three days throughout the experimental period. The biotherium was maintained under constant temperature conditions (23±2°C) and under a 12-hour light-dark cycle, with the light phase beginning at 7 a.m. Animals in groups LDX-I and LDX-T received LDX at a dose of 11.3 mg/kg/day diluted in 0.9% saline solution in a volume of 500 µl, via gavage, for forty consecutive days. This dose is equivalent to the administration of a 70mg capsule of the drug and corresponds to 1.13% of the oral LD_{50} for rats (oral LD_{50} for rats >1000 mg/kg) [16,17]. Groups C-I and C-T received only the vehicle (0.9% saline solution) in an equal volume. The treatment period occurred between postnatal day (PND)25 and PND65, which correspond to the juvenile and peripubertal phase of Wistar rats [18,19]. After the treatment period, the C-T and LDX-T groups were kept in the biotherium for a free-drug period of 65 days, receiving only water and food *ad libidum*.

The experimental protocol was approved by the Animal Use Ethics Committee of the State University of Londrina. (CEUA-UEL/ OF. CIRC. CEUA nº 82/2019).

2.2 Material collection

Euthanasia of rats in groups C-I and LDX-I occurred on PND66, while euthanasia of groups C-T and LDX-T occurred on PND131. On their respective days, the animals were anesthetized by intraperitoneal injection of Ketamine (90 mg/kg of weight) and Xylazine (10 mg/kg of weight) and euthanized by cardiac puncture to collect blood in a heparinized tube (sodium heparin).

The right epididymis were collected, weighed on a precision analytical balance and used to determine the number of mature spermatids, histopathological and stereological analyzes (n=10/group). The left epididymides were collected, weighed and used for oxidative stress analyzes (n=10/group). Spermatozoa were collected from the vas deferens and used to assess sperm motility and mitochondrial activity.

2.3 Histological processing

The epididymis were collected and fixed in Methacan for 8 hours. After fixation, the epididymis were included in Paraplast® and semi-serial sections were obtained at 5μm. The sections were stained with hematoxylin and eosin (HE) and examined under light microscopy for stereological and histopathological analysis [20].

2.3.1 Stereological, histopathological analysis and cell frequency of the epididymis

For stereological analysis, 10 random fields were photodocumented, per animal, from the caput (Region 2A) and cauda (Region 5A/B) regions of the epididymis [21]. Images were captured using a photomicroscope (Opton) and

BELView software version 6.2.3.0 (BEL Engineering) for Windows under 100x magnification. This analysis was performed using the multifunctional Weibel grid with 168 points [22] to quantify the tissue components of the epididymis (epithelium, stroma and lumen).

Epididymal histopathological analysis was performed qualitatively using transverse sections of the caput and cauda regions using an Opton microscope (100x magnification) [20].

Cell frequency in the epididymal duct was determined by counting in the caput and cauda regions [23]. Cells were counted in 10 sections from each region of the epididymis per animal. The cell count result was corrected by applying the formula Amann (1962):

$$\text{True Count} = \text{Cells number} \times \frac{\text{Section thickness}}{\text{Section thickness} + \sqrt{\left[\frac{\text{Nuclear diameter}}{2}\right]^{2} - \left[\frac{\text{Nuclear diameter}}{4}\right]^{2}}}$$

After obtaining the corrected numbers of cells present in the epididymal epithelium, general means per region were obtained, with the final results expressed as a percentage.

2.4 Oxidative stress biomarkers

2.4.1 Tissue preparation

The epididymis were sectioned into caput and cauda. Then, the epididymis segments were homogenized in 1 mL of phosphate buffer (pH 7.4) and centrifuged at 9500 rpm for 10 minutes at 4°C. Protein quantification of samples was determined by the Bradford method, using bovine serum albumin as standard [25]. Samples were then normalized to 1 mg protein/mL and used for subsequent analyses.

2.4.2 Lipid peroxidation

Lipid peroxidation was measured indirectly by quantifying the peroxides produced. The result reflects the intensity of lipid peroxidation [26]. Dosages were performed by the method of thiobarbituric acid reactive substances (TBARS) with absorbance of 535 nm and 572 nm in relation to the standard curve for malondialdehyde (MDA), the main byproduct of cellular lipid peroxidation [27]. To prepare the test, 50 µL of each normalized sample was pipetted in duplicate into a microplate, followed by the addition of $FeCl_3$ (1M), ascorbic acid, TCA (2.8%) and TBA (1.0%). The microplate was shaken and placed in a water bath at 90 °C for 15 min. It was then cooled to stop the reaction, and readings were taken at 535 nm and 572 nm. Lipid peroxidation was estimated by correcting the amount of protein, and the results were expressed as nmol of TBARS per mg of protein.

2.4.3 Reduced glutathione

Reduced glutathione (GSH) levels were determined as proposed by Rahman et al., (2007), with some modifications. For this, the sulfhydryl reagent 5,5'-dithio-bis (2-nitrobenzoic acid) DTNB was added to the supernatant of the epididymis homogenate to oxidize GSH. The result of this reaction is the formation of 5′-thio-2-nitrobenzoic acid evidenced by the formation of yellow color. GSH levels were measured at 412 nm and results expressed as micromoles/mg of protein.

2.4.4 Glutathione S-transferase activity

The enzymatic activity of glutathione S-transferase (GST) in the epididymis was determined through the formation of a thioether from the interaction of GSH with CDNB. The increase in absorbance through thioether formation was monitored at 340 nm (RS: 100 mM potassium phosphate buffer pH 6.5; 1.5 mM GSH; 2 mM CDNB) for 5 min at 40 s intervals [29]. Values were expressed as μM Thioether formed/min/mg of protein.

2.4.5 Total glutathione

Total glutathione was quantified through the oxidation of glutathione by the sulfhydryl reagent 5,5'-dithio-bis (2-nitrobenzoic acid) DTNB to form the yellow derivative 5'-thio-2-nitrobenzoic acid. The glutathione disulfide formed was recycled into glutathione by glutathione reductase in the presence of NADPH. Total glutathione content in the samples was determined by comparison with a predetermined glutathione standard curve [28].

2.4.6 Superoxide dismutase activity

Assessment of superoxide dismutase (SOD) enzyme activity was performed as described by Senthilkumar et al. (2021), with some modifications. The enzyme comes from homogenates normalized to 1 mg/mL. A reaction mixture was prepared containing sodium carbonate buffer (50 mM, pH 10.2), nitroblue tetrazoilium (NBT) (96 uM) and Triton X-100 (0.6%), which was incubated for 2 min with hydroxylamine hydrochloride (20 mM $NH_2OH{\cdot}HCl$, pH 6.0). The final volume was adjusted to 200 μl. The reaction consists of quantifying the complexes formed by superoxide anions with the addition of NBT and NH2OH·HCl of a yellowish color with the reduction of NBT, forming a bluish color read at 560 nm for 2 min at intervals of 15 s.

2.5.7 Catalase activity

The enzymatic activity of catalase (CAT) was determined by the degradation of hydrogen peroxide into oxygen and water. After determining the protein concentration (normalized to 1.0 mg/mL in PBS), the sample was added to 297 µl of reaction medium in a UV4 microplate (in triplicate) and reading was performed at 240 nm for 60 s [31].

2.5 Sperm Transit Time Calculation through the Epididymis

To evaluate sperm transit time, the epididymis divided into caput+corpus and cauda were then weighed and homogenized, as described by Fernandes et al. (2007). After diluting the homogenate, a small volume of sample was transferred to a Neubauer chamber (four fields per animal) to count spermatids resistant to homogenization (stage 19 of spermatogenesis). To calculate sperm transit time through the epididymis, the number of sperm present in the epididymis was divided by the daily sperm production value of each animal.

2.6 Sperm motility

Sperm motility was evaluated according to the methods described by Siervo et al. (2015). The vas deferens was washed with 0.3 mL of modified HTF containing gentamicin (Irvine Scientific) at 34ºC-36ºC to obtain spermatozoa. A 10 µL aliquot of the spermatozoa-containing solution was transferred to a heated Makler counting chamber (Sefi-Medical, Haifa, Israel). Sperm motility was assessed by visual estimation (100 sperm per animal) under a light microscope (Motic) at 100x magnification. Spermatozoa were classified as motile or immobile.

2.7 Sperm mitochondrial activity analysis

To evaluate the mitochondrial activity of spermatozoa a method based on the selective incorporation of the dye diaminobenzidine by the active mitochondria present in the intermediate part of the sperm was used [33]. Spermatozoa were collected from the vas deferens in 300µL PBS. A 100µL aliquot of the solution was added to 200µL of DAB (3,3' Diaminobenzidine tetrahydrochloride) dye solution. The mixture was incubated for one hour at 37ºC. After incubation, a smear was prepared, fixed in PBS with formaldehyde (10%) and 200 cells were analyzed and classified into DAB class I (completely stained intermediate piece), DAB class II (partially stained intermediate piece) and DAB class III (absence coloring in the middle piece).

2.9 Statistical analysis

Data distribution was evaluated using the Shapiro-Wilk normality test and homogeneity of variances was evaluated using Levene's test. Parametric data were evaluated by Student's t test for independent samples and data were expressed as mean (±SEM). Non-parametric data were evaluated using the Mann-Whitney test and data were expressed as median (Q1-Q3). Values were considered significantly different when $p<0.05$.

3 Results

3.1 Epididymis weight

LDX administration during the postnatal development period did not change the weight of the right and left epididymis of animals in the LDX-I group when compared to C-I. Similarly, 65 days after treatment interruption, the weight of the right and left epididymis remained similar between the LDX-T and C-T groups (data not shown).

3.2 Stereological, histopathological analysis and cell frequency of the epididymis

Stereological analysis revealed that LDX administration does not change the proportion between the luminal, stromal and epithelial compartments of the caput epididymis region (Table 1).

In contrast, the cauda of the epididymis was sensitive to the effects of LDX. In this region, an increase in the proportion of the epithelial compartment and a reduction in the proportion of the luminal compartment were observed in the LDX-I group compared to the C-I group (Table 1). Even after treatment interruption, the increase in the proportion of the epithelial compartment and reduction of the luminal compartment persisted in the cauda of the epididymis of the animals in the LDX-T group (Table 1).

Despite this, LDX administration did not induce histopathological changes in the caput and cauda of the epididymis (data not shown). In all experimental groups, the epithelial tissue and stromal region showed a normal morphological appearance, as well as only spermatozoa were present in the ductal lumen.

The frequency of cells in the caput region was similar between the experimental groups (Figures 1A, 1C and 1E). However, in the cauda region there was a reduction in the percentage of basal cells and a consequent increase in the percentage of principal cells in the LDX-I and LDX-T groups when compared to the C-I and C-T groups, respectively (Figures 1D and 1B).

3.3 Oxidative stress biomarkers

Malondialdehyde concentrations were similar between experimental groups C-I and LDX-I, both in the caput and cauda regions of the epididymis (Figures 2A). After 65 days of treatment interruption, the LDX-T group showed a significant increase in malondialdehyde concentration only in the cauda epididymis region (Figure 2B).

Catalase and superoxide dismutase activity remained equivalent in the caput and cauda regions of the epididymis of animals in groups C-I and LDX-I, as well as in groups C-T and LDX-T (data not shown). However, the use of LDX significantly reduced glutathione S-transferase activity and the concentrations of total glutathione and reduced glutathione in the caudal region of the epididymis of animals in the LDX-I group (Figures 2D, 2F and 2H). After 65 days of treatment interruption, the concentrations of total glutathione and reduced glutathione in the cauda epididymis of the LDX-T group returned to levels similar to those of the C-T group (Figures 2F and 2H). Despite that, glutathione S-transferase activity in this area remained reduced in the LDX-T group (Figure 2D).

3.4 Sperm parameters

Sperm transit time through the epididymis, both in the caput and corpus regions and in the cauda region, was significantly accelerated in the LDX-I group compared to the C-I group (Figure 3A and 3B). Consequently, the LDX-I group showed a reduction in the number of motile sperm (Figure 3C). Furthermore, there was a loss in the percentage of active mitochondria in sperm from the LDX-I group (Table 2). In this group there was a reduction in the percentage of spermatozoa whose intermediate piece contained fully active mitochondria (DAB class I) and an increase in the percentage of spermatozoa whose intermediate piece had partially active mitochondria (DAB class II).

After 65 days of treatment interruption with LDX, sperm transit time through the epididymis was reestablished in the LDX-T group, both in the caput and corpus regions and in the cauda region (Figures 3A and 3B). However, the number of motile sperm remained reduced in this experimental group (Figure 3C). In the same way, the percentage of fully active mitochondria remained reduced in sperm from the LDX-T group (Table 2).

4 Discussion

This study provides information not yet available in the literature which indicates that the administration of LDX during the period of postnatal development of the epididymis of Wistar rats delays its maturation and leads to irreversible changes responsible for impairing the functioning of this organ.

At birth, the epididymal duct of rats is composed of undifferentiated columnar cells and undergoes a postnatal development process divided into 3 periods: undifferentiation (birth until PND 15), differentiation (PND 16–44) and expansion (>PND 44) [12]. The differentiation period is considered critical for development, since at this stage undifferentiated columnar cells undergo differentiation into basal, main, apical, narrow, clear and halo cells stimulated by testosterone and growth factors present in the seminal fluid. [34,35]. The expansion period, in turn, is characterized by an increase in the diameter of the epididymal duct, acquiring an adequate proportion store sperm from the testicles. [34]. Our data revealed that LDX administration to Wistar rats during the period of postnatal epididymis development delays the differentiation and expansion of the cauda region evidenced by basal cell hypoplasia and a reduction in the proportion of the luminal compartment in this region.

Basal cells appear for the first time in the epididymal epithelium from PND28, undergo multiple mitotic divisions until PND44 and lose proliferative capacity from that moment on, not acting as stem cells [34,36]. For this reason, the basal cell population is not reestablished in adult animals that received LDX during the postnatal development period of the epididymis.

In addition to delaying the development of the epididymis, LDX administration accelerates sperm transit time in all regions of the epididymis, contributing to a reduction in mitochondrial activity and a consequent reduction in sperm motility. When leaving the testicles, spermatozoa are morphologically complete cells, however, they are immobile and incapable of fertilizing an oocyte [37]. The transit of spermatozoa through the lumen of the epididymal duct is essential for sperm maturation, a process characterized by nuclear compaction, changes in the composition of the plasma membrane and the structure of the cytoskeleton, as well as changes in the load of proteins and non-coding RNA [38]. Several studies have revealed that the acceleration of sperm transit time through the epididymis is an important contributor to the reduction of sperm motility in mammals, by reducing the contact time of spermatozoa with epididymosomes, epithelial cells and luminal ions [38-40].

Sperm transit time through the epididymis is coordinated by the rhythmic contraction of the smooth muscle layer that surrounds the entire epididymal duct [41]. The muscle and interstitial cells of the rat epididymis have α1-adrenergic receptors responsible for stimulating the contraction of the epididymal duct [42]. Therefore, drugs that inhibit norepinephrine reuptake, such as LDX [7], increase contractility of the epididymis through increased signaling of this neurotransmitter in α1-adrenergic receptors [43].

Although the acceleration of transit time is an important contributor to impairments in sperm maturation, our data reveal that this is not the only factor involved in the reduction of sperm motility in rats that received LDX, since GST activity was reduced in the cauda of the epididymis of these animals. GSTs correspond to an important family of antioxidant enzymes that catalyze reduced glutathione-dependent reactions [44]. The cauda region of the epididymis presents the GSTM3 and GSTP1 isoforms, released into the seminal fluid together with the epididymosomes. These enzymes act not only to protect sperm against reactive oxygen species, but also to regulate signaling pathways important for sperm maturation [45]. GSTP1 forms a heterocomplex with c-Jun N-terminal kinase (JNK), blocking the JNK binding site to C-Jun and its subsequent phosphorylation. During spermatozoa storage in the cauda of the epididymis, loss of stability of the GSTP1-JNK heterocomplex impairs mitochondrial integrity, resulting in reduced sperm motility [46]. Thus, the reduction of GST activity in the cauda epididymis of animals receiving LDX is an important contributor to reduced mitochondrial activity and reduced sperm motility.

After 65 days of interruption of treatment, mitochondrial activity and sperm motility remained impaired despite the reestablishment of sperm transit time. Basal cells perform several functions in the epididymis, including secretion of prostaglandin E2 which regulates the transport of water and electrolytes from seminal fluid by principal cells [47], immune defense [48] and protection of epithelium and spermatozoa against reactive oxygen species [49]. The epididymis predominantly presents the GST-P1 subunit, which in the epithelium of the cauda region is located exclusively in basal cells [50]. Thus, as basal cells are unable to proliferate after PND44, the basal cell hypoplasia observed in adult animals is an important contributor to the reduced GST activity after treatment interruption. Furthermore, reduced antioxidant activity resulted in increased lipid peroxidation in the cauda epididymis of adult animals. Spermatozoa are highly sensitive to oxidative damage since proteins cannot be translated by this cell type and their antioxidant protection is dependent on enzymes acquired during spermatogenesis and provided by epithelial cells of the epididymis during maturation. [51]. Oxidative stress is capable of damaging the plasma membrane, mitochondria and DNA of sperm, consequently impairing mitochondrial activity, motility and the ability of sperm to fertilize oocytes [52].

5 Conclusion

In conclusion, LDX administration to rats during the period of postnatal development of the epididymis, it irreversibly delays the differentiation and expansion of the cauda region resulting in failure in the maintenance of mitochondrial integrity during the sperm storage period. These results describe for the first time the effects of LDX administration on postnatal development of the epididymis in an animal model.

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Interest conflicts

The authors declare there are no conflicts of interest.

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Table 1. Epididymal stereology of Wistar rats

	Experimental groups	
Parameters (%)	**C-I (n=10)**	**LDX-I (n=10)**
Caput		
Lumen	55.55 ±1.5	56.26 ±1.4
Stroma	15.28 ±0.7	13.74 ±1.7
Epithelium	29.17 ±1.6	30.00 ±1.5
Cauda		
Lumen	54.94 ±0.7	51.92 ±0.7*
Stroma	30.75 ±1.0	30.29 ±1.0
Epithelium	14.31 ±0.6	17.79 ±1.0*

	C-T (n=10)	**LDX-T (n=10)**
Caput		
Lumen	61.55 ±0.8	61.44 ±1.0
Stroma	14.07 ±0.6	14.17 ±0.8
Epithelium	24.38 ±0.5	24.39 ±0.5
Cauda		
Lumen	69.80 ±1.0	64.83 ±1.3**
Stroma	22.02 ±0.9	24.32 ±1.1
Epithelium	8.18 ±0.4	10.85 ±0.6**

Values expressed as mean ± S.E.M. Student's t test. *p<0.05; **p<0.005. C-I= immediate control group; LDX-I= immediate lisdexamfetamine dimesylate group; C-T= tardy control group; LDX-T= tardy lisdexamfetamine dimesylate group.

Table 2. Sperm mitochondrial activity

	Experimental groups	
Parameters (%)	**C-I (n=10)**	**LDX-I (n=10)**
DAB I	72.5 ±6.8	54.7 ±11.3*
DAB II	21 ±5.6	32.8 ±8.2*
DAB III	6.5 ±3.2	12.5 ±6.4
	C-T (n=10)	**LDX-T (n=10)**
DAB I	94.2 ±1.0	85.7 ±2.4*
DAB II	5.7 ±1.0	13.9 ±2.3*
DAB III	0.1 ±0.1	0.4 ±0.1

Values expressed as mean ± S.E.M. Student's t test. *p<0.05. C-I= immediate control group; LDX-I= immediate lisdexamfetamine dimesylate group; C-T= tardy control group; LDX-T= tardy lisdexamfetamine dimesylate group.

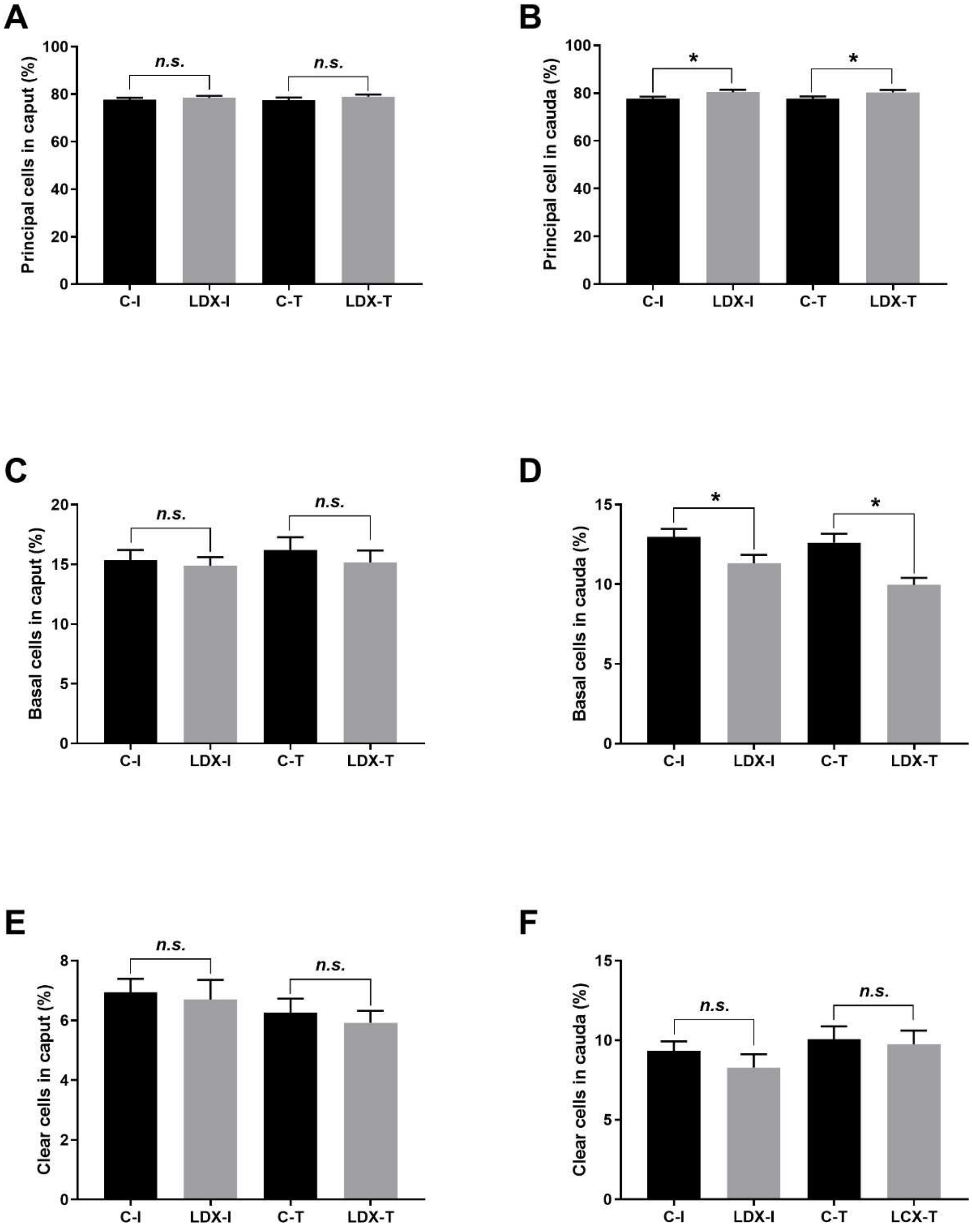


Figure 1. Epithelial cell frequency of the epididymis in the caput and cauda regions. Percentage of principal cells in the caput (A) and cauda (B) region. Percentage of basal cells in the caput (C) and cauda (D) region. Percentage of clear cells in the caput (E) and cauda (F) region. Values expressed as mean ± S.E.M. Student's t test. *n.s.*= not significant; *p<0.05. C-I= immediate control group; LDX-I= immediate lisdexamfetamine dimesylate group; C-T= tardy control group; LDX-T= tardy lisdexamfetamine dimesylate group.

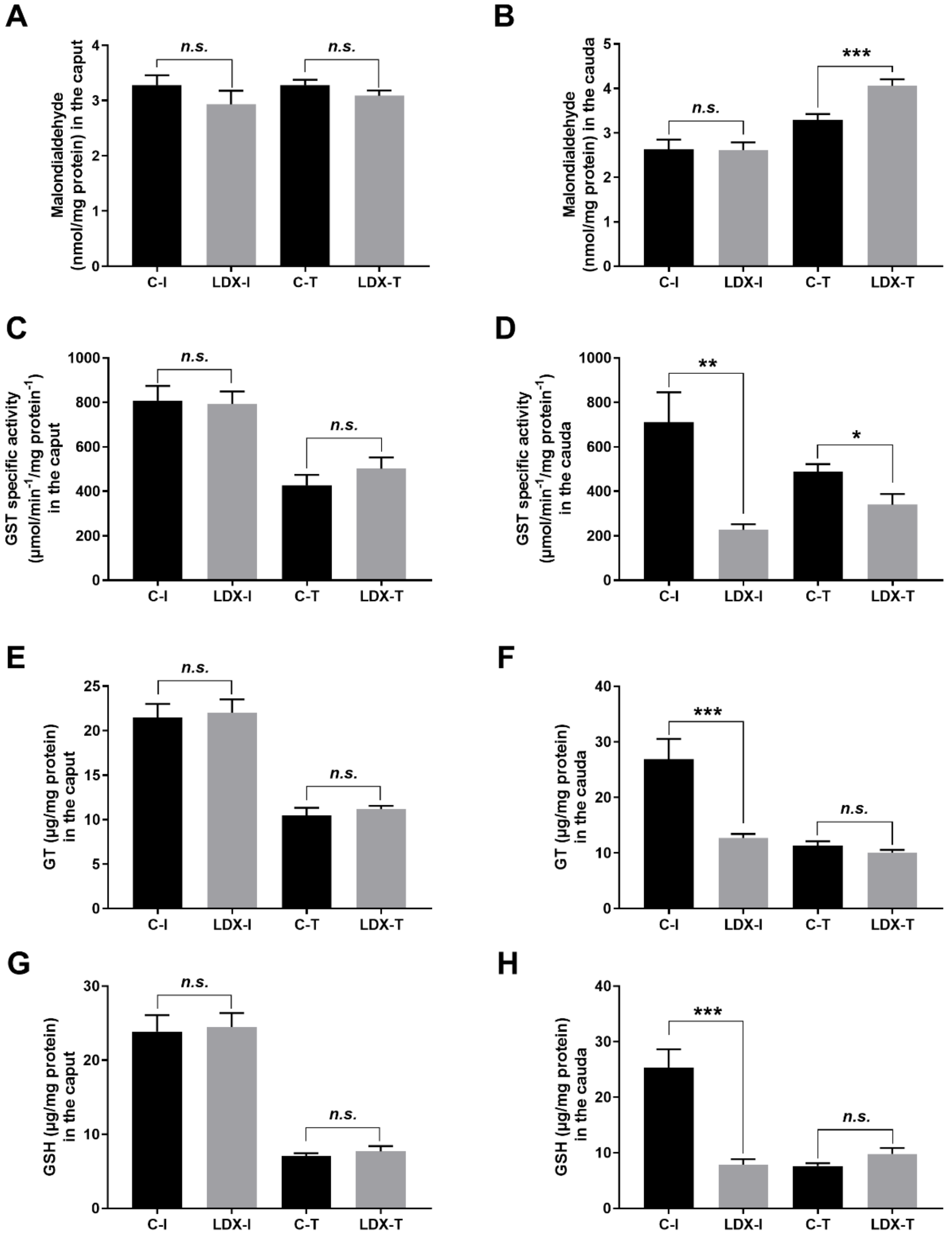


Figure 2- Oxidative stress biomarkers in the caput and cauda of the epididymis. (A e E) Malondialdehyde concentration. (B e F) Glutathione S-transferase activity. (C e G) Total glutathione concentration. (D e H) Reduced glutathione concentration. Values expressed as mean ± S.E.M. Student's t test. *n.s.*= not significant; *p<0.05; **p<0.005; ***p<0.001. C-I= immediate control group; LDX-I= immediate lisdexamfetamine dimesylate group; C-T= tardy control group; LDX-T= tardy lisdexamfetamine dimesylate group.

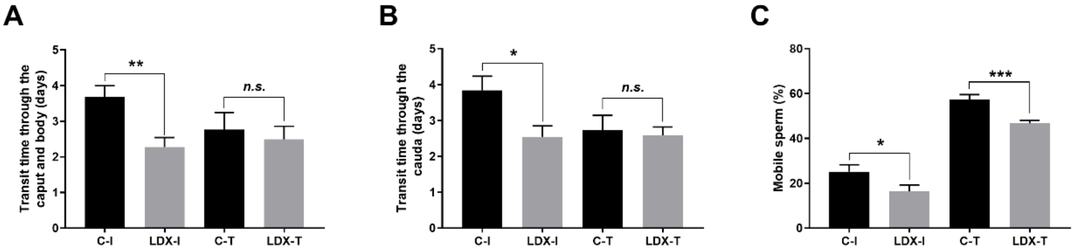


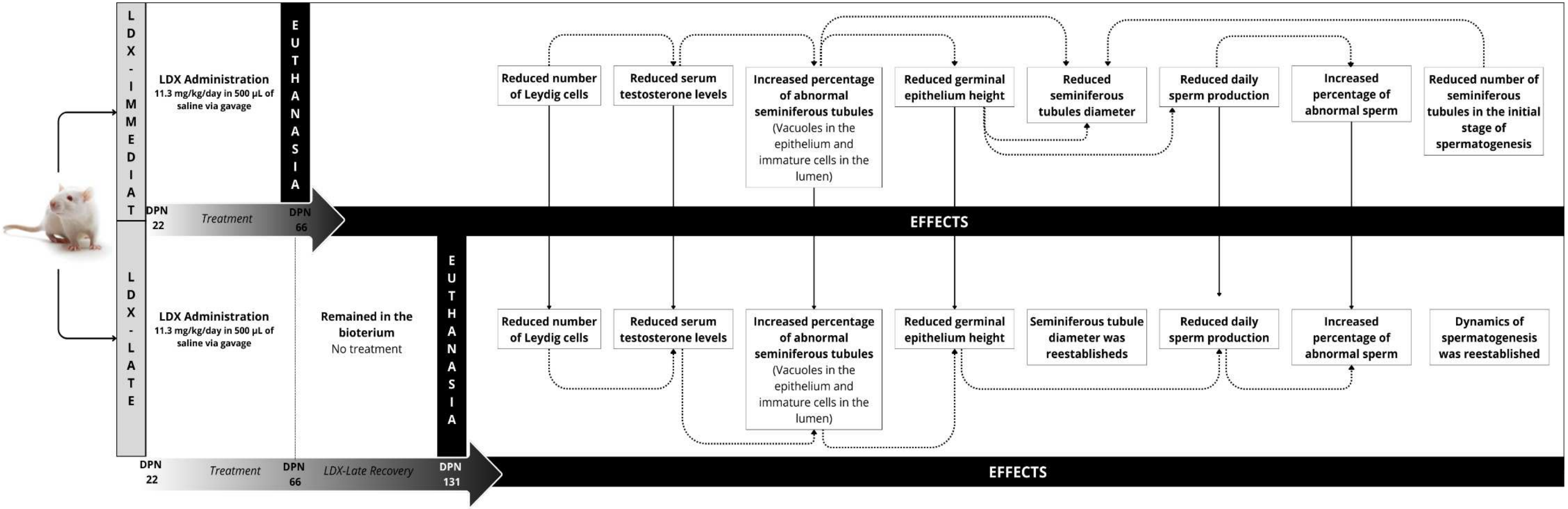
Figure 3- Sperm parameters. (A) Sperm transit time through the caput and corpus of the epididymis. (B) Sperm transit time through the cauda of the epididymis. (C) Percentage of mobile sperm. Values expressed as mean ± S.E.M. Student's t test. *n.s.*= not significant; *p<0.05; **p<0.005; ***p<0.001. C-I= immediate control group; LDX-I= immediate lisdexamfetamine dimesylate group; C-T= tardy control group; LDX-T= tardy lisdexamfetamine dimesylate group.

6 CONSIDERAÇÕES FINAIS

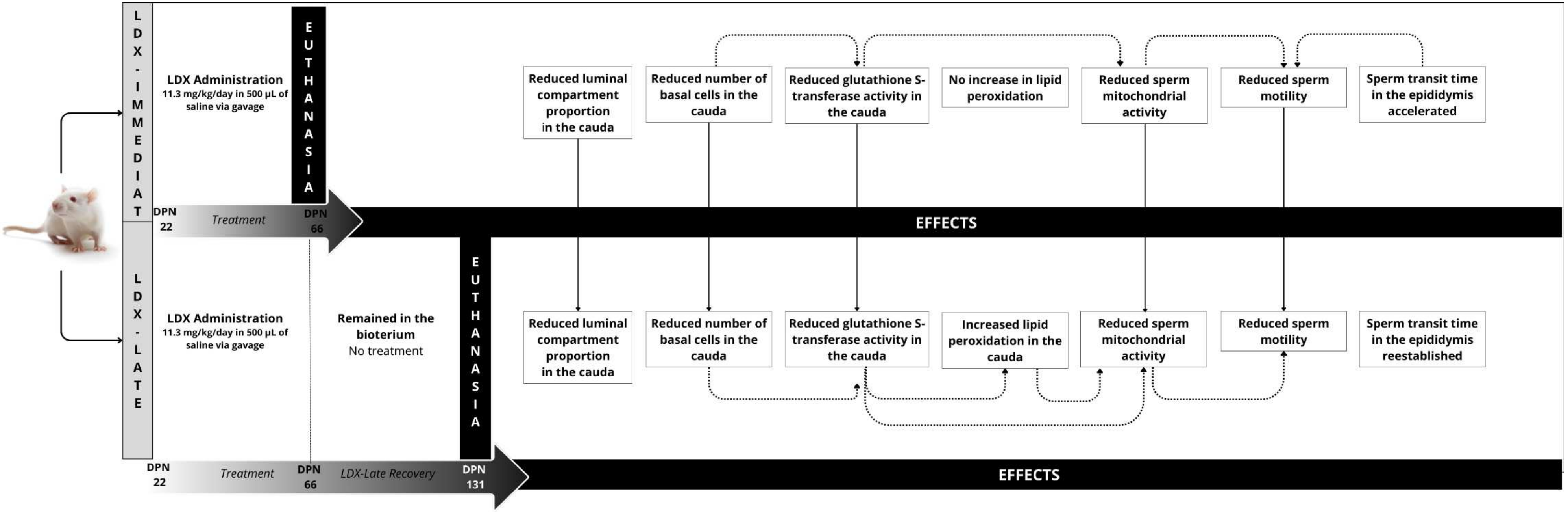
Ao desenvolver este trabalho buscou-se assim preencher uma lacuna existente na literatura, uma vez que, trabalhos que avaliem efeitos adversos e toxicidade do LDX são escassos. A hipótese testada nesse trabalho mostrou-se válida, ou seja, a administração de LDX a ratos Wistar durante o período de desenvolvimento pós-natal do sistema genital masculino retarda o desenvolvimento do testículo, da cauda do epidídimo e gera prejuízos à qualidade espermática. Além disso, após a interrupção da administração de LDX as alterações morfológicas, histopatológicas e funcionais permanecem nesses órgãos.

Em resumo o LDX induziu hipoplasia de células de Leydig resultando em redução dos níveis plasmáticos de testosterona. A baixa disponibilidade de testosterona favoreceu o desprendimento de células imaturas do epitélio germinativo, culminando em aumento do percentual de túbulos seminíferos contendo vacúolos no epitélio e células imaturas na luz. Esses fatores contribuíram para redução da altura do epitélio germinativo, do diâmetro dos túbulos seminíferos e da PDE. A hipoplasia de células de Leydig estava presente após a interrupção do tratamento, causando atrofia gonadal e persistência da testosterona plasmática reduzida. Consequentemente, houve persistência do desprendimento de células imaturas do epitélio germinativo, da redução da altura do epitélio germinativo e da PDE nos animais adultos. No epidídimo, a administração de LDX gerou hipoplasia de células basais e retardou a expansão do ducto epididimário na região de cauda, irreversivelmente. O tempo de trânsito espermático pelo epidídimo foi acelerado e a atividade de glutationa-S-transferase reduzida na região de cauda resultando em prejuízo na atividade mitocondrial e motilidade espermática. Após a interrupção do tratamento o tempo de trânsito foi reestabelecido, mas a atividade da glutationa S-transferase na cauda se manteve reduzida levando ao aumento da peroxidação lipídica nessa região e persistência da redução da atividade mitocondrial e motilidade nos espermatozoides dos animais adultos.

Ao trazer as primeiras evidencias dos efeitos do LDX sobre o desenvolvimento pós-natal do sistema genital de ratos, abre-se uma janela de oportunidades para se compreender os efeitos adversos e complicações da administração desse fármaco sobre a maturação dos órgãos sexuais masculinos e fertilidade.



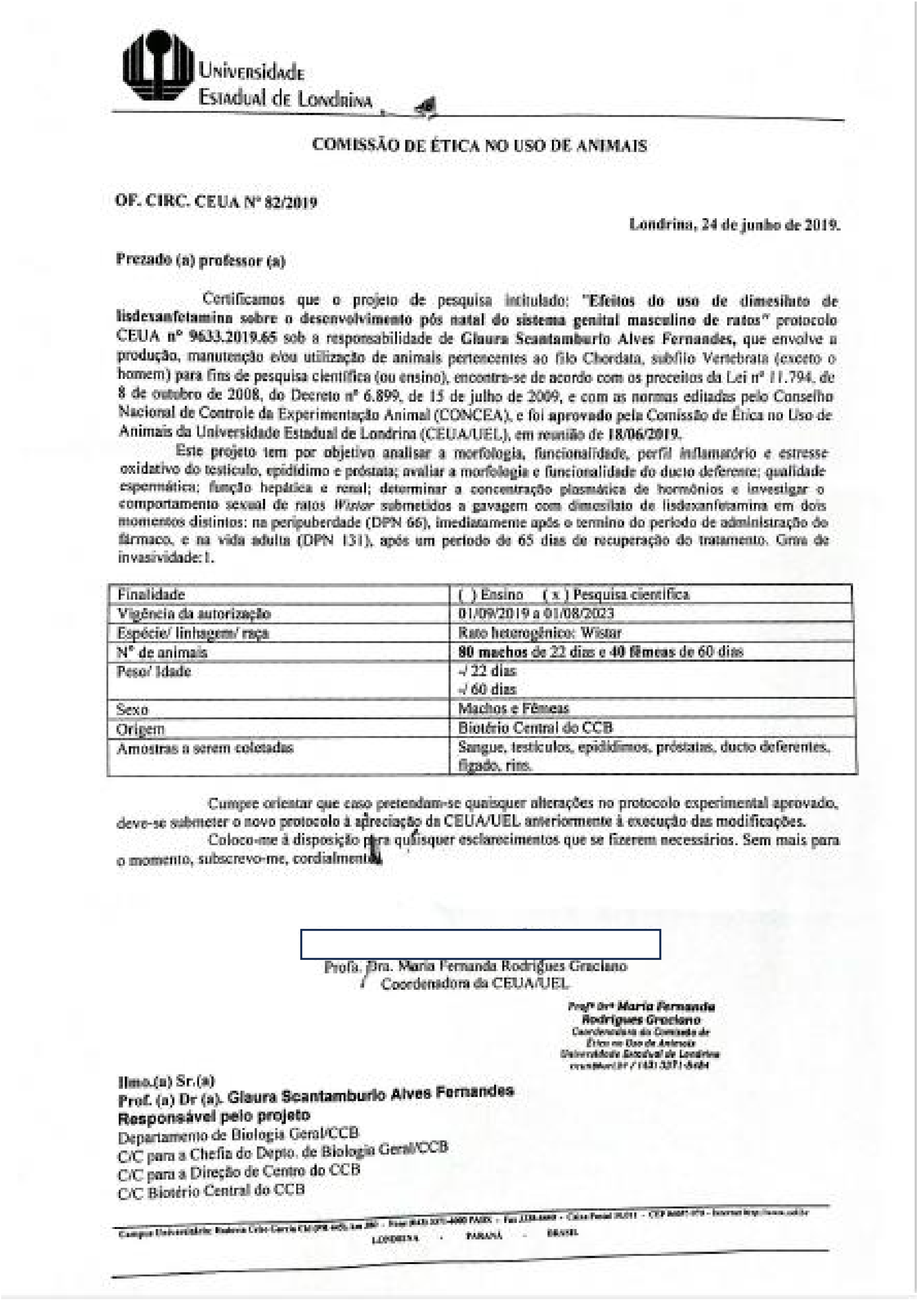
Graphical Abstract 1- Effects of LDX administration on postnatal testicular development in Wistar rats.



Graphical Abstract 2- Effects of LDX administration on postnatal epididymal development in Wistar rats.

ANEXO A

Oficio Circular CEUA Nº82/2019.



Universidade Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 82/2019

Londrina, 24 de junho de 2019.

Prezado (a) professor (a)

Certificamos que o projeto de pesquisa intitulado: "**Efeitos do uso de dimesilato de lisdexanfetamina sobre o desenvolvimento pós natal do sistema genital masculino de ratos**" protocolo CEUA **nº 9633.2019.65** sob a responsabilidade de **Glaura Scantamburlo Alves Fernandes**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL), em reunião de **18/06/2019**.

Este projeto tem por objetivo analisar a morfologia, funcionalidade, perfil inflamatório e estresse oxidativo do testículo, epidídimo e próstata; avaliar a morfologia e funcionalidade do ducto deferente; qualidade espermática; função hepática e renal; determinar a concentração plasmática de hormônios e investigar o comportamento sexual de ratos *Wistar* submetidos a gavagem com dimesilato de lisdexanfetamina em dois momentos distintos: na peripuberdade (DPN 66), imediatamente após o término do período de administração do fármaco, e na vida adulta (DPN 131), após um período de 65 dias de recuperação do tratamento. Grau de invasividade:1.

Finalidade	( ) Ensino (x) Pesquisa científica
Vigência da autorização	01/09/2019 a 01/08/2023
Espécie/ linhagem/ raça	Rato heterogênico: Wistar
Nº de animais	**80 machos** de 22 dias e **40 fêmeas** de 60 dias
Peso/ Idade	-/ 22 dias -/ 60 dias
Sexo	Machos e Fêmeas
Origem	Biotério Central do CCB
Amostras a serem coletadas	Sangue, testículos, epidídimos, próstatas, ducto deferentes, fígado, rins.

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações.

Coloco-me à disposição para quaisquer esclarecimentos que se fizerem necessários. Sem mais para o momento, subscrevo-me, cordialmente.

[redacted]

Profa. Dra. Maria Fernanda Rodrigues Graciano
Coordenadora da CEUA/UEL

Profª Drª **Maria Fernanda Rodrigues Graciano**
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Estadual de Londrina
[illegible]

Ilmo.(a) Sr.(a)
Prof. (a) Dr (a). Glaura Scantamburlo Alves Fernandes
Responsável pelo projeto
Departamento de Biologia Geral/CCB
C/C para a Chefia do Depto. de Biologia Geral/CCB
C/C para a Direção de Centro do CCB
C/C Biotério Central do CCB

Campus Universitário: Rodovia Celso Garcia Cid (PR 445), km 380 - Fone [illegible] PABX - Fax [illegible] - Caixa Postal [illegible] - CEP [illegible] - Internet [illegible]
LONDRINA - PARANÁ - BRASIL

ANEXO B

Guide for Authors

Reproductive Sciences:
https://link.springer.com/journal/43032/submission-guidelines