UNIVERSIDADE FEDERAL DA GRANDE DOURADOS FACULDADE DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

Influência da exposição *in utero* e lactacional ao tamoxifeno: repercussão tardia em parâmetros reprodutivos e comportamentais, em ratos machos adultos

BEATRIZ DE MATOS MANOEL

Dourados-MS
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ATA DA DEFESA DE DISSERTAÇÃO DE MESTRADO APRESENTADA POR **BEATRIZ DE MATOS MANOEL** DO PROGRAMA DE PÓS-GRADUAÇÃO *STRICTO SENSU* EM CIÊNCIAS DA SAÚDE, ÁREA DE CONCENTRAÇÃO "FARMACOLOGIA".

Aos trinta dias do mês de agosto de dois mil e vinte e um, às 13 horas e 30 minutos, em sessão pública, realizou-se por videoconferência a defesa de dissertação de Mestrado intitulada **"Influência da exposição *in utero* e lactacional ao tamoxifeno: repercussão tardia em parâmetros reprodutivos e comportamentais, em ratos machos adultos"** apresentada pela aluna **Beatriz de Matos Manoel**, do Programa de Pós-Graduação em Ciências da Saúde, à Banca Examinadora constituída pelos membros: Dr.ª Arielle Cristina Arena - PPGCS/UFGD/UNESP (presidente), Dr.ª Cândida Aparecida Leite Kassuya - PPGCS/UFGD e Dr.ª Daniela Cristina Ceccatto Gerardin/UEL. Iniciados os trabalhos, a presidência deu a conhecer à candidata e aos integrantes da Banca as normas a serem observadas na apresentação da dissertação. Após a candidata ter apresentado a sua explanação, os componentes da Banca Examinadora fizeram suas arguições. Terminada a Defesa, a Banca Examinadora, em sessão secreta, passou aos trabalhos de julgamento, tendo sido a candidata considerada APROVADA, fazendo *jus* ao título de **MESTRE EM CIÊNCIAS DA SAÚDE**. **A presidente da banca abaixo-assinado atesta que as doutoras** Cândida Aparecida Leite Kassuya e Daniela Cristina Ceccatto Gerardin **participaram de forma remota desta defesa de dissertação, conforme o** § 3º do Art. 1º da Portaria RTR/UFGD n. 200, de 16/03/2020 e a Instrução Normativa PROPP/UFGD Nº 1, de 17/03/2020, **considerando a candidata APROVADA, conforme declarações anexas**. Nada mais havendo a tratar, lavrou-se a presente ata, que vai assinada pelo presidente da Comissão Examinadora.

Dourados, 30 de agosto de 2021.

Dr.ª Arielle Cristina Arena - PPGCS/UFGD/UNESP

Dr.ª Cândida Aparecida Leite Kassuya - PPGCS/UFGD (participação remota)

Dr.ª Daniela Cristina Ceccatto Gerardin/UEL (participação remota)

ATA HOMOLOGADA EM: ____/___/_____, PELA PRÓ-REITORIA DE ENSINO DE PÓS-GRADUAÇÃO E PESQUISA / UFGD.

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DEDICATÓRIA

Aos meus pais e a toda minha família por todo o apoio recebido, meu muito obrigada. Este trabalho é dedicado a vocês.

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EPÍGRAFE

"Nada na vida deve ser temido,
somente compreendido. Agora é hora
de compreender mais para temer
menos. "

(MARIE CURIE)

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SÍMBOLOS

AP-1	Ativador de proteína-1
APO	Área pré-optica
DE	Desreguladores endócrinos
DMT	Dose Máxima Tolerada
DNA	Ácido Desoxirribonucleico - Deoxyribonucleic acid
E2	Estradiol
ERE	Elementos de Resposta ao Estrogênio
FDA	Food and Drug Administration
GABA	Ácido gama-aminobutírico - Gamma aminobutyric acid
IGF-1	Fator de crescimento tipo insulina I - Insulin-like growth factor-1
LBD	Domínio de ligação com o ligante - ligand-binding domain
MAPK	Proteína quinase ativada por mitógeno - Mitogen-activated protein kinase
MSRE	Moduladores Seletivos do Receptor de Estrógeno
NOAEL	No Observed Adverse Effect Level - Nível Sem Efeitos Adversos Observáveis
PGE2	Prostaglandina E2
RE	Receptores de Estrógeno
SCB	Sistema de Classificação Biofarmacêutica
SDN-POA	Núcleo sexualmente dimórfico da área pré-optica
SNC	Sistema Nervoso Central
Sp1	Proteína de especificidade 1
T_3	Triiodotironina
TAM	Tamoxifeno
TGF- α	Fator de crescimento transformador alfa - Transforming growth factor alpha
TGF-β	Fator de crescimento transformante beta - Transforming growth factor beta
TSH	Hormônio estimulante da tiroide
µg	Micrograma

Influência da exposição *in utero* e lactacional ao tamoxifeno: repercussão tardia em parâmetros reprodutivos e comportamentais, em ratos machos adultos.

RESUMO

O tamoxifeno, modulador seletivo do receptor estrogênico não esteroidal, é utilizado como terapia endócrina adjuvante em pacientes com câncer de mama positivo para receptores hormonais. Como muitas pacientes com câncer de mama estão em idade reprodutiva, a gestação é adiada até o fim do tratamento, devido ao potencial efeito teratogênico da droga. No entanto, informações sobre o risco do uso do tamoxifeno na gestação ainda são escassas, especialmente se a exposição ocorrer no período de diferenciação sexual do cérebro, o qual é sensível ao estradiol. Além disso, esta droga é um potencial desregulador endócrino com capacidade de interferir na reprodução em organismos não-alvo. Como o tamoxifeno possui ação anti-estrogênica, o objetivo deste estudo foi avaliar os efeitos da exposição materna ao tamoxifeno sobre parâmetros reprodutivos, na prole masculina de ratos. Dessa forma, ratas Wistar prenhes (n=32) foram expostas a três doses de tamoxifeno (0.12; 0.6 ou 3 µg/kg) entre a última semana de gestação (Dias gestacionais 15-21) e o final da lactação (Dia pós-natal 20) por gavagem. Durante o tratamento, foram monitorados o consumo de água e ração, massa corporal, bem como o comportamento materno das ratas expostas. Ao final do tratamento, coletou-se o sangue das mães para análises bioquímicas e órgãos para registro do peso. Após o nascimento, os filhotes machos foram avaliados através dos seguintes parâmetros: determinação do peso corporal, distância anogenital e idades de separação prepucial, descida testicular e play behavior. Na vida adulta, estes mesmos animais foram investigados em relação ao: pesos de órgãos reprodutores, contagem, morfologia, motilidade espermática, histologia do testículo e epidídimo, expressão de receptores de andrógeno no hipotálamo, comportamento e preferência sexual. O tratamento com tamoxifeno não interferiu nos parâmetros maternos avaliados. Na avaliação do desenvolvimento inicial da prole masculina, observou-se alterações no peso corporal e atraso na idade de separação prepucial (biomarcador de início de puberdade). Na vida adulta, a exposição ao tamoxifeno não alterou o peso relativo de órgãos, a morfologia espermática, o número, parâmetros histológicos do testículo, expressão de receptores de andrógeno ou a fertilidade, entretanto, causou alterações na motilidade, contagem espermática e na histopatologia do epidídimo. No entanto, os parâmetros comportamentais avaliados não foram alterados. Conclui-se que a exposição materna ao tamoxifeno foi capaz de interferir com

a maturação sexual e comprometeu a qualidade e a quantidade espermática dos descendentes machos.
Palavras-chaves: Estradiol, tamoxifeno, puberdade, diferenciação sexual hipotalâmica.

Influence of *in utero* and lactational exposure to tamoxifen: later repercussion on reproductive and behavioral parameters, in adult male rats.

ABSTRACT

Tamoxifen, selective non-steroidal estrogen receptor modulator, is used as adjuvant endocrine therapy in patients with breast cancer positive for hormone receptors. Since many breast cancer patients are of reproductive age, pregnancy is postponed until the end of treatment due to the potential teratogenic effect of the drug. However, information on the risk of using tamoxifen during pregnancy is still scarce, especially if the exposure occurs during the period of sexual differentiation of the brain, which is sensitive to estradiol. Furthermore, this drug is a potential endocrine disruptor capable of interfering with reproduction in non-target organisms. Since tamoxifen has an anti-estrogenic action, the aim of this study was to evaluate the effects of maternal exposure to tamoxifen on reproductive parameters in male rats. Therefore, pregnant Wistar rats (n=32) were exposed to three doses of tamoxifen (0.12; 0.6; or 3 µg /kg), from the last week of gestation (Gestational days 15-21) to the end of lactation (Postnatal day 20), via gavage. During treatment, water and food consumption, body mass and maternal behavior of exposed rats were monitored. At the end of the treatment, blood was collected from the mothers for biochemical analyzes and organs for recording the weight. After birth, male offspring were evaluated using the following parameters: determination of body weight, anogenital distance, ages of preputial separation, testicular descent and play behavior. In adult life, these same animals were investigated regarding: reproductive organ weights, sperm count, sperm morphology and motility, histological analysis of the testis and epididymis, expression of androgen receptors in the hypothalamus, sexual behavior and sexual preference. Tamoxifen treatment did not interfere with the evaluated maternal parameters. In the assessment of the initial development of male offspring, changes in body weight and delay in the age of preputial separation (biomarker of puberty onset) were observed. In adult life, exposure to tamoxifen did not change the relative organs weight, sperm morphology, testicular histological parameters, expression of androgen receptors or fertility, although it did cause alterations in sperm motility, sperm count and histopathological alterations were found in the epididymis. However, there was no change in behavioral parameters. Therefore, maternal exposure to tamoxifen was able to affect sexual maturation and compromised the sperm quality and quantity of male offspring.

***Keywords*:** Estradiol, tamoxifen, puberty, hypothalamic sexual differentiation.

SUMÁRIO

1 INTRODUÇÃO

O câncer de mama é a neoplasia maligna mais comum em mulheres em idade reprodutiva (Bray et al., 2018). No Brasil, a estimativa para cada ano do triênio 2020-2022 é de 66 mil novos casos de câncer de mama (De Oliveira Santos, 2020). Consequentemente, os médicos são confrontados cada vez mais com câncer de mama durante a gestação ou em mulheres que ainda querem engravidar, devido ao aumento da incidência de câncer de mama em mulheres jovens e a tendência em adiar a maternidade (IKNL, 2018). A maioria das pacientes com câncer de mama será submetida a tratamento sistêmico com quimioterapia, e as que tiverem doença positiva para receptores hormonais seguirão com a terapia endócrina. O tratamento endócrino padrão consiste no uso do tamoxifeno (TAM) por 5 anos com a possibilidade de estender até 10 anos no câncer de mama com características de alto risco (Davies et al., 2013).

Em 1977, após extensos testes clínicos, a Food and Drug Administration (FDA) aprovou o uso do TAM, um supressor de estrogênio usado para tratar o câncer de mama (Burstein et al., 2019). Esta droga, pertencente a classe dos Moduladores Seletivos do Receptor Estrogênico não esteroidal (MRSE), exibe diferenças interespécies em relação aos seus efeitos estrogênicos/antiestrogênicos (Hengstler et al., 1999). Em ratos e em humanos tem ação tanto estrogênica como antiestrogênica, já em camundongos possui ação estrogênica completa e em galinhas age como antiestrogênico (Jordan; Robinson, 1987).

Devido à sua longa meia-vida de 14 dias, a completa eliminação do TAM bem como de seus metabólitos da circulação, leva aproximadamente de 6 a 8 semanas. Seus principais metabólitos são o N-desmetiltamoxifeno (formado pela CYP3A4), o 4-hidroxitamoxifeno e o endoxifeno (formado pela CYP2D6). Em humanos, a afinidade dos estrogênios naturais pelo receptor de estrogênio é muito maior que a do TAM. No entanto, a afinidade dos metabólitos ativos do TAM, endoxifeno e 4-OH-tamoxifeno é muito maior que a do próprio TAM, e mais forte que dos estrogênios circulantes (Scripture; Sparreboom; Figg, 2005).

Em pacientes que desejam engravidar, é recomendado interromper o uso deste medicamento devido ao seu potencial efeito teratogênico. Desse modo, muitas mulheres serão obrigadas a decidir entre o tratamento anti-câncer e o desejo de ter filhos, com o risco de uma anormalidade congênita ou um efeito teratogênico. Apesar das evidências serem limitadas, o consenso geral é parar o uso do TAM pelo menos dois meses antes da

concepção, em razão da longa meia-vida de seus metabólitos ativos (Berger; Clericuzio, 2008). Dessa forma, estudos sobre o risco do uso de TAM na gestação são necessários e devem ser encorajados.

As advertências sobre o uso do TAM durante a gestação baseiam-se principalmente em estudos com modelos animais, os quais demonstraram toxicidade fetal. Tais alterações foram semelhantes aos causados pelos estrogênios, demonstrando efeitos tróficos no útero e na vagina. Anormalidades na diferenciação sexual da prole feminina também foram observadas (Hines et al., 1987). Entretanto, há vários estudos que não confirmaram uma incidência aumentada de anormalidades fetais nos filhotes de animais tratados com TAM (Furr; Valcaccia; Challis, 1976). No entanto, esses resultados conflitantes são antigos e necessitam de uma atualização.

Dessa forma, as implicações clínicas do uso do TAM na gestação ainda são incertas. No entanto, com base no mecanismo de ação do TAM, a suposição de que essa droga pode exercer um efeito negativo sobre o feto em desenvolvimento não deve ser descartada, principalmente se a exposição ocorrer em períodos críticos do desenvolvimento, altamente sensíveis a hormônios, como é o caso do período da diferenciação sexual hipotalâmica.

Em mamíferos, para que aconteça o comportamento sexual tipicamente masculino, o hipotálamo precisa ser masculinizado, uma vez que, durante a vida pré-natal, esta região está organizada intrinsecamente do tipo feminino, determinando na vida adulta um comportamento sexual típico de fêmea (Maclusky; Naftolin, 1981). Sendo assim, a diferenciação do cérebro em machos é determinada por dois processos distintos: a defeminização e a masculinização. Em modelos experimentais, o processo de defeminização compreende a indução de esterilidade anovulatória e a redução da capacidade de apresentar lordose, já a masculinização, é definida como a capacidade de apresentar comportamento de monta (Mcewen et al., 1977).

O processo de masculinização do hipotálamo é dependente de testosterona, que por ação da enzima citocromo P450 aromatase, é metabolizada originando estrógeno no cérebro (Erskine; Tobet; Baum, 1988; Rhoda; Corbier; Roffi, 1984). Em ratos machos, ocorrem dois picos de testosterona de origem testicular, sendo o primeiro no 18º e 19º dia de gestação (Weisz; Ward, 1980), e o segundo, durante as primeiras horas após o nascimento (Baum et al., 1988; Lalau et al, 1990). Em um estudo comparativo, demonstrou-se que ocorre também uma elevação nos níveis de testosterona, após as primeiras horas do nascimento, numa variedade de mamíferos machos, corroborando a

importância dos andrógenos na diferenciação sexual do cérebro nesta classe de vertebrados (Corbier; Edwards; Roffi, 1992).

Evidências sugerem que a conversão de testosterona a estradiol pela aromatase neural, é o mecanismo responsável por regular diversos processos fisiológicos e comportamentais, como a ativação do comportamento sexual masculino, a diferenciação sexual hipotalâmica e efeitos na retroalimentação negativa sobre a secreção de gonadotrofinas (Balthazart; Ball, 1998). Dessa forma, substâncias capazes de suprimir ou retardar o pico de testosterona neonatal podem alterar o processo de masculinização e/ou defeminização do hipotálamo (Gore, 2010). Neste sentido, estudos demonstram que o estresse, a exposição a contaminantes ambientais, bem como a administração de fármacos, como o tamoxifeno, durante o período crítico de diferenciação sexual hipotalâmica pode comprometer esse processo, acarretando em alterações na fisiologia reprodutiva e no comportamento sexual (Arena; Pereira, 2002; Marques Pereira; Piffer, 2005; Negri-Cesi, 2015), as quais muitas vezes só são detectadas mais tarde, na vida adulta reprodutiva.

Desse modo, estudos que abrangem o período gestacional e pós-natal são importantes para a obtenção de informações sobre efeitos adversos que possam ocorrer durante a gestação, lactação e no recém-nascido, podendo ser prolongado até a idade de maturação sexual na presença de efeitos adversos (EMA, 2017).

2 REVISÃO DE LITERATURA

2.1 Diferenciação sexual hipotalâmica

A diferenciação do cérebro e das gônadas em masculino ou feminino acontece através de sinalizações hormonais, uma vez que, estes são órgãos bipotenciais durante o desenvolvimento embrionário (Schwarz; Mccarthy, 2008). Desse modo, o processo de determinação sexual dos mamíferos é fundamental, uma vez que o sexo genético determina o sexo gonadal e os hormônios gonadais determinam o sexo do cérebro (MCcarthy; Arnold, 2011).

Nos machos (XY), a região determinante de sexo do cromossomo Y (SRY) contém os genes necessários para induzir a formação do testículo (Koopman et al., 1990). Em fêmeas (XX), com a ausência do gene SRY, a gônada bipotente torna-se um ovário (Sinclair et al., 1990). Os testículos em desenvolvimento começam a produzir quantidades significativas de testosterona durante os últimos dias de gestação nos roedores, e já no

segundo trimestre em primatas (Rhoda et al. 1984 e Weisz; Ward 1980), responsável por formar características sexuais secundárias, incluindo o epidídimo, os vasos deferentes e o pênis (Jost, 1948). Além disso, a testosterona é convertida em estradiol, encarregado de induzir mudanças em regiões do hipotálamo, fazendo com o que o sexo gonadal seja o mesmo do cérebro (Schwarz; MCcarthy, 2008).

Antes do período crítico de diferenciação sexual hipotalâmica, o hipotálamo dos mamíferos está organizado intrinsecamente do tipo feminino, levando a um comportamento sexual típico de fêmea e um padrão cíclico de secreção de gonadotrofinas na vida adulta. Para que ocorra o comportamento sexual tipicamente masculino e um padrão tônico de secreção de gonadotrofinas, em machos, o hipotálamo precisa ser masculinizado (Maclusky; Naftolin, 1981). Desta maneira, dois processos distintos são responsáveis pela diferenciação do sistema nervoso central (SNC) em machos: a defeminização e a masculinização. A defeminização é estabelecida através da perda da capacidade de apresentar comportamento sexual feminino (lordose), ou seja, de responder aos efeitos do estradiol e da progesterona, já a masculinização é definida através da capacidade de apresentar comportamento sexual tipicamente masculino (monta) (MCEwen et al., 1977) (Figura 2).

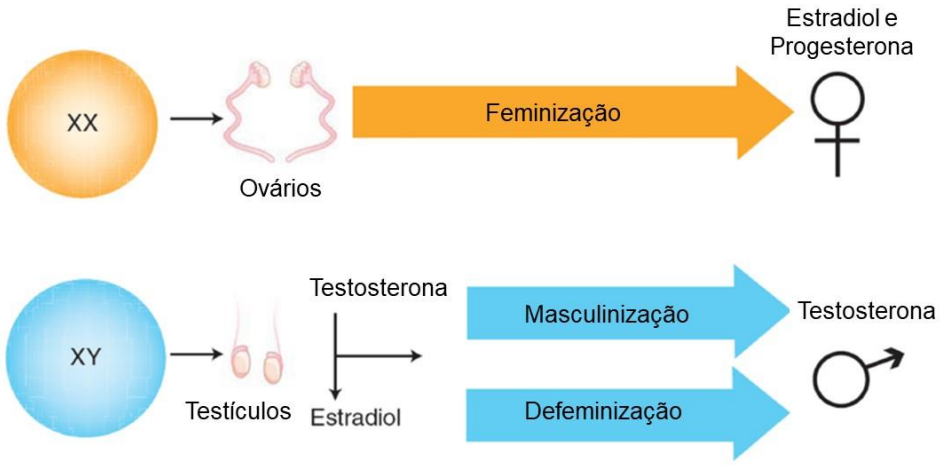

Figura 1. Processo de diferenciação sexual, no qual o sexo cromossômico determina o sexo gonadal, estabelecendo o sexo do cérebro (Adaptado de McCarthy e Arnold, 2011).

A masculinização do hipotálamo depende de testosterona e, através da ação da enzima citocromo P450 aromatase, é metabolizada e origina o estrógeno no SNC

(Erskine; Tobet; Baum, 1988; Rhoda; Corbier; Roffi, 1984). Após este processo, o estrógeno liga-se a dois subtipos de receptores de estrógeno (α e β), sendo que o α está envolvido principalmente na masculinização e o β com maior função na defeminização (Kudwa et al., 2006). A exposição à testosterona ocorre em um momento em que o cérebro está particularmente sensível à exposição a este hormônio, ou seja, definindo o período crítico para a diferenciação sexual do cérebro (Schwarz; MCCarthy, 2008).

Deste modo, tanto a testosterona como o estrógeno participam da organização cerebral masculina. Entre a sexta e oitava semana de gestação, ocorre o primeiro pico de testosterona através da gônada masculina, sendo responsável pela diferenciação da genitália. No cérebro, ocorre sua conversão em di-hidrotestosterona, responsável por estruturar as conexões cerebrais que darão origem a uma grande variedade de comportamentos masculinos (Auyeung et al., 2009). Além disso, a testosterona atua na área pré-optica (APO), que é rica em aromatase e em receptores dos esteroides sexuais, o que possibilita a conversão da testosterona em estrógeno, crucial para o processo de masculinização. Essa região também é responsável pelo comportamento sexual masculino e feminino (Hillarp; Olivecrona; Silfverskiöld, 1954; Wright et al., 2010). O estrógeno, por meio de um mecanismo complexo, participa do processo de defeminização, suprimindo as funções cerebrais femininas no homem e promovendo atitudes tipicamente masculinas.

Evidências sugerem que o papel do estrógeno no processo de masculinização ocorre mais para o fim da gestação e nos primeiros dez dias pós natal (De Vries et al., 2002; MCcarthy; Wright; Schwarz, 2009). O segundo pico hormonal ocorre na puberdade, chamado de fase de “refinamento”, uma vez que os circuitos neurais estão se organizando para facilitar a expressão do comportamento sexual (Schulz et al., 2009). Em ratos, a primeira onda de testosterona testicular ocorre entre o 18º e 19º dia de gestação e a segunda durante as primeiras horas após o nascimento (Figura 3) (MCCarthy; Wright; Schwarz, 2009; Weisz; Ward, 1980).

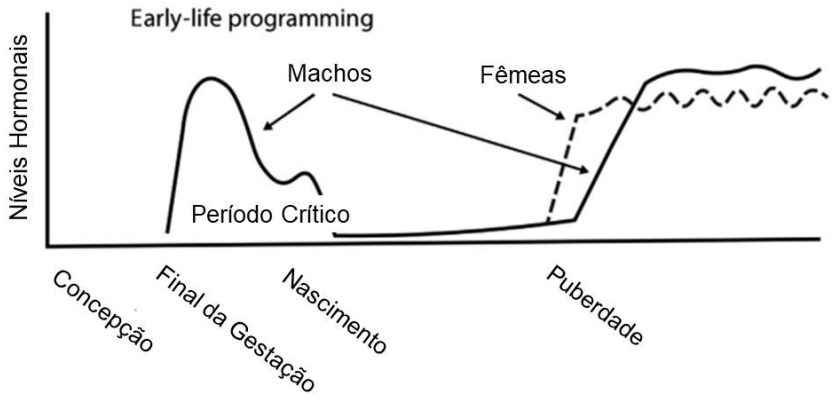

Figura 2. Níveis hormonais, definindo o período crítico para a diferenciação sexual hipotalâmica (Adaptado de McCarthy e DeVries, 2010).

Sabe-se que a aromatização da testosterona a estrógeno é um evento necessário para que ocorra a masculinização das estruturas cerebrais do SNC dos roedores, de modo que o dimorfismo sexual cerebral possa ser estabelecido (Hrabovszky; Hutson, 2002). Desse modo, durante o período crítico de desenvolvimento, o estradiol atua em diferentes regiões do cérebro induzindo mudanças permanentes relacionadas à masculinização do hipotálamo, com mecanismos distintos (Figura 4). Tais mecanismos envolvem a indução da apoptose celular, que causa diferenças sexuais em núcleos hipotalâmicos; altera a morfologia celular dos neurônios (aumentando as espinhas dendríticas); através da prostaglandina E2 (PGE2), induz diferenças sexuais no núcleo sexualmente dimórfico da área pré-óptica (SDN-POA); no núcleo ventromedial, região central do hipotálamo mediobasal, altera a função neurotransmissora; aumenta a complexidade dos astrócitos no núcleo arqueado de machos, aumentando a síntese e liberação de ácido gama-aminobutírico (GABA).(Schwarz; MCcarthy, 2008).

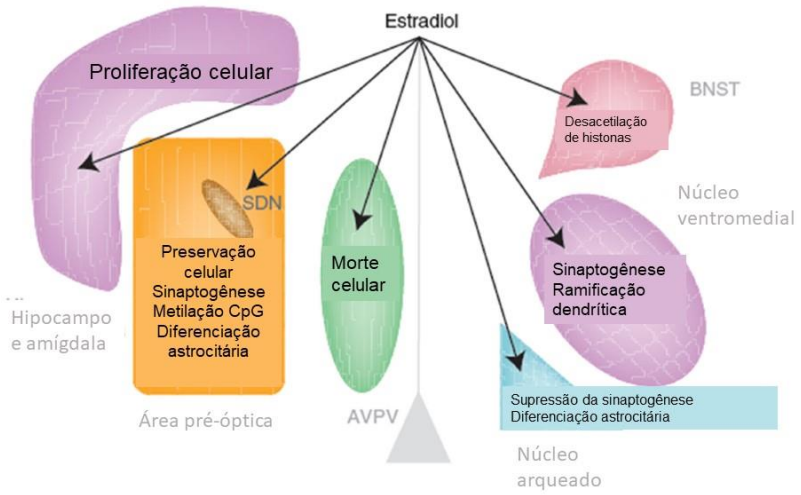

Figura 3. Mecanismos de diferenciação induzidos pelo estradiol (Adaptado de McCarthy & Arnold, 2011).

Neste sentido, o processo de masculinização e defeminização do hipotálamo pode ser alterado por substâncias que podem conseguir retardar ou suprimir os picos de testosterona neonatal. O estresse, a exposição a poluentes e o uso de medicamentos no decorrer dos períodos críticos de desenvolvimento podem comprometer esses processos, alterando o comportamento sexual e a fisiologia reprodutiva, os quais muitas vezes só serão observada na idade adulta reprodutiva (Gerardin et al., 2008; Negri-Cesi, 2015).

2.2 Desreguladores endócrinos e sua ação através de receptores de estrógeno

Os desreguladores endócrinos (DE) são compostos que interferem na síntese, secreção, transporte, recepção ou eliminação de hormônios naturais no corpo que são responsáveis pela manutenção da homeostase, reprodução, desenvolvimento e/ou comportamento (Kavlock et al., 1996). Podem ser divididos em duas categorias: substâncias naturais (estrógenos presentes no organismo, 17βestradiol, estrona e estriol, os fitoestrógenos) e os sintéticos (agentes farmacêuticos, pesticidas, fungicidas) (Diamanti-Kandarakis et al., 2009).

Os DE exibem as mesmas características de hormônios, podendo interferir ou bloquear hormônios naturais ou seus receptores, agir como antagonistas, ligando nos receptores hormonais endógeno dentro de uma célula ou imitar a ação de hormônios

naturais no corpo como estrógenos, andrógenos e hormônios da tireoide (Bergman et al., 2012).

Uma variedade de distúrbios pode estar relacionada à desregulação endócrina, tais como criptorquidia, hipospádia, oligospermia, câncer de testículo, distúrbios da ovulação, mioma uterino, entre outros. A diferenciação sexual masculina depende de androgênios (e potencialmente dependente de estrógenos), em contrapartida a diferenciação feminina ocorre independentemente de estrógenos e androgênios. Desse modo, diferentes distúrbios podem ser observados em homens e mulheres como resultado de efeitos causados por DE que, em geral, mimetizam os estrógenos e/ou antagonizam os androgênios (Sharpe, 2006).

Originalmente, achava-se que os DE exerciam suas ações principalmente por meio de receptores nucleares de hormônios, incluindo receptores de estrógeno, receptores de andrógeno, receptores de progesterona, receptores tireoidianos e receptores de retinóides, entre outros (Kabir; Rahman; Rahman, 2015). Atualmente, sabe-se que os mecanismos são muito mais amplos do que o foi primeiramente estabelecido. Podem agir através de receptores nucleares, receptores não nucleares de hormônios esteróides (RE de membrana), receptores não esteróides (receptores de neurotransmissores, como o receptor de serotonina, receptor de dopamina, receptor de norepinefrina), receptores órfãos e vários outros mecanismos que tem ação nos sistemas endócrino e reprodutivo (Diamanti-Kandarakis et al., 2009).

Muitos DE apresentam atividade estrogênica e interferem na sinalização do estrógeno, mediada por dois receptores: REα e Reβ, com papéis fisiológicos únicos na mediação da sinalização hormonal, além de serem altamente dependentes do tecido e do tipo de célula (Taylor; Al-Azzawi, 2000). A sinalização dos RE pode ser dependente de ligante ou independente de ligante. Eventos mediados por sinalizações estrogênicas são regulados pela via genômica (nuclear), seja por interações diretas RE-DNA ou através da amarração do RE ao DNA por meio de outros fatores de transcrição, e pela via não genômica (não nuclear), na qual a exposição ao estrógeno leva à rápida ativação das cascatas de sinalização da quinase (Figura 4) (Shanle; Xu, 2011).

Na via genômica, RE medeiam a regulação do gene alvo através da ligação direta aos Elementos de Resposta ao Estrogênio (ERE) ou por fatores de transcrição, como proteína de especificidade 1 (Sp1) ou proteína ativadora-1 (AP-1) (Kushner et al., 2000; Saville et al., 2000). Na sinalização genômica clássica, os ligantes se ligam ao receptor no domínio de ligação com o ligante (LBD), induzindo mudanças conformacionais,

permitindo a dimerização e a ligação ao DNA. Os ligantes podem induzir conformações que recrutam preferencialmente cofatores específicos, induzindo assim respostas diferenciais. Os cofatores de receptor nuclear mais descritos são a família de coativadores p160, SRC-1, SRC-2 e SRC-3 (Lonard; O'malley, 2007).

As ações não genômicas geralmente estão associada à ativação de várias cascatas da proteína-quinase, que podem levar a mudanças indiretas na expressão gênica por meio da fosforilação de fatores de transcrição (Vrtačnik et al., 2014). Além disso, são mais associadas a um subconjunto de RE ligado à membrana, causando a mobilização de cálcio intracelular, estimulando a atividade de adenilato ciclase e produção de AMP cíclico, ativação da via de sinalização da proteína quinase ativada por mitógeno (MAPK) e ativação de receptores de membrana de tirosina quinase (Ajj et al., 2013; Björnström; SjöBerg, 2005).

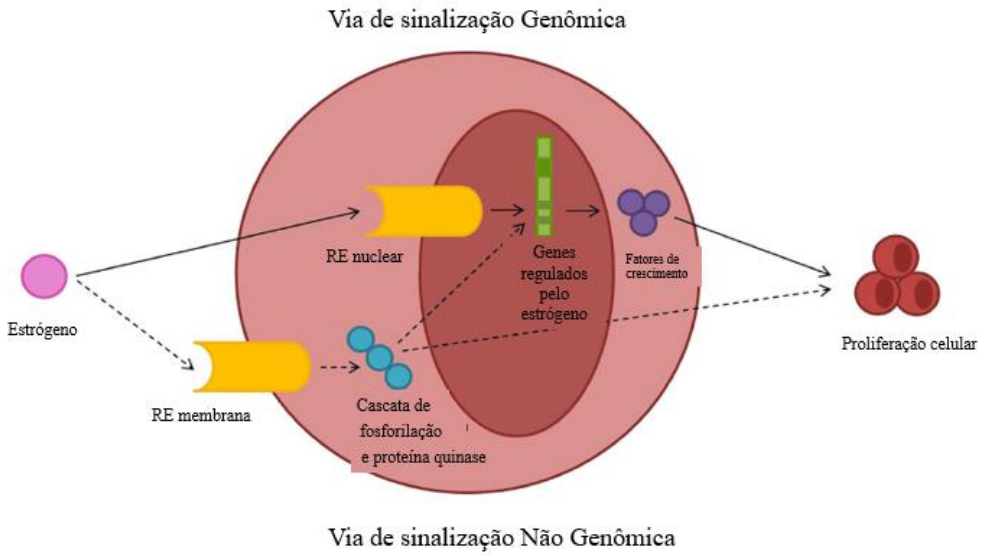

Figura 4. Ações genômicas e não genômicas do estrógeno via receptor de nuclear e associado à membrana (adaptado Schuurman et al 2019).

Além das ações genômicas e não genômicas, vários correguladores celulares são expressos, podendo aumentar ou diminuir a atividade transcricional dos receptores hormonais. Várias etapas do processo de expressão gênica envolvem correguladores, como modificação e remodelação da cromatina, iniciação da transcrição, alongamento de cadeias de RNA, splicing de mRNA, tradução de mRNA, processamento de miRNA e degradação dos complexos correguladores ativados por receptores nucleares (Lonard; O'malley, 2007). Correguladores são proteínas capazes de atuar como integradores de

sinais de hormônios esteróides e têm sido associados a muitas doenças que são afetadas por hormônios sexuais, como o câncer (Lonard; O'malley, 2006).

Moduladores seletivos do receptor de estrógeno (MSRE) são capazes de atuar em diferentes tecidos tanto como agonistas quanto como antagonistas dos receptores de estrógeno (Martinkovich et al., 2014). Além disso, são compostos adequados para compreender a sinalização específica de DE induzida por RE (Shanle; Xu, 2011). A nível molecular, os MSRE exercem sua ação como antagonista competindo com o estradiol pela ligação a uma bolsa hidrofóbica interna dentro do domínio de ligação ao ligante de ERα (Wärnmark et al., 2002). Alguns dos MSRE mais importantes incluem tamoxifeno, raloxifeno, clomifeno, ormeloxifeno e toremifeno (Farooq, 2015).

O TAM é eficaz para o tratamento de câncer de mama positivo para REα e contribui para o declínio nas taxas de mortalidade desta doença. É um antagonista do RE em células da mama, inibindo a progressão do câncer de mama mediado por REα, ademais, age como agonista deste receptor no endométrio, aumentando o risco de câncer endometrial após exposição prolongada (Swaby; Sharma; Jordan, 2007). Os efeitos específicos do TAM nos tecidos são devidos, em parte, à disponibilidade do coativador e ao contexto celular (Shang; Brown, 2002).

2.3 Tamoxifeno

O tamoxifeno (Z)-2-[4-(1,2-difenilbut-1-enil)fenoxil]-N,N-dimetil-etamina (Figura 5), fórmula molecular $C_{26}H_{29}NO.C_6H_8O_7$ e peso molecular de 563,63808 g/mol, é um modulador seletivo dos receptores de estrogênio (MSRE), com um metabolismo complexo, vários mecanismos de ação em diferentes níveis e longa meia-vida. Apresenta-se como um pó cristalino branco (Abbasalipourkabir; Salehzadeh; Abdullah, 2012; Pregnancy, 2011).

Este medicamento pertence à classe II do Sistema de Classificação Biofarmacêutica (SCB), apresentando baixa solubilidade aquosa e elevada permeabilidade membranar. Disponível na dose de 10 mg, duas vezes ao dia, ou 20 mg uma vez ao dia, por via oral, na forma de comprimido (Moy et al. 2012; Altmeyer et al., 2016). Essa droga foi aprovada pela primeira vez em 1977 pela FDA para o tratamento de mulheres com câncer de mama metastático, e nos anos seguintes, para o tratamento adjuvante do câncer de mama (Burstein et al., 2019).

Originalmente, foi desenvolvido como agente contraceptivo, mas estimulou o crescimento folicular e provou ser tão eficaz quanto o clomifeno na indução da ovulação

em pacientes com infertilidade anovulatória (Tsuiki et al., 1984). Em seguida, foi usado em conjunto com o misoprostol para induzir ao aborto na fase inicial da gestação, mas não trouxe efeitos adicionais a este medicamento (Jain et al., 1999). Atualmente, é usado no tratamento de todos os estágios do câncer de mama positivo para receptores de estrógeno em mulheres na pré e pós-menopausa, além do tratamento hormonal no câncer de mama masculino (Hayes, 2009; Hughes-Davies et al., 2009). A terapia endócrina adjuvante contra o câncer de mama com o TAM é realizada a longo prazo (por 5 anos, podendo estender para 10 anos), reduzindo o risco de recorrência da doença em 12% (45% para 33,2%) e a mortalidade em 9% (33% para 24%) (Abe et al., 2011).

Este medicamento pertence à classe dos Moduladores Seletivos do Receptor de Estrógeno (MSRE), uma vez que bloqueia a ligação do estrógeno a células específicas e pode ser classificado como agonista ou antagonista, dependendo de seu efeito e localização (MCDonnell, 1999). Vários fatores como estrutura, parceiros de ligação, unidades promotoras de genes responsivos e várias moléculas que entrarão em contato são os responsáveis por prever o efeito de um MSRE (Yang et al., 2013). Como antagonista, o TAM inibe a proliferação das células cancerígenas nos tecidos mamários; sua ação como agonista se dá a nível ósseo, prevenindo a osteoporose; atua como agonista no útero, elevando o risco de hiperplasia endometrial e malignidade (Park; Jordan, 2002).

No tecido mamário, agindo como antagonista, o TAM se liga aos receptores de estrógeno (RE) das células cancerígenas e induz mudanças na estrutura tridimensional do receptor, impedindo a ligação do estrógeno com o estrógeno responsivo do DNA. Desse modo, estimula o aumento da produção celular de TGF-β, inibidor do crescimento tumoral, e diminui tanto a síntese de IGF-1, fator de crescimento das células tumorais da mama, como a produção de TGF-α (SANTEN *et al.*, 1990; YAMAMOTO, 1985). Outros mecanismos envolvem a inibição da proteína quinase C e da calmodulina (Scholar, 2010). Além disso, pode bloquear a fase G1 do ciclo celular das células cancerígenas que estavam em divisão (Altmeyer et al., 2016).

Após a administração oral, este fármaco é rapidamente absorvido, adquirindo concentrações séricas máximas entre 3-7 horas e estado de equilíbrio dinâmico entre 4 a 6 semanas de uso. O TAM possui uma meia-vida de sete dias, porém a de seus metabólitos é mais longa, cerca de 14 dias (Astrazeneca Pharmaceuticals Lp, 2004).

Sua metabolização ocorre no fígado, através de um longo metabolismo de primeira passagem, por meio de vários metabólitos secundários e primários. Alguns dos seus metabólitos exibem um efeito antiestrogênico com mais afinidade nas células de

câncer de mama do que o próprio tamoxifeno. A principal via é a N-desmetilação do tamoxifeno pelas enzimas CYP3A4 e CYP3A5, resultando na formação de N-desmetiltamoxifeno. O N-desmetiltamoxifeno pode ser hidroxilado pelo CYP2D6 em endoxifeno, um metabólito muito potente em termos de atividade antiestrogênica com altos níveis plasmáticos. Outra via metabólica é a hidroxilação do tamoxifeno em 4-OH-tamoxifeno pelo CYP2D6. A atividade antiestrogênica do 4-OH-tamoxifeno é aproximadamente 30 a 100 vezes maior que a do TAM e comparável à do endoxifeno, no entanto, as concentrações plasmáticas de 4-OHtamoxifeno em humanos são cerca de seis vezes menores que as do endoxifeno (Desta et al., 2004; Shagufta; Ahmad, 2018).

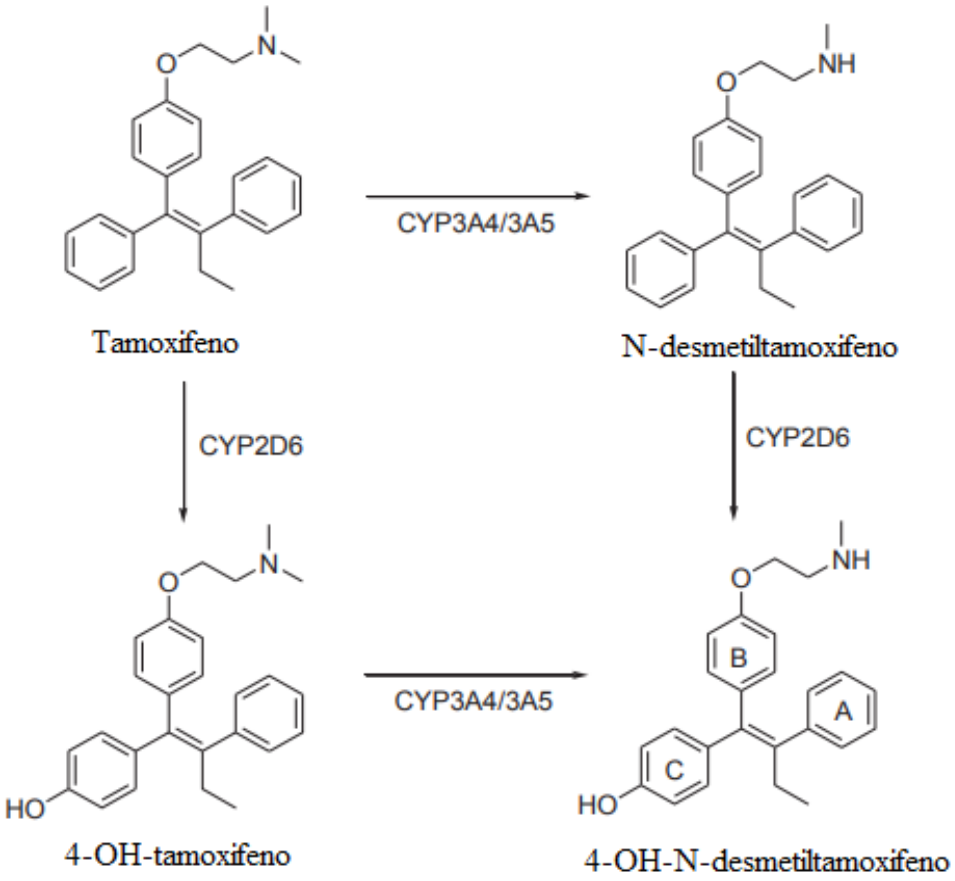

Figura 5. Tamoxifeno e seus metabólitos (Adaptado de Shagufta, 2018).

Logo após a circulação entero-hepática, os metabólitos que foram conjugados em ácido glicurônico são excretados no intestino através do ducto biliar, com a reabsorção e recirculação entero-hepática de seus metabólitos hidrolisados e conjugados em TAM ocorrendo logo em seguida. A excreção dos metabólitos que não foram reabsorvidos são

excretados pelas fezes, com taxas mínimas pela urina (Craig Jordan, 2006; Dickschen et al., 2012).

Embora tenha efeitos benéficos para o tratamento de câncer de mama, suas reações adversas incluem: sintomas da menopausa, ondas de calor, menstruação irregular, toxicidade ocular, eventos tromboembólicos, trombocitopenia ou leucopenia, complicações ginecológicas, câncer endometrial (baixo grau), hiperplasia e pólipos endometriais, cistos ovarianos (Osborne, 1998).

O uso deste fármaco não é recomendado durante a gestação e lactação, uma vez que apresenta níveis clinicamente significativos do composto original e seus metabólitos no leite humano (Alessandro Peccatori et al., 2020). Quando a gravidez acontece acidentalmente durante o tratamento, os efeitos no feto e na gravidez são incertos, aumentando o risco de malformação fetal (Burstein et al., 2019).

A recomendação para interromper o tratamento com tamoxifeno durante a gestação é baseada nos efeitos encontrados em três relatos de caso (Berger; Clericuzio, 2008; Cullins; Pridjian; Sutherland, 1994; Tewari et al., 1997). A exposição ao fármaco durante o primeiro e segundo trimestre resultou em uma criança com síndrome de Goldenhar, microtia do lado direito e microssomia hemifacial (Cullins; Pridjian; Sutherland, 1994). Além disso, foram relatados 11 bebês com malformações congênitas, como hipertrofia clitoriana leve, adenoma vaginal, fenda palatina, trissomia do cromossomo 21 e defeitos craniofaciais (Braems et al., 2011; Hartmann et al., 2008). No entanto, há casos relatados de bebês saudáveis, mesmo com exposição durante ou após o primeiro trimestre (Ishizuka; Satou, 2016; Jyoti et al., 2016; Koca et al., 2013).

Não foi observado indícios de teratogenicidade em ratos, coelhos e saguis em um estudo conduzido pelo fabricante. No entanto, a exposição a este medicamento durante o início da gravidez resultou em um aumento na taxa de aborto espontâneo em saguis e coelhos prenhes (Astrazeneca Pharmaceuticals Lp, 2004; Furr; Valcaccia; Challis, 1976). Quando administrado após o primeiro trimestre, o TAM causou alterações tóxicas no desenvolvimento do trato genital em algumas espécies de animais (Hines et al., 1987; Nguyen et al., 1986).

No ensaio de toxicidade subaguda (Test No. 407: Repeated Dose 28-day Oral Toxicity Study in Rodents, 2008), a Dose Máxima Tolerada (DMT) do TAM, tanto em machos quanto em fêmeas, em ratos é de 200 µg/kg, o que reduz o ganho de peso corporal, além de leves alterações nos sinais clínicos, nos padrões hematológicos e sorológicos e alterações na histologia de tecidos endócrinos. Essa dose também foi capaz de aumentar

o peso relativo do testículo e epidídimo, e diminuir o peso relativo do ovário e utero. Em machos, 5 µg/kg representa o NOAEL (No Observed Adverse Effect Level - Nível Sem Efeitos Adversos Observáveis) (Cho et al., 2003; Kennel et al., 2003).

Além disso, utilizando o protocolo para detectar o potencial como desregulador endócrino em ratas imaturas durante o período de maturidade sexual, a dose de 200 µg/kg de TAM alterou o ciclo estral das fêmeas, prolongando os dias em diestro e diminuiu o peso do ovário e do útero. Doses mais baixas (10 e 50 µg/kg) alteraram os padrões hormonais de E_2, TSH e T_3 (Kim et al., 2002).

Desse modo, as consequências do uso do TAM durante a gestação ainda são desconhecidas. Levando em consideração o mecanismo de ação do TAM, é de extrema importância avaliar os efeitos resultantes do seu uso durante este período crítico de desenvolvimento.

3 OBETIVO

GERAL

Avaliar os efeitos resultantes da exposição materna ao tamoxifeno sobre o desenvolvimento sexual e as possíveis repercussões tardias sobre parâmetros reprodutivos e comportamentais em ratos machos.

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5 APÊNDICE

Artigo 1: Maternal exposure to tamoxifen: late repercussion on reproductive and behavioral parameters in adult male rats

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Maternal exposure to tamoxifen: late repercussion on reproductive and behavioral parameters in adult male rats

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Abbreviations

AGD	Anogenital distance
AR	Androgen receptor
BnST	Bed nucleus of the stria terminalis
CNS	Central nervous system
EDCs	Endocrine disruptor chemicals
ER	Estrogen receptor
FSH	Follicle stimulating hormone
GABA	Gamma aminobutyric acid
GD	Gestational day
LD	Lactational day
LH	Luteinizing hormone
mPOA	Medial preoptic area
NMDR	non-monotonic dose response
PGE2	Prostaglandin E2
PND	Postnatal day
POA	Preoptic area
PVH	Paraventricular nucleus of the hypothalamus
SERM	Selective estrogen receptor modulator
SDN-POA	Sexually dimorphic nucleus of the preoptic area
TAM	Tamoxifen
VMH	Ventromedial nucleus of the hypothalamus

Highlights

- Tamoxifen exposure delayed puberty installation.
- There was an increase in sperm count of cauda epididymis.
- Sperm motility was adversely affected.
- Increase in the number of hyperplasia of epithelial clear cells in cauda epididymis.

Abstract

Tamoxifen, a selective non-steroidal estrogen receptor modulator, is used in patients with breast cancer positive for hormone receptors as an adjuvant endocrine therapy. Since information on the risk of using tamoxifen during pregnancy is still scarce, further studies are required. The aim of this study was to evaluate the possible effects of tamoxifen on *in utero* and lactational exposure and its late repercussions on reproductive parameters in male rats. Pregnant rats were exposed to three doses of tamoxifen (0.12; 0.6; 3 µg /kg), via gavage, from gestational day 15 to postnatal day 20. After birth, the following parameters were used to evaluate the male offspring: body weight, anogenital distance (AGD), ages of preputial separation and testicular descent, and play behavior. In adult life, organs weight, sperm parameters, histological procedures and histopathological evaluation, fertility test, male and female sexual behavior, sexual preference and androgen receptor expression in hypothalamic neurons were analyzed. Tamoxifen exposure did not altered the AGD in the male offspring; however, there was a significant increase in the body weight in the 0.12 µg/kg dose and a decrease in the 0.6 µg/kg dose. The male offspring treated with the highest dose exhibited a delay in the onset of puberty, evidenced by an increase in the age of preputial separation. Regarding sperm parameters, there was an increase in the sperm count in the cauda epididymis in the intermediate and highest dose groups, in addition to an increase in the number of static sperm and a decrease in the progressive sperm in the same groups. Moreover, histopathological alterations were observed in the epidydimis. These results indicated that maternal exposure to tamoxifen compromised the sexual development and altered reproductive parameters of the male descendants, acting as an endocrine disruptor.

Keywords: Hypothalamic sexual differentiation, sexual development, reproductive parameters.

1. Introduction

Breast cancer is the most common cancer in women worldwide and a major public health concern [1]. It is predominantly a disease of aging, with increased diagnoses in women under 40 in several countries, approximately 11% of new cases diagnosed in women 45 years of age or younger [2]. Progress in diagnosis and treatment has caused a sharp decline in the death rate, especially in young women, leading to younger breast cancer survivors [3]. The tendency to delay pregnancy and this rising incidence is increasing the number of women with the dilemma of dealing with a potentially fatal condition and the desire to have children.

Tamoxifen (TAM), a selective estrogen receptor modulator, is used routinely as an adjuvant endocrine treatment in hormone receptor-positive breast cancer, with the recommended treatment taking up to 5 years [4]. This drug is not recommended during pregnancy and/or lactation, as it has clinically significant levels of the parent compound and its metabolites in human milk [5].Therefore, doctors are required to advise the use of contraceptives for women taking this medication, alerting about its teratogenicity and/or fetal adverse effects. If unintended pregnancy occurs while using TAM, the consensus is to stop treatment immediately and the risks should be discussed. In women who wish to become pregnant, it is necessary to wait for the total excretion of TAM, which takes about three months [6,7].

This drug has mixed agonist/antagonist actions with estrogen receptor [8]. Estrogens play an important role in regulating the structure and function of many neuronal systems, contributing to sexual differentiation of the brain, mediated by estrogen receptors (ER), with two subtypes ERα and ERβ [9]. ERα and ERβ are present in the arcuate nucleus and in the preoptic area, while ERα is present in the ventromedial nucleus of the hypothalamus (VMH) and ERβ in the paraventricular nucleus of the hypothalamus (PVH), important regions for reproduction and sexual behavior [10].

Gonadal hormones act on the neonatal brain, mainly in the hypothalamus, causing sexual differences that ground reproductive physiology and adult behavior [11]. Two distinct processes are needed to differentiate the central nervous system in order to have a typically male brain: defeminization and masculinization. Defeminization is the loss of the ability to respond to the effects of estradiol and progesterone, that induces female sexual behavior (lordosis), whereas masculinization is defined through the ability to exhibit typically male sexual behavior (mounts) [12]. This process depends on testosterone. In male rats, two testosterone peaks of testicular origin are important, the first between the 18 and 19 gestational day (GD), and the second, during the first hours after birth [13,14]. Testosterone, through the action of aromatase cytochrome P450 enzyme, is metabolized to estrogen in the central nervous system (CNS) [15].

Through different mechanisms, estradiol induces permanent changes in different regions of the brain during critical periods of development. A possible mechanism is restricted to pre-existing cell types or signaling pathways in each brain region: PGE2 in the preoptic area (POA), Gamma Aminobutyric Acid (GABA) in the arcuate and glutamate in the ventromedial nucleus (VMN) of the hypothalamus. In addition, multiple mechanisms of exposure to estradiol during development are necessary to establish multiple functional outcomes in adulthood [16].

Therefore, substances capable of interfering with the action of estradiol during critical periods of development, such as TAM, may alter reproductive physiology and sexual behavior. The aim of this study was to evaluate the consequences of maternal exposure to tamoxifen on sexual development and possible late repercussions on reproductive and behavioral parameters in the male offspring.

2. Materials and Methods

2.1 Animals

Ten adult males (90 days old, weighing approximately 350g) and 32 females (80 days old, weighing approximately 250g) *Wistar* rats were obtained from the Central Biotherium of São Paulo State University (UNESP), and kept under controlled conditions (23°C, 12 hours light/12 hours dark cycle) with food (commercial chow phytoestrogen-free; Nuvilab CR1- Nuvital-Paraná, Brazil) and water *ad libitum*. The experimental procedures were in accordance with the Ethical Principles on Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) and were approved by the Ethics Committee for Animal Experimentation (CEEA) of the Biosciences Institute of Botucatu (UNESP) (protocol number: 1157/2019). The experimental protocol is in Figure 1.

Two nulliparous female rats were mated with one male, at the dark cycle of photoperiod. Gestational day (GD) 0 was considered when the presence of sperm was detected in the vaginal smear of the females. Pregnant rats were weighed and distributed in the experimental groups and maintained in individual cages. The method of determining sample size was based in the "tradition" or common sense", confirmed by the resource equation [17].

2.2 Experimental groups and treatment

Pregnant rats were distributed into four experimental groups (n=8/group). For the evaluation of the effects of tamoxifen (Z)-2-[4-(1,2-difenilbut-1-enil)fenoxil]-N,N-dimetil-etamina, Cruz Vermelha Pharmacy (98% purity), three groups were treated with doses of 0.12; 0.6; or 3.0 µg/kg/day, and the control group received only the vehicle (distilled water), from GD 15 until the end of lactation, orally by gavage. The offspring

were exposed to tamoxifen via placenta and through breastfeeding. The treatment period is equivalent to the critical period of hypothalamic sexual differentiation [9]. The doses were based on a previous study that used similar doses and demonstrated a delay in the onset of puberty [18]. During treatment, pregnant and lactating rats, maintained in individual cages, were weighed on alternate days to calculate the volume of TAM to be administered and clinical and behavioral signs of toxicity (body weight, diarrhea, piloerection, bleeding, abnormal breathing, tremors, convulsions and changes in gait, posture and reaction to manipulation), as well as the average daily intake of water and food. After birth, the number of pups per litter was reduced to eight, four males and four females. The males were kept until adulthood to assess sexual development and reproductive parameters.

2.3 Maternal parameters

2.3.1 Maternal behavior

This parameter was evaluated according to the Montagnini et al. (2016)[19]. On the lactacional day (LD) 5, the pups were removed from the cage and the nest destroyed. After 30 min, they were returned to the cage and the mother-pup interaction recorded on video for 30 min. Latency for retrieval behavior and total time of grouping, pup grooming, off pups (defined as the time the rat spent without any type of interaction with the pups, regardless of their position in the cage, were evaluated), and the building of the nest were analyzed.

2.3.2 Hematological parameters and maternal biochemical analysis (n=8/group)

After weaning, dams were anesthetized with sodium thiopental 50 mg/kg (i.p.) and the blood collected by cardiac puncture. The serum obtained after centrifugation

(1200 rpm for 20 minutes) was used to measure Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Gamma-glutamyl transferase (Gamma-GT), Alkaline phosphatase (FA), Urea, Creatinine, Calcium, Cholesterol, Total Protein and Albumin. These parameters were determined using the BioPlus 2000 semiautomatic equipment, using Bioclin kits. For hematological analysis, the total and differential count of leukocytes, erythrocytes and platelets were performed, in addition to hemoglobin, hematocrit and erythrocyte distribution widths. With the exception of hemoglobin levels, which were determined using BioPlus 2000, the remaining hematological parameters were analyzed manually. The organs (thyroid, heart, lung, liver, kidney, adrenal, spleen, uterus, and ovary) were also collected, weighed and analyzed for external morphology.

2.5 Male offspring parameters

2.5.1 Initial sexual development and external examination at puberty (n=8 litters/group)

The male pups were weighed and the anogenital distance (AGD) measured at PND 1, 13 and 22. The relative AGD distance was calculated by the ratio between the AGD and the cube root of body weight [20]. To confirm the beginning of sexual maturity, the bilateral descent of the testis was inspected through visualization and palpation of the scrotum, from PND 15. From PND 30, preputial separation was manually checked to verify the retraction of the prepuce.

2.5.2 Play behavior (n=8/litter/group)

This test was performed according to Zaccaroni et al. (2018) [21]. The animals were evaluated between PND 40 and 45. Four males per litter were observed during the dark cycle, under red light, and their behavior recorded on video for 15 minutes. Social behaviors and non-social behaviors were evaluated.

2.5.3 Organ Weights (n= 8/group)

At PND 90, male rats from each group (1/litter) were anesthetized with sodium thiopental 50 mg/kg (i.p.). Through cardiac puncture, blood was collected for hormonal dosage and organs such as testis, epididymis, seminal vesicle, vas deferens, prostate, thyroid, liver and kidney were removed and weighed on analytical balance. The right testis and epididymis were stored for sperm analysis.

2.5.4 Sperm count in the testis and epididymis (n=8-9/group)

As described previously by Robb et al. (1978) [22] and with adaptations adopted by Fernandes et al. (2007) [23], homogenization-resistant sperm and sperm in the caput/corpus and cauda of the epididymis were counted. The testis was decapsulated and weighed after collection, and homogenized in 5 mL of 0.9% NaCl containing 0.5% Triton X 100 0.5%. The samples were diluted 10 times and transferred to Neubauer chambers, counting mature spermatids. To calculate the daily sperm production (DSP), the number of spermatids in stage 19 was divided by 6.1 (number of days in the seminiferous cycle in which these spermatids are present in the seminiferous epithelium). Portions of the caput/corpus and cauda epididymis portions were cut into fragments and homogenized, and the sperm count was proceeded as described for the testis. The transit time of sperm through the epididymis was determined by dividing the number of sperm in each portion of the DSP.

2.5.5 Sperm morphology (n=8-9/group)

To evaluate the sperm morphology, the interior of the right vas deferens of rats was washed with the aid of a needle and syringe, with 1.5 mL of saline solution. Smears were performed on histological slides, and 200 sperm per animal were evaluated under

light microscopy (400 X magnification). The morphological abnormalities found in the sperm were classified into abnormalities of the head and tail [24].

2.5.6 Sperm motility (n=5/group)

Analysis of motility from cauda epididymis sperm was performed as described by Perobelli et al. (2010) [25]. A 10 mL aliquot of sperm suspension was immediately transferred to a Makler chamber maintained at 34 ºC. Using a phase contrast microscope (400X magnification), 100 sperm were counted and classified as Type A (motile with progressive movement), Type B (motile without progressive movement), or Type C (static).

2.5.7 Histological procedures and histopathological evaluation (n=5/group)

Left testis and epididymis were fixed in Alfac fixative solution (alcohol, acetic acid and formaldehyde) for 24 h and processed as described by Balin et al. [26].

Leydig cell nucleus volume and count: Leydig cell nuclei were counted in 10 random fields in each histological section of the testis. The mean core diameter of the Leydig cells were measured for the calculation of their volume. For this, 50 random nucleus (circular or elliptical) were measured per animal [27]. The larger (D) and smaller (d) diameter of the cell nuclei were obtained using a Nikon E-200 (X40) Microscope coupled to a digital camera and computer with NisElements software (version 4.20 for Windows). The mean diameter (M) was calculated using the formula $M = (D \times d)/2$, the nuclear area (A) and the volume (V) were obtained using the following formulas: $A = \pi \times ¼ \times M2$ and $V = \pi \times ⅙ \times M3$, respectively [28].

The histopathological analysis of the epididymis was performed quantitatively, classifying 100 histological sections of the epididymal tubules into normal and abnormal.

The abnormalities evaluated were classified according to De Grava Kempinas and Klinefelter [29], evaluating the appearance of the epithelium and interstitium as well as the luminal content.

2.5.8 Fertility test (n=8/group)

Male rats at PND 80 were paired with untreated females in their cages (1 female/male), late in the afternoon. On the following morning, vaginal smears were collected, and the day on which the presence of sperm was found in the smear was considered the GD 0. On GD20, the females were killed for the collection of the uterus and ovaries to record the number of corpora lutea, implants, reabsorptions, live and dead fetuses. From these results, these parameters were calculated: the fertility potential (efficiency of implantation): implantation sites/corpora lutea x100; gestation rate: number of pregnant females/number of inseminated females x100; pre-implantation loss rate (number of corpora lutea - number of implantations/number of corpora lutea X100); post-implantation loss rate (number of implantations - number of live fetuses/number of implantations x100), and sex ratio (number of male fetuses/number of female fetuses).

2.5.9 Sexual behavior

Two weeks after fertility test, adult rats, now sexually experienced, were anesthetized with Xylazine (10 mg/kg) and Ketamine (25 mg/kg) and bilaterally castrated. The experiments started after a period of 15 days for adaptation of the animals to the inverted light/dark cycle and recovery from surgery. To assess sexual behavior, the animals were placed in observation cages, during the dark period of the light-dark cycle, under light with a red filter.

The castrated animals received testosterone propionate 1mg/day (s.c.), three times a week, for 15 days, the first injection was administrated the day after the surgery and the last one was given the day before the male sexual behavior. The female sexual behavior test started 15 days after the male sexual behavior, with the same animals.

2.5.10 Male sexual behavior (n=9/group)

Male rats were placed individually in cages of polycarbonate crystal, staying for at least 5 min for adaptation. Then, a female rat in natural estrus was introduced into the same cage. The evaluation was concluded if, at the end of 10 min, the males did not mount and it was considered that this animal was sexual inactive. The following parameters were observed for 30 min: latency for the first mount, intromission, and ejaculation; latency to the first mount and intromission after first ejaculation; number of mounts and intromissions until the first ejaculation, total number of mounts, intromissions and ejaculations during the test [30].

2.5.11 Female copulatory behavior in the male offspring (n=9/group)

Experimental male rats were treated with estradiol 17-benzoate 20 µg/Kg (i.p.), 24 hours prior to the test [31]. Untreated and sexually experienced males were placed individually in acrylic cages, where they remained for 5 min for adaptation. Then, the male from an experimental group was placed in the cage. For 10 min the presentation of lordosis and acceptance of the mount by the males of the experimental groups was evaluated in the presence of an untreated and sexually experienced male.

2.5.11 Sexual partner preference (n=9/group)

This test was performed in a rectangular arena (50 x 50 x 100) with two small arenas (25 × 15 cm) positioned on opposite sides, where the stimulus animals were positioned. One arena contained one sexually experienced adult male and the other an oestrus female rat. The tested rats did not have direct contact with the stimulus animals and the incentive was the odor that the stimulus animals exhale. The arena floor in front of each stimulus animal was demarcated in male and female incentive zones (30 × 20 cm). The experimental animal was placed in the center of the arena and the following parameters measured for 20 minutes: number of visits to each of the sexual incentive zone, total time spent in each of the zones, duration of each visit to each incentive zone. After the end of the test, a sexual preference pattern score was calculated for each animal (time spent in female zone/total time spent in both incentive zones) [32].

2.5.12 Androgen receptor (AR) expression in hypothalamic neurons (n=8/group)

2.5.12.1 Extraction and quantification of protein

Hypothalamus frozen samples were mechanically homogenized with RIPA buffer, plus proteases inhibitor (Sigma-Aldrich®, USA), in a Tureaux type homogenizer (Ultra Stirrer-Ultra80) in 3 cycles of 10 s around 4 °C. The homogenate was centrifuged at 14,000 rpm for 20 min at 4 °C, and the supernatant was collected. Protein concentration was determined by the Bradford method [33] on 96-well polystyrene plates and reading of absorbance was performed on Biochrom microplate reader (Holliston, Massachusetts, USA).

2.5.12.2 Western blotting

Aliquots containing 5 μg of proteins samples (eight samples) were separated on SDS-PAGE. Following the electrophoresis, the proteins were transferred to nitrocellulose

membranes or Polyvinylidene Difluoride (PVDF) (only Caspase-3-cleaved). The nonspecific binding of proteins was blocked by incubating the membrane in 5% non-fat milk in TBST buffer for 90 min at room temperature. The membranes were incubated with the respective primary antibody in 1% non-fat milk or 3% BSA (only Caspase-3-cleaved) in TBST (1: 350-1,000) overnight at 4 °C: AR (N20) (sc-816- Santa Cruz® Biotechnology, Inc., USA) and ß-Actin (sc-47778-Santa Cruz® Biotechnology, Inc., USA).

The membranes were then incubated with a specific secondary antibody conjugated with peroxidase, which was diluted (1:10,000–20,000) in TBST for 1 h (IgG goat-antirabbit, ab97051 and IgG goat-anti-mouse, ab97023, Abcam® Inc., USA). The immunoreactive components were revealed by GE Amersham ECL chemiluminescent substrate (GE Healthcare). Analyses were done in eight different biological samples per group. The optic density of band was used as the unit of measure with software Image J (version 1.33u—National Institutes of Health, USA), and normalized by β-actin values.

2.6 Statistical Analysis

Values are expressed as mean ± SEM or median (Q1 – Q3). Statistical analysis of variance tests - ANOVA were used to compare the results between the experimental groups, with a post hoc Tukey-Kramer test, or the Kruskal-Wallis, followed by a post hoc Dunn test. The differences were considered significant with $p<0.05$, performed on the GraphPad InStat (version 3.02).

3. Results

Females treated with TAM during pregnancy and lactation showed no signs of toxicity. In addition, there was no statistical differences in water and food consumption, in weight gain, as well as in maternal behavior, organ weights or biochemical parameters in relation to the control group (Supplementary material).

Exposure to TAM *in utero* and during lactation did not show alterations in the anogenital distance (AGD) (Figure 2). However, there was a significant increase in the body weight of the male offspring exposed to the lowest dose (0.12 µg/kg), and a decrease in the intermediate dose group (0.6 µg/kg) (Figure 3). The age of testicular descent did not change among groups, but the animals exposed to the highest dose (3 µg/kg) had a delay in the age of preputial separation (Figure 4A). No statistical difference was observed in the evaluation of play behavior among experimental groups (Figure 5).

At PND 90, the exposure to TAM did not interfere with body weight, relative and absolute organs weight (Table 1), and sperm morphology (Table 3). However, there was an increase in sperm number in the cauda of the epididymis (0.6 µg/kg and 3 µg/kg) (Table 2) and in the percentage of static sperm in the intermediate dose group, as well as a decrease in progressive sperm, which was also demonstrated in the group highest dose group (Figure 6). Histopathological analysis of the epididymis showed an increase in the number of hyperplasia of epithelial clear cells (0.6 µg/kg and 3 µg/kg) (Figure 7). TAM exposure did not interfere with Leydig cell count mean in ten units of interstitial tissue, as well as the volume and area of these cells (Figure 8).

The other parameters evaluated such as fertility test (Table 4), male and female sexual behavior (Table 5), and sexual preference (Table 6), did not present differences among groups. In addition, none of the animals were considered sexually inactive. TAM

exposure did not influence the expression of androgen receptors in the hypothalamus (Figure 9).

4. Discussion

Since there are a limited number of case demonstrating potential adverse effects of TAM on the fetus, its use still is considered contraindicated during pregnancy. However, the disadvantage of delaying or discontinuing TAM for maternal prognosis is unclear [4]. Our results carry an important contribution in this subject, once it showed that TAM exposure in the brain sexual differentiation period compromised important reproductive parameters in the male offspring.

Despite the benefits for the treatment of breast cancer, TAM causes a number of side effects, such as hot flashes, irregular menstruation, ocular toxicity, sleep disorders, depression, gynecological complications and, occasionally, adverse events such as endometrial hyperplasia or endometrial cancer and venous thromboembolic disease [34]. However, it is also important to evaluate the maternal toxic effects during a possible pregnancy.

Milder signs of toxicity may appear during treatment with several chemical compounds, including loss of body mass, diarrhea, piloerection, and changes in behavior, while more severe signs can lead to the death [35]. In our study, the treatment with different doses of TAM during pregnancy and lactation did not result in maternal toxicity, since no clinical signs or symptoms of toxicity were observed. Studies that associated exposure to TAM with maternal drug toxicity have shown similar results [36,37], reinforcing the maternal safety in the use of this medication in similar doses. However, the absence of signs of maternal toxicity does not necessarily reflect on the protection of the fetus [38], which needs to be further investigated.

AGD in rats may be a biomarker of androgen exposure, being an important parameter for assessing early sexual development [39]. Testosterone is responsible for sexual differences in animals through several parameters, including AGD [40]. Shorter

AGD can indicate an inadequate action or release of testosterone and can be an early indication of impaired sexual activity in adulthood [41]. In this study, TAM exposure during both testosterone peaks was not able to alter the AGD at any of the ages evaluated. However, alterations in body weight of the offspring in different groups were observed, which was normalized on the day of the preputial separation. A study with lasofoxifene, a drug of the same class (SERM) of TAM, demonstrated a decrease in the body weight of the offspring in all groups treated from the first week of life until the post-weaning period [42]. Other authors have not observed differences in the body weight of the offspring that were exposed to doses similar to ours via placenta or milk [18,36], demonstrating a controversy in the results. Nonetheless, non-monotonic dose-response relationships (NMDR) are regularly associated with endocrine disruptors (EDCs) [43].

Preputial separation in rats is an androgen-dependent event and an external indicator of the onset of sexual maturity [44]. Therefore, a delay in this event, observed in this study, is connected to antiestrogenic substances with consequences for fertility [45]. Alterations on the day of preputial separation were also detected in rats that received several chemicals related to the endocrine system, including estrogens and androgens [46–49]. Delayed puberty in rodents is usually related to delayed maturation or inhibition of the function of the hypothalamic-pituitary-gonadal axis, with consequences for spermatogenesis, concentration or action of LH (Luteinizing Hormone) and FSH (Follicle-Stimulating Hormone), and sexual behavior [50]. Therefore, it was possible to observe an impairment of TAM in the installation of puberty in male offspring when administered in higher doses in pregnant and lactating rats, evidencing the need to investigate other reproductive parameters.

Sex steroid hormones act mostly via nuclear receptors, such as ER and androgen receptor (AR), controlling the development of neural circuits and regulating the

hypothalamic-pituitary-gonadal axis [51]. ER-α are present in the prenatal development of the reproductive tract in rodents, also in the non-differentiated gonad, testicular cells, vas deferens and epididymis, showing its importance for sexual development [52–54]. In addition, ER-β is highly present throughout the male reproductive tract and in almost all cell types in the testis, efferent ducts and epididymis [55]. Besides being present in the primordial stages of the development of the male reproductive tract of rats, estrogen participates in the negative feedback that controls the hypothalamic-pituitary-testis axis, regulating the secretion of LH and FSH [56]. The control of the levels of these hormones is essential for spermatogenesis, therefore, the excess or deficit of the action of estrogen can cause deregulations in this process [57].

Sperm count is a good marker of impaired testicular function or alterations in epididymal capacity [58]. The epididymis has several roles, such as sperm maturation, sperm transport, and formation of a sperm reserve [59]. The sperm transit time through this organ has a major importance for the production of functional gametes, since during sperm maturation the gametes acquire motility and the ability to suffer acrosome reaction [60]. In this study, the exposure to TAM increased the number of sperm in the cauda epididymis in the intermediate and higher dose groups. Adult rats that received diethylstilbestrol (a non-steroidal estrogenic compound) had a decrease in the sperm reserves of the epididymis, showing that alterations mediated by estrogen can cause changes in sperm transit [61]. Since diethylstilbestrol is a drug that mimics the effects of estrogen, it is possible to conclude that the decrease in tamoxifen-mediated activation of estrogen receptors may have influenced the increase in sperm reserve in treated rats.

Knockout mice for the estrogen receptor showed a decline in sperm motility, demonstrating that modifications in the action of this hormone can cause problems in the spermatogenesis process [62]. Additionally, estrogen affects the transport and maturation

of sperm in the epididymis, essential processes for sperm to gain motility and ability to fertilize [63]. In the present study, exposure to different doses of TAM significantly altered the pattern of sperm motility obtained from the cauda epididymis. Therefore, treatment with a selective estrogen receptor modulator can influence sperm formation and maturation. Furthermore, defects in fluid reabsorption from the efferent duct may be responsible for altering the epididymal fluid milieu, which could have negatively affected sperm function in our study, since ER-α knockout epididymis failed to acidify the luminal milieu which led to defects on intracellular pH and sperm motility [64].

In the intermediate and highest doses groups, the epididymis showed histopathological alterations, such as clear cell hyperplasia, especially found in the epididymis cauda. Clear cells are responsible for phagocytosing the cytoplasmic droplets, eliminated from the maturing sperm, as well as possibly phagocytosing other luminal debris [29]. The hyperplasia of these cells may be associated with an increase in the number of lysosomes as they are undergoing more endocytosis, in addition to being associated with a delay in the development of this organ [65].

Leydig cells are the main sites of testicular androgen production and its maintenance of normal functions is crucial for the reproductive capacity and fertility of males [66,67]. There is evidence that the steroidogenesis of leydig cells is directly inhibited by treatment with substances that mimic the effect of estradiol and exposure of male neonatal rats to estrogen leads to an interruption in the development or absence of leydig cells in the mature animal [68–70]. Nevertheless, the exposure of the male offspring to TAM did not cause deleterious effects to these testicular cells, by not altering their structure or number.

In utero and lactational exposure to tamoxifen did not affect the fertility of rats in adulthood after natural mating, despite changes in sperm motility. Fertility assessments

in animals have limited sensitivity as a direct measure of reproductive injury [50]. In some rodent species, the production of normal sperm can be reduced by up to 90% or more without compromising fertility; however, small changes in sperm parameters in men can have serious consequences for fertility [71].

Play behavior is sensitive to prenatal and neonatal exposure to chemical factors, and is a useful behavioral marker of neurodevelopment [72]. In addition, it is sexually differentiated, as males exhibit a higher frequency in this parameter than females, these differences are determined by the medial amygdala [73,74]. AR expression in several brain nuclei is also different according to the sex species, such as the BnST and MPOA, both being involved in the control of male sexual behavior [75]. Moreover, other regions of the hypothalamus are sexually dimorphic, such as the sexually dimorphic nucleus of the preoptic area (SDN-POA), region that controls male sexual behavior and is 5 times larger in males than in females [76]. In contrast, the ventromedial nucleus (VMH) regulates female sexual behavior [77]. In view of these data, neonatal exposure to TAM possibly did not interfere with the development of these regions, since behavioral parameters and AR expression in the hypothalamus were not altered. However, an imbalance in the hypothalamic-pituitary-gonadal axis may explain the sperm alterations, indicating interference in different brain regions.

In conclusion, the present study demonstrated that maternal exposure to TAM affected the installation of puberty of the male offspring and compromised important reproductive parameters in the adult life, acting as an endocrine disruptor. These effects were particularly observed in the intermediate and highest dose groups. Considering these results, future studies are important for a better understanding of the effects of this drug on the male reproductive tract, since the use of this drug impacts on the lives of many women.

5. Conflict of interest

The authors report no conflicts of interest.

6. Acknowledgements

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Figures and tables

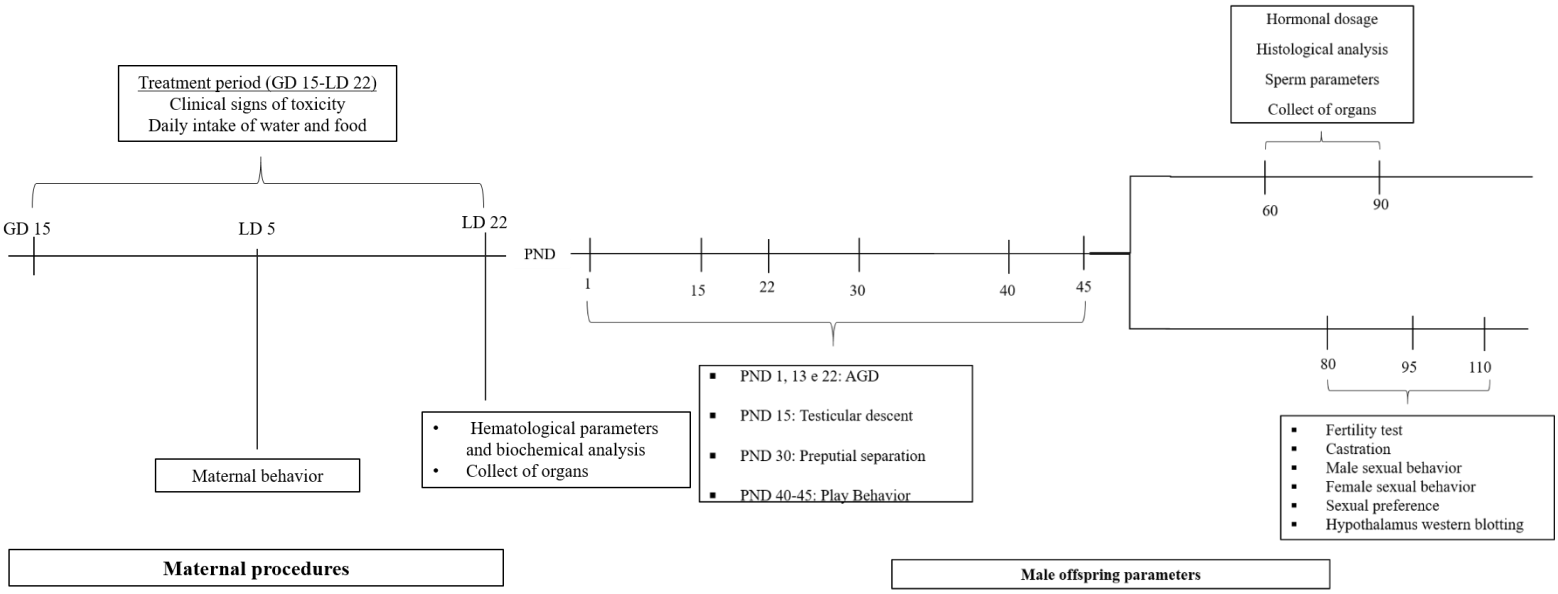


Figure 1. Diagram of the experimental design

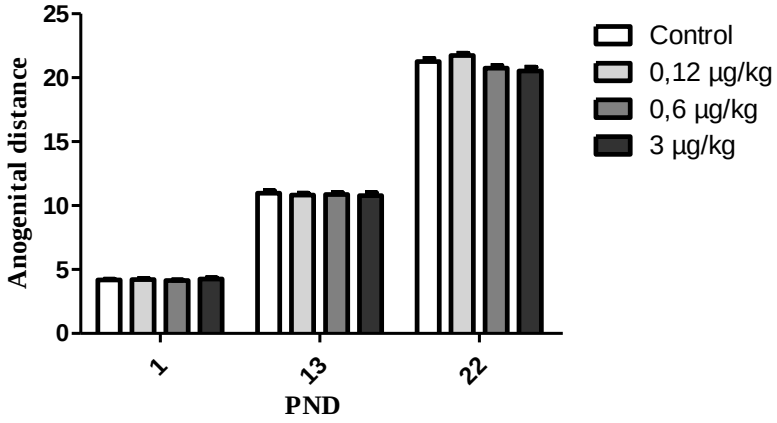


Figure 2. Relative anogenital distance in PND 1, 13 and 22 of the male offspring exposed to tamoxifen. Values expressed as mean ± standard error of the mean (SEM). n= 8 litters/group. ANOVA. $p > 0.05$.

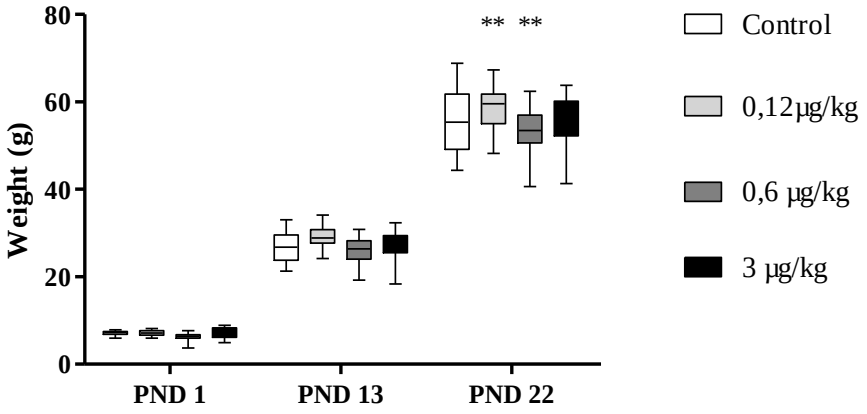


Figure 3. Body weight (g) of the male offspring at PND 1, 13 e 22. Values expressed in median-interquartile range (Q1-Q3). n= 8 litter/group. Kruskal-Wallis with posterior test in Dunn**p<0.05.

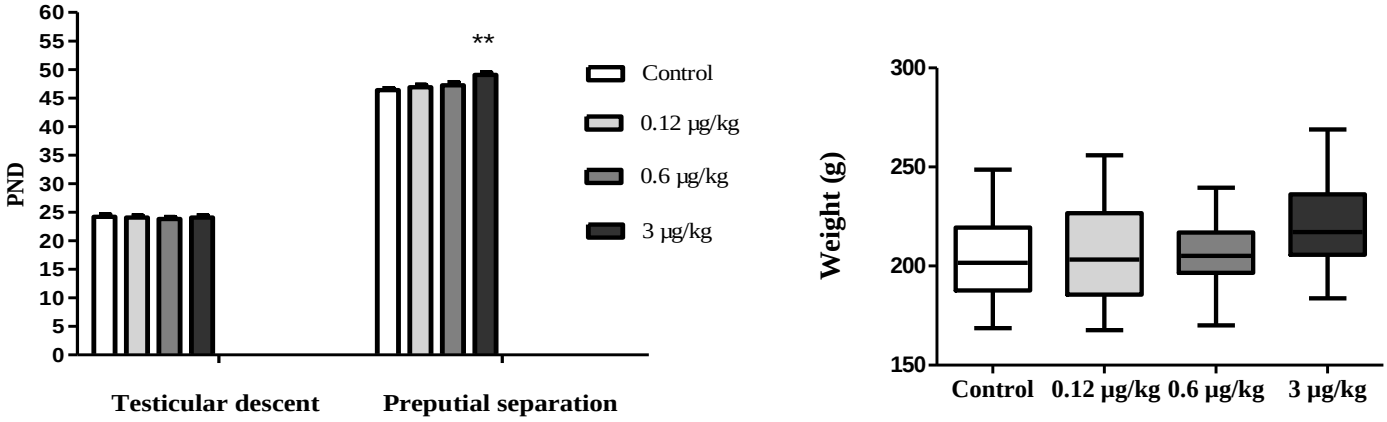


Figure 4. Ages at the time of testicular descent and preputial separation (A). Body weight (g) at the time of preputial separation. Values expressed as mean ± standard error of the mean (SEM). ANOVA with posterior test Dunnet (B). Values expressed as median-interquartile range (Q1-Q3). n= 8 litter/group. Kruskal-Wallis with posterior test in Dunn ** p <0.05.

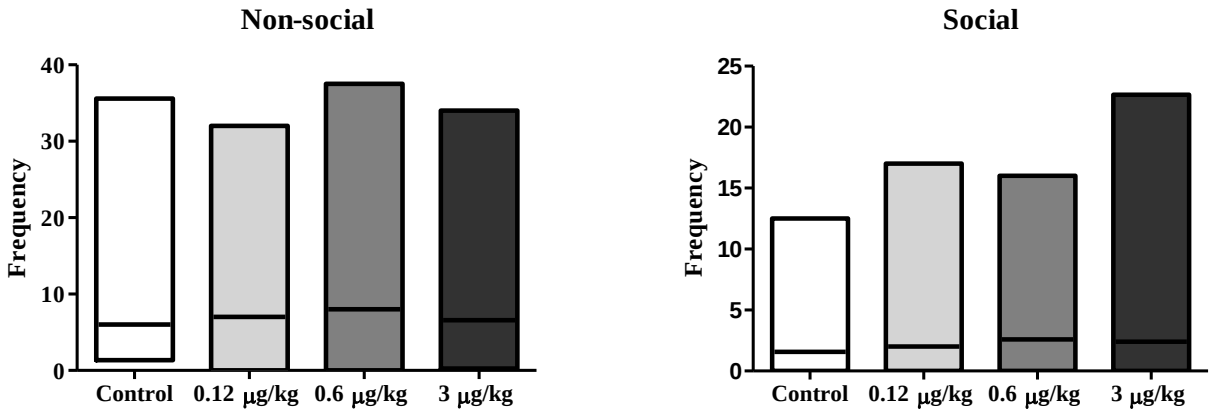


Figure 5. Play Behavior of male offspring exposed to tamoxifen, behavioral parameters grouped into Sociable and Non-sociable. Values expressed as median-interquartile range (Q1-Q3). n= 8 litter/group. Kruskal-Wallis with posterior test in Dunn. p> 0.05

Table 1. Relative weight of organs of adult males exposed to tamoxifen *in utero* and during lactation.

Parameters	**Experimental Groups**			
	Control	**0.12 µg/kg**	**0.6 µg/kg**	**3 µg/kg**
Body weight (g)	408.72 ± 17.73	395.11 ± 13.57	372.76 ± 11.66	408.87 ± 17.94
Liver (g)	3.81 ± 0.08	3.72 ± 0.09	3.70 ± 0.09	3.73 ± 0.09
Kidney (g)	0.39 ± 0.00	0.39 ± 0.01	0.41 ± 0.01	0.41 ± 0.01
Adrenal Gland (mg)	8.87 ± 0.76	7.37 ± 1.10	7.87 ± 0.63	7.87 ± 0.66
Spleen (mg)	174.8 ± 4.87	197.39 ± 9.95	192.37 ± 6.29	200.83 ± 10.44
Heart (mg)	333.48 ± 12.16	340.25 ± 12.81	334.97 ± 16.26	330.41 ± 8.36
Lungs (g)	0.43 ± 0.03	0.42 ± 0.02	0.50 ± 0.04	0.44 ± 0.02
Thyroid (mg)	3.57 ± 0.48	3.37 ± 0.26	4.00 ± 0.37	4.37± 0.46
Testis (mg)	401.8 ± 8.6	406.84 ± 11.53	431.25 ± 19.42	412.62 ± 20.23
Epididymis (mg)	137.87 ± 4.8	138.1 ± 1.61	151.22 ± 7.16	142.02 ± 4.74
Vas deferens (mg)	22.14 ± 1.29	22.50 ± 0.77	21.25 ± 1.26	19.62 ± 1.13
Seminal gland (g)	0.30 ± 0.03	0.33 ± 0.02	0.34 ± 0.01	0.33 ± 0.02
Prostate (mg)	82.24 ± 6.4	82.15 ± 6.29	81.83 ± 8.75	79.93 ± 6.65

Values expressed as mean ± SEM. n = 8/group. ANOVA with posterior test Dunnet. p> 0.05

Table 2. Sperm counts of adult male rats exposed to tamoxifen.

Parameters	Experimental groups			
	Control	**0.12 µg/kg**	**0.6 µg/kg**	**3 µg/kg**
Testis				
#Mature spermatids number (x10^6/testis)	102.16 (96.82 – 172.21)	124.43 (96.72 – 144.51)	126.52 (83.1 – 149.52)	131.74 (104.46 – 143.04)
#Relative mature spermatids number (x10^6g/testis)	78.58 (67.24 – 125.98)	103.94 (83.16 – 127.78)	93.48 (70.83 – 147.01)	95.91 (78.66 – 103.23)
#Daily sperm production (x10^6testis/day)	16.74 (15.87 – 28.23)	20.4 (15.86 – 23.7)	20.74 (13.62 – 24.51)	21.6 (17.12 – 23.45)
#Relative daily sperm production (x10^6/testis/g/day)	12.88 (11.02 – 20.65)	17.04 (13.63 – 20.95)	15.32 (11.61 – 24.1)	15.72 (12.89 – 16.92)
Epididymis (caput/corpus)				
Sperm number (x10^6organ)	56.51 ± 3.03	63.65 ± 3.07	61.57 ± 1.75	65.38 ± 3.27
Relative sperm number (x10^6g/organ)	234.86 ± 7.91	239.16 ± 6.92	229.21 ± 3.69	237.22 ± 8.54
Sperm transit time (days)	3.00 ± 0.26	3.17 ± 0.27	3.27 ± 0.22	3.16 ± 0.21
Epididymis (cauda)				
Sperm number (x10^6organ)	109.77 ± 9.62	130.01 ± 6.43	139.80 ± 10.01*	139.79 ± 2.27*
Relative sperm number (x10^6/g/organ)	665.00 ± 8.66	673.33± 19.52	686.11 ± 15.04	655.27 ± 17.97
Sperm transit time (days)	3.00 ± 0.26	3.17 ± 0.27	3.27 ± 0.22	3.16 ± 0.21

Values expressed as mean ± SEM of 8/group. ANOVA with posterior test Dunnet. # Values expressed as median as median-interquartile range (Q1-Q3). Kruskal-Wallis with posterior test in Dunn. *p>0.05

Table 3. Sperm morphology of cauda epididymis sperm of adult male rats exposed to tamoxifen.

Parameters (%)	Experimental groups			
	Control	**0.12 µg/kg**	**0.6 µg/kg**	**3 µg/kg**
Normal Sperm	196 ± 1.33	196.37 ± 0.68	196.14 ± 0.86	194.62 ± 1.84
Straight Head Sperm	0.00	0.00	0.12 ± 0.12	0.00
Isolated Head Sperm	4.71 ± 1.46	3.37 ± 0.6	3.28 ± 0.68	5.37 ± 1.44
Curled Tail Sperm	0.00	0.1 ± 0.1	0.28 ± 0.18	0.00
#Broken Tail Sperm	0.00 (0.00 – 0.00)	0.00 (0.00 – 1.00)	0.00 (0.00 – 1.00)	0.00 (0.00 – 2.00)
Cytoplasmatic droplet	18.5 ± 4.48	12.5 ± 2.35	8.75 ± 2.34	11.14 ± 1.96

Values expressed as mean ± SEM. ANOVA with posterior test Dunnet. #Values expressed as median-interquartile range (Q1-Q3). Kruskal-Wallis with posterior test in Dunn. n= 8 litter/group. p>0.05

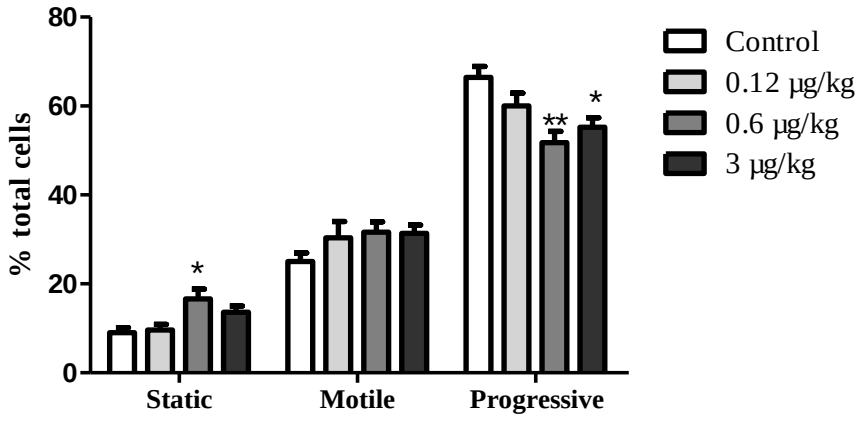


Figure 6. Sperm motility of cauda epididymis sperm of male adult rats exposed to tamoxifen. Values expressed as mean± SEM. n= 5 animals/group. ANOVA with posterior test Dunnet. *p<0.05 **p<0.01.

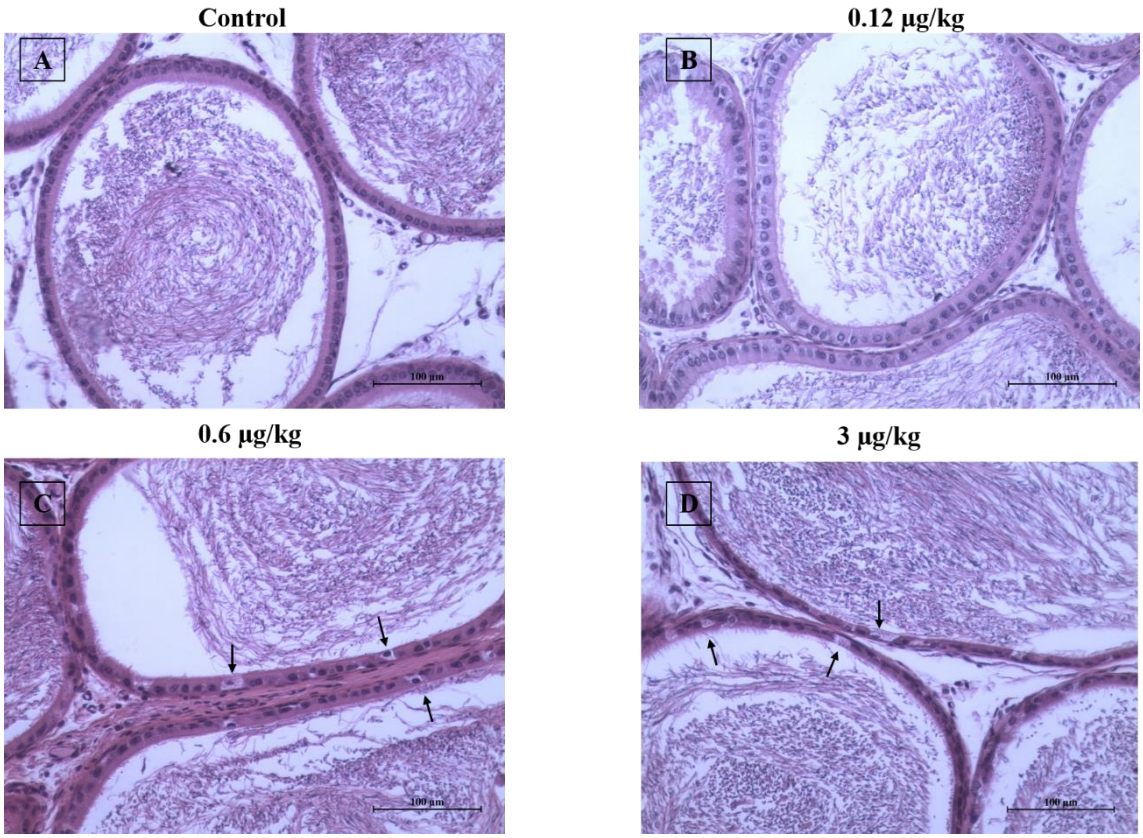


Figure 7. Photomicrographs of cauda epididymidis. (A-B) Normal aspect. (C-D) Hyperplasia of clear cells (arrows). H&E, 20x.

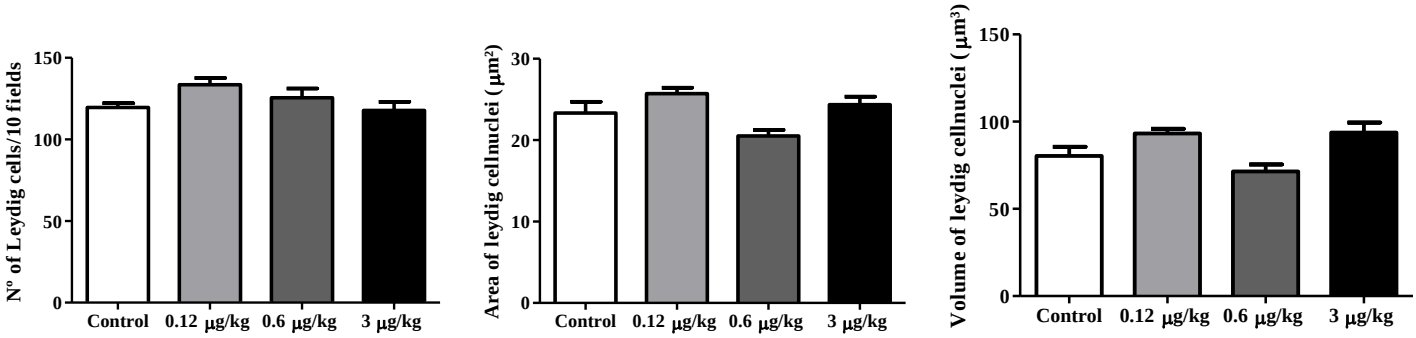


Figure 8. Leydig cell nuclei count and measurements (area and volume) in the interstitial tissue of male rats exposed to tamoxifen. Values expressed as mean ± SEM. n= 5 animals/group. ANOVA with Dunett's posterior test. p>0.05.

Table 4. Fertility test of adult male rats exposed to tamoxifen.

Parameters	Experimental groups			
	Control	**0.12 µg/kg**	**0.6 µg/kg**	**3 µg/kg**
Fertility potential (%)	100 (86 – 100)	91 (71 – 100)	91 (75 – 100)	100 (90 – 100)
#Weight gain (g)	144.92 ± 9.23	130.60 ± 5.89	127.60 ± 9.22	134.82 ± 6.85
Uterine + fetal weight (g)	85.3 (54.4 – 88.73)	69.57 (54.9 – 77.38)	73.58 (48.23 – 88.18)	70.08 (63.27 – 93.74)
Placenta weight (g)	0.58 (0.56 – 065)	0.66 (0.54 – 0.76)	0.62 (0.52 – 0.76)	0.57 (0.53 – 0.68)
#Placenta efficiency	7.26 ± 0.30	7.03 ± 0.23	6.85 ± 0.49	7.57 ± 0.24
Number of live fetuses	13 (9 – 14)	10 (9 – 12)	12 (8 – 15)	11 (11 – 14)
Number of implantations	12.85 ± 0.79	12.00 ± 0.42	12.33 ± 1.08	11.87 ± 0.58
Number of corpora lutea	14 (10 – 15)	12 (11 – 14)	13 (12 – 17)	11.5 (10 – 15)
Number of resorptions	0 (0 – 1)	1 (0 – 1)	0 (0 – 2)	0
Pre-implantation loss (%)	0 (0 - 13)	8 (0 – 28)	8 (0 – 25)	0 (0 – 9)
Post-implantation loss (%)	0 (0 – 10)	8 (0 - 10)	0 (0 – 11)	0 (0 – 0)
#Sex ratio (M:F)	0.71 ± 0.12	1.18 ± 0.26	1.25 ± 0.22	1.76 ± 0.40

Values expressed as mean ± SEM. n = 8/group. #ANOVA with posterior test Dunnet. Values expressed as median – interquartile range (Q1-Q3). Kruskal-Wallis/Dunn's test. $p>0.05$.

Table 5. Male sexual behavior of adult male rats exposed to tamoxifen.

Parameters	Experimental group			
	Control	**0.12 µg/kg**	**0.6 µg/kg**	**3 µg/kg**
Latency to the first mount (s)	45.62 ± 10.04	64.62 ± 22.65	79.78 ± 25.12	65.43 ± 13.44
Number of mounts	7.33 ± 1.75	6.55 ± 1.94	6.00 ± 1.43	6.58± 0.61
Latency of the first intromission (s)	55.55 ± 15.01	63.22 ± 23.71	91.67 ± 22.07	35.17 ± 13.69
Number of intromissions	21.11 ± 2.95	16.89 ± 2.39	16.12 ± 2.46	17.86 ± 2.41
Latency to the first ejaculation (s)	723.71 ± 167.23	454.75 ± 105.95	656.50 ± 127.59	836.43 ± 171.95
Latency of the first post-ejaculatory mount (s)	962.33 ± 168.43	811.87 ± 114.62	909.57 ± 156.15	1025.17 ± 157.24
Number of post-ejaculatory mounts	1.88 ± 0.70	3.33 ± 1.00	1.78 ± 0.55	2.86 ± 0.86
Latency of the first post-ejaculatory intromission (s)	905.33 ± 126.17	614.86 ± 59.11	943.37 ± 139.95	996.50 ± 153.93
Number of post-ejaculatory intromissions	5.11 ± 1.83	6.78 ± 1.20	6.44 ± 1.32	8.29 ± 2.45
#Number of total ejaculations	2 (0 – 4)	3 (2 – 4)	3 (0 – 4)	2 (1 – 4)

Values expresses as mean ± SEM. n=8/group. ANOVA/Dunnet's test. #Kruskal-Wallis/Dunn's test. $p>0.05$

Table 6. Sexual preference score of adult male rats exposed to tamoxifen. Values expressed as mean ± SEM. n= 9/group. ANOVA with posterior test Dunnet. p>0.05

Parameters	Experimental groups			
	Control	0.12 µg/kg	0.6 µg/kg	3 µg/kg
Time spent in male zone (s)	205.22 ± 27.09	144 ± 28.62	191.77 ± 34.52	136.75 ± 23.75
Time spent in female zone (s)	362.33 ± 73.42	384.33 ± 49.43	289.67 ± 38.23	362 ± 47.32
Number of visits in male zone	20 ± 2.03	17.89 ± 3.85	20.44 ± 1.75	17.38 ± 3.3
Number of visits in female zone	22.56 ± 2.99	25.22 ± 3.1	24.89 ±2.12	24.5 ± .28
Preference score	0.6 ± 0.05	0.73 ± 0.05	0.64 ± 0.02	0.69 ± 0.02

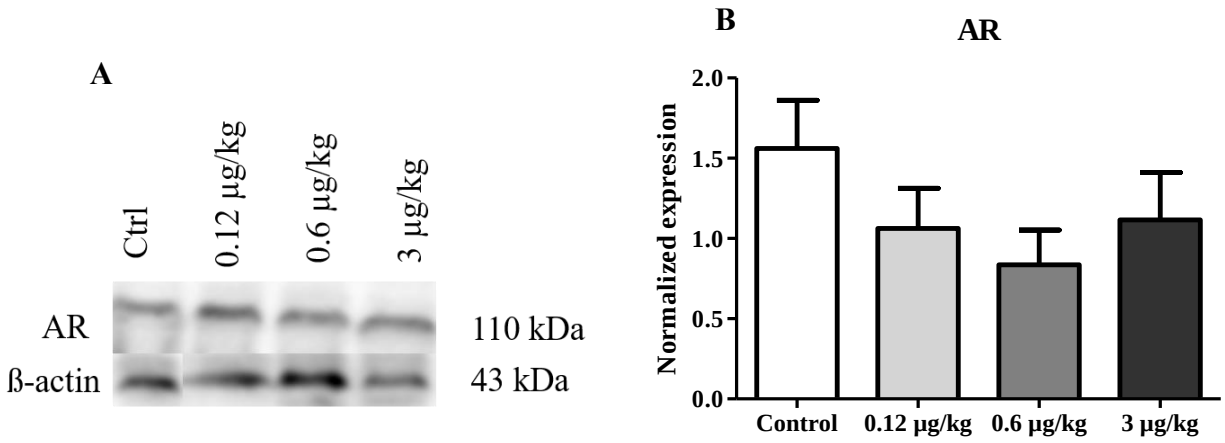


Figure 9. Androgen receptor (AR) expression in hypothalamic neurons of rats exposed to tamoxifen in utero and via lactation (image (**A**) and graph (**B**)). Values expressed as mean ± SEM. n= 9/group. ANOVA with posterior test Dunnet. p>0.05

Supplementary material

Table 1 - Body weight, water consumption and food intake of the dams treated with tamoxifen.

Parameters	Experimental groups			
	Control	**0,12 µg/kg**	**0,6 µmg/kg**	**3 µg/kg**
Initial weight (g)	298.28 ± 6.92	286.65 ± 6.69	296.25±11.27	299.11±9.44
Final weight (g)	295.78 ± 6.56	290.16 ± 5.97	301.17 ± 8.44	290.03 ± 10.19
Weight gain (%)	-0.74 ± 1.68	1.31 ± 1.171	2 ± 1.86	1.20 ± 0.96
Food intake (g/day)	48.43 ± 3.47	50.26 ± 4.18	53.05 ± 5.21	49.53 ± 5.25
Water consumption (mL/day)	89.97 ± 8.21	88.82 ± 7.84	86.24 ± 6.05	85.76 ± 7.73

Values expressed as mean ± SEM. n = 8/group. ANOVA with posterior test Dunnet.p> 0.05

Table 2 - Maternal behavior of lactating females treated with tamoxifen.

Parameters	Experimental groups			
	Control	**0,12 µg/kg**	**0,6 µg/kg**	**3 µg/kg**
Grouping (s)	990.00 ± 212.34	1101.33 ± 206.51	554.87 ± 98.65	648.12 ± 197.60
Total time grouping (s)	32.00 ± 14.73	82.28 ± 39.49	38.25 ± 16.64	145.37 ± 76.97
Pup grooming (s)	159.28 ± 65.87	40.25 ± 22.75	135.87 ± 24.82	45.57 ± 21.36
Off pups (s)	1387.28 ± 99.01	1588.00 ± 61.99	1384.87 ± 97.31	1549.62 ± 80.17
Nest	1.28 ± 0.42	1.00 ± 0.37	2.25 ± 0.31	1.12 ± 0.29

Values expressed as mean ± SEM. n = 8/group. ANOVA with posterior test Dunnet.p> 0.05

Table 3 – Final body weight and relative organ weight (g/100g body weight) of pregnant and lactating rats treated with tamoxifen.

Parameters	**Experimental groups**			
	Control	**0,12 µg/kg**	**0,6µg/kg**	**3 µg/kg**
Body weight (g)	281.04 ± 5.19	273.91 ± 6.45	283.45 ± 7.50	287.10 ± 12.63
Liver (g)	4.600 ± 0.14	4.56 ± 0.24	5.12± 0.24	4.73 ± 0.28
Kidney (g)	0.37 ± 0.01	0.40 ± 0.00	0.40 ± 0.01	0.38 ± 0.01
Adrenal gland (mg)	15.22 ± 1.09	17.21 ± 1.31	13.94 ± 1.39	15.92 ± 1.67
Spleen (g)	0.30 ± 0.01	0.27 ± 0.00	0.27 ± 0.01	0.29 ± 0.01
Heart (g)	0.37 ± 0.00	0.39 ± 0.01	0.38 ± 0.01	0.36 ± 0.01
Lung (g)	0.51 ± 0.01	0.51 ± 0.00	0.54 ± 0.02	0.48 ± 0.02
Ovaries (mg)	29.46 ± 1.74	28.14 ± 1.11	32.72 ± 3.57	34.96 ± 4.12
Left ovary (mg)	13.11 ± 1.11	13.04 ± 0.48	15.12 ± 2.48	18.27 ± 2.54
Uterus (g)	0.17 ± 0.00	0.16 ± 0.01	0.17 ± 0.02	0.14 ± 0.01

Values expressed as mean ± SEM. n = 8/group. ANOVA with posterior test Dunnet.p> 0.05

Table 4 - Hematological and biochemical parameters of rats treated with tamoxifen.

Parameters	Experimental groups			
	Control	**0.12 µg/kg**	**0.6 µg/kg**	**3 µg/kg**
Hematological				
Platelets (10^3/µL)	28.14±3.7	22.44±2.04	25.87±2.10	18.25±2.12
Erythrocytes (10^6/µL)	5.00±1.11	6.48±0.33	5.22±0.92	4.55±0.83
Hematocrit (%)	43.86±2.4	41.44±1.64	44.12±1.75	39.87±1.30
Leukocytes (10^3/µL)	6.62±0.41	7.58±0.65	7.54±0.57	6.77±0.46
Hemoglobin	18.83±0.6	19.41±0.76	18.30±0.81	17.89±0.75
MCV (fL)	61.83±3.8	65.37±4.30	69.06±4.19	67.18±3.67
MCH (pg)	26.57±1.4	30.44±1.82	28.74±2.10	30.06±1.62
MCHC (%)	45.03±1.1	47.22±2.23	42.10±2.86	44.80±0.65
Lymphocyte (%)	57.14±2.8	55.67±3.68	56.50±2.09	59.12±1.43
Neutrophilis (%)	23.86±2.0	22.11±1.51	22.12±1.40	23.75±1.35
Monocytes (%)	13.00±0.6	13.89±1.05	13.62±1.08	12.50±0.68
Biochemical				
AST (U/L)	35.86±6.69	33.88±9.34	26.12±7.58	37.88±10.92
#ALT (U/dL)	30 (17 – 40)	10 (5 – 38)	16.5 (3 – 34)	17 (6 – 66)
#Gamma glutamyltransferase (U/L)	0 (0 – 4)	0 (0 – 2)	0	0 (0 – 2)
Total protein (g/dL)	6.87±0.17	7.42±0.16	7.48±0.23	7.29±0.26
Albumin (g/dL)	2.43±0.19	1.99±0.17	2.14±0.08	2.14±0.22
Urea (mg/dL)	58.86±2.8	60.62±2.39	63.38±2.60	59.62±4.54
Creatinine (mg/dL)	1.33±0.04	1.36±0.06	1.25±0.06	1.19±0.04
Calcium (mg/dL)	16.31±1.1	15.05±0.93	16.82±0.86	17.15±1.80
Cholesterol (mg/dL)	74.43±2.7	77.12±4.80	73.00±5.56	73.37±4.45
Uric Acid	9.61±0.66	9.45 ± 0.91	10.59 ± 1.08	8.88 ± 1.04
Alkaline Fosfatase (U/L)	289.86±22	273.44±29.32	230.12±24.57	252.87±39.93

MCV – mean corpuscular volume; MCH – mean corpuscular hemoglobin; MCHC – mean corpuscular hemoglobin concentration; AST- aspartate aminotransferase; ALT- alanine transaminase.

Values expressed as mean ± SEM. n=8/group. ANOVA with posterior test Dunnet. Kruskal-Wallis with posterior test in Dunn. $p > 0.05$

6 CONCLUSÃO

Nossos resultados demonstraram que, neste modelo experimental, o tamoxifeno agiu como um desregulador endócrino quando administrado no período gestacional e lactacional. Isso foi particularmente evidenciado uma vez que a exposição a essa droga foi capaz de provocar efeitos adversos na maturação sexual e comprometer os parâmetros reprodutivos dos descendentes machos, o que pode estar relacionado a ação anti-estrogênica desta droga. Futuras análises serão importantes para complementar estes resultados, como dosagem hormonal e maiores análises no epidídimo.

7 ANEXOS

As figuras presentes nesta seção não foram incluídas no manuscrito, entretanto as análises foram realizadas durante a execução do projeto de pesquisa.

Aprovação do CEUA- Comitê de ética no uso de animais

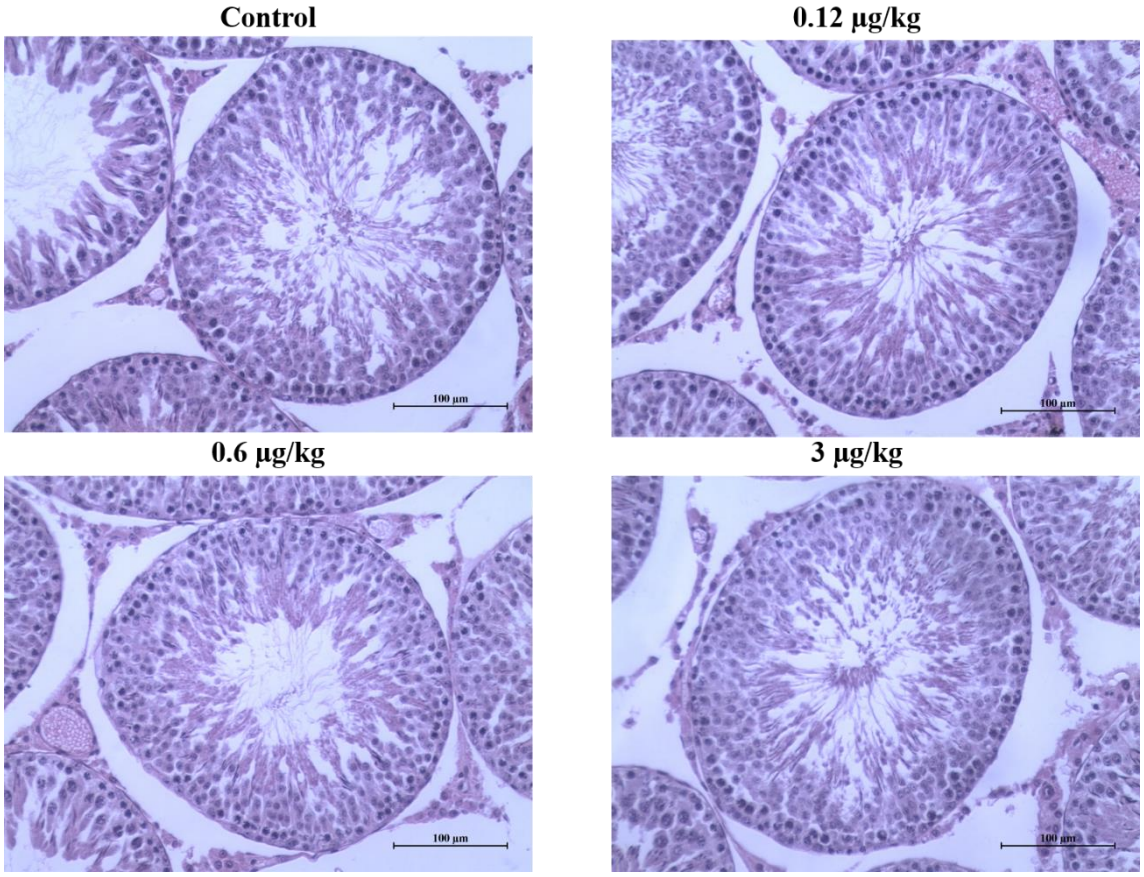
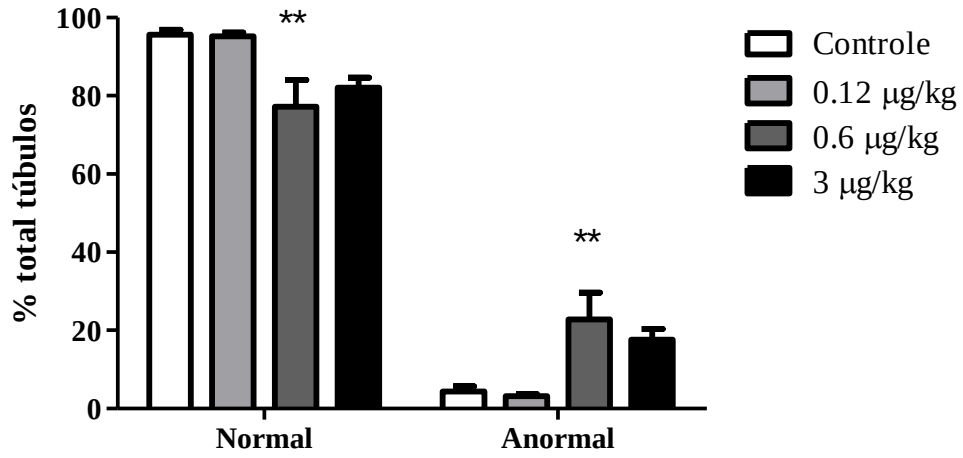


Figura 1. Fotomicrografias de cortes transversais de testículo de ratos adultos expostos a diferentes doses de tamoxifeno. Coloração em HE. 20x.

Figura 2. Porcentagem de células normais e anormais no epidídimo



Values expressed as mean ± SEM. n = 5/group. ANOVA with posterior test Dunnet. **p< 0.01.

PARECER DE APROVAÇÃO DO COMITÊ DE ÉTICA

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

Instituto de Biociências de Botucatu - Unesp

Certificado

Certificamos que o projeto intitulado "Influência da exposição in utero e lactacional ao tamoxifeno: repercussão na vida adulta em parâmetros reprodutivos e comportamentais, em ratos machos", Protocolo nº **1157-CEUA**, sob a responsabilidade de **Arielle Cristina Arena**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 9 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela ***COMISSÃO DE ÉTICA NO USO DE ANIMAIS*** (CEUA), nesta data.

Finalidade:	( ) Ensino	(x) Pesquisa Científica
Vigência do Projeto:	Início: 2/7/2019	Término: 31/1/2020
Espécie/linhagem:	*Rato Wistar*	
Nº de animais:	*125*	
Peso:	*300g*	Idade: 80 dias
Sexo:	*Macho e fêmea*	
Origem	*Biotério Central da Unesp-Câmpus de Botucatu/SP.*	

Botucatu, 10 de maio de 2019.

Prof. Assoc. Wellerson Rodrigo Scarano
Coordenador da CEUA

Comissão de Ética no Uso de Animais IBB-Unesp

Comissão de Ética no Uso de Animais - CEUA
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