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

PG-BGA

EFEITOS DA EXPOSIÇÃO DE RATOS MACHOS *WISTAR* ADULTOS A ESTATINAS E INIBIDORES DE RECAPTURA DE NEUROTRANSMISSORES SOBRE PARÂMETROS REPRODUTIVOS E A FERTILIDADE

PATRÍCIA VILLELA E SILVA

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

Orientadora: Prof.ª Dra. Wilma De Grava Kempinas

BOTUCATU – SP
2018

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1. Rosuvastatina cálcica. 2. Sinvastatina. 3. Sibutramina. 4. Bupropiona. 5. Reprodução. 6. Fecundidade.

Palavras-chave: Bupropiona; Reprodução; Rosuvastatina; Sibutramina; Sinvastatina.

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Epígrafe

"A vida só pode ser compreendida olhando-se para trás; mas só pode ser vivida olhando-se para frente"

Soren Kierkegaard

Sumário

Resumo

A obesidade é um problema mundial de saúde pública associada ao aumento do risco de síndrome metabólica, dislipidemia, entre outras comorbidades. Entre os fármacos utilizados no tratamento de pacientes obesos estão as estatinas, como a rosuvastatina e sinvastatina, as quais reduzem os níveis séricos de colesterol, precursor de testosterona, e os agentes inibidores de recaptura de neurotransmissores, como a sibutramina e a bupropiona, utilizados na redução do peso corpóreo. Estudos anteriores relataram efeitos adversos da exposição isolada a estes fármacos sobre parâmetros reprodutivos masculinos. Considerando a exposição de pacientes obesos a estatinas e inibidores da recaptura de neurotransmissores, o presente estudo objetivou investigar os efeitos da co-exposição à rosuvastatina e sibutramina, bem como a co-exposição à sinvastatina e bupropiona, sobre os parâmetros reprodutivos em ratos adultos. Para isso, foram realizados dois experimentos. No primeiro experimento, ratos adultos (90 dias) alocados nos grupos controle (salina e dimetilsulfóxido), rosuvastatina (5 mg/kg de rosuvastatina), sibutramina (10 mg/kg de sibutramina) e rosuvastatina associada à sibutramina (n = 26-28/grupo) foram tratados via oral por 70 dias. No segundo experimento, ratos adultos (70 dias) alocados nos grupos controle (água destilada), sinvastatina (50 mg/kg de sinvastatina), bupropiona (30 mg/kg de bupropiona) e sinvastatina associada à bupropiona (n = 20/grupo) foram tratados via oral por 52 dias. No primeiro experimento, o tratamento com rosuvastatina, isolado ou associado à sibutramina, resultou em hiperplasia nas células claras do epidídimo. A exposição à sibutramina, isolada ou associada à rosuvastatina, resultou em redução do consumo alimentar, do peso corpóreo e de órgãos reprodutores, dos níveis séricos de testosterona e aumento do índice de espermatozoides com gota citoplasmática, comparado aos grupos controle e rosuvastatina. As reservas e o tempo de trânsito espermático pelo epidídimo também foram reduzidos nesses grupos, sendo potencializados pela co-exposição aos fármacos. Não foi observado alteração na morfologia testicular, na morfologia e motilidade espermática, bem como na expressão da enzima 3β-HSD, porém, a co-exposição resultou em atraso na ejaculação e redução do potencial fértil após acasalamento natural, comparado aos grupos controle e rosuvastatina, sugerindo possível sinergismo entre os fármacos. No segundo experimento, todos os grupos experimentais apresentaram redução nos níveis de testosterona sérica e intratesticular, comparado ao grupo controle. Alteração na motilidade espermática e redução da síntese de testosterona pelas células de Leydig foram observadas no grupo tratado com sinvastatina. A exposição à sinvastatina, isolada ou associada à bupropiona, resultou em hiperplasia de células claras no epidídimo e aumento no índice de espermatozoides com gota citoplasmática. Não foi observado alteração no peso dos órgãos reprodutores, na morfologia espermática, na morfologia e morfometria testicular, bem como na expressão da enzima 3β-HSD, porém, a co-exposição resultou em redução do potencial fértil, sugerindo possível sinergismo entre os fármacos. Considerando que ratos apresentam maior eficiência reprodutiva comparada a humanos, os resultados obtidos nos dois experimentos podem indicar riscos reprodutivos em homens co-expostos a estatinas e inibidores de recaptura de neurotransmissores.

Palavras-chave: rosuvastatina, sinvastatina, sibutramina, bupropiona, reprodução.

Abstract

Obesity is a global public health problem associated with an increased risk of metabolic syndrome, dyslipidemia, and other comorbidities. The drugs used to treat obese patients include statins, as rosuvastatin and simvastatin, which reduce serum levels of cholesterol, a precursor of testosterone, and neurotransmitter reuptake inhibitors, such as sibutramine and bupropion, used to reduce body weight. Previous studies reported adverse effects of the isolated exposure to these drugs on male reproductive parameters. Considering the exposure of obese patients to statins and neurotransmitter reuptake inhibitors, the present study aimed to investigate the effects of the co-exposure to rosuvastatin and sibutramine, as well as the co-exposure to simvastatin and bupropion, on reproductive parameters in adult male rats. For this, two experiments were conducted. In the first experiment, adult male rats (90 days) allocated into control (saline and dimethylsulfoxide), rosuvastatin (5 mg/kg rosuvastatin), sibutramine (10 mg/kg sibutramine) and rosuvastatin combined with sibutramine (n = 26-28 / group) were treated orally for 70 days. In the second experiment, adult male rats (70 days) allocated into control (distilled water), simvastatin (50 mg/kg simvastatin), bupropion (30 mg/kg bupropion) and simvastatin combined with bupropion (n = 20 / group) were treated for 52 days. In the first experiment, treatment with rosuvastatin, alone or in combination with sibutramine, resulted in hyperplasia in the clear cells of epididymis. Exposure to sibutramine, alone or in combination with rosuvastatin, resulted in reduced food intake, body and reproductive organ weights, serum testosterone levels, and increased index of spermatozoa with cytoplasmic droplets compared to the control and rosuvastatin groups. Sperm reserves and transit time through the epididymis were also reduced in these groups and potentiated by co-exposure to the drugs. No alterations in the testicular morphology, sperm morphology and motility, as well as expression of 3β-HSD enzyme were observed, however, the co-exposure resulted in delayed ejaculation and reduction of fertile potential after natural mating, compared to the control and rosuvastatin groups, suggesting possible synergism between the drugs. In the second experiment, all the experimental groups presented reduction in serum and intratesticular testosterone levels, compared to the control group. Alteration in sperm motility and reduction of testosterone synthesis by Leydig cells were observed in the simvastatin-treated group. Exposure to simvastatin, isolated or associated with bupropion, resulted in hyperplasia of clear cells from the epididymis and increased index of sperm with cytoplasmic droplet. No alterations in the reproductive organ weights, sperm morphology, testicular morphology and morphometry, and expression of 3β-HSD enzyme were observed, however, the co-exposure resulted in reduced fertility potential, suggesting synergism between the drugs. Considering that rats have a higher reproductive efficiency compared to humans, the results obtained in these two experiments may indicate reproductive risks in men co-exposed to statins and neurotransmitter reuptake inhibitors.

Keywords: rosuvastatin, sinvastatin, sibutramine, bupropion, reproduction.

Introdução

1. Obesidade e dislipidemias

O sobrepeso e a obesidade estão entre os maiores problemas de saúde pública que vem crescendo a nível mundial nos últimos 30 anos (Ananthapavan *et al*., 2014). De acordo com a *World Health Organization* (WHO), a prevalência mundial de obesidade dobrou entre 1980 e 2014, sendo as taxas de 1,9 bilhões de adultos (39%) com sobrepeso e 600 milhões (13%) de adultos obesos em 2014 (WHO, 2016). Nos Estados Unidos, mais de dois terços da população adulta (70,2%) encontra-se em condições de sobrepeso ou obesidade e, entre os indivíduos obesos (37,7%), 7,7% encontra-se em situação de obesidade mórbida (NIDDK, 2017).

Pode-se definir o sobrepeso e a obesidade como o acúmulo anormal ou excessivo de gordura no tecido adiposo decorrente do desequilíbrio entre calorias ingeridas e gastas (Nammi *et al*., 2004), sendo mensurados pelo índice de massa corporal (IMC), o qual é obtido através da divisão da massa corporal (quilogramas, Kg) pelo quadrado da estatura (metros ao quadrado, m^2) (Antipatis & Grill, 2001). Em adultos, índices entre 25 a 29,9 são categorizados como sobrepeso, enquanto índices maiores ou iguais a 30 são considerados obesidade (Antipatis & Grill, 2001), e valores maiores ou iguais a 40 classificam-se como obesidade severa (NIDDK, 2017).

Considerada uma pandemia global (Ng, 2014), a obesidade é uma doença crônica multifatorial, de origem genética, metabólica, hormonal, psicológica, sociocultural e funcional (Hillegass & Sadowsky, 2001; Ananthapavan *et al.*, 2014; NHLBI, 2017), e está associada a diversas morbidades, como câncer, hipertensão pulmonar, problemas gastrointestinais e pulmonares, osteoartrites, diabetes, síndrome metabólica, doenças cardíacas e depressão (Hillegass & Sadowsky, 2001; Withrow & Alter, 2011; Bray, 2013; NHLBI, 2017), levando à redução da expectativa de vida (Whitlock *et al*., 2009). Uma análise de 57 estudos realizados em uma população de 894.576 adultos revelou que a obesidade reduz a expectativa de vida em 2 a 4 anos, enquanto a obesidade mórbida leva à redução em até 10 anos (Whitlock *et al.*, 2009).

Além de representar um alto custo para a sociedade, a obesidade pode levar ao aumento do risco de doenças cardiovasculares devido a vários fatores, incluindo a elevação da pressão arterial, dos níveis séricos de glicose e insulina, da superprodução hepática de apolipoproteína B e dislipidemias (Klop *et al.*, 2013).

As dislipidemias consistem em alterações nos níveis séricos de lipídios (colesterol, triglicerídeos ou ambos), sendo a hiperlipidemia caracterizada pela elevação dos níveis de colesterol total, triglicerídeos, lipoproteínas de baixa densidade (*Low Density Lipoproteins*;

LDL), bem como redução dos níveis de lipoproteínas de alta densidade (*High Density Lipoproteins*; HDL) (Hillegass & Sadowsky, 2001; Klop *et al*., 2013). O tratamento da dislipidemia é recomendado quando os níveis de LDL e colesterol total apresentam-se maiores que 190 mg/dL e 240 mg/dL, respectivamente (Hillegass & Sadowsky, 2001).

A hiperlipidemia, evento extremamente comum na população obesa, é considerada um fator de risco para doenças cardiovasculares, devido aos elevados níveis de colesterol, sendo responsável por mais de 25% dos casos de mortes no mundo (Grover *et al*, 2017). Altos níveis de colesterol podem resultar em acúmulo de gordura na parede arterial, levando à aterosclerose e a várias patologias cardiovasculares, como trombose, infarto, entre outras (Ciccone, 2011).

Segundo a WHO (2017), um terço da doença isquêmica do coração é resultado dos altos níveis de colesterol. Estudos relatam que a redução em 10% do colesterol em homens adultos com 40 anos de idade pode resultar em 50% da redução de doenças cardíacas em até cinco anos, sendo que a mesma taxa de redução dos níveis de colesterol em homens com 70 anos pode levar à redução de 20% de doenças cardíacas (WHO, 2017).

Várias intervenções podem ser utilizadas no controle do peso corpóreo e dos níveis lipídicos de pacientes obesos, como a dieta, exercício físico, cirurgia e medicamentos. Entretanto, alguns pacientes apresentam dificuldade no controle desses parâmetros através de métodos não farmacológicos, sendo necessária a intervenção com medicamentos (Ciccone, 2011). Entre os fármacos que promovem redução dos níveis lipídicos e do peso corpóreo estão, respectivamente, as estatinas (ex: rosuvastatina e sinvastatina) e os inibidores de recaptura de neurotransmissores (ex: sibutramina e bupropiona) (Svacina *et al*., 2007; Nylén *et al*, 2013; Benaiges *et al*., 2017; Golden, 2017).

2. Trato genital masculino

O trato genital masculino do rato, como na maioria dos mamíferos é constituído pelo pênis, um par de testículos, epidídimos e ductos deferentes, além de glândulas sexuais acessórias, incluindo a próstata e vesícula seminal (Allegrini, 2000; Junqueira & Carneiro, 2011).

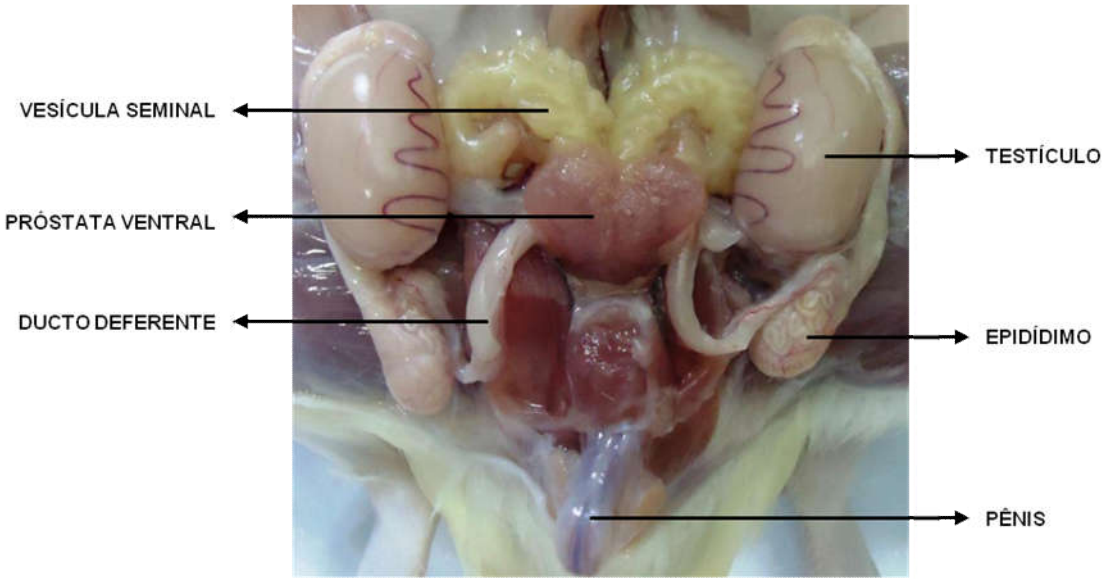

Figura 1: Trato genital masculino de rato. Fonte: Laboratório ReproTox.

2.1. Testículos

Os testículos são as gônadas masculinas, responsáveis pela espermatogênese e pela síntese de andrógenos, como a testosterona, que auxilia no processo espermatogênico e no desenvolvimento das características sexuais secundárias (Walker & Homberger, 1997; Junqueira & Carneiro, 2011). Envolto por uma camada espessa de tecido conjuntivo fibroso, denominada túnica albugínea, ele encontra-se localizado fora da cavidade abdominal, no interior do escroto, o qual contribui para a manutenção do órgão a uma temperatura abaixo da temperatura abdominal (Junqueira & Carneiro, 2011).

Os testículos são formados por túbulos seminíferos longos e enovelados, separados por septos da túnica albugínea, formando lóbulos (O'Donnell *et al*., 2017). Entre os túbulos seminíferos existe um tecido intersticial formado por vasos sanguíneos e linfáticos, nervos, células livres, como os macrófagos e linfócitos, células do tecido conjuntivo, como os fibroblastos, e células de Leydig (Junqueira & Carneiro, 2011; O'Donnell *et al*., 2017). A atividade e o número das células intersticiais são dependentes de estímulo hormonal (Junqueira & Carneiro, 2011). As células de Leydig, com núcleo ovalado e nucléolo evidente, constituem o tipo celular mais abundante do tecido intersticial, sendo responsáveis pela síntese de testosterona (O'Donnell *et al*., 2017).

Os túbulos seminíferos, envoltos por uma lâmina basal e tecido conjuntivo, são constituídos pelo epitélio seminífero, onde são encontradas células somáticas denominadas células de Sertoli e células germinativas em diferentes estágios de maturação (espermatogônias,

espermatócitos, espermátides e espermatozoides), e pelo lúmen, região para onde os espermatozoides são lançados após serem produzidos. Em rato adulto há aproximadamente 20 túbulos seminíferos em cada testículo (Allegrini, 2000).

O processo espermatogênico, regulado pelo eixo neuroendócrino hipotalâmico-hipofisário-gonadal (Cheng & Mruk, 2010), torna-se ativo na puberdade, sendo continuo até a senescência (Lan *et al*., 2013). Ele ocorre no epitélio dos túbulos seminíferos, através de sucessivas divisões mitóticas das espermatogônias, meioses dos espermatócitos, e do remodelamento e diferenciação celular das espermátides (espermiogênese), gerando os espermatozoides (Haschek *et al.*, 2010). A duração deste processo varia conforme a espécie, sendo aproximadamente 38,7 dias em camundongos, 58 dias em ratos e 64 dias em humanos (França *et al*., 2005). Em ratos, este processo consiste em 4,5 ciclos de aproximadamente 12,9 dias, sendo dividido em 14 estágios, que ocorrem distintamente ao longo do túbulo seminífero, demarcando segmentos (França *et al*., 1998; França *et al*., 2005).

As células de Sertoli, com núcleo piramidal e nucléolo evidente, localizam-se próximo à membrana basal dos túbulos seminíferos e exercem importantes funções na espermatogênese (Junqueira & Carneiro, 2011). Sua população é estabelecida durante a puberdade, sendo que a quantidade dessas células determina o tamanho do testículo e a magnitude da produção espermática (Figueiredo *et al*., 2016). Elas possuem várias reentrâncias em seu citoplasma que envolvem parcialmente as células germinativas, fornecendo suporte (Foley, 2001).

Além de fornecer suporte, as células de Sertoli protegem as células germinativas contra o sistema imune, através da formação de uma barreira, denominada barreira hemato-testicular, formada pela comunicação entre células de Sertoli adjacentes através de junções de oclusão, junções de aderência e junções gap, separando o epitélio seminífero em dois compartimentos: basal e adluminal (Foley, 2011; Junqueira & Carneiro, 2011). Com a formação dessa barreira, as células germinativas mais maduras são protegidas contra substâncias nocivas presentes no sangue e contra o próprio sistema imune (Foley, 2011; Junqueira & Carneiro, 2011).

Outro papel importante das células de Sertoli é coordenar a sincronização do processo espermatogênico. Elas possuem comunicação iônica e química com as células germinativas e, assim, controlam a maturação e migração dessas células em direção à luz do túbulo seminífero, estabelecendo o padrão centrípeto da espermatogênese. Além disso, elas possuem funções de nutrição, fagocitose e secreção. Entre as substâncias secretadas por essas células incluem-se proteínas, como a inibina e a proteína ligante de andrógeno, a qual contribui para a concentração de andrógenos nos túbulos seminíferos, essenciais para a espermatogênese.

Apesar de os espermatozoides saírem morfologicamente prontos dos testículos, eles ainda são imaturos e não apresentam motilidade progressiva e capacidade de fertilizar, que são adquiridos ao longo de sua passagem pelo epidídimo (Cornwall, 2009).

2.2. Epidídimos

Os epidídimos são órgãos constituídos por um túbulo único altamente enovelado, responsáveis pela maturação e armazenamento espermático (Cornwall, 2009; Kempinas & Klinefelter, 2014). Conforme as diferenças histológicas e ultraestruturais, o epidídimo pode ser dividido em quatro regiões: segmento inicial, cabeça, corpo e cauda (Figura 2) (Cornwall, 2009). Cada região apresenta funções diferentes, sendo a cabeça e corpo responsáveis pela maturação espermática, enquanto a cauda é responsável, principalmente, pelo armazenamento espermático (Cornwall, 2009).

O processo de maturação ocorre durante a passagem dos espermatozoides ao longo deste órgão, através da adição e remoção de substâncias, resultando em modificações fisiológicas, bioquímicas e morfológicas, permitindo a aquisição da motilidade progressiva e capacidade fértil (Cornwall, 2009). Entre as modificações que ocorrem estão as alterações na dimensão e aparência do acrossomo e núcleo, na migração da gota citoplasmática ao longo da cauda e modificações estruturais em organelas (Olson *et al*., 2002).

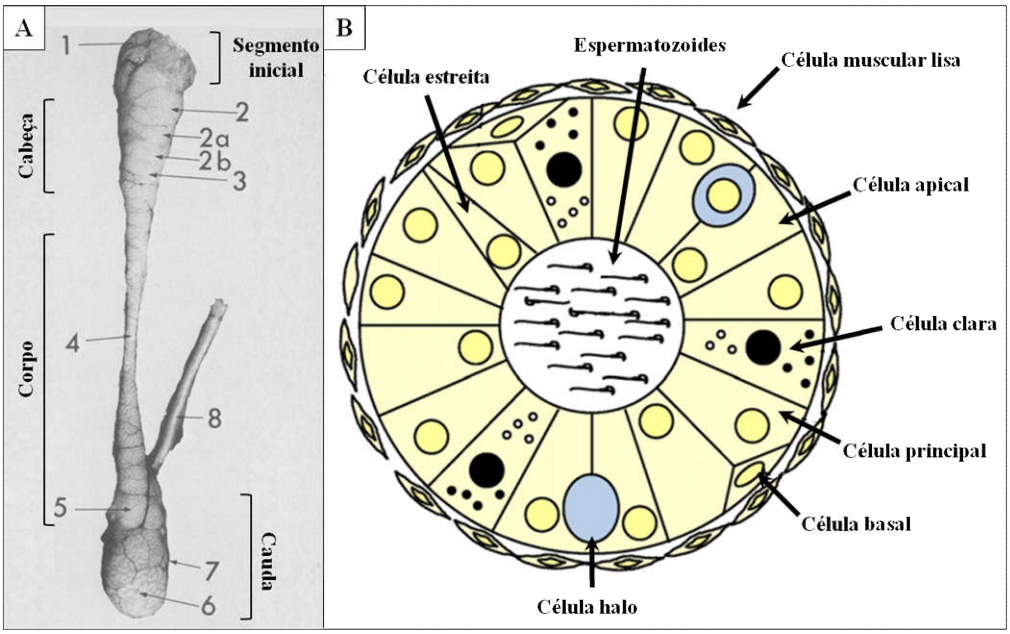

Figura 2. Divisões do epidídimo e sua composição celular. (A) Fotografia de epidídimo de rato mostrando suas sub-regiões: segmento inicial (1), cabeça proximal (2), cabeça distal (3), corpo proximal (4), corpo distal (5), cauda proximal (6), cauda distal (7); (B) Esquema ilustrativo de

um corte transversal do epidídimo, mostrando sua composição celular. Fonte: Adaptado de El-Hajjaji *et al.* (2011) e Hinton *et al*. (1979).

A maturação espermática é um processo dependente de andrógenos, como testosterona e dihidrotestosterona, bem como da interação entre o espermatozoide e a lâmina própria do epitélio epididimário e do microambiente do órgão (Kempinas & Klinefelter, 2014). Assim, compostos que atuam sobre o controle do eixo hipotalâmico-hipofisário-gonadal ou sobre o microambiente epididimário podem interferir no desenvolvimento normal do sistema genital masculino, bem como na maturação espermática (Cornwall, 2009; Luccio-Camelo & Prins, 2011).

A parede do epidídimo é formada por um epitélio pseudoestratificado, cuja altura é maior nas regiões proximais e menor nas regiões distais do órgão (Kempinas & Klinefelter, 2014). Este epitélio é constituído por vários tipos celulares (Figura 2), incluindo as células principais, basais, claras, estreitas, apicais e halo, cuja característica, prevalência e função se difere ao longo das diferentes regiões do órgão (Cornwall, 2009).

As células principais correspondem a 80% do epitélio e são responsáveis pela secreção da maior parte das proteínas do lumen epididimário. Além disso, elas se ligam umas às outras, formando uma barreira epididimária que cria um local imunoprotetor no lúmen, necessário para a maturação espermática (Cyr *et al*., 2007). As células halo também possuem função de proteção imune, enquanto as células estreitas, apicais e claras participam da acidificação do lumen (Pietrement *et al*., 2006; Kujala *et al*., 2007; Shum *et al*., 2009).

As células basais não entram em contato com o lúmen e estão intimamente associadas às células principais, o que indica possível papel na regulação da função dessas células (Veri *et al*., 1993; Seiler *et al*., 1999). As células apicais participam da endocitose de componentes luminais, função também exercida pelas células claras, as quais são responsáveis por fagocitar as gotas citoplasmáticas presentes na cauda dos espermatozoides (Kempinas & Klinefelter, 2014).

O epitélio epididimário é circundando por uma camada de células musculares lisas que apresentam receptores adrenérgicos e respondem a estímulos da inervação simpatico, controlando o processo contrátil e auxiliando no trânsito espermático pelo epidídimo, bem como na expulsão dos espermatozoides da região da cauda em direção ao ducto deferente (Kempinas *et al.,* 1998; Ricker, 1998). Assim, compostos que aumentam a disponibilidade de neurotransmissores catecolaminérgicos, como a noradrenalina, podem interferir no controle nervoso do sistema genital masculino (Nojimoto *et al.,* 2009; Jurkiewicz *et al.,* 2012). Estudos realizados em nosso laboratório relataram aumento da contratilidade epididimária *in vitro*

(Borges *et al*., 2013) e *ex vivo* (Cavariani *et al*., 2015), acompanhado por redução da qualidade espermática após a exposição à sibutramina e bupropiona, fármacos simpatomiméticos.

2.3. Glândulas sexuais acessórias

As glândulas acessórias consistem em: vesícula seminal, próstata, glândula coaguladora, glândula bulbouretral e glândula prepucial. Localizadas ao longo da uretra, elas produzem secreções essenciais para a função reprodutiva masculina (Junqueira & Carneiro, 2011).

As vesículas seminais estão localizadas dorsolateralmente à bexiga urinária e se desembocam na ampola da uretra (Junqueira & Carneiro, 2011). Elas são formadas por tubos tortuosos que produzem uma secreção amarelada viscosa alcalina que corresponde de 50 a 80% do ejaculado, constituída frutose, citrato, inositol, prostaglandinas e várias proteínas importantes para os espermatozoides (Junqueira & Carneiro, 2011).

A próstata é constituída por um conjunto de glândulas túbulo-alveolares ramificados, com ductos que desembocam na uretra, e revestida por uma cápsula fibroelástica que a penetra formando lóbulos (Junqueira & Carneiro, 2011). Em ratos, a próstata consiste em três lobos bilaterais, os quais se localizam próximo à vesícula seminal (lobo anterior ou glândula coaguladora) e na superfície ventrolateral (lobo ventral) e dorsolateral (lobo dorsolateral) da bexiga urinária (Abbott *et al.,* 2003). Ela secreta fluido rico em enzimas proteolíticas, zinco, inositol, transferrina e ácido cítrico (Haschek *et al.*, 2010).

2.4. Pênis

O pênis é órgão copulador masculino. Em ratos, é constituído pela uretra e três estruturas eréteis: dois corpos cavernosos, localizados na região dorsal do órgão, e o corpo esponjoso, que envolve ventralmente a parte esponjosa da uretra (Junqueira & Carneiro, 2011).

Este órgão recebe inervação simpática e parassimpática que controlam a ereção e ejaculação através de impulsos nervosos que chegam no músculo liso das artérias e das trabéculas que circundam os espaços vasculares dos corpos cavernosos (Junqueira & Carneiro, 2011). O processo de ereção se inicia com estímulos parassimpáticos que resultam em vasodilatação, causando o relaxamento da musculatura lisa. Ao mesmo tempo, ocorre inibição de impulsos vasoconstritores do estímulo simpático. A vasodilatação aumenta o fluxo sanguíneo, que preenche os corpos cavernosos, promovendo rigidez ao pênis. Após a ejaculação, o pênis retorna ao seu estado flácido, devido à redução da atividade parassimpática (Junqueira & Carneiro, 2011).

3. Lipídios, colesterol e os andrógenos

Os lipídios pertencem a uma família de compostos que possuem baixa solubilidade em água e alta solubilidade em solventes orgânicas, como álcool, éter e acetona (Nelson & Cox, 2013). Eles desempenham importante papel no armazenamento de energia, isolamento térmico, proteção mecânica e composição de membranas, além de apresentarem funções como sinalizadores celulares (metabólitos, mensageiros, hormônios) e cofatores enzimáticos (Nelson & Cox, 2013).

O colesterol, principal esterol sintetizado no tecido animal, é um dos principais componentes da membrana celular dos organismos eucariontes e possui papel essencial no crescimento, diferenciação e proliferação celular (Russel, 1992; Nelson & Cox, 2013; Singh *et al*., 2013), além de ser precursor de sais biliares, vitamina D e hormônios esteroides, como os andrógenos (Russel, 2009). Nos mamíferos, ele pode ser obtido via exógena (30% dos colesteróis), através do metabolismo de alimentos ricos em partículas lipoproteicas, ou via endógena, pela biossíntese a partir do Acetil-CoA (70% dos colesteróis) (Gotto, 1990; Russel, 2009), sendo encontrado tanto nos tecidos quanto no plasma sanguíneo, na sua forma livre ou combinado a ácidos graxos de cadeia longa, resultando em ésteres de colesterol, sua forma mais hidrofóbica (Nelson & Cox, 2013).

3.1. Biossíntese do colesterol

Os principais órgãos que sintetizam colesterol são o intestino delgado e o fígado, sendo o último responsável pela produção de aproximadamente um terço do colesterol do organismo (Nelson & Cox, 2013).

A biossíntese do colesterol é um processo que envolve mais de 20 enzimas (Singh *et al*., 2013). Tem início com a condensação de duas moléculas de acetil-CoA, formando acetoacetil-CoA que, por sua vez, condensa-se com outra molécula de acetil-CoA, produzindo 3-hidroxi-3-metilglutaril-CoA (HMG-CoA) que, através da enzima HMG-CoA redutase, é reduzida a mevalonato (Figura 3) (Nelson & Cox, 2013; Oesterle *et al*., 2017). Esta enzima é uma proteína de membrana localizada no retículo endoplasmático liso, representando o principal ponto de regulação da via do mevalonato, precursor do colesterol e outros isoprenoides (Nelson & Cox, 2013).

O mevalonato, é submetido a fosforilações, produzindo o 5-pirofosfomevalonato que, através de reações de descarboxilação, origina o isopentenil-pirofosfato. Nas etapas seguintes ocorre a produção de geranil-pirofosfato, farnesil-pirofosfato e esqualeno, o qual passa por uma

série de reações (oxidações, remoções ou migrações de grupos metil) resultando na síntese do colesterol (Nelson & Cox, 2013).

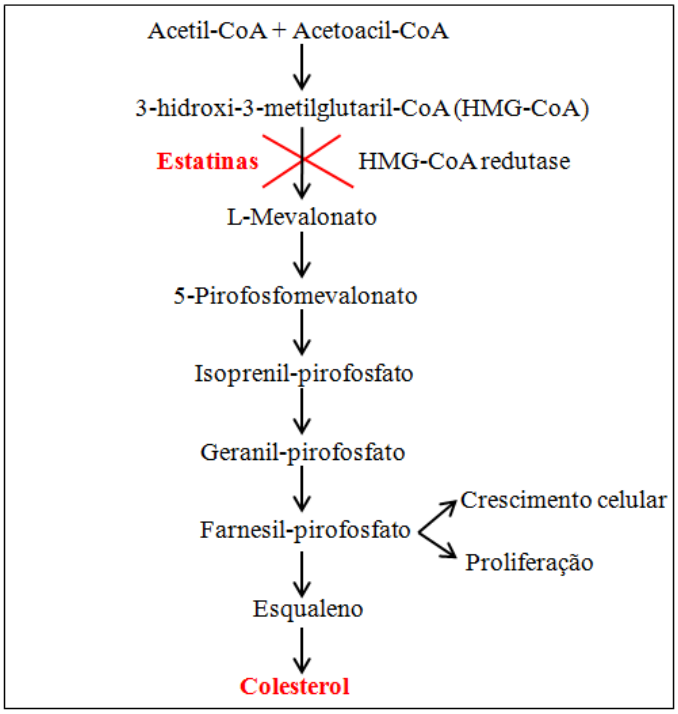

Figura 3: Via de biossíntese do colesterol e isoprenoides. Fonte: Adaptado de Oesterle *et al.* (2017).

3.2. Transporte e excreção do colesterol

Pequena fração de colesterol sintetizado no fígado é incorporada às membranas dos hepatócitos. A maioria do colesterol biossintetizado é exportada para os demais órgãos através de três formas: colesterol biliar, ácidos biliares ou ésteres de colesterol (Nelson & Cox, 2013). Os sais biliares são derivados de colesterol relativamente hidrofílicos, que auxiliam na digestão de lipídios, enquanto os ésteres de colesterol representam a sua forma mais hidrofóbica (Nelson & Cox, 2013).

Por serem insolúveis em água, o colesterol e os ésteres de colesterol são transportados pelo plasma através da sua ligação a proteínas transportadoras específicas, as apolipoproteínas, e outros lipídios, como fosfolipídios e triglicerói, formando complexos macromoleculares denominados lipoproteína plasmática. As diferentes combinações de lipídios e proteínas resultam na formação de lipoproteínas de diferentes densidades (Nelson & Cox, 2013). No plasma humano, já foram identificadas, pelo menos, nove apolipoproteínas (Nelson & Cox, 2013).

Conforme a densidade, as lipoproteínas podem ser separadas em cinco classes: quilomícrons, lipoproteínas de densidade muito baixa (*Very-low-density lipotrotein*, VLDL), lipoproteínas de baixa densidade (*Low-density lipoprotein*, LDL), lipoproteínas de densidade intermediária (*Intermediate-density lipoprotein*, IDL) e lipoproteínas de alta densidade (*High-density* lipoprotein, HDL) (Nelson & Cox, 2013).

Os quilomícrons são as partículas de maior tamanho e menor densidade, que transportam colesterol, ésteres de colesterol e, em maior quantidade, triglicerói̇s provenientes da dieta, do intestino para os músculos e outros tecidos, onde são consumidos ou estocados. O excesso desses lipídios é transportado pelos quilomícrons em direção ao fígado (Nelson & Cox, 2013).

Antes de deixar os hepatócitos, os triglicerídeos, colesterol e ésteres de colesteróis se ligam a apolipoproteínas, formando as VLDLs. As lipoproteínas VLDLs transportam estes lipídios sintetizados pelo fígado (fonte endógena), para os músculos e tecido adiposo. Quando perdem triglicerói̇s, são convertidos em LDL (Nelson & Cox, 2013).

As LDLs são lipoproteínas responsáveis pelo transporte de cerca de 70% do colesterol que circula no plasma, e seus altos níveis na circulação sanguínea estão associados a riscos de doenças cardiovasculares, uma vez que são partículas pequenas e densas o suficiente para se ligarem ao endotélio dos vasos sanguíneos, levando à deposição de placas lipídicas nas paredes de artérias, denominada aterosclerose (Huxley *et al.*, 2002; Catapano *et al.*, 2017; O'Keefe *et al*., 2017), sendo assim, conhecido como "colesterol ruim" (Nelson & Cox, 2013).

As HDLs, por sua vez, conhecidas como "colesterol bom" (Nelson & Cox, 2013), transportam o excesso de colesterol dos tecidos de volta para o fígado, onde é reciclado ou eliminado. Assim, elevado nível de HDL tem sido inversamente associado a riscos de doenças cardiovasculares (Nelson & Cox, 2013; Lee *et al*., 2017).

As células utilizam vários mecanismos para eliminar o excesso de colesterol. A maior parte do colesterol degradado (90%) é convertida em ácidos biliares por enzimas no fígado, enquanto uma fração menor é convertida em hormônios esteroides pelas glândulas endócrinas, vitamina D_3 (pela pele, fígado e rins), e oxiesterois pelo pulmão e cérebro (Russel, 2009).

3.3. Biossíntese dos andrógenos

Os andrógenos são sintetizados através de sucessivas reações de enzimas esteroidogênicas, que consistem em enzimas específicas do citocromo P450 (CYPs), hidroxiesteroides desidrogenases (HSDs) e esteroides redutases (Miller, 1988; Sanderson, 2006).

A biossíntese dos andrógenos tem início na mitocôndria. O colesterol é inicialmente transportado da membrana externa para a membrana interna da mitocôndria através da ação da proteína reguladora aguda da esteroidogênese (*Steroidogenic acute regulatory protein*, StAR) (Miller & Bose, 2011). Uma vez na membrana interna, o colesterol é convertido a pregnenolona através da enzima P450ssc (*Cholesterol side-chain cleavage;* produto do gene CYP11A), que compõe um subgrupo de enzimas do citocromo P450 (Parker & Schimmer, 1995; Sanderson, 2006; Carey *et al.*, 2007; Rone *et al.*, 2009) (Figura 4). A pregnenolona é transferida para o retículo endoplasmático, onde é convertida em progesterona pela 3β-hidroxiesteroide desidrogenase (3β-HSD) tipo 2, enzima predominantemente expressa nas glândulas adrenais, testículos e ovários (Sanderson, 2006).

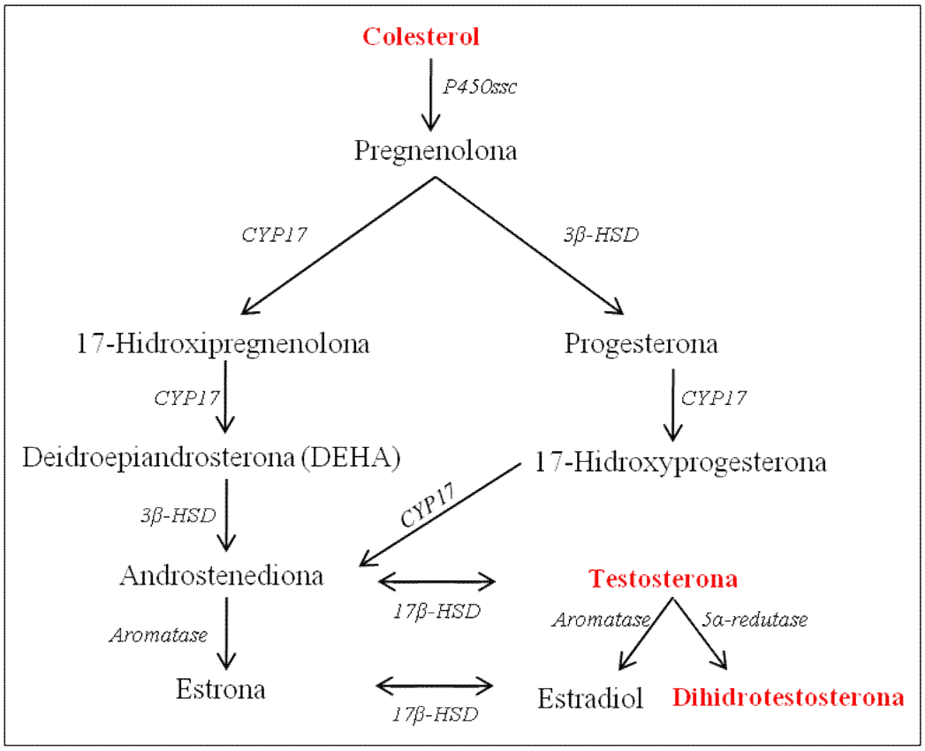

Figura 4. Síntese dos hormônios sexuais esteroides. Fonte: Adaptado de Carey *et al*. (2007).

Tanto a pregnenolona quanto a progesterona são hidroxiladas pela CYP17 para formar suas respectivas 17α-hidroxiesteroides, que darão origem à deidroepiandrosterona (DHEA) e androstenediona, respectivamente (Sanderson, 2006). A androstenediona é convertida em testosterona pela enzima 17β-hidroxiesteroide dehidrogenase (17β-HSD), a qual é expressa nas células de Leydig (Mindnich *et al*., 2004). Pouca fração da testosterona produzida nas células de Leydig é convertida em estradiol a partir da aromatase, a qual também é encontrada nas células

de Sertoli e células germinativas (Carreau *et al*., 2003). Essa conversão é necessária para a iniciação da espermatogênese e a mitose das espermatogônias (Carreau *et al.,* 2003).

Nos epidídimos, na próstata e, em menor quantidade, nos testículos, a 5α-redutase converte a testosterona em dihidrotestosterona (DHT). A DHT é o principal metabólito ativo da testosterona, responsável pelo desenvolvimento da próstata e virilização da genitália externa masculina, enquanto a testosterona é responsável pelo desenvolvimento do epidídimo, ductos deferentes e vesícula seminal (Goodman *et al.*, 2001; Sanderson, 2006; Bahie & Klinefelter, 2015).

A testosterona é produzida pelas células de Leydig nos testículos e, em pequena fração, pelo córtex da glândula adrenal (Sanderson, 2006). Além da testosterona, os testículos também secretam pequena quantidade de DHEA, androstenediona, DHT e estradiol. O estradiol é secretado tanto pelas células de Leydig quanto pelas células de Sertoli (Bahie & Klinefelter, 2015). Entre os esteroides androgênicos sintetizados pela glândula adrenal, pode-se destacar a testosterona, a DHEA e a androstenediona (Sanderson, 2006).

A produção dos andrógenos pelas glândulas adrenais é regulada pelos hormônios adrenocorticotróficos (ACTH), também conhecidos como corticotrofina (Parker, 1991). Já nos testículos, é controlada pela ação do hormônio liberador de gonadotrofina (GnRH), que é sintetizado pelo hipotálamo. Este hormônio atua sobre a adenohipófise, promovendo a liberação dos hormônios gonadotróficos, o hormônio folículo estimulante (FSH) e hormônio luteinizante (LH), os quais atuam sobre as gônadas (Rang *et al*., 1997; Bahie & Klinefelter, 2015).

O FSH encontra receptores expressos nas células de Sertoli, onde se liga, estimulando a via de mensageiros secundários mediados pela cAMP, que ativa vários fatores necessários para o processo da espermatogênese (Sanderson, 2006). O LH, por sua vez, se liga aos receptores nas células de Leydig, os quais também são acoplados à via de sinalizadores cAMP, estimulando a produção de testosterona (Bahie & Klinefelter, 2015). No sexo masculino, o LH também é denominado hormônio de estimulação de célula intersticial (ICSH) (Rang *et al*., 1997).

Uma vez sintetizados, os hormônios androgênicos circulam no plasma, onde se ligam a proteínas carreadoras, como a globulina de ligação dos hormônios sexuais (*Sex hormone binding globulin*; SHBG) e, em menor quantidade, a albumina (Goodman *et al.*, 2001), ou podem ser encontrados circulando livremente, na sua forma ativa. No homem, 40 a 65% da testosterona circulante encontra-se associada à SHBG, enquanto o restante encontra-se livre, na sua forma ativa (Keyser *et al.*, 2015).

3.4. Mecanismo de ação dos andrógenos

Por serem moléculas lipofílicas, os andrógenos atravessam facilmente a membrana plasmática em direção ao citosol. A testosterona e DHT atuam sobre receptores androgênicos (AR) intracelulares que se encontram inicialmente estabilizados pelas proteínas do choque térmico hsp90 (Cadwallader *et al.*, 2011; Jadhavar *et al.*, 2016).

Tanto a testosterona quanto a DHT ligam-se a esses receptores formando um complexo hormônio-receptor, que resulta no desligamento das hsp90, permitindo o deslocamento do complexo em direção ao núcleo onde se liga ao DNA em uma região específica denominada elementos de resposta androgênica (*Androgen response elements*, ARE) (Cadwallader *et al*., 2011). Essa interação, juntamente com o recrutamento de co-ativadores ou co-repressores, levam ao aumento ou inibição da transcrição de genes específicos, que resultam nos efeitos fisiológicos do hormônio (Cadwallader *et al*., 2011).

Grande fração da testosterona que entra no citosol é convertida em DHT, o qual possui maior afinidade pelo receptor de andrógeno sendo, assim, o principal responsável pela modulação da transcrição do DNA (Sanderson, 2006; Bahie & Klinefelter, 2015).

Além da sua função na regulação da espermatogênese, a testosterona tem importante papel na libido, distribuição de gordura, massa muscular e produção de células sanguíneas (Keyser *et al.*, 2015).

4. Estatinas

Entre os fármacos utilizados na regulação da hiperlipidemia estão os sequestradores de ácido biliar, niacina, ácidos fíbricos, ômega-3 e as estatinas (Ciccone, 2011; Sanin *et al.*, 2017).

As estatinas, descobertas pelo microbiologista Dr. Akira Endo na década de 1970, são uma das classes de drogas mais utilizadas no mundo e os fármacos mais utilizados no tratamento de dislipidemias (Endo, 1992; Feingold & Grunfeld, 2016). Comercializadas desde 1987, elas constituem uma notável classe de medicamentos redutores de colesterol, sendo considerados os agentes mais eficazes para a redução das concentrações plasmáticas de LDL (Oesterle *et al*., 2017). Devido a este efeito, estão associadas com uma expressiva diminuição da morbidade e mortalidade cardiovascular de pacientes em prevenção primária ou secundária de doenças coronárias (Ballantyne, 1998; Ichiki *et al*., 2001; Arca & Gaspardone, 2007), sendo prescritas no tratamento das hiperlipidemias em pacientes obesos (Nylén *et al.*, 2013; Klop *et al.*, 2013).

Kokkinos e colaboradores (2013) relataram que o tratamento de pacientes com dislipidemia utilizando-se estatinas combinado a exercício físico levou à redução nas taxas de

mortalidade. Outros ensaios clínicos também demonstraram que as estatinas podem aumentar a expectativa e qualidade de vida, retardando a progressão do diabetes mellitus e doença renal crônica, além de melhorar o fluxo sanguíneo central e periférico (Shepherd, 2006).

A família das estatinas compreende nove compostos, sendo que oito foram aprovados para a comercialização a nível mundial nos últimos 20 anos: lovastatina (Mevacor® e Altoprev®), mevastatina (Compactin®), pravastatina (Pravachol®) e sinvastatina (Zocor®), derivadas de fungos, e atorvastatina (Lipitor®), rosuvastatina (Crestor®, anteriormente conhecida como ZD4522), fluvastatina (Lescol®), cerivastatina (Baycol®, retirada do mercado em 2001) e pitavastatina (Livalo®), que são compostos sintéticos (FDA, 2013; Ciurleo *et al*., 2014). Estes compostos diferem pelos mecanismos de biodisponibilidade, tempo de meia-vida, metabolismo mediado pelo citocromo P-450, bem como pela forma com que são transportados nas células, pelo potencial de redução do colesterol e pela lipossolubilidade (Vaughan & Gotto, 2004; Schachter, 2004; Ciurleo *et al.*, 2014; Sirtori, 2014).

Entre as estatinas de última geração disponíveis no mercado, a rosuvastatina é a que apresenta maior eficiência na redução dos níveis circulantes de LDL, seguida pela atorvastatina, sinvastatina e pravastatina (Schachter, 2004), sendo capaz de promover redução em 60% quando administrada na sua maior dose (40 mg) atualmente disponível no mercado (McTaggart *et al*., 2001; Davidson, 2002; Feingold & Grunfeld, 2016). Também comercializada como Trezor®, a rosuvastatina está disponível nas doses de 5, 10, 20 e 40 mg (Vaughan & Gotto, 2004).

Estudo realizado em uma população composta por 17.082 pacientes, relatou redução nos níveis de LDL-C em 89,2% nos indivíduos tratados por um ano com 20 mg/kg de rosuvastatina, sendo que 46,3% (3.640 indivíduos) apresentou redução maior ou igual a 50% dos níveis basais de LDL-C (Ridker *et al.*, 2016). Em outro estudo realizado em uma população etnicamente diversificada, com 12.705 indivíduos de 21 países, foi relatado redução significativa do risco de eventos cardiovasculares após cinco anos de tratamento com 10 mg de rosuvastatina, quando comparado ao grupo placebo (Yusuf *et al.*, 2016).

A sinvastatina, por sua vez, disponível nas doses de 5, 10, 20, 40 e 80 mg (FDA, 2012), está entre as estatinas mais utilizadas por adultos a partir de 40 anos de idade nos Estados Unidos, segundo estudo realizado com dados do *National Health and Nutrition Examination Survey* (NHANES) (Gu *et al*., 2014).

Estudo realizado em pacientes com diabetes tipo 2 e baixos níveis de HDL relatou melhora no perfil lipídico após 6 semanas de tratamento com 40 e 80 mg de sinvastatina, de forma dose-dependente (Miller *et al*., 2006). Em ambas as doses foi observado redução nos níveis de VLDL-C, VLDL3 e IDL, bem como quatro subclasses de LDL, comparado ao grupo

placebo. Em outro estudo clínico, realizado com 20.536 pacientes adultos (40 a 80 anos) do Reino Unido que apresentavam doença vascular ou diabetes, recebendo 40 mg de sinvastatina ou placebo durante 5 anos, relatou redução nas taxas de mortalidade, principalmente devido a doença vascular, bem como nas taxas de ataques cardíacos não fatais, enfartes e procedimentos de revascularização em pacientes que receberam a droga, comparado ao placebo (HPSCG, 2005).

4.1. Mecanismo de ação das estatinas

As estatinas atuam como inibidores seletivos e competitivos da enzima HMG-CoA redutase, ligando-se ao sítio ativo desta enzima, o que dificulta a ligação do seu substrato, resultando na redução dos metabólitos isoprenoides, como farnesil-pirofosfato e geranil-geranilpirofosfato, e de colesterol, sintetizados pelos hepatócitos (Schachter, 2004; Sirtori, 2014; Oesterle *et al.*, 2017). A redução do colesterol leva à queda dos níveis séricos de VLDL, bem como o aumento da expressão dos receptores de LDL no fígado, aumentando a remoção do LDL circulante na corrente sanguínea em até 60% (Davidson, 2002; Sirtori, 2014; Feingold & Grunfeld, 2016), o que contribui para a redução dos eventos cardiovasculares ateroscleróticos (Feingold & Grunfeld, 2016).

Estudos tem demonstrado que as estatinas também promovem um pequeno aumento nos níveis de HDL (Maron *et al.*, 2000; Adams *et al.*, 2014) e reduzem os níveis de IDL e da apolipoproteína B (Bos *et al*., 2014). Jones e colaboradores (2003) relataram que o tratamento de pacientes hipercolesterolêmicos com rosuvastatina na dose de 10 a 40 mg apresentaram a maior taxa de aumento dos níveis de HDL-C (7,7-9,6%), seguida pela sinvastatina nas doses de 10 a 80 mg (5,2-6,8%), pravastatina nas doses de 10 a 40 mg (3,2-5,6%) e atorvastatina nas doses de 10 a 80 mg (2,1-5,7%).

Além da redução dos níveis de LDL e aumento dos níveis de HDL, as estatinas podem ser prescritas no tratamento de hipergliceridemia, uma vez que promovem redução dos níveis de triglicérides no sangue, a qual está correlacionada com a queda dos níveis de LDL (Feingold & Grunfeld, 2016). Estudos clínicos relataram que pacientes hipercolesterolêmicos tratados com rosuvastatina nas doses de 10 a 40 mg apresentaram redução nos níveis de LDL-C em 52 a 63% e de triglicérides em até 28%, bem como aumento nos níveis de HDL-C em até 14% (Rosenson, 2003).

4.2. Efeitos pleiotrópicos das estatinas

Além da sua eficiência como agentes hipolipemiantes, as estatinas possuem efeitos pleiotrópicos, ou seja, podem agir de forma independente da redução dos lipídios (Oesterle *et al*., 2017). Acredita-se que esses efeitos ocorram devido à inibição da síntese de compostos isoprenoides (não esteroidais), também produzidos a partir do mevalonato (Schachter, 2004; Oesterle *et al*., 2017) (Figura 3). Entre os efeitos pleiotrópicos relatados, pode-se citar sua propriedade antioxidante pela regulação da produção de óxido nítrico, anti-apoptótica, imunomoduladora, anti-inflamatória, antitrombogênica, nefroprotetora e neuroprotetora, além de melhorar a função endotelial e aumentar os níveis de vitamina D (Ichiki *et al.*, 2001; LaRosa, 2001; Meroni *et al.*, 2012; Violi *et al.*, 2013; Heeba & Hamza, 2015; Verdoia *et al.*, 2017).

Estudos demonstraram que as estatinas reduzem a produção de espécies reativas de oxigênio induzida nas células musculares lisas dos vasos pela angiotensina II, através do seu efeito inibitório sobre a oxidação do NADPH mediada pelo Rac 1, e da redução da expressão dos receptores angiotensina AT1 (Wassmann *et al*., 2001). A ativação do Rac 1 no endotélio tem sido associada à aterosclerose e disfunção endotelial (Gregg *et al.*, 2003).

Esses efeitos pleiotrópicos promovidos pelas estatinas têm estimulado o desenvolvimento de diversos estudos, que demonstraram novas aplicações terapêuticas, como no tratamento da doença de Alzheimer e Parkinson, esclerose múltipla, acidente vascular cerebral, lesão cerebral traumática, epilepsia, esclerose, encefalomielite, supressão de neoplasias e no tratamento de osteoporose (Puglielli, 2003; Gao *et al*., 2015; De Oliveira *et al*., 2015; Scicchitano *et al*., 2015; Gao *et al*., 2016; Ghasami *et al*., 2016; Kang *et al*., 2017).

4.3. Farmacocinética das estatinas

A estrutura química das estatinas determina a sua lipossolubilidade que, por sua vez, influencia a sua absorção, distribuição, metabolismo e excreção (Schachter, 2004), além de ser a principal característica que confere a seletividade de atuação das estatinas (Schachter, 2004; Oesterle *et al*., 2017). Todas as estatinas (exceto a pitavastatina) apresentam intenso efeito de primeira passagem no fígado e são transportadas (com exceção da pravastatina) pelas proteínas plasmáticas, sendo albumina a principal proteína transportadora (Corsini *et al*., 1999). Assim, a concentração do fármaco livre na circulação sistêmica é baixa (Schachter, 2004).

Estatinas mais lipofílicas, como atorvastatina, sinvastatina, lovastatina, fluvastatina, cerivastatina e pitavastatina, conseguem atravessar facilmente a membrana plasmática por difusão passiva, além de serem mais susceptíveis ao metabolismo pelo sistema do citocromo

P450. Já as estatinas como a pravastatina e a rosuvastatina, são relativamente hidrossolúveis, o que requer transporte ativo através de polipeptídios de transporte de ânions orgânicos (*Organic Anion Transporting Polypeptides*, OATPS), sendo assim, mais hepatoseletivas comparadas às demais estatinas, e menos metabolizados pelas enzimas do citocromo P450, apresentando um potencial reduzido de absorção pelas células periféricas (Schachter, 2004; McTaggart *et al.*, 2001; Oesterle *et al*., 2017).

Lovastatina, mevastatina e sinvastatina são pró-fármacos que possuem anéis de lactona e necessitam ser hidrolisadas no fígado para serem ativadas. Por serem lactonas, são menos hidrossolúveis (Serajuddin *et al*., 1991; Schachter, 2004). Já a atorvastatina e rosuvastatina encontram-se na sua forma ativa, sendo administradas como β-hidroxiácidos (Corsini *et al*., 1999; Schachter, 2004).

A CYP3A4 é a principal enzima do citocromo P450 que metaboliza a maioria das estatinas, como lovastatina, sinvastatina, cerivastatina e atorvastatina (Corsini *et al.*, 1999). Estudos relataram que a lovastatina e sinvastatina apresentam interação farmacocinética, como o aumento da biodisponibilidade, quando associadas a fármacos inibidores da enzima CYP3A4, como antifúngicos azóis (cetoconazol, itraconazol), antibióticos macrólidos (eritromicina) e antidepressivos, como a nefazodona, o qual atua como inibidor dos receptores de serotonina e da recaptação da serotonina, dopamina e noradrenalina (Jacobson *et al*., 1997; Richelson, 1998; Corsini *et al.*, 1999).

As estatinas são rapidamente absorvidas após a sua administração, atingindo a concentração máxima no plasma em 3 a 4 horas, e apresentam meia-vida de até 20 horas (McTaggart *et al*., 2001). A lovastatina, pravastatina e simvastatina (derivadas de metabólitos de fungos) têm meia-vida de eliminação de uma a três horas, enquanto as estatinas sintéticas variam de uma hora (fluvastatina) a 19 horas (rosuvastatina) (Schachter, 2004).

A principal rota de eliminação das estatinas e seus metabólitos ocorre via sais biliares em fezes, após a sua metabolização no fígado (Schacher, 2004), sendo que quantidades insignificantes são excretadas como medicamentos originais, sem alteração na sua estrutura (Corsini *et al.*, 1999).

4.4. Efeitos adversos das estatinas

Apesar dos seus efeitos terapêuticos, as estatinas podem apresentar efeitos secundários, como hepatotoxicidade, perturbações da cavidade oral, problemas gastrointestinais (dores gástricas, náusea, diarréia, gases e azia), neuropatias e problemas musculares, como dores

musculares, câimbras e severa fraqueza e fadiga, que podem indicar miosite, a qual leva a severa desagregação e destruição do músculo esquelético (rabdomiólise) (Ciccone, 2011; Schooling *et al.*, 2013; Sirtori, 2014; FDA, 2017a). Além disso, estudo tem demonstrado que as estatinas apresentam efeitos adversos sobre o sistema genital (Leite *et al*., 2017; Pons-Rejraji *et al*., 2014; Keyser *et al*., 2015).

4.5. Efeitos das estatinas sobre o sistema genital

Os processos da espermatogênese e maturação espermática são eventos regulados pela testosterona. Assim, diante do efeito das estatinas na redução do colesterol, precursor da testosterona, vários estudos tem sido desenvolvido para investigar os seus efeitos sobre o sistema genital masculino.

Estudo *in vitro* demonstrou, em ratos, que células de Leydig cultivadas com atorvastatina, mevastatina e sinvastatina, sob estímulo de LH, apresentaram redução na síntese de testosterona em 40% (Klinefelter *et al*., 2014). Outro estudo, realizado em uma população adulta de Rotterdam (Holanda) composta por 4166 homens, investigou a associação entre o uso de estatinas e os níveis de testosterona sérica. Entre os pacientes que utilizaram estatinas por 1 a 6 meses, foi relatado redução significativa dos níveis de testosterona sérica total ligada a proteínas transportadoras (SHBG) e de testosterona livre no plasma (Keyser *et al.*, 2015). Schooling e colaboradores (2013) também observaram redução nos níveis de testosterona em 501 homens hipercolesterolêmicos tratados com estatinas. Azzarito e colaboradores (1996) relataram que pacientes hipercolesterolêmicos tratados com 20 mg de sinvastatina apresentaram redução nos níveis séricos de testosterona livre após 6 e 12 meses de exposição à droga.

Em estudo realizado com 151 homens diabéticos (diabetes tipo 2) e hiperglicêmicos, tratados com 5 mg/kg de rosuvastatina (Hsieh & Huang, 2016), foi observado redução nos níveis de colesterol total e LDL após seis meses de tratamento, acompanhado pela redução dos níveis séricos de testosterona. Seis meses após o término do tratamento, houve aumento tanto nos níveis de colesterol quanto testosterona.

Pons-Rejraji e colaboradores (2014) relataram que homens inicialmente saudáveis apresentaram alteração nos níveis plasmáticos de lipídios, nos parâmetros espermáticos e na composição do fluido seminal após a exposição a 10 mg de atorvastatina durante 5 meses. Os níveis de LDL-C e a concentração de colesterol total foram reduzidos em 42% e 24%, respectivamente. Durante o tratamento e 3 meses após a sua interrupção, foram observadas várias alterações nos parâmetros espermáticos, como redução no número de espermatozoides e

na vitalidade em 31% e 9.5%, respectivamente, além de alterações na cinética da reação acrossômica, motilidade e morfologia espermática (anormalidades de cabeça, pescoço e peça intermediária). As concentrações seminais de fosfatases ácidas, α-glucosidase e L-carnitina também foram reduzidas durante a terapia, indicando alterações nas funções prostáticas e epididimárias.

Em macacos, a exposição a 80 mg/kg de rosuvastatina resultou na redução do epitélio seminífero, presença de células gigantes e vacuolização celular (FDA, 2003). Em cães, também foi observado presença de células gigantes, além de degeneração moderada dos túbulos seminíferos após o tratamento por um mês com 90 mg/kg de rosuvastatina (FDA, 2003).

Recentemente, relatamos que ratos adultos expostos a 3 e 10 mg/kg de rosuvastatin durante a pré-puberdade apresentaram atraso na instalação da puberdade e no desenvolvimento do epidídimo, bem como alterações na morfologia testicular e epididimária e, na maior dose, depleção nos níveis séricos de testosterona (Leite *et al*., 2017).

Zhang e colaboradores (2017) relataram que ratos expostos a 4, 8 ou 16 mg/kg de sinvastatina durante 20 e 40 dias apresentaram redução nos níveis séricos de colesterol total, LDL, triglicérides, testosterona, estradiol e progesterona, de forma dose e tempo-dependente, além de aumento nos níveis de FSH e LH, indicando regulação por feedback.

Beverly e colaboradores (2014) relataram que a exposição *in utero* à sinvastatina resultou em redução dos níveis de triglicérides, LDL, HDL, colesterol, da produção de testosterona, e da expressão de dois genes no testículo fetal em ratos.

Além das alterações nos parâmetros reprodutivos masculinos, estudos têm relatado que a exposição a estatinas pode levar ao rompimento da placenta, afetar a embriogênese e causar efeitos teratogênicos, como morte fetal, retardo no crescimento intrauterino, malformações e abortos (Dostal *et al*, 1994; Edison & Muenke, 2004; Kenis *et al*., 2005). Mulheres tratadas com diferentes estatinas durante a gestação apresentaram parto prematuro e redução do peso fetal ao nascimento (Taguchi *et al.*, 2008). Ratas expostas a estatinas durante a prenhez apresentaram fetos com malformações esqueléticas (lovastatina), diminuição do peso corpóreo e atraso no desenvolvimento (atorvastatina) (Minsker *et al.*, 1983; Henck *et al.*, 1998). Camundongos expostos à atorvastatina e lovastatina apresentaram indução do aumento de apoptose de células germinativas primordiais em fetos, sugerindo potencial efeito sobre a fertilidade (Ding *et al.*, 2008).

Alguns estudos, porém, relatam efeitos protetores das estatinas sobre o sistema genital. Heeba & Hamza (2015) reportaram que as alterações testiculares promovidas pela diabetes induzida em ratos foram atenuadas após a administração de baixas doses de rosuvastatina (5 e 10

mg/kg), via oral, por oito semanas. Entre os efeitos observados estão a recuperação do peso testicular e epididimário reduzidos pela diabetes, bem como aumento da contagem e motilidade espermática na cauda epididimária, com redução dos níveis testiculares de óxido nítrico e malondialdeído e da atividade da mieloperoxidase, além do aumento nos níveis de glutationa e superóxido dismutase, que se apresentaram reduzidos pela diabetes. Esse efeito protetor da rosuvastatina ocorreu de forma dose-dependente, através de mecanismos antioxidantes, anti-inflamatórios e anti-apoptóticos, como a redução na atividade MOP testicular e supressão da expressão de óxido nítrico sintase induzível (iNOS), NF-κB p65 e caspase-3 (Heeba & Hamza, 2015).

As células de Sertoli possuem uma comunicação química e iônica entre si através de junções celulares, formando uma barreira no epitélio testicular, denominada barreira hemato-testicular, que protege as células germinativas em desenvolvimento contra o sistema imunológico, espécies reativas de oxigênio e agentes xenobióticos que podem alcançar os testículos pela corrente sanguínea (Robaire *et al*., 2006). Estudo *in vitro* relatou efeito protetor da sinvastatina contra a toxicidade promovida nas células de Sertoli pela exposição à cisplatina (Wang *et al*., 2015). Tal efeito foi mediado pela redução na fosforilação da conexina 43, promovendo aumento das junções gap (Wang *et al*, 2015).

Estudos *in vitro* também relataram evidências de que as estatinas possuem potencial efeito terapêutico no tratamento de endometriose, um quadro clínico caracterizado pelo crescimento ectópico de tecido endometrial (Piotrowski *et al.*, 2006; Vitagliano *et al.*, 2015).

Esfandiari e colaboradores (2007) relataram efeito inibidor da lovastatina sobre a proliferação celular e a angiogênese, em cultura de células endometriais de 40 mulheres com ovulação normal, submetidas ao tratamento de infertilidade. Tal efeito apresentou-se de forma concentração-dependente. Na presença de 1 μM de lovastatina, foi observado redução da angiogênese, porém, a proliferação celular não foi alterada. Já nas concentrações 5 e 10 μM, ambos os parâmetros foram inibidos.

Piotrowski e colaboradores (2006) demonstraram redução na proliferação e viabilidade de células do estroma endometrial humano cultivadas por 48 horas com mevastatina e sinvastatina (1-30 μmol/L). Da mesma forma, Sokalska e colabradores (2010) também relataram redução na proliferação e viabilidade de células do estroma endometrial humano cultivadas com 10 μmol/L de sinvastatina, de forma temporal (efeito máximo após 48 horas de exposição) e dose-dependente (10 e 30 μmol/L). Além da proliferação celular, Nasu e colaboradores (2009) também demonstraram que a sinvastatina promoveu a inibição da contratilidade de células do estroma endometrial humano.

5. Fármacos utilizados no tratamento da obesidade

Entre os fármacos que promovem redução do peso corpóreo em pacientes obesos estão os supressores simpatomiméticos de apetite, como bupropiona, benzafetamina, dietilpropiona, metanfetamina, fendimetrazin e fentermina, que agem através da diminuição da ingestão calórica, aumentando a influência da noradrenalina e dopamina no centro regulador da fome localizado no hipotálamo lateral; os inibidores de lipase, como o orlistat, que agem sobre as enzimas pancreáticas que digerem a gordura, levando à redução da sua absorção no trato gastrointestinal; e os inibidores de recaptura de serotonina e noradrenalina, como a sibutramina, que inibe o apetite através do aumento dos efeitos desses neurotransmissores sobre o sistema nervoso central (Ciccone, 2011).

5.1. Sibutramina

Comercializada como Meridia® (Estados Unidos e Brasil) e Reductil™ (Europa e Brasil), nas doses de 1 mg, 10 mg e 15 mg, a sibutramina foi sintetizada pela primeira vez em 1980 e descoberta como um potente agente antidepressivo em 1984, quando foi inicialmente administrada em humanos (Nisoli & Carruba, 2000; Barkeling *et al.*, 2003; FDA, 2005). Em 1997, foi aprovada pela *Food and Drug Administration* (FDA) para a redução do peso corpóreo e manutenção da perda de peso, em pacientes obesos e pacientes com sobrepeso que apresentavam outros fatores de risco para doença cardiovascular (FDA, 2010).

Membro da classe de fármacos denominados inibidores da recaptura de serotonina-noradrenalina (*Serotonin-norepinephrine reuptake inhibitors*; SNRIs), a sibutramina e seus metabólitos atuam através da inibição da recaptura desses neurotransmissores e, em menor quantidade, dopamina, no sistema nervoso central, principalmente no hipotálamo (Jackson *et al.*, 1997; Padwal & Majumdar *et al*., 2007; Fletcher *et al.*, 2010). Este bloqueio na recaptura leva ao aumento da sua disponibilidade na fenda sináptica, resultando no aumento do gasto energético e da saciedade e, consequente redução da ingestão alimentar (Nisoli & Carruba, 2000).

A sibutramina possui o substrato β-feniltilamina, também encontrado em várias classes de fármacos anorexígenos, como anfetamina, fenfluramina e fentermina (Nisoli & Carruba, 2000), e compartilha algumas propriedades farmacológicas com o fármaco antidepressivo venlafaxina (Nisoli & Carruba, 2000). Seu potencial inibidor seletivo da recaptura de noradrenalina e serotonina são propriedades também encontradas nos antidepressivos desipramina e fluoxetina, respectivamente (Nisoli & Carruba, 2000).

Vários estudos clínicos demonstraram a eficácia da sibutramina como agente redutor do peso corpóreo. Pacientes tratados por um ano com 10 mg de sibutramina apresentaram redução significativa do peso corpóreo, quando comparados com o grupo placebo (Jordan, 2013). Nisoli & Carruba (2000) demonstraram, em ensaios clínicos, que existe uma relação entre a dose de sibutramina administrada e a perda do peso corpóreo após o tratamento, podendo ser observado redução em 11% e manutenção desta taxa ao longo de 18 meses de tratamento. A análise de 11 estudos clínicos com pacientes tratados com doses entre 1 e 30 mg de sibutramina por 12 a 52 semanas apresentou redução significativa do peso corpóreo de forma dose-dependente nas doses entre 5 e 20 mg quando comparado ao grupo placebo (FDA, 2005).

Jackson e colaboradores (1997) relataram que ratos expostos a 10 mg/kg de sibutramina durante a fase escura do fotoperíodo apresentaram significativa redução no consumo alimentar oito horas após a administração da droga. Esse efeito, porém, foi antagonizado quando expostos ao prazosin (antagonista α_1-adrenoceptor), e parcialmente antagonizado quando expostos ao metoprolol (antagonista β_1 adrenoceptor) e metergolina (antagonista do receptor 5-$HT_{2A/2C}$), o que demonstrou que α_1 e β_1 adrenoceptores e 5-HT2A/2C estão envolvidos nos efeitos da sibutramina sobre o consumo alimentar (Jackson *et al*., 1997).

Estudo realizado em ratos tratados com 10 mg/kg de sibutramina e seus metabólitos, relataram alterações na termogênese e metabolismo (Connoley *et al*., 1999). Após o tratamento, foi observado aumento do consumo de oxigênio e da taxa metabólica em 20 e 30%, respectivamente, e da temperatura corpórea, quando comparados ao grupo controle, eventos que foram relacionados com a ativação do sistema nervoso simpático eferente do tecido adiposo marrom, através da estimulação dos β_3-adrenoceptores (Connoley *et al.*, 1999).

Além de induzir a redução do peso corpóreo, estudos relataram que a sibutramina apresenta efeitos sobre vários fatores cardiometabólicos, como os níveis sanguíneos de glicose e lipídios (Lean, 1997; Nisole & Carruba, 2000).

Em um estudo realizado com 83 pacientes obesos e diabéticos recebendo 15 mg de sibutramina por 12 semanas, foi observado redução da concentração máxima de glicose no sangue, em 1,1 mmol/L (Finer *et al.*, 2000). Outro estudo realizado em uma população de pacientes hispânicos obesos com diabetes tipo 2, foi relatado redução do peso corpóreo, IMC e na concentração plasmática de glicose após o tratamento com 10 mg de sibutramina por um ano (Sánchez-Reyes *et al*., 2004). Weeke e colaboradores (2010) também relataram que pacientes com sobrepeso e obesos tratados com 10 mg de sibutramina por seis semanas apresentaram redução nos níveis de triglicerídeos, colesterol total, LDL, VLDL e HDL.

5.1.1. Farmacocinética da sibutramina

A sibutramina é uma amina terciária que, após passar por desmetilação, produz seis aminas secundárias como metabólitos (Nisoli & Carruba, 2000), sendo os metabólitos mono e dimetil-sibutramina (M1 e M2, respectivamente) as formas predominantes de sua ação. A ativação desses metabólitos ocorre pelo metabolismo de passagem, mediado pela isoenzima 3A4 do citocromo P450 (CYP 3A4) presente no fígado (Padwal & Majumdar, 2007).

A sibutramina é rapidamente absorvida pelo trato gastrointestinal, apresentando pico de concentração plasmática em uma a duas horas após a administração oral, e é submetida ao metabolismo de primeira passagem no fígado para formar os metabólitos M1 e M2, que apresentam picos entre três a quatro horas, respectivamente (FDA, 2005). Aproximadamente 77% do fármaco é absorvido após administração oral, sendo sua meia-vida de 14 a 16 horas em humanos (FDA, 2005; Silva *et al*., 2010). *In vitro*, a maior fração da sibutramina e os metabólitos M1 e M2 (97%, 94% e 94%, respectivamente) encontra-se ligada a proteínas plasmáticas humanas, sendo a maior parte da excreção (77%) realizada via renal, com tempo de excreção de uma hora, 14 horas e 16 horas, respectivamente (FDA, 2005; Padwal & Majumdar, 2007).

5.1.2. Efeitos adversos da sibutramina

Entre os efeitos adversos da sibutramina estão boca seca, aumento da pressão sanguínea, insônia, náusea, tontura e alterações na fertilidade (Ciccone, 2011). Além disso, há relatos de casos de transtornos psiquiátricos como mania, crise de pânico e psicose (Binkley *et al*., 2002; Cordeiro & Vallada, 2002; Taflinski & Chojnacka, 2000).

Estudos relatam que a sibutramina também possui efeito genotóxico. Silva e colaboradores (2010) demonstraram, em camundongos, que a sibutramina apresenta danos clastogênicos e/ou aneugênicos de forma dose-dependente. Chakrabarti e colaboradores (2017) demonstraram efeito genotóxico da sibutramina sobre linfócitos humanos cultivados com 250, 500 e 1000 µg/mL da droga.

A comercialização da sibutramina na União Europeia foi inicialmente aprovada em 1999, sendo disponível em forma de cápsulas contendo 10 mg ou 15 mg da droga (EMA, 2010). Devido a relatos de efeitos cardiovasculares, incluindo aumento da pressão arterial e da frequência cardíaca, ela foi revisada em 1999 e 2002 (EMA, 2010). Baseados nos resultados, a *European Medicines Agency* (EMA) concluiu que os benefícios da sibutramina no tratamento de pacientes sobrepesos e obesos superavam os riscos, porém, foi solicitado à companhia fabricante

do Reductil® que realizasse um estudo com um tempo maior de exposição, incluindo um número maior de pacientes com fatores de riscos cardiovasculares (EMA, 2010).

Em 2002, iniciou-se o estudo denominado *Sibutramine Cardiovascular Outcome Trial* (SCOUT), composto por 9.800 pacientes obesos ou com sobrepeso que apresentavam doença cardiovascular, hipertensão e diabetes tipo 2, tratados por seis anos com sibutramina (EMA, 2010). Ao final desse estudo, concluiu-se que a administração da sibutramina por período prolongado, em pacientes que apresentavam fatores de risco cardiovasculares pré-existentes, aumentou o risco de infarto do miocárdio e acidente vascular cerebral não fatal, o que levou à retirada da droga do comércio na Europa e dos Estados Unidos em 2010 (EMA, 2010; FDA, 2010; Krentz *et al.*, 2016).

5.1.3. Efeitos da sibutramina sobre o sistema genital

Além dos efeitos adversos promovidos pela exposição à sibutramina acima citados, estudos relataram alterações no sistema genital e na fertilidade (Bellentani *et al*., 2011; Borges *et al*., 2013).

O epidídimo é um órgão responsável pela maturação e armazenamento espermático. A maturação espermática ocorre pela adição e remoção de substâncias durante o trânsito dos espermatozoides pelo epidídimo, levando a alterações nas suas características morfológicas e fisiológicas (Orgebin-Crist, 1969) que permitem adquirir habilidade fértil e apresentar movimento progressivo (Robaire *et al*., 2006; Kempinas & Klinefelter, 2014). Esse transporte é mediado pela atividade contrátil espontânea da musculatura lisa presente na cabeça e corpo do epidídimo e pela atividade contrátil induzida da musculatura lisa que compõe a parede da cauda epididimária, região altamente inervada do órgão (Kempinas & Klinefelter, 2018; Bellentani *et al*., 2011).

Sabe-se que o epidídimo recebe inervação autônoma proveniente do gânglio do mesentério inferior (Ricker, 1998), e que esta inervação controla os processos de contração muscular epididimária através da liberação de noradrenalina, a qual age via ativação de α_1-adrenoceptores (Ventura & Pennefather, 1991). Assim, fármacos que interferem no sistema simpático ou na contratilidade epididimária podem alterar o tempo de trânsito espermático pelo órgão, e essa alteração pode afetar a maturação espermática (Kempinas *et al*., 1998; Fernandez *et al.*, 2008).

Estudos realizados em ratos demonstraram que a sibutramina promove alterações na contratilidade de órgãos reprodutores masculinos, como a próstata, ducto deferente, vesícula

seminal e epidídimo, bem como alterações na ejaculação, na qualidade espermática e na fertilidade (Nojimoto *et al*., 2009; Fernandez *et al*., 2011; Borges *et al*., 2013). Em homens, também foi reportado que o tratamento com o fármaco resultou em ejaculação anormal (FDA, 2010).

Estudo realizado em ratos tratados com 5, 20 e 50 mg/kg de sibutramina demonstrou que, dependendo da dose, a droga pode facilitar ou inibir a ejaculação (Nojimoto *et al*., 2009). Nas menores doses (5 e 20 mg/kg), foi observado redução na latência da ejaculação em 48%, acompanhada pela redução no número de intromissões até a primeira ejaculação quando comparado com o grupo controle, enquanto a maior dose promoveu aumento na latência da ejaculação em aproximadamente 50%. Ensaio farmacológico *in vitro* demonstrou que a exposição à sibutramina nas doses de 3 a 30 μM promoveu aumento na sensibilidade da vesícula seminal e do ducto deferente à noradrenalina, com consequente aumento na contratilidade da musculatura lisa desses órgãos. Porém, foi observado redução da contração máxima induzida pela noradrenalina, carbacol e $CACl_2$ quando os tecidos foram expostos a concentrações maiores que 10 μM de sibutramina. Além disso, foi demonstrado que o transiente intracelular de Ca^{2+} induzido pelo carbacol foi inibido pela sibutramina nas concentrações entre 10 e 100 μM. Assim, a facilitação da ejaculação foi explicada pelo aumento da sensibilidade do tecido muscular liso à noradrenalina, enquanto o atraso na ejaculação foi explicado pela redução intracelular de íons de cálcio.

Bellentani e colaboradores (2011) relataram alterações nos parâmetros espermáticos de ratos adultos tratados com 10 mg/kg de sibutramina via oral durante 28 dias consecutivos, bem como na contratilidade, *in vitro*, do ducto epididimário. Após o tratamento, foi observado redução no peso corpóreo e no peso absoluto da próstata ventral e epidídimo, bem como redução do tempo de trânsito e da reserva espermática no epidídimo. Os ensaios farmacológicos *in vitro* demonstraram que a sibutramina inibe a recaptura da noradrenalina, promovendo aumento da contratilidade do tecido muscular do ducto da cauda epididimária. Esse efeito explica o trânsito espermático acelerado, a redução da reserva espermática e a consequente redução do peso do órgão observada nos animais tratados com sibutramina. Embora não tenha sido realizado estudo farmacológico com o tecido da próstata, a redução do peso deste órgão também foi explicada pela possível alteração na contratilidade possivelmente promovida pela sibutramina (Bellentani *et al.,* 2011). Apesar das alterações observadas neste estudo, o tratamento com sibutramina não apresentou efeito sobre a fertilidade, investigada após a realização de acasalamento natural (Bellentani *et al*., 2011).

Borges e colaboradores (2013) também relataram aumento da contratilidade epididimária *in vitro* e alterações nos parâmetros espermáticos de ratos adultos após o tratamento com 10 mg/kg de sibutramina durante 30 dias, porém, diferentemente de Bellentani e colaboradores (2011), foi relatado redução do potencial fértil após a realização de inseminação artificial intrauterina. Entre as alterações observadas estão a redução do peso corpóreo e dos pesos absoluto e relativo da próstata e do epidídimo, bem como redução do peso absoluto da vesícula seminal, aceleração no trânsito espermático e redução das reservas espermáticas. Ensaios farmacológicos *ex vivo* demonstraram que o tratamento por 30 dias com a sibutramina não promoveu alterações na contratilidade epididimária, porém, a exposição dos tecidos à tiramina apresentou evidências de que as reservas de noradrenalina endógena epididimárias possam ter sido reduzidas após o tratamento (Borges *et al*., 2013).

Além do sistema genital masculino, há relatos de efeitos adversos da sibutramina sobre o desenvolvimento embrionário. Estudo realizado em nosso Laboratório demonstrou que ratas expostas a 6 mg/kg de sibutramina durante 15 dias antes da prenhez, até o dia gestacional 15, apresentaram redução do peso materno, bem como aumento na porcentagem de perdas pós-implantação e no índice placentário (Francia-Farje *et al.,* 2010). Em humanos, a análise dos dados do Registro Sueco de Nascimento Médico entre 1998 e 2011 revelou significativo aumento do risco de malformação em 242 bebês nascidos de mães expostas à sibutramina (Källén, 2014).

5.1.4. Comercialização da sibutramina no âmbito nacional e internacional

Apesar dos efeitos adversos promovidos pelo tratamento com sibutramina, demonstrados pelo estudo clínico SCOUT, este fármaco continua sendo comercializado no Brasil, sendo o medicamento emagrecedor com registro válido mais antigo no país, datado de 1998 (ANVISA, 2017).

Em 2011, a Agência Nacional de Vigilância Sanitária (ANVISA) retirou do mercado três inibidores de apetite derivados da anfetamina: mazindol, femproporex e anfepramona. Nessa época, a sibutramina também foi reavaliada, mas como os benefícios superavam o seu risco, sua comercialização permaneceu liberada no mercado brasileiro, porém com certas restrições, devendo ser prescritas apenas para determinados perfis de pacientes (Anvisa, 2017; Mariotti *et al.*, 2013). Assim, foi criada uma receita especial para reforçar o controle da sua venda (Anvisa, 2017). Em 22 de junho de 2017, apenas 13 empresas possuíam registro e eram autorizadas a

fabricar e comercializar a sibutramina no Brasil, sendo 22 medicamentos disponíveis no mercado do país.

Em 23 de junho de 2017, Rodrigo Maia, Presidente da Câmara dos Deputados em exercício, sancionou o projeto de Lei número 13.454, sendo legalizado "a produção, a comercialização e o consumo, sob prescrição médica no modelo B2, dos anorexígenos sibutramina, anfepramona, femproporex e mazindol" no Brasil (BRASIL, 2017). Assim, a venda e o uso desses fármacos são autorizados, porém, sob prescrição médica no modelo específico para medicamentos psicotrópicos anorexígenos.

Apensar da proibição da sibutramina em vários países, ela tem sido adquirida sem prescrição médica e utilizada indiscriminadamente (Silva *et al.*, 2010). Além disso, vários produtos para emagrecimento são adulterados com sibutramina para aumentar seu efeito na perda do peso corpóreo, sendo vendidos sem apresentar indicações da sua presença na composição (Jordan, 2013; Krivohlavek *et al*., 2016; Jin *et al*., 2017; Neves & Caldas, 2017). Nos Estados Unidos, a FDA tem constantemente relatado a confirmação de produtos adulterados com sibutramina. Em 2014, 2015, 2016, e 2017, foi relatado a detecção da droga em, respectivamente, 32, 27, 30 e 5 produtos vendidos como naturais (FDA, 2017b).

Jin e colaboradores (2017) investigaram a presença de quatro compostos sintéticos em suplementos de emagrecimento. Entre estes compostos, a sibutramina foi o mais utilizado, sendo detectada nos produtos denominados Bihais, Galong e Aolist, os quais continham 12,4 mg/g, 3,6 mg/g e 20,3 mg/g do fármaco, respectivamente. Krivohlavek e colaboradores (2016) analisaram 123 produtos de emagrecimento vendidos como suplementos alimentares à base de plantas na Croácia, no período de 2009 a 2014, incluindo chá verde, café, chocolate em pó, comprimidos, cápsulas e xaropes. Um quinto das amostras testadas (22%) apresentou resultados incompatíveis com as declarações do produto, sendo detectado 0,014 mg/kg ou mais de sibutramina no seu conteúdo.

A adulteração de suplementos alimentares pode levar à interação de fármacos em pacientes submetidos a certas terapias, podendo resultar em toxicidade grave ou até mesmo levar à redução da eficácia da terapia (Jordan, 2013). Evidências sugerem que o uso indiscriminado de fármacos de emagrecimento, associado ao aumento do período de exposição, pode levar ao aumento das taxas de danos genéticos (Silva *et al*., 2000).

Estudos recentes sugerem que fármacos inibidores seletivos de recaptura de serotonina (SSRIs), como antidepressivos e ansiolíticos, estão associados à inibição da via metabólica do citocromo P450 (CYP), sendo responsáveis por várias interações farmacocinéticas (Hemeryck & Belpaire, 2002). Al-Asmari e colaboradores (2017) relataram interação entre sinvastatina e

fluoxetina em humanos, sendo observado aumento da biodisponibilidade da sinvastatina e dos níveis de neurotransmissores cerebrais, após a co-exposição.

Evidências sugerem que a co-administração da sibutramina com inibidores da enzima CYP3A4, como cimetidina, cetoconazol, eritromicina, sinvastatina, omeprazol e ciclosporina, pode levar a interações farmacológicas, como aumento moderado da concentração plasmática da sibutramina (Krivohlavek *et al.*, 2016).

5.2. Bupropiona

A bupropiona é um fármaco que atua sobre o sistema nervoso central como antagonista do receptor nicotínico, bem como inibidor seletivo da recaptura de noradrenalina, dopamina e, em menor quantidade, serotonina, pelos neurônios pré-sinápticos, aumentando a disponibilidade desses neurotransmissores na fenda sináptica (Ghanbari *et al*., 2011; Tek, 2016), sendo amplamente utilizado no tratamento da depressão, que acomete vários pacientes obesos e em sobrepeso, além da cessação do tabagismo (Onyike *et al*., 2003; Luppino *et al*., 2010; Pratt & Brody, 2014; Tek, 2016; Saunders *et al*., 2018). Comercializada como Wellbutrin® para o uso no tratamento de depressão, ela é administrada via oral, sendo a dose usual em adultos de 300 mg/dia, administrada em três doses de 100 mg (FDA, 2011). Sua absorção é rapida, atingindo a concentração plasmática máxima em aproximadamente cinco horas (Tek, 2016). Para o uso na cessasão do tabagismo, ela é comercializada sob o nome de Zyban®, sendo disponível na dose de 150 mg (FDA, 2014). Inicialmente é prescrito dose de 150 mg por dia, durante três dias. Após esse período, a dose é aumentada para 300 mg por dia, sendo 150 mg duas vezes ao dia, em um intervalo de aproximadamente 8 horas.

Além de suas propriedades terapêuticas como antidepressivo e na cessação do tabagismo, vários estudos demonstraram redução do peso corpóreo após a sua utilização (Weisler *et al*., 1994; Gadde *et al*., 2001; Jain *et al*., 2002; Tek, 2016). O mecanismo pelo qual ela promove redução do peso ainda não foi totalmente elucidado, porém, está possivelmente associado aos seus efeitos sobre a recaptura das catecolaminas no sistema nervoso central, levando à saciedade, ou ao possível efeito direto sobre o hipotálamo, no controle de apetite (Tek, 2016). Weisler e colaboradores (1994) relataram que pacientes tratados por seis semanas com 225 a 450 mg de bupropiona apresentaram perda no peso de aproximadamente 1,13 kg (Weisler *et al*., 1994). Em mulheres obesas ou em sobrepeso tratadas por oito semanas com 100 a 200 mg de bupropiona acompanhado a uma dieta alimentar, foi observado redução de 4,9% do peso corpóreo, comparado a 1,6% no grupo placebo (Gadde *et al*., 2001). Jain e colaboradores (2002) relataram

que pacientes obesos tratados por 26 semanas com 300 a 400 mg de bupropiona (n = 193) combinado a uma dieta alimentar apresentaram redução significativa do peso corpóreo, de 4,6%, comparado ao placebo, que apresentou redução de 1,8% (n = 191).

Recentemente, o FDA e a *European Medicines Agency* (2014 e 2015, respectivamente) aprovaram a utilização da bupropiona no tratamento crônico de pacientes obesos para reduzir o peso corpóreo, sob a administração combinada ao naltrexona, um antagonista opioide (Tek, 2016; Halseth *et al*., 2017). Os fármacos, comercializados como Contrave® nos Estados Unidos e Mysimba™ na Europa, são disponíveis em capsulas contendo 8 mg de naltrexona e 90 mg de bupropiona, sendo inicialmente prescrito apenas uma cápsula por dia, aumentando uma cápsula semanalmente, até atingir a dose terapêutica de 360 mg de bupropiona por dia (Tek, 2016; Saunders *et al*., 2018). Esses fármacos possuem efeito sobre o comportamento alimentar, possivelmente relacionado ao sistema de recompensa alimentar, porém apenas a bupropiona induz a redução do peso corpóreo em pacientes obesos, enquanto o naltrexona não apresenta esse efeito (Tek, 2016). Estudos também relataram que a exposição combinada a esses fármacos promoveu redução nos níveis séricos de triglicérides em 10-12% e de LDL em 2-6%, bem como aumento dos níveis de HDL em 3-8% (Feingold & Grunfeld, 2015).

Apesar do sucesso terapêutico da bupropiona no tratamento da depressão, obesidade e na cessação do tabagismo, ela apresenta vários efeitos colaterais, incluindo convulsões, taquicardia, glaucoma, aumento da pressão arterial, erupção cutânea e, em casos raros, síndrome de Stevens-Johnson, ansiedade, agitação e psicose (Tek, 2016).

5.2.1. Efeitos da bupropiona sobre o sistema genital

Fármacos antidepressivos apresentam ação sobre o sistema nervoso central, modulando vias excitatórias e inibitórias envolvidas no desejo sexual (Croft, 2017). Estudos relatam que a utilização de medicamentos antidepressivos, especialmente os inibidores seletivos da recaptura de serotonina (SSRIs), e de serotonina e noradrenalina (SNRIs), geralmente é acompanhada por disfunção sexual (Clayton *et al*., 2002; Higgins *et al*., 2010), entre elas, redução do desejo sexual, da excitação sexual, do orgasmo, da ereção, bem como atraso no orgasmo ou na ejaculação (Higgins *et al*., 2010).

O uso da bupropiona, porém, tem sido associada à baixa incidência de disfunção sexual, uma vez que aumenta os níveis de dopamina, uma catecolamina que influencia positivamente a resposta sexual masculina, e exerce baixa influência na recaptura de serotonina, neurotransmissor inibidor do comportamento sexual masculino (Andersson, 2011).

Em um estudo clínico realizado em 4534 mulheres e 1763 homens adultos tratados com antidepressivos, a exposição à bupropiona e nefazodona foi associada a menores taxas de disfunção sexual (22-25% e 25%, respectivamente), enquanto mirtazapina e venlafaxina foram associados a maiores taxas (36-43%) (Clayton *et al*., 2002).

Em um estudo realizado com 248 pacientes com depressão severa, Kavoussi e colaboradores (1997) relataram problemas no orgasmo em 41% das mulheres e 61% dos homens tratados com sertralina durante 16 semanas, enquanto o tratamento com bupropiona apresentou menores taxas, de 7 e 10%, respectivamente. Segraves e colaboradores (2000) também investigaram os efeitos desses dois fármacos na função sexual em 240 pacientes com depressão moderada. Foram observadas maiores taxas de disfunção sexual em mulheres e homens (41 e 63%, respectivamente) tratados com sertralina, quando comparado ao tratamento com bupropiona (7 e 15%, respectivamente).

Sukoff Rizzo (2008) relatou redução no número de ereção peniana em ratos Sprague-Dawley tratados com 20 mg/kg de bupropiona (8%), seguido pela desipramina e fluoxetina (53 e 71%, respectivamente) administradas na dose de 10 mg/kg.

Além dos relatos de disfunção sexual, recentemente demonstramos, em ratos, outras alterações nos parâmetros reprotuditvos promovidas pela exposição à bupropiona. Após a exposição a 15 mg/kg de bupropiona durante 30 dias, foi observado aumento nos níveis séricos de LH, bem como na contratilidade epididimária de ratos *Wistar* adultos (Cavariani *et al*., 2014). Na maior dose testada, de 30 mg/kg, além do aumento da contratilidade epididimária, também foi observado redução na qualidade espermática, através do aumento do número de espermatozoides que apresentavam movimento não progressivo, possivelmente associado a modificação do microambiente epididimário.

Estudos também relataram alterações na prole após a exposição intrauterina à bupropiona. Gopalakrishnan e colaboradores (2017) relataram distúrbios cardíacos na prole de ratas prenhes tratadas com 13 mg/kg de bupropiona duas vezes ao dia. Entre as alterações, foi observado aumento significativo da frequência cardíaca e diminuição do débito cardíaco, volume sistólico e porcentagem de engrossamento da parede posterior do ventrículo esquerdo, comparado ao grupo controle. Em um estudo clínico realizado com dados de 38.074 famílias, foi relatado uma associação significativa entre a exposição a antidepressivos durante a gestação e o aumento do risco de déficit de atenção/distúrbio hiperativo em crianças de até 5 anos de idade (Figueroa, 2010). De Long e colaboradores (2010) relataram adiantamento da instalação da puberdade, indicada pela abertura vaginal precoce nas proles F1 e F2 de ratas expostas a 10

mg/kg de bupropiona durante a prenhez e a lactação. Além disso, foi observado redução no peso fetal da prole F1.

Justificativa e Relevância do Tema

A obesidade é um problema de saúde pública que vem crescendo a nível global, e está fortemente associada a disfunções metabólicas, dislipidemias, depressão, entre outras comorbidades. Devido a limitações de estratégias utilizadas para reduzir o peso corpóreo e controlar os níveis séricos de lipídios, e devido à associação entre obesidade e depressão, pacientes obesos estão expostos a agentes hipolipemiantes, como estatinas, e inibidores de recaptura de neurotransmissores, como sibutramina e bupropiona.

As estatinas são uma classe de fármacos mais utilizados mundialmente no tratamento de dislipidemias. Elas possuem ação inibitória sobre a enzima HMG-CoA redutase, levando à redução da síntese de colesterol, precursor da testosterona. A bupropiona e a sibutramina, por sua vez, são fármacos que promovem aumento da disponibilidade de neurotransmissores na fenda sináptica, auxiliando na perda de peso e no controle de sintomas da depressão.

Sabe-se que os andrógenos e a inervação simpática controlam a aquisição da motilidade e capacidade fértil dos espermatozoides, além da função das glândulas sexuais acessórias. Estudo realizado em nosso laboratório relatou depleção dos níveis de andrógenos em ratos pré-púberes expostos à rosuvastatina, resultando em atraso na instalação da puberdade e alterações na histologia testicular e epididimária. Em ratos adultos expostos à sibutramina, foi relatado aumento na contratilidade de órgãos reprodutores, bem como aumento do trânsito espermático pelo epidídimo e redução nas reservas espermáticas e no potencial fértil. Outro estudo, também realizado em nosso laboratório, relatou aumento nos níveis de LH e na contratilidade do ducto epididimário, além de alteração na motilidade espermática, em ratos adultos tratados com bupropiona.

Diante da ampla utilização de estatinas e inibidores de recaptura de neurotransmissores no tratamento de pacientes obesos, associado aos relatos de alterações nos parâmetros reprodutivos promovidas pela exposição isolada a esses fármacos, justifica-se a realização de estudos que avaliem possível interação entre essas classes de fármacos e seus efeitos sobre os parâmetros reprodutivos.

Objetivos

Objetivo geral:

O presente estudo teve como objetivo fundamental avaliar os efeitos da exposição a estatinas (rosuvastatina ou sinvastatina) e inibidores de recaptura de neurotransmissores (sibutramina ou bupropiona), administrados isoladamente ou em associação, sobre parâmetros reprodutivos e a fertilidade de ratos machos adultos.

Objetivos específicos:

1) Avaliar, em ratos machos adultos, os efeitos da exposição aos fármacos rosuvastatina e sibutramina, administrados isoladamente ou em associação, sobre:

a) o consumo alimentar e o peso corpóreo e de órgãos reprodutores;

b) o comportamento sexual;

c) o perfil hormonal e lipídico;

d) a histologia testicular, epididimária e a imunolocalização testicular da enzima 3β-HSD;

e) a qualidade espermática e a fertilidade.

2) Avaliar, em ratos machos adultos, os efeitos da exposição aos fármacos sinvastatina e bupropiona, administrados isoladamente ou em associação, sobre:

a) o peso corpóreo e de órgãos reprodutores;

b) as concentrações séricas e intratesticulares de testosterona;

c) a síntese *in vitro* de testosterona pelas células de Leydig;

d) a histologia testicular, epididimária e a imunolocalização testicular da enzima 3β-HSD;

e) a qualidade espermática e a fertilidade.

Os resultados obtidos no presente trabalho deram origem a dois manuscritos, apresentados a seguir, em forma de capítulos (Capítulos I e II).

Capítulo I

O Capítulo I apresenta a avaliação dos efeitos da exposição subcrônica à sibutramina e rosuvastatina, administrados isoladamente ou em associação, sobre parâmetros reprodutivos de ratos machos adultos. Os resultados obtidos deram origem ao manuscrito intitulado "Effects of the co-exposure to sibutramine and/or rosuvastatin on reproductive parameters of adult male rats", que será submetido para publicação no periódico *Food and Chemical Toxicology*. Fator de impacto (2016): 3,778.

EFFECTS OF THE EXPOSURE TO SIBUTRAMINE AND/OR ROSUVASTATIN ON REPRODUCTIVE PARAMETERS OF ADULT MALE RATS

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HIGHLIGHTS

- Exposure to rosuvastatin, isolated or with sibutramine, resulted in impairment of epididymal morphology in adult male rats.
- Sibutramine, alone or with rosuvastatin, affected sperm parameters, androgen levels and reproductive organ weights.
- Delayed ejaculation and decreased fertility potential observed in the co-exposed rats indicate synergism between the drugs.

ABSTRACT

Obese patients are exposed to statins and sibutramine, which are hypolipemiant and anorexigenic agents, respectively. Previous studies reported adverse effects of isolated exposure to these drugs on male rat reproductive parameters. In the present work we further investigated male reproductive toxicity of these two drugs, administered isolated or in combination. For this, adult male rats (90 days) from control (saline and dimethyl sulfoxide), sibutramine (10 mg/kg sibutramine), rosuvastatin (5 mg/kg rosuvastatin) and rosuvastatin associated with sibutramine (5 mg/kg rosuvastatin + 10 mg/kg sibutramine) were treated for 70 days by gavage. The exposure to sibutramine, alone or associated with rosuvastatin, resulted in reduced food intake, body weight gain, absolute and relative weights of ventral prostate and seminal vesicle with fluid, absolute weight of epididymis, sperm reserves and transit time through the epididymis, testosterone serum levels and increased index of cytoplasmic droplet in spermatozoa, compared to control and rosuvastatin groups. Treatment with rosuvastatin, alone or associated with sibutramine, resulted in hyperplasia of clear cells in the epididymis. Reduced absolute weight of seminal vesicle without fluid, increased relative weight of testis, delayed ejaculation and decreased fertility potential after natural mating were also observed in the co-exposed group, compared to control and rosuvastatin groups, suggesting synergistic effect of these drugs and raising concern of possible fertility impairment in men taking these drugs.

Keywords: rosuvastatin, sibutramine, fertility, reproductive toxicity, male rat.

INTRODUCTION

Obesity is a major public health concern with a rising prevalence all over the world in the past 30 years (Bray, 2013; Withrow and Alter, 2011). Considered a global pandemy (Bray, 2013), it is associated with several comorbidities such as diabetes mellitus, osteoarthritis, metabolic syndrome, cardiovascular diseases, cancer, depressive disorder and dyslipidemia, with important impact in life quality and expectancy (Bray, 2013; Klop et al., 2013; Toups et al., 2013; Withrow and Alter, 2011), in addition to result in high costs to society (Withrow and Alter, 2011).

The success in the treatment of obesity typically requires a combination of interventions, including diet and exercise. However, some patients have been unable to lose weight and control the blood lipid levels through non-pharmacological interventions, which makes the use of drugs an important intervention (Ciccone, 2011; Safeer and Lacivita, 2000).

Statins are the first line of drugs prescribed to treat dyslipidemia and the largest selling class of drugs around the world (Endo, 2010; Feingold and Grunfeld, 2000). They act as selective and competitive inhibitors of HMG-CoA reductase activity, a key-enzyme of cholesterol biosynthesis (Ciurleo et al., 2014). Clinical trials indicated that statins reduce serum levels of low-density lipoproteins (LDL), intermediate-density lipoproteins (IDL), very-low-density lipoproteins (VLDL), triglycerides, and increase HDL levels (Adams et al., 2014; Ginsberg, 1998; Maron et al., 2000). Moreover, they reduce isoprenoid metabolites, such as farnesyl and geranylgeranyl pyrophosphates, which results in pleiotropic effects, including immunomodulatory, anti-inflammatory and antithrombogenic properties, in addition to improve endothelial function, contributing to prevent cardiovascular diseases (Ciurleo et al., 2014). Rosuvastatin (Crestor®), a new synthetic statin, has been shown to have greater efficiency in the reduction of LDL circulating levels (by 50%) and longer elimination half-life (20 hours) compared to the other statins (Bener et al., 2014; Davidson, 2002; McTaggart et al., 2001).

Despite the therapeutic success of statins, they have been associated to reproductive toxicity (Leite et al., 2017a; Leite et al., 2017b; Pons-Rejraji et al., 2014). Klinefelter *et al*. (Klinefelter et al., 2014) reported decrease in testosterone synthesis after *in vitro* culture of Leydig cells with statins (atorvastatin, simvastatin and mevastatin) in the presence of luteinizing hormone. In addition, we previously reported that prepubertal male rats exposed to 3 and 10 mg/kg/day of rosuvastatin showed delayed puberty installation and development of the epididymis, as well as changes in testicular and epididymal morphology and decreased testosterone levels in the highest dose (Leite et al., 2017b). In another study, juvenile male rats exposed to 3 and 10 mg/kg of rosuvastatin from postnatal day (PND) 23 until PND 53, showed decreased sperm quality and, in the highest dose, reduced level of testosterone (Leite et al., 2017a).

Sibutramine is a drug initially developed as an antidepressant and approved by the Food and Drug Administration (FDA) in 1997 to treat obesity, after the discovery of its anorexigenic effect (FDA, 2010a). Member of the serotonin-norepinephrine reuptake inhibitor (SNRI) class of drugs, it acts on the central nervous system inhibiting the presynaptic reuptake of these neurotransmitters and, to a lesser extent, dopamine, increasing their levels in the synaptic clefts, promoting appetite suppression, weight loss and antidepressant effects (Hofbauer et al., 2007; Nisoli and Carruba, 2000; Slovacek et al., 2009). Moreover, studies reported that sibutramine can maintain weight loss after prolonged exposure (Apfelbaum et al., 1999; Hofbauer et al., 2007; James et al., 2000).

In 2010, sibutramine was withdrawn from the markets in Europe and the United States due to reports of increased risk of cardiovascular diseases (EMA, 2010; FDA, 2010a), however, it is still prescribed in some countries, including Brazil, and it has been detected as an additive in a large number of adulterated weight loss supplements that are marketed around the world (da Silva et al., 2010; Kim et al., 2014; Mota et al., 2014).

In addition to cardiovascular diseases, studies have reported adverse effects of sibutramine on the reproductive parameters (Bellentani et al., 2011; Borges et al., 2013; Nojimoto et al., 2009). Nojimoto *et al.* (Nojimoto et al., 2009) reported that rats treated with sibutramine showed increase in the vas deferens and seminal vesicle contractility, as well as alteration in the ejaculation pattern. Abnormal ejaculation was also observed in men exposed to sibutramine (FDA, 2010b). In previous studies, we reported alterations in reproductive parameters of adult rats exposed to 10 mg/kg of sibutramine, including reduced reproductive organ (seminal vesicle, epididymis and ventral prostate) weights, decreased sperm reserves and transit time through the epididymis, as well as decreased sperm quality and fertility after artificial intrauterine insemination and increased contractility of epididymis, ventral prostate and seminal vesicle, observed by *in vitro* assays (Bellentani et al., 2011; Borges et al., 2013).

Some studies reported the use of statins associated with sibutramine (Hayes et al., 2015; Svacina et al., 2007), however, the effects on reproductive parameters were not investigated. Obese patients are still exposed to statins and other SNRI drugs, widely prescribed as antidepressants, such as venlafaxine, duloxetine and tramadol (Islin et al., 2017; Toups et al., 2013). Thus, considering the exposure of obese patients to statins and SNRIs, the present study aimed to investigate the effects of rosuvastatin and sibutramine (isolated or in association) on sperm quality and fertility of adult male rats.

2. MATERIALS AND METHODS

2.1. Animals

Adult male (90 days of age) and female (70 days of age) *Wistar* rats were supplied by the Central Biotherium of São Paulo State University (UNESP), Brazil, and maintained on reversed phase lighting under controlled conditions (23±2 ^{o}C, 30 % air humidity, 12:12-h photoperiod) with standart dorent chow (Purina Labina, Agribrands do Brasil Ltda, Paulínia/SP, Brazil) and

filtered water provided *ad libitum*. The animals were housed in polypropylene cages (43 cm × 30 cm × 15 cm) with laboratory grade pine shavings as bedding. The experimental protocol followed the Ethical Principles in Animal Research of the Brazil College of Animal Experimentation and was approved by the Bioscience Institute/UNESP Ethics Committee on Use of Animals (CEUA; protocol # 631).

2.2. Drugs and experimental design

Rosuvastatin (rosuvastatin calcium), purchased from Farmácia Botica Oficinal (Botucatu, Brazil), was dilluted in saline (vehicle 1), while sibutramine (sibutramine hydrochloride monohydrate; Jiangyin Eas, China) was dilluted in 33.3 % dimethyl sufoxide (DMSO) and 66.7 % of saline (vehicle 2).

The adult male rats were allocated into four experimental groups (n = 26-28/group) and treated daily for 70 days by two consecutive oral administrations (gavages) of vehicle or the drug diluted in its correspondent vehicle (Table 1), as follows: control (C; vehicle 1 followed by vehicle 2), rosuvastatin (R; 5 mg/kg of rosuvastatin followed by vehicle 2), sibutramine (S; vehicle 1 followed by 10 mg/kg of sibutramine) and rosuvastatin combined with sibutramine (R+S; 5 mg/kg of rosuvastatin followed by 10 mg/kg of sibutramine). The treatment was performed during the dark phase of the photoperiod, when rodents consume most of their food, and hence, the effects of drugs that suppress food intake can be better detected (Jackson et al., 1997). The duration of exposure was chosen based in the minimum length time necessary to cover the complete process of spermatogenesis and epididymal maturation in rat (Kempinas and Klinefelter, 2014).

The dose of sibutramine was chosen as the minimum anorexigenic effect in this experimental model (Borges et al., 2013), while rosuvastatin was based in the highest dose available in the market to reduce total cholesterol and serum LDL levels (Vaughan and Gotto,

2004), adjusted for rats (Bachmann et al., 1996). The total volume of the solutions administered was 1.5 mL/Kg of vehicle 1 or rosuvastatin (dissolved in vehicle 1), and 0.5 mL/kg of vehicle 2 or sibutramine (dissolved in vehicle 2), according to the treatment above described.

The study was conducted into three experiments. In experiment 1 (n = 5-6/group), the food intake, testicular and epididymal histology and immunoreactivity of the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) were evaluated; in the experiment 2 (n = 10/group), sexual behavior and fertility after natural mating were investigated; and in experiment 3 (n = 11-12/group), the hormonal levels and lipid profile, reproductive organ weights and sperm parameters were assessed. The treatment regimen and doses were the same for all experiments.

During treatment, the body weight of animals from the three experiments was recorded in order to adjust the doses and evaluate the evolution of body weight, as well as the body weight gain (final weight subtracted from the initial weight).

2.3. Experiment 1

2.3.1. Food intake

During the treatment period, the food consumption was weekly recorded in five cages per group (two animals per cage) and the individual food intake was estimated using the mean of each cage (n = 5/group).

2.3.2. Histological procedures

Approximately two hours after the last treatment, the animals (C, n = 6; R, n = 5; S, n = 6; R+S, n = 5) were euthanized by CO_2 anesthesia inhalation followed by decapitation and the left epididymis and testis were excised, trimmed, fixed in Bouin's solution (75% picric acid saturated aqueous solution, 25% formalin formulated with 40% aqueous formaldehyde, and 5% glacial acetic acid in de-ionized water) and embedded in Paraplast®. Three transversal sections of

testis and longitudinal sections of epididymis (4 μm) were obtained with a distance of 50 μm among them and stained with hematoxylin and eosin (HE) for the histopathological evaluation of testis and epididymis and spermatogenic kinetics analysis in the testis. Other three transversal sections of testis were placed in silanized slides for the immunohistochemical analysis of 3β-HSD.

The investigation of histopathology and spermatogenic kinetic was performed using the Pannoramic Viewer software (3DHISTECH Ltd.) in digitalized slides, while the immunostaining for 3β-HSD was evaluated under a light microscope (Zeiss, Axiostar plus, Oberkochen, Germany).

2.3.2.1. Histopathological analysis

One hundred random tubular sections were evaluated in three testicular cross-sections per animal and classified as normal (presence of concentric and normally organized germ cell layers in seminiferous epithelium) or abnormal (presence of acidophilic or multinucleated cells, vacuoles in the epithelium, sperm retention, degeneration of germ or Sertoli cells, germ cells and cellular debris in the lumen). The Leydig cells and peritubular myoid cells morphology as well as the appearance of blood vessels were qualitatively analyzed. Each region of the epididymis was also qualitatively evaluated, according to the epithelium, lumen and interstitial tissue morphological appearance.

2.3.2.2. Spermatogenesis kinetics analysis

The stage frequencies in the cycle of the seminiferous epithelium can be used to estimate the rhythm or duration of the spermatogenic process and is a criteria for assessing effects of treatment on spermatogenesis (Clermont and Harvey, 1965; Hess et al., 1990). In order to assess spermatogenesis kinetics, the same one hundred tubular sections evaluated in the

histopathological analysis were classified into four categories: stages I-VI (two generations of spermatids), VII-VIII (late spermatids lined along the luminal edge), IX-XIII (only one generation of spermatid) and XIV (secondary spermatocites).

2.3.2.3. Immunohistochemical analysis of the 3β-HSD enzyme

The 3β-hydroxysteroid dehydrogenase (3β-HSD) is an enzyme that converts pregnenolone to progesterone, a precursor of testosterone. In the testis, this enzyme is exclusively expressed in the Leydig cells (Payne and Youngblood, 1995).

For immunoreactivity avaliation of 3β-HSD, three sections per animal, mounted in silanized slides, were deparaffinized in xylene and hydrated using decreasing concentrations of ethanol. After that, the slides were immersed in citrate buffer (0.01 M sodium buffer, 0.05 % Tween 20, pH 6.0) for antigen recovery under microwave (1 min), washed two times with Dulbecco's phosphate-buffered saline (DPBS), followed by incubation with blocking buffer (DPBS containing 3 % protease-free BSA and 0.3% Tween 20) for 1 h at 34 ^{o}C to prevent non-specific reactions. Subsequently, endogenous peroxidase activity was blocked with hydrogen peroxide (3 % in distilled water) for 15 min, washed two times with DPBS and incubated overnight at 4 ^{o}C with rabbit anti-3β-HSD receptor antibody (ab167417; Abcam) diluted 1:100 in blocking buffer. Negative controls were incubated with blocking buffer only (absence of primary antibody) in order to confirm the specific reaction. After this step, the sections were washed two times with DPBS, incubated 1 h at 34 ^{o}C with biotinylated universal horse anti-rabbit/mouse (BP-1400, Vector Laboratories) diluted 1:200 in blocking buffer, washed two times in DPBS and incubated 1 h with Avidin-Peroxidase Complex (ABC-HRP Reagent, PK-7100, VECTASTAIN® Elite®) at 34 ^{o}C. After two washes with DPBS, peroxidase activity was revealed at room temperature using 3,3′-diaminobenzidine (DAB) until the reaction product was visible (approximately 30 min). The sections were counterstained with Vector Hematoxylin QS (15

min), dehydrated via graded ascending series of ethanol, cleared with xylene and coverslipped with Vectamount permanent mounting medium. The sections were qualitatively analyzed under light microscope according to the intensity of staining.

2.4. Experiment 2

2.4.1. Male sexual behavior

Approximately two hours after the last treatment, ten male rats from each experimental group were placed individually in polycarbonate crystal cages (44 x 31 x 16 cm) during the dark phase of the photoperiod. After 5 min, one adult female rat in natural estrus (sexually receptive) determined by vaginal smears was introduced in the cage. For the next 40 min, the sexual behavior was analyzed as described by (Favareto et al., 2011). For this, the following measures were recorded: latency to the first mount, intromission and ejaculation, number of mounts and intromissions until the first ejaculation, latency of the first post-ejaculatory intromission, number of post-ejaculatory intromissions, and total number of ejaculations. Males that did not mount or intromit in the first 10 min were considered sexually inactive.

2.4.2. Fertility test after natural mating

In the end of the sexual behavior investigation, the same paired animals were kept together for an additional 4 hours. Rats considered pregnant, determined by the presence of ejaculate or spermatozoa in the vaginal smear, were separated and maintained until the gestational day 20 (GD 20), when they were euthanized by CO_2 anesthesia inhalation followed by decapitation for the analysis of fertility. For this, the uterus and ovaries were removed and the numbers of live and dead fetuses, corpora lutea, implants and resorptions were determined and used to calculate the proportions of implants per corpus luteum (fertility potential) and resorptions per implantation site.

2.5. Experiment 3

2.5.1. Reproductive organ weights

Other males (C: n = 12, R: n = 12, S: n = 11, R+S: n = 11, respectively) were treated following the same protocol from experiment 1 and, approximately two hours after the last treatment, they were weighed and euthanized by CO_2 inhalation followed by decapitation. The ventral prostate, seminal vesicle (with and without its fluid, without the coagulating gland) and right testis, epididymis and vas deferens were removed and their absolute weights were recorded. The final body weight was used to calculate the relative weights of these organs.

2.5.2. Hormonal levels and lipid profile

Serum levels of FSH, LH and testosterone, as well as intratesticular concentration of testosterone were determined by double-antibody radioimmunoassay, while the lipid profile (total cholesterol, triglycerides, HDL and LDL) was determined by enzymatic colorimetric method. For this, the serum was obtained by centrifugation (2400 rpm, 4 ^{o}C for 20 min) of the blood collected between 9:00 and 12:00 AM from the ruptured cervical vessels, while the testicular fluid was obtained by centrifugation (3000 rpm, 4 ^{o}C for 30 min) of the left decapsulated testis, and stored at -20 ^{o}C until the assay. FSH and LH levels were determined using specified kits provided by the National Institute of Arthritis, Diabetes and Kidney Diseases (NIADDK), while the concentration of testosterone (serum and intratesticular) was assessed using 172 ImmuChem™ Double Antibody Testosterone 125I RIA Kit. All samples were measured in duplicate and in the same assay to avoid inter-assay error, and the intra-assay variability was 2.8% for FSH, 3.4% for LH and 4% for testosterone. Cholesterol, triglycerides, HDL and LDL levels were determined using specified kits supplied from Bioclin (K083, K117, K071 and K088, respectively).

2.5.3. Sperm motility and morphology

In order to investigate sperm motility and morphology, the proximal cauda of the left epididymis from each experimental group was nicked and sperm sample was collected in 2 ml of modified human tubal fluid (HTF) medium (Irvine Scientific, Santa Ana, CA, USA). An aliquot of 10 µl of sperm suspensions was transferred to a Makler chamber and 100 spermatozoa were counted and classified according to their motility as Type A (mobile with progressive movement), Type B (mobile without progressive movement), and Type C (immobile). Additionally, an aliquot of 100 µl of the sperm sample was fixed in 900 µl of formol saline for sperm morphology analysis. For this, smears were prepared on histological slides, and 200 spermatozoa per animal were classified into the three followed categories: normal spermatozoa (without abnormalities in the head and tail), spermatozoa with abnormal head (head without characteristic curvature, pin head or isolated form, i.e. no tail attached) spermatozoa with abnormal tail (tail broken or rolled into a spiral) (Filler, 1993). The presence or absence of the cytoplasmic droplet was also evaluated. Both motility and morphology analysis were performed under a phase-contrast microscope (400 x magnification).

2.5.4. Sperm counts, daily sperm production (DSP) and epididymal sperm transit time

Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) were counted in the same testis parenchyma used to obtain the intratesticular fluid for testosterone assay. The samples were processed based on (Robb et al., 1978), with adaptations. Briefly, the testicular parenchyma was homogenized for 3 min in 5 mL of NaCl 0.9% containing Triton X 100 0.5%, followed by 30 sec sonication. After a 10-fold dilution, one sample was transferred to a Neubauer chamber and mature spermatids were counted in 4 fields per animal.

In order to determine the DSP, the number of spermatids in stage 19 was divided by 6.1, which corresponds to the number in days of the cycle, during which the spermatozoa are present

in the seminiferous epithelium (Robb et al., 1978), and the relative DSP was obtained by dividing the DSP by the testis weight. In the same manner, the left epididymis caput/corpus and cauda were cut into small fragments and homogenized, and the sperm was counted as described for the testis.

The sperm reserves in the epididymal cauda were determined by the sum of the cells counted in the sperm suspension used for the artificial insemination and the sperm count obtained from the homogenized tissue. The sperm transit time through the epididymis was determined by dividing the number of sperm in each region of the epididymis by the DSP.

2.6. Statistical Analysis

For comparison of intergroup results, one-way analysis of variance (One-way ANOVA) with *a posteriori* Tukey's test, Kuskal-Wallis with *a posteriori* Dunn's test or Chi-square were used, according to the characteristics of each variable. Results were expressed as mean ± SEM or median (Q1-Q2) for parametric or nonparametric values, respectively. Differences were considered significant when $p \leq 0.05$. Statistical analyzes were performed by GraphPad Prism, version 5.

3. RESULTS

There was a statistically significant decrease in the body weight gain (Figure 1A) as well as in the final body weight (Figure 1B) of the animals from the three experiments, treated with sibutramine alone or associated with rosuvastatin, when compared to control and rosuvastatin groups.

3.1. Experiment 1

The weekly food intake (g, mean ± SEM, ANOVA followed by Tukey's test) was significantly reduced ($p < 0.001$) in the groups exposed to sibutramine alone (177.5 ± 3.21) or associated with rosuvastatin (181.00 ± 4.19), compared to the rosuvastatin (198.30 ± 1.41) and control (202.30 ± 1.33) groups.

No difference among groups was observed in the testicular histopathological analysis (Figure 2) and in the spermatogenesis kinetics (Table 2). However, the animals treated with rosuvastatin alone or associated with sibutramine exhibited hyperplasia of clear cells in the proximal epididymis cauda (Figure 3). Moreover, the histological analysis also showed fewer spermatozoa in the epididymis cauda of the sibutramine-treated group and an apparent reduction, with an intermediate amount of spermatozoa, in the co-exposed group (Figure 3).

The Leydig cells stained positively for the 3β-HSD enzyme (Figure 4), but no difference among groups was observed in the staining intensity by qualitative analysis.

3.2. Experiment 2

The experimental groups showed increased latency to the first ejaculation, assessed by sexual behavior test, compared to the control group (Table 3), with statistical significance in the co-exposed group. A significant decrease in fertility after natural mating was also observed in the co-exposed animals, when compared to control and rosuvastatin groups (Table 4).

3.3. Experiment 3

All the experimental groups showed reduced final body weight, compared to the control group (Table 5), with statistical significance in the sibutramine and co-exposed groups. S and R+S-treated groups also showed a decrease in the absolute and relative weights of ventral prostate and seminal vesicle with fluid and reduced absolute weight of epididymis, compared to

control and rosuvastatin groups (Table 5). In addition, the co-exposed group showed decreased absolute weight of seminal vesicle without fluid and increased relative weight of testis, when compared to control group (Table 5).

No alterations were observed in sperm motility and morphology (Table 2), serum levels of FSH, LH, total cholesterol, triglycerides, HDL and LDL (Table 6). Despite the trend toward a decrease in the intratesticular levels of testosterone observed in the S and R+S-treated groups, compared to R and C groups, no statistical significance was observed. However, these groups showed statistically significant decrease in serum levels of testosterone, when compared to R and C groups (Table 6).

The exposure to sibutramine alone or associated with rosuvastatin resulted in significant decrease of sperm reserves and transit time through the epididymal cauda when compared to control and rosuvastatin groups, with more significance in the co-exposed than sibutramine-exposed group (Table 2). These animals also showed significant increase in the percentage of sperm with cytoplasmic droplet, compared to control and rosuvastatin groups (Table 2).

4. DISCUSSION

Statins are hypolipemiant drugs that were shown to reduce testosterone biosynthesis by inhibiting the activity of HMG-CoA reductase, a key-enzyme in the biosynthesis of cholesterol, a precursor of testosterone (Klinefelter et al., 2014; Leite et al., 2017b). Sibutramine, a SNRI drug used in the treatment of obese patients and as additive in dietary supplements illegally marketed in many countries to lose weight, was shown to increase the contractility of reproductive organs by inhibiting the reuptake of neurotransmitters by the presynaptic neurons (Bellentani et al., 2011; Borges et al., 2013; Nojimoto et al., 2009). Given the exposure of obese patients to statins and SNRIs, and the previous reports of adverse effects of the isolated administration of these drugs on male rat reproductive parameters, the present study aimed to investigate the effects of

the subchronic treatment of adult male rats with rosuvastatin and sibutramine, administered isolated or in association, on the reproductive parameters. Our results showed adverse effects of sibutramine in the reproductive parameters after 70 days of isolated or combined exposure with rosuvastatin, affecting sperm quality and reserves, serum levels of testosterone and reproductive organ weights. The exposure to rosuvastatin isolated or associated with sibutramine resulted in impaired epididymal morphology. In addition, possible synergistic effect was observed between the drugs, evidenced by the impaired sexual behavior and fertility promoted by the co-exposure to these drugs.

The treatment with sibutramine alone or associated with rosuvastatin resulted in reduced body weight gain, compared to the control and rosuvastatin groups, which can be explained by the decreased food intake observed in these animals, confirming the efficacy of the treatment, once sibutramine is an anorexigenic drug that reduces food intake and appetite, and increases energy expenditure, reducing body weight (Hofbauer et al., 2007; Nisoli and Carruba, 2000).

There was a significant decrease in the testosterone serum levels of the animals exposed to sibutramine alone or associated with rosuvastatin, compared to the control and rosuvastatin groups, but no alterations were detected in the rosuvastatin-treated group. The serum levels of FSH and LH, cholesterol, triglycerides, HDL and LDL were similar among the groups, indicating that the decreased testosterone levels was not a result of alteration in cholesterol or in the function of the hypothalamic-pituitary-gonadal axis. In a previous study, we reported decreased serum levels of triglycerides and testosterone in adult rats exposed to 10 mg/kg of rosuvastatin during prepuberty and reduced cholesterol levels in the animals treated with the doses 3 and 10 mg/kg (Leite et al., 2017b). In addition, these animals showed delayed puberty installation and impaired testicular and epididymal morphology, which were associated with the testosterone depletion (Leite et al., 2017b). In another study, we also reported decreased testosterone levels in adult rats exposed to 10 mg/kg of rosuvastatin from postnatal day (PND)

23 until PND 53, in addition to reduced sperm quality with decreased sperm motility, increased sperm abnormality and fragmentation in the DNA of spermatozoa from the animals treated with 3 and 10 mg/kg of rosuvastatin (Leite et al., 2017a). The decrease in the levels of testosterone observed by Leite *et al.*, compared to the absence of alterations in this parameter in the present study, may in part be explained by the age of the animals, once Leite *et al*. investigated, in adult rats, the effects of the exposure to the drug during prepuberty, which is a critical period in the male reproductive development and, consequently, more sensitive to endocrine disrupters (Favareto et al., 2011; Stoker et al., 2000), while the animals from the present study were treated during adulthood. Moreover, the dose used in the present study, for adult rats, may not have been enough to detect alterations in the lipid profile as well as the androgen levels.

Previous studies reported absence of alterations in androgen levels of adult male rats treated with 10 mg/kg of sibutramine (Bellentani et al., 2011; Borges et al., 2013). However, these rats were investigated after 30 days of exposure to the drug, which may have been insufficient to detect alterations in testosterone levels. Moreover, the treatment in the present study was conducted during the dark phase of the photoperiod, when the effects of drugs that suppress food intake can be better detected, since rodents consume most of their food during the nocturnal period (Jackson et al., 1997). On the other hand, decreased levels of testosterone were reported in men treated with venlafaxine, a SNRI drug (Bell and Shipman, 2000; Shebak and Varma, 2014). After the medication was discontinued, these levels were increased (Bell and Shipman, 2000).

The biosynthesis of testosterone involves the activity of numerous enzymes, including specific cytochrome P450 enzymes (CYPs), hydroxysteroid dehydrogenases (HSDs), and steroid reductases (Miller, 1988). The 3β-hydroxysteroid dehydrogenase (3β-HSD), is an enzyme expressed in the testicular interstitium, detected in the mitochondria of Leydig cells, responsible to convert pregnenolone to progesterone, which are precursors of testosterone (Payne

and Youngblood, 1995). In the present study, the Leydig cells of all groups were positively stained for 3β-HSD enzyme, however, no difference was observed among groups in the qualitative analysis.

A recent study performed *in vitro* reported that venlafaxine, duloxetine, and tramadol, three widely used drugs from the same class of sibutramine (SNRIs), showed endocrine disrupting effects, with the enzyme CYP17 being the main target (Islin et al., 2017). This enzyme is responsible to convert pregnenolone and progesterone to 17-α-hydroxypregnenolone and 17-α-hydroxyprogesterone, and the subsequent lyase formation of dehydroepiandrosterone (DEHA) and androstenedione, respectively, which are precursors of testosterone. Thus, the decrease in the testosterone levels, even with no significant alteration in the lipid profile and 3β-HSD activity, may be an effect of sibutramine in the activity of the enzyme CYP17.

It is known that testosterone plays an important role in the epididymis, accessory glands function and reproductive behavior (Kempinas and Klinefelter, 2014; Klinefelter, 2015), affecting the central and peripheral aspects of the ejaculatory process (Corona et al., 2012), and that the effects of endocrine disrupters in the reproductive tract occur in a time-exposure manner (Blystone et al., 2007). Nojimoto *et al*. (Nojimoto et al., 2009) reported alterations in the ejaculation pattern, in a dose-dependent manner, in adult rats exposed to sibutramine, and showed evidences that reductions of intracellular Ca^{2+} may be involved in the delayed ejaculation observed in high doses of sibutramine (Nojimoto et al., 2009). Abnormal ejaculation was also observed in men exposed to sibutramine (FDA, 2010b). In the present study, the co-exposure to the drugs resulted in significant increase in the latency to the first ejaculation, compared to control group, which may be explained by the decreased levels of testosterone associated with possible effects of sibutramine in the intracellular levels of Ca^{2+}. However, no alterations were observed in the animals treated with sibutramine and rosuvastatin alone, what suggests possible synergic effect of the drugs.

The epididymis is the organ responsible for sperm maturation and storage (Kempinas and Klinefelter, 2014). The maturation process occurs by the addition and removal of substances during sperm transit through this organ, leading to changes in their morphological and physiological characteristics (Orgebin-Crist, 1969), allowing the acquisition of motility and fertility capacity (Kempinas and Klinefelter, 2014; Robaire et al., 2006). It is known that the innervation of the epididymis plays an important role on sperm maturation due to its influence on the sperm transit through this organ (Kempinas and Klinefelter, 2018), and that this transit is mediated by the spontaneous contractile activity promoted by the smooth muscle present in the caput and corpus regions and by the induced-contractile activity promoted by the smooth muscle present in the wall of the epididymal cauda, a highly innervated region of this organ (Bellentani et al., 2011).

Previous studies reported increase of epididymis, vas deferens, seminal vesicle and prostate contractility by *in vitro* assays (Bellentani et al., 2011; Borges et al., 2013; Nojimoto et al., 2009) and decrease of ventral prostate, epididymis and seminal vesicle weights, in addition to decrease in sperm reserves and transit time through the epididymal cauda of adult rats treated with 10 mg/kg of sibutramine, which were explained by the increased contractility of the reproductive organs observed *in vitro* (Bellentani et al., 2011; Borges et al., 2013). In the present study, sperm reserve was significantly reduced in the animals treated with sibutramine alone or associated with rosuvastatin, compared to control and rosuvastatin groups, which may be explained by the reduced sperm transit time through the epididymal cauda observed in these animals, associated with possible increase in the contractility of this organ. Interestingly these parameters were more significantly reduced in the co-exposed group.

It is known that sperm content is responsible for approximately 50% of the epididymal weight and that decreased weights of seminal vesicle and ventral prostate are sensitive indicators of reduced androgen status, particularly due to a reduction in the stored secretion (Creasy and

Chapin, 2013). The animals treated with sibutramine alone or associated with rosuvastatin showed decreased absolute weight of the epididymis, which may be associated with the reduced sperm reserves and accelerated sperm transit through this organ, in addition to the reduced final body weight, androgen levels and possible increase in the contractility, reducing its stored content, alterations that can also explain the decreased absolute weight of seminal vesicle with fluid and ventral prostate observed in these groups. The relative weights of ventral prostate and seminal vesicle with fluid were also reduced in these animals, reinforcing the impact of testosterone depletion. The co-exposed group showed increased relative weight of testis, which can be explained by the decreased body weight, once testes are conservative organs (Creasy and Chapin, 2013). In addition, this group also showed decreased absolute weight of the seminal vesicle without fluid compared to control group, which reinforces the possible synergic effect of the drugs.

The excess of cytoplasm in the spermatozoa is generally removed by Sertoli cells before spermiation. However, an excess of residual cytoplasm occasionally remain in the sperm found in the epididymis (Toshimori, 2003), which is phagocytosed by the clear cells present in the epididymal wall, by the time sperm transit to the proximal cauda epididymis (Cooper, 2011; Hermo et al., 1988). In the present study, a significant increase in the index of cytoplasmic droplet in the spermatozoa was observed in the animals treated with sibutramine alone or associated with rosuvastatin, compared to rosuvastatin and control groups. The exposure to rosuvastatin isolated or associated with sibutramine resulted in hyperplasia of clear cells in the proximal cauda of epididymis, however, no alterations were observed in the clear cells of the sibutramine group, which suggests that the increased index of cytoplasmic droplet in the spermatozoa observed in the animals treated with sibutramine alone or associated with rosuvastatin may be explained by the acceleration in sperm transit trough the epididymis, resulting in insufficient time necessary for the removal of the excess of residual cytoplasm.

Many studies associated the excess of residual cytoplasm on spermatozoa with poor sperm function. In human, the presence of cytoplasmic droplet was associated with infertility in smokers (Mak et al., 2000) and in men with varicocele (Zini et al., 2000), as well as decreased fertility capacity (Keating et al., 1997), reduced sperm motility (Zini et al., 1998) and reduced binding to the zona pellucida (Ergur et al., 2002; Liu and Baker, 1992). Aitken and Krausz reported that the excess of residual cytoplasm was also associated with increase in DNA damages, resulted from oxidative stress (Aitken and Krausz, 2001). Silva *et al*. reported increase in the frequency of genotoxic damage in Swiss mice exposed to sibutramine, assessed by comet assay in the peripheral blood cells, which was explained by the indirect action through the induction of oxidative stress (da Silva et al., 2010).

In the present study, the co-administration of sibutramine and rosuvastatin resulted in reduced fertility potential after natural mating, compared to rosuvastatin and control groups, which may be explained by a combination of the effects observed in these animals: (1) reduced sperm transit time through the epididymis, which may have enabled the complete sperm maturation; (2) increased index of cytoplasmic droplet in the spermatozoa, which may have resulted in oxidative stress and DNA damage in the spermatozoa and/or reduction of binding to the zona pellucida; (3) reduced testosterone levels, which may be involved in the delayed ejaculation, once this hormone is essential for the accessory glands function and reproductive behavior (Klinefelter, 2015).

5. CONCLUSIONS

In conclusion, our results revealed adverse effects of exposure to sibutramine, alone or combined with rosuvastatin, on male rat reproductive parameters, including alterations in the reproductive organ weights, testosterone serum levels and index of cytoplasmic droplet in the spermatozoa. Sperm reserves and transit time through the epididymis were also adversely

affected, more markedly in the co-exposed group, suggesting a synergic effect of the drugs. This synergism was reinforced by the delayed ejaculation and decreased fertility potential after natural mating observed in the co-exposed group. Moreover, the exposure to rosuvastatin alone or associated with sibutramine resulted in impairment of epididymal morphology. These results raise concern of possible impairment of fertility in men exposed to SNRIs and statins, such as sibutramine and rosuvastatin, respectively.

To the best of our knowledge, our study is pioneer showing the effects of co-exposure to hypolipemiant and SNRI drugs on the adult male reproductive tract and possible synergistic effect. Further studies are encouraged to investigate the effects of the co-exposure to different doses of these drugs on male and female reproductive parameters and possible effects of paternal treatment on the offspring development.

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8. CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

9. LEGENDS OF FIGURES

Figure 1. Body weight gain (final weight subtracted from the initial weight) (A) and evolution of body weight (B) during the treatment in the control (C), rosuvastatin (R), sibutramine (S) and rosuvastatin associated with sibutramine (R+S) groups (n = 28, n = 27, n = 27, n = 26, respectively) from the three experiments. Values expressed as mean ± SEM (One-way ANOVA followed by Tukey's test); ***$p < 0.001$, compared to control and rosuvastatin groups.

Figure 2. Photomicrographs illustrating testicular transversal sections of animals from the experiment 1. H & E, 100x (scale bar = 200 μm) and 200x (scale bar = 100 μm).

Figure 3. Photomicrography illustrating the epididymal sections in the animals from the experiment 1. Note the presence of hyperplasia of clear cells in the proximal cauda of epididymis from the animals treated with rosuvastatin alone or associated with sibutramine (inserted images). H & E, 100x, scale bar = 200 μm.

Figure 4. Cellular localization of 3β-HSD enzyme, a Leydig cell marker, in the transversal sections of testes from control (A), rosuvastatin (B), sibutramine (C) and rosuvastatin associated with sibutramine (D) groups, from the experiment 2. Negative control (without primary antibody) is inserted in the image A. 400x, scale bar = 50 μm.

Table 1. Overview of the treatment design.

Experimental Groups	1st Gavage	2nd Gavage
Control (C)	vehicle 1	vehicle 2
Rosuvastatin (R)	rosuvastatin	vehicle 2
Sibutramine (S)	vehicle 1	sibutramine
Rosuvastatin + Sibutramine (R+S)	rosuvastatin	sibutramine

Vehicle 1 = saline; vehicle 2 = saline + 33.3% of dimethyl sufoxide (DMSO).

Table 2. Sperm counts, motility and morphology, index of cytoplasmic droplet in the spermatozoa of animals from the experiment 3 (n = 8/group) and spermatogenesis kinetics of animals from the experiment 1 (Control: n = 6; Rosuvastatin: n = 5; Sibutramine: n = 6; Rosuvastatin+Sibutramine: n = 5).

	Experimental Groups			
Parameters	Control	Rosuvastatin	Sibutramine	Rosuvastatin + Sibutramine
[1]*Spermatogenesis kinetics*				
Stages I–VI (%)	42.50 (39.00-47.25)	40.00 (37.00-45.00)	45.50 (42.50-49.50)	38.00 (35.00-40.50)
Stages VII–VIII (%)	29.50 (24.75-33.25)	25.00 (24.00-30.00)	29.50 (23.50-30.25)	33.00 (28.50-37.50)
Stages IX–XIII (%)	23.00 (20.75-25.50)	26.00 (22.00-30.00)	24.50 (20.00-27.00)	24.00 (18.50-30.00)
Stage XIV (%)	4.00 (2.00-6.00)	6.00 (5.50-8.00)	3.00 (2.50-4.50)	6.00 (3.00-6.50)
[1]*Sperm motility*				
Type A	62.50 (59.25-63.75)	62.00 (55.00-64.84)	58.00 (50.00-68.00)	58.00 (56.50-61.50)
Type B	29.00 (24.50-30.75)	29.00 (24.25-31.00)	27.00 (23.00-31.00)	24.50 (20.75-28.75)
Type C	9.00 (7.00-12.75)	10.50 (7.25-13.75)	13.00 (9.00-22.00)	16.00 (10.50-23.00)
[1]*Sperm morphology*				
Normal	197.50 (195.30-199.00)	197.00 (196.80-199.00)	197.00 (196.00-199.00)	196.00 (195.80-199.00)
Abnormal	2.50 (1.00-4.75)	3.00 (1.00-3.25)	3.00 (1.00-4.00)	4.00 (1.00-4.25)
[2]*Index of perm with/without cytoplasmic droplet*	1.32	1.21	1.51^{a*b***}	$1.55^{a**b***}$
[3]*Sperm counts in the testis*				
Mature spermatid number ($\times 10^6$/testis)	260.70 ± 13.60	271.80 ± 6.49	253.50 ± 17.77	276.60 ± 16.70
Relative mature spermatid number ($\times 10^6$/g testis)	191.80 ± 7.92	196.80 ± 7.10	190.90 ± 18.13	202.60 ± 9.69
Daily sperm production ($\times 10^6$/testis/day)	42.74 ± 2.23	44.55 ± 1.06	41.55 ± 2.91	45.34 ± 2.74
Relative daily sperm production ($\times 10^6$/g testis/day)	31.45 ± 1.30	32.26 ± 1.16	31.30 ± 2.97	33.22 ± 1.59
[3]*Sperm counts in the epididymal caput/corpus*				
Sperm number ($\times 10^6$/organ)	141.60 ± 6.23	163.30 ± 4.51	137.80 ± 8.09	139.70 ± 10.10
Relative sperm number ($\times 10^6$/g organ)	531.90 ± 33.17	541.30 ± 22.16	519.40 ± 12.96	495.60 ± 21.52
Sperm transit time (days)	3.33 ± 0.08	3.68 ± 0.12	3.36 ± 0.15	3.15 ± 0.29
[3]*Sperm counts in the epididymal cauda*				
Sperm number ($\times 10^6$/organ)	285.40 ± 23.91	284.30 ± 13.68	$188.00 \pm 9.65^{a**b**}$	$157.20 \pm 17.94^{a***b***}$
Sperm transit time (days)	6.74 ± 0.62	6.39 ± 0.29	$4.59 \pm 0.20^{a**b*}$	$3.46 \pm 0.31^{a***b***}$

[1]Values expressed as median and interquartile intervals (Kruskal–Wallis followed by Dunn's test). [2]Chi-square test. [3]Values expressed as mean ± SEM (One-way ANOVA followed by Tukey's test).
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; differences among groups are indicated by superscripts as follows: a ≠ control, b ≠ rosuvastatin.

Table 3. Sexual behavior of male rats from the experiment 2.

Parameters	Experimental Groups			
	Control (n=10)	Rosuvastatin (n=10)	Sibutramine (n=10)	Rosuvastatin + Sibutramine (n=10)
[1]Animals that showed sexual behavior (%)	90 (n=9)	80 (n=8)	100 (n=10)	100 (n=10)
[2]Latency to the first mount (s)	95.00 (28.50-174.50) (n=9)	193.50 (50.75-362.80) (n=8)	84.00 (20.50-226.00) (n=9)	138.00 (96.00-279.00) (n=9)
[2]Number of mounts until the first ejaculation	6.00 (2.00-8.50) (n=9)	3.00 (1.00-10.00) (n=8)	4.00 (1.50-8.50) (n=9)	2.00 (1.50-4.50) (n=9)
[2]Latency to the first intromission (s)	162.50 (59.25-288.50) (n=8)	350.00 (28.00-379.00) (n=3)	54.50 (31.50-115.00) (n=4)	140.00 (136.00-275.00) (n=7)
[2]Number of intromissions until the first ejaculation	19.50 (11.75-25.75) (n=8)	21.00 (7.00-29.00) (n=3)	12.00 (6.50-19.75) (n=4)	8.00 (6.00-26.00) (n=7)
[2]Latency to the first ejaculation (s)	219.30 (1.21-555.00) (n=8)	420.50 (1.06-840.00) (n=2)	654.00 (635.00-864.00) (n=3)	949.00 (694.50-1555.00)* (n=4)
[2]Latency to the first post-ejaculatory intromission (s)	343.00 (311.00-378.00) (n=7)	323.50 (320.00-327.00) (n=2)	374.00 (332.00-406.00) (n=3)	313.00 (292.00-322.00) (n=3)
[2]Number of post-ejaculatory intromissions	8.00 (8.00-13.00) (n=7)	7.50 (6.00-9.00) (n=2)	8.00 (6.00-10.00) (n=3)	7.00 (6.00-10.00) (n=3)
[2]Total number of ejaculations	2.00 (1.25-2.00) (n=8)	2.50 (2.00-3.00) (n=2)	3.00 (2.00-3.00) (n=3)	2.00 (1.25-2.75) (n=4)

[1]Chi-square test. [2]Values expressed as median and interquartile intervals (Kruskal–Wallis followed by Dunn's test).
*$p < 0.05$ compared to control group.

Table 4. Fertility parameters after natural mating of adult male rats from the experiment 2.

	Experimental Groups			
Parameters	Control (n=9)	Rosuvastatin (n=10)	Sibutramine (n=9)	Rosuvastatin + Sibutramine (n=10)
[1]Inseminated per sperm positive females (%)	100.00	100.00	100.00	100.00
[2]Body weight of dams (g)	348.70 (330.00-411.80)	384.90 (363.90-416.30)	368.00 (344.00-374.60)	361.30 (332.40-375.50)
[2]Fetal body weight (g)	3.23 (3.04-3.42)	3.35 (3.01-3.49)	3.50 (3.24-3.55)	3.38 (3.29-3.49)
[2]Uterus weight with fetuses (g)	58.86 (53.01-70.90)	68.01 (64.82-75.68)	63.01 (49.05-69.81)	57.62 (39.80-66.21)
[2]Placental weight (mg)	0.53 (0.51-0.56)	0.57 (0.51-0.61)	0.59 (0.52-0.65)	0.60 (0.53-0.65)
[1]Sex ratio (M/F)	59/52	64/61	46/51	45/49
Corpora lutea				
Total number of corpora lutea	119	136	115	121
[2]Number of corpora lutea per litter	13.00 (11.00-15.00)	14.00 (12.75-14.25)	13.00 (11.50-14.00)	12.00 (9.75-13.25)
Implantation sites				
Total number of implantation sites	114	130	103	99
[2]Number of implantion sites per litter	12.00 (10.50-14.50)	13.00 (12.00-14.00)	12.00 (10.00-13.00)	10.50 (8.25-12.25)
[1]Implantion sites per corpora lutea (%)	95.80	95.59	89.56	81.82[a***b***]
Resorptions				
Total number of resorptions	3	6	6	7
[2]Number of resorptions per litter	0.00 (0.00-1.00)	0.00 (0.00-1.25)	1.00 (0.00-1.00)	1.00 (0.00-1.00)
[1]Resorptions per implantation sites (%)	2.63	4.61	5.82	7.07
Live fetuses				
Total number of live fetuses	111	124	97	94
[2]Number of live fetuses per litter	11.00 (10.00-14.50)	12.00 (11.75-14.00)	11.00 (9.00-12.50)	10.50 (7.25-12.00)
[1]Live fetuses per implantation sites (%)	97.37	95.38	94.17	94.95

[1]Chi-square test. [2]Values expressed as median median and interquartile intervals (Kruskal-Wallis test followed by Dunn's test).
[***]$p < 0.001$; differences among groups are indicated by superscripts as follows: a ≠ control, b ≠ rosuvastatin.

Table 5. Final body weight and absolute and relative weights of reproductive organs of adult rats from the experiment 3.

	Experimental Groups			
Reproductive organs	Control (n=12)	Rosuvastatin (n=12)	Sibutramine (n=11)	Rosuvastatin + Sibutramine (n=11)
Final body weight (g)	478.90 ± 9.99	450.80 ± 17.23	425.60 ± 14.39^{a*}	414.20 ± 6.18^{a**}
Absolute organ weights				
Testis (g)	1.78 ± 0.05	1.80 ± 0.06	1.65 ± 0.05	1.71 ± 0.10
Epididymis (mg)	681.70 ± 16.73	675.00 ± 13.30	608.80 ± 14.65^{a**b*}	605.80 ± 14.14^{a**b*}
Vas deferens (mg)	107.90 ± 3.24	110.20 ± 4.79	112.10 ± 4.85	109.10 ± 2.89
Ventral prostate (mg)	723.40 ± 30.74	703.90 ± 35.37	419.20 ± 31.68^{a***b***}	401.40 ± 25.07^{a***b***}
Seminal vesicle with fluid (g)	1.56 ± 0.05	1.67 ± 0.05	1.06 ± 0.05^{a***b***}	1.03 ± 0.11^{a***b***}
Seminal vesicle without fluid (mg)	550.90 ± 23.96	537.10 ± 28.31	495.70 ± 22.09	450.90 ± 28.42^{a*}
Relative organ weights				
Testis (g/100g BW)	0.37 ± 0.01	0.40 ± 0.02	0.39 ± 0.02	0.43 ± 0.01^{a*}
Epididymis (mg/100g BW)	139.80 ± 3.92	145.50 ± 8.25	142.70 ± 6.81	149.60 ± 4.66
Vas deferens (mg/100g BW)	22.85 ± 0.87	24.73 ± 1.28	26.79 ± 1.68	25.89 ± 0.57
Ventral prostate (mg/100g BW)	151.30 ± 6.77	156.00 ± 5.20	98.83 ± 7.48^{a***b***}	99.10 ± 4.63^{a***b***}
Seminal vesicle with fluid (g/100g BW)	0.33 ± 0.01	0.36 ± 0.02	0.25 ± 0.01^{a*b***}	0.25 ± 0.02^{a*b***}
Seminal vesicle without fluid (mg/100g BW)	116.00 ± 6.49	120.30 ± 7.28	117.30 ± 5.45	110.80 ± 4.68

Bw = body weight.
Values expressed as mean ± SEM (One-way ANOVA followed by Tukey's test).
$^{*}p < 0.05$, $^{***}p < 0.001$; differences among groups are indicated by superscripts as follows: a ≠ control, b ≠ rosuvastatin.

Table 6. Hormonal levels (ng/mL) and biochemical measurements (mg/dL) in the adult male rats from the experiment 2.

	Experimental Groups			
Parameters	Control (n)	Rosuvastatin (n)	Sibutramine (n)	Rosuvastatin + Sibutramine (n)
Hormonal levels (ng/mL)				
FSH	6.16 ± 0.58 (12)	6.58 ± 0.40 (12)	7.56 ± 0.81 (11)	7.64 ± 1.14 (11)
LH	2.30 ± 0.26 (11)	2.15 ± 0.25 (11)	1.92 ± 0.21 (10)	1.97 ± 0.30 (11)
Testosterone	1.49 ± 0.22 (11)	1.26 ± 0.14 (11)	$0.48 \pm 0.09^{a***b**}$ (9)	$0.66 \pm 0.12^{a**b*}$ (10)
Intratesticular testosterone	138.80 ± 19.63 (11)	133.40 ± 17.13 (9)	106.40 ± 13.70 (9)	118.10 ± 16.20 (10)
Biochemical measurements (mg/dL)				
Total cholesterol	51.90 ± 3.39 (10)	49.20 ± 1.81 (10)	49.70 ± 2.20 (10)	54.20 ± 1.98 (10)
Triglycerides	60.40 ± 6.46 (10)	52.90 ± 6.65 (10)	49.00 ± 3.35 (10)	43.10 ± 5.68 (10)
HDL	5.56 ± 0.47 (9)	6.30 ± 0.49 (10)	7.80 ± 0.74 (10)	7.10 ± 1.00 (10)
LDL	9.20 ± 0.86 (5)	8.00 ± 0.55 (5)	7.25 ± 0.95 (4)	9.50 ± 0.64 (4)

Values expressed as mean ± SEM (One-way ANOVA followed by Tukey's test).
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; differences among groups are indicated by superscripts as follows: a ≠ control, b ≠ rosuvastatin.

Figure 1.

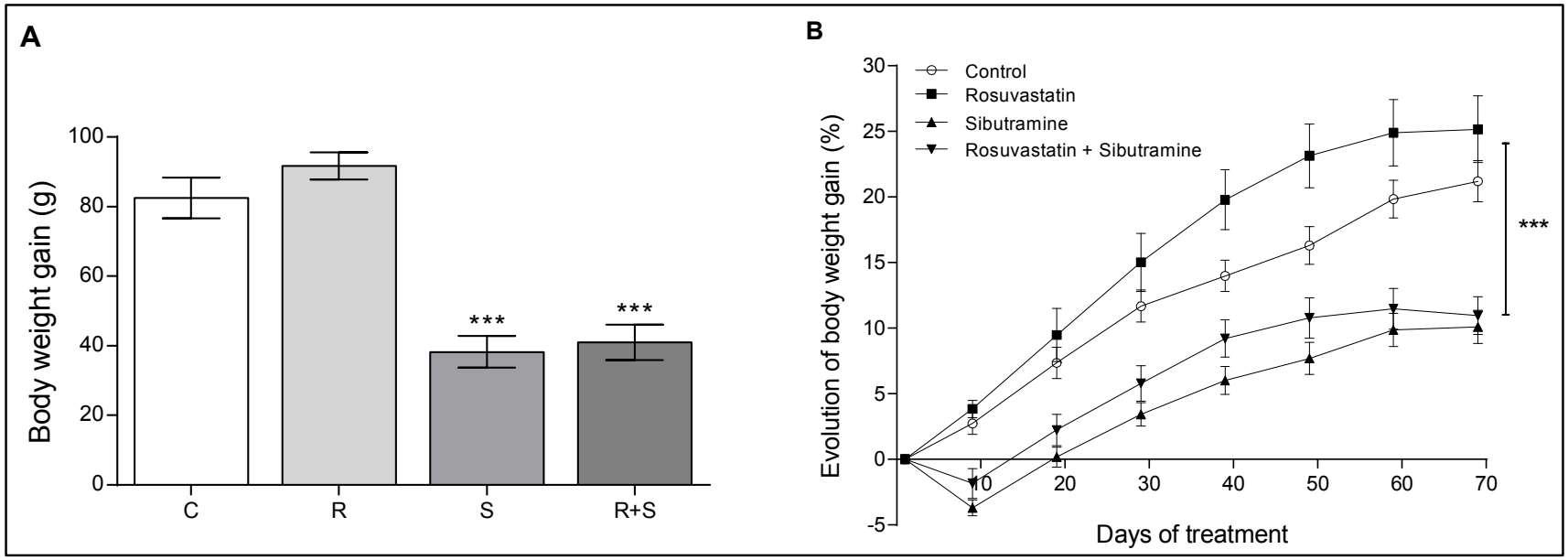

A
Body weight gain (g)
100
80
60
40
20
0
C
R
S
R+S
***
***
B
Evolution of body weight gain (%)
30
25
20
15
10
5
0
-5
Control
Rosuvastatin
Sibutramine
Rosuvastatin + Sibutramine
***
10
20
30
40
50
60
70
Days of treatment

Figure 2.

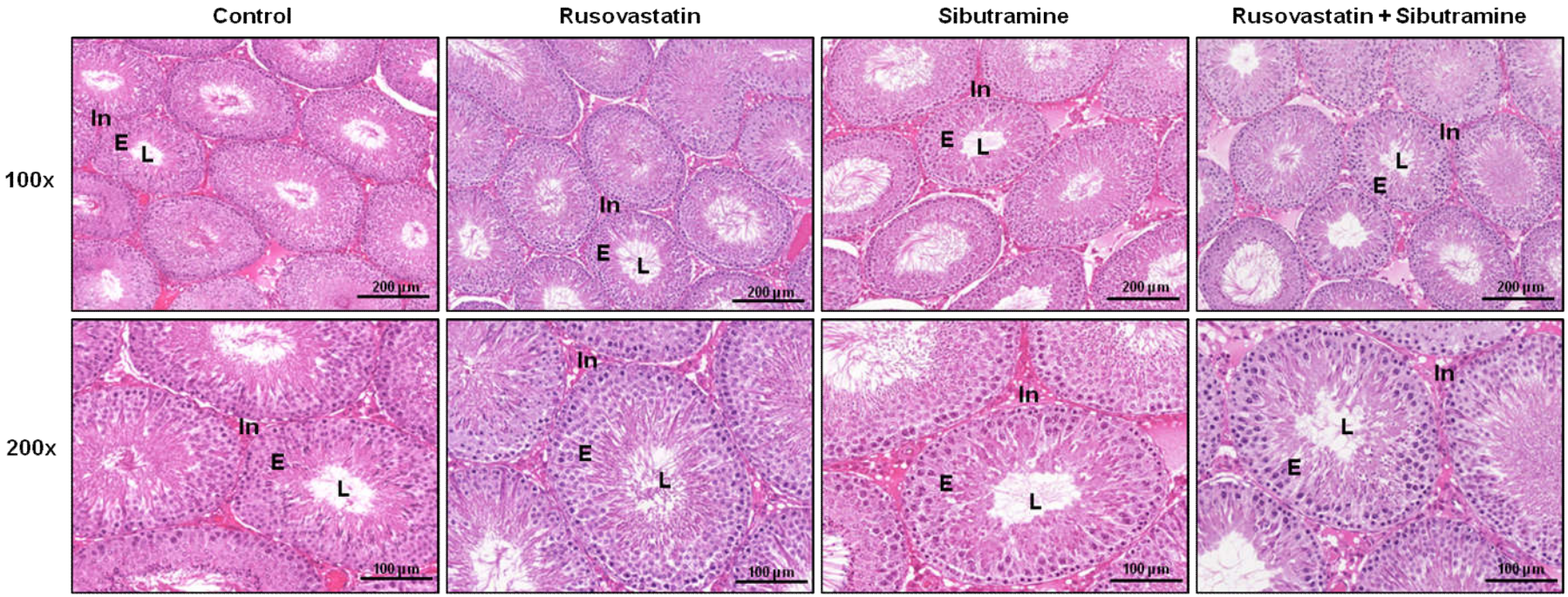

Control
Rusovastatin
Sibutramine
Rusovastatin + Sibutramine
100x
200x
In
E
L
200 µm
100 µm

Figure 3.

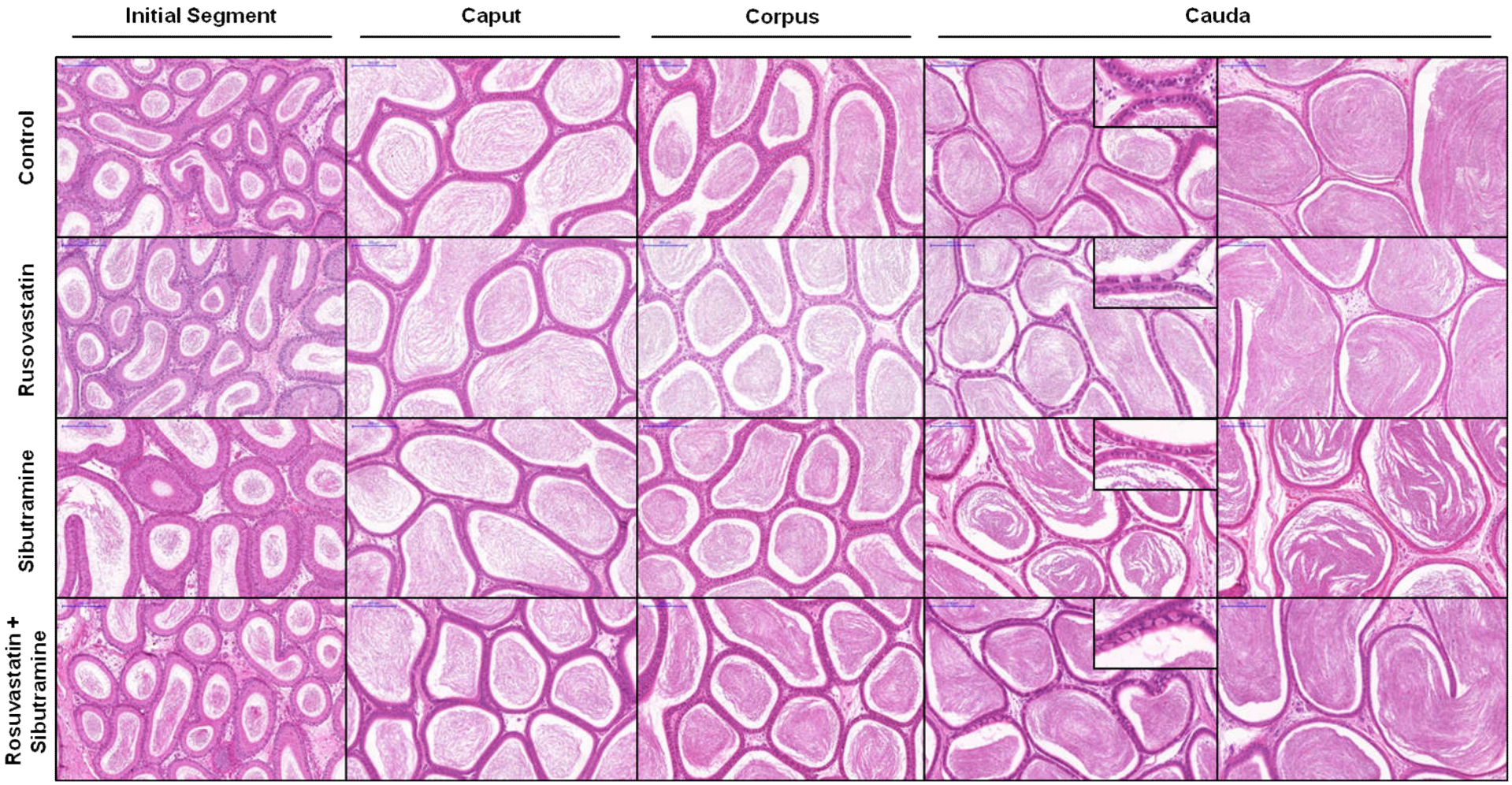
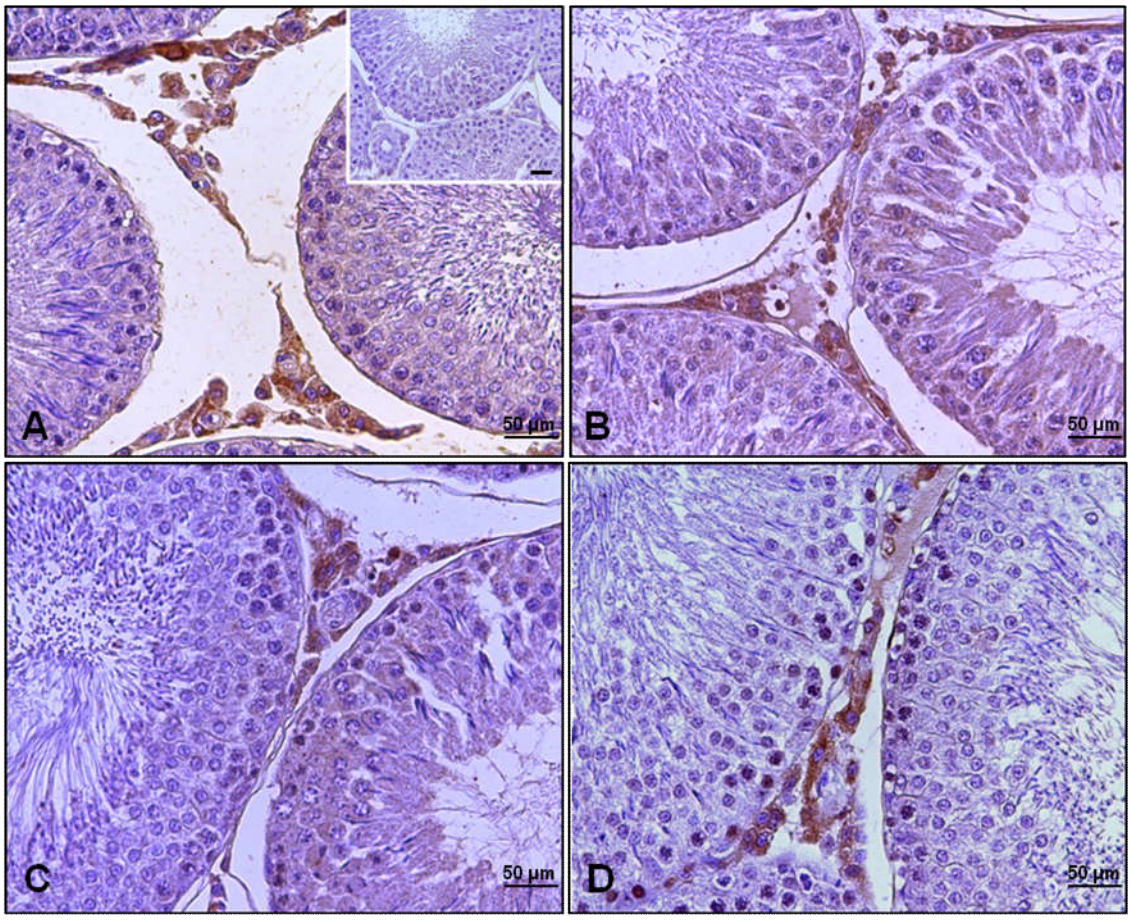

Initial Segment
Caput
Corpus
Cauda
Control
Rusovastatin
Sibutramine
Rosuvastatin + Sibutramine

Figure 4.



Capítulo II

O Capítulo II apresenta a avaliação dos efeitos da exposição à sinvastatina e bupropiona, administrados isoladamente ou em associação, sobre os parâmetros reprodutivos de ratos machos adultos. Os resultados obtidos deram origem ao manuscrito intitulado "Androgen depletion and alterations in the reproductive tract of adult male rats exposed to bupropion and simvastatin", que será submetido para publicação no periódico *Reproductive Toxicology*. Fator de impacto (2017): 2,341.

ANDROGEN DEPLETION AND ALTERATIONS IN THE REPRODUCTIVE TRACT OF ADULT MALE RATS EXPOSED TO BUPROPION AND SIMVASTATIN

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ABSTRACT

Obesity, a global health problem, is strongly associated with metabolic syndrome, dyslipidemia and depression disorder. Obese patients are exposed to several drugs, such as statins and bupropion. Previous studies reported alterations in reproductive parameters after isolated exposure to these drugs. Thus, the present study aimed to investigate the effects of simvastatin and bupropion, isolated or in association, on male rats reproductive parameters. For this, adult *Wistar* male rats (70 days) from control (C; distilled water), simvastatin (S; 50 mg/kg), bupropion (B; 30 mg/kg), and S+B groups were treated orally for 52 days. All experimental groups showed decreased levels of serum and intratesticular testosterone, compared to C group. Impaired sperm motility and decreased synthesis of testosterone by the Leydig cells were observed in S-treated group. Impaired epididymal morphology and increased index of sperm with cytoplasmic droplet were observed in the S and S+B groups. Moreover, impaired fertility was observed in S+B, suggesting a synergism between the drugs. These results raise concern of possible impairment of fertility in men co-exposed to simvastatin and bupropion.

Keywords: fertility, simvastatin, bupropion, reproductive toxicology, testosterone.

1. INTRODUCTION

Obesity is a complex and multifactorial chronic disease that has been growing worldwide in the last 30 years, affecting, along with overweight, more than one third of the world's population [1-3]. Besides representing high cost to society, it is strongly associated with other diseases, such as diabetes, cancer, metabolic syndrome, depression disorder and cardiovascular diseases, increasing the rates of morbidity and mortality [1, 4, 5].

Many factors are involved in the cardiovascular disease, including the elevated glucose and insulin serum levels, blood pressure, and dyslipidemia, which affects 60-70% of the obese patients, consisting of changes in the lipid levels, characterized by elevated triglycerides, low density lipoproteins (LDL) and reduced levels of high density lipoproteins (HDL) [6]. Studies indicate that weight loss can decrease serum triglyceride and LDL levels and increase HDL levels [6-9]. As obese patients are at an increased risk of developing cardiovascular disease, treatment of their dyslipidemia is often indicated [7].

Given the limitation of nonpharmacological strategies to reduce body weight and treat dyslipidemia in obese patients, and given the strong association between obesity and depression, the use of medications, such as statins and bupropion, is a common indicated intervention [3, 10, 11].

In recent years, statins have been highlighted among the drugs used to treat dyslipidemia and reduce cardiovascular risks. Considered the most effective LDL-lowering agents and termed as "drugs of 21st century", these hypolipemiant compounds act as selective and competitive inhibitors of HMG-CoA reductase, an enzyme responsible to convert HMG-CoA to mevalonate, a precursor of cholesterol [6, 12-14]. Besides reducing the levels of cholesterol, studies reported that statins promote decrease in the levels of very low density lipoproteins (VLDL) and increase in the expression of receptors for low density lipoprotein (LDL) in the liver, increasing their removal from blood up to 60 %, which contributes to reduce the risks of atherosclerosis [11, 14,

15]. Clinical studies have also shown that statins can increase life expectancy slowing the progression of diseases such as diabetes mellitus and chronic kidney disease, in addition to reduce the mortality rates in patients with dyslipidemia [16, 17].

Among the cholesterol-lowering medications prescribed in the United States for adults aged 40 and over, simvastatin (Zocor®) is the most commonly used drug [18]. Miller *et al*. [19] reported that treatment of type 2 diabetic patients with 40 and 80 mg of simvastatin for 6 weeks promoted improvement in the lipoprotein profile in a dose-dependent manner. Both drugs promoted reduction in the levels of VLDL, IDL and LDL, compared to the placebo group. Another study, conducted in 20,536 adult patients (aged 40-80 years) from the UK suffering from vascular disease or diabetes and randomly receiving 40 mg of simvastatin or placebo for 5 years, reported reduction in the rates of mortality, particularly from vascular disease, as well as decreased rates of non-fatal heart attacks, strokes and revascularization procedures in patients that received the drug, compared to the placebo [20].

Despite the therapeutic success of statins in the treatment of dyslipidemia, many studies reported alterations in male reproductive parameters after exposure to these drugs. Klinefelter *et al.* [21] reported that Leydig cells incubated *in vitro* with atorvastatin, simvastatin and mevastatin, under stimulation with LH for 18 hours, showed significant reduction (40%) in the synthesis of testosterone. In a previous study [22] we reported that adult male rats exposed to 3 and 10 mg/kg/day of rosuvastatin during prepuberty showed delayed puberty installation and development of the epididymis, alterations in testicular and epididymal morphology, in addition to androgen depletion in the highest dose. Reduced sperm quality with decreased sperm reserves and motility and increased DNA fragmentation and abnormalities in the spermatozoa were observed in rats treated with 3 and 10 mg/kg of rosuvastatin from postnatal day (PND) 23 until PND 53, in addition to reduced androgen levels in the group treated with the highest dose [23]. Zhang *et al*. [24] reported that rats treated for 20 and 40 days with 4, 8 and 16 mg/kg/day of

simvastatin showed reduced serum levels of testosterone, estradiol, progesterone, total cholesterol, LDL and triglycerides, as well as increased levels of FSH and LH, in a dose- and time-dependent manner. Beverly *et al*. [25] reported that *in utero* exposure to simvastatin resulted in reduced fetal testosterone production, serum levels of triglycerides, LDL, HDL, cholesterol and downregulation of two genes in the fetal testis in rat. In a clinical study, Azzarito *et al*. [26] reported decrease in the serum levels of testosterone of hypercholesterolemic patients treated with 20 mg/kg/day of simvastatin for 6 and 12 months.

Bupropion, a norepinephrine-dopamine reuptake inhibitor (NDRI) widely used in the treatment of major depressive disorder and smoke cessation, acts inhibiting the reuptake of these neurotransmitters, increasing their levels in the synaptic cleft [3, 27-30]. Studies have also shown that this drug can promote weight loss [3, 31-33]. The mechanisms by which this drug reduces body weight is still not fully understood, however, it is possibly related to its action on these neurotransmitters and may have direct effect on hypothalamus, regulating appetite and reducing food intake [34].

Recently, the Food and Drug Administration and the European Medicines Agency (2014 and 2015, respectively) approved the use of bupropion to reduce weight in obese patients, under the co-administration with naltrexone, an opiate antagonist [3, 35]. These drugs have an effect on dietary behavior, possibly related to the food reward system, but only bupropion was reported to induce weight loss in obese patients [3].

Despite the success of bupropion in the treatment of depression, smoke-cessation and obesity, some studies reported adverse effects on the reproductive parameters. In a study conducted with Sprague-Dawley male rats to evaluate a model of sexual dysfunction, the exposure to 20 mg/Kg of bupropion promoted reduction in the number of penile erection in 8% when compared to the control group [36]. In a clinical study conducted in 1763 men and 4534 women, Clayton *et al*. [37] reported sexual dysfunction in 22% of patients treated with

bupropion immediate release and 25% of patients treated with bupropion sustained release. Another study conducted in 248 patients with severe depression reported orgasm dysfunction in men and women (10 and 7%, respectively) exposed to bupropion for 16 weeks [38]. Segraves *et al*. [39] also reported sexual dysfunction in men (15%) and women (7%), in a population of 248 patients with moderate depression, exposed to bupropion. In a recent study we reported that adult male rats treated with 15 mg/Kg of bupropion showed increased serum levels of luteinizing hormone and epididymal contractility. At a higher dose, 30 mg/Kg of bupropion, the animals showed impaired sperm quality, with an increase in the incidence of non-progressive sperm [40].

The use of statins and antidepressants has increased in recent years [41] and, in the United States, they are the most prescribed drugs [42]. Many studies have shown the effects of hypolipemiant and neurotransmitter reuptake inhibitor drugs, however, to the best of our knowledge, there is no study investigating the effects of the treatment with the combination of these drugs on male reproductive parameters. Given the exposure of obese patients to these drugs and their increased use worldwide, the present study aimed to investigate the effects of the coexposure to simvastatin and bupropion on the reproductive parameters of adult male rats.

2. MATERIAL AND METHODS

2.1. Animals

Adult male (70 days old; n = 95) and female (70 days old; n = 60) *Wistar* rats provided from Charles River Laboratories (Raleigh, NC) were allowed to acclimate for two weeks to room conditions of 12 h light/dark, 22 ± 1 °C, 50 % ± 10 % relative humidity, housed one per cage, receiving water and rodent chow *ad libitum*. All the procedures used in this experiment were approved by the U.S. EPA NHEERL Institutional Animal Care and Use Committee (IACUC) and followed the guidelines from the National Institute of Health (NIH) related to animal welfare. The surgeries conducted in this study were performed under isoflurane inhalation

anesthesia (Henry Schein; Dublin, OH, USA) and the euthanasia was performed by decapitation. All efforts were made to minimize the animals suffering.

2.2. Drugs and treatment

Adult male rats were ranked by weight and randomly distributed into the following experimental groups (n = 20/group): control (distilled water; C), simvastatin (50 mg/kg of simvastatin; S), bupropion (30 mg/kg of bupropion; B), and simvastatin associated with bupropion (S+B). The drugs were diluted in distilled water and the treatment was performed by gavage in the light phase of photoperiod, for 52 days, the approximate time to complete spermatogenesis cycle in male rats [43].

The dose of simvastatin was chosen based on the literature [44, 45], while the dose of bupropion was chosen based on the dose effective in enhancing noradrenergic neurotransmission in rat hippocampus [40, 46]. Both drugs were purchased from Sigma-Aldrich (bupropion: PHR1730; simvastatin: S6196; St. Louis, MO, USA).

2.3. Experimental design

The study was conducted into two cohorts, described as follows (Figure 1).

The first cohort (n = 10/group) was performed in two steps. In the first step, after one hour from the last treatment, 5 animals per group were anesthetized and euthanized by decapitation to obtain both testes for the analyses of *ex vivo* synthesis of testosterone by the Leydig cells. In the second step, after one to three hours from the last treatment, other 5 animals per group were anesthetized with isoflurane inhalation and fixed by perfusion with Bouin's solution for histological analysis of the right epididymis and testis and testicular morphometric analysis. The right testis was also used for immunohistochemical analysis.

In the second cohort, one to three hours after the last treatment, the animals (n = 10/group) were anesthetized with isoflurane inhalation and euthanized by decapitation to obtain the sperm from both epididymal cauda to perform sperm motility and morphology analysis and to access the fertility after intrauterine artificial insemination (IUI). The right testis was collected to determine the concentration of testosterone in the testicular interstitial fluid. The blood was collected to investigate the serum levels of testosterone and the reproductive organs had the absolute and relative weights analyzed.

2.4. Description of the analyses

2.4.1. Body and reproductive organ weights

The body weight of the animals during the treatment period was recorded and the evolution of body weight as well as the body weight gain (calculated by subtracting the final body weight from the initial weight) were evaluated in both Cohorts.

In Cohort 2, the absolute and relative weights of the reproductive organs (ventral prostate, seminal vesicle with fluid and without coagulating glands, right and left testes and epididymis) were recorded.

2.4.2. *Ex vivo* synthesis of testosterone by Leydig cells

Both testes were removed and a semi-purification of Leydig cells was performed as previously described [21], with modifications. Briefly, both testes from each animal were placed in a warm media for dissociation of testicular parenchyma (DM; dissociation media) consisting of Medium-199 (Gibco, 31100-035) containing Earle's salts and phenol red, buffered with 0.71 g/L sodium bicarbonate and added 2.1 g/L HEPES, 25 mg/L trypsin inhibitor and 1.0 g/L BSA. The testicular artery was perfused by flush of 0.5 mL DM containing freshly added collagenase and dispase (2 mg/mL each) to remove blood. After removal of the tunica albuginea, the

parenchyma from both testes was placed into a 50-ml centrifuge tube containing 5 ml of DM. Another 5 mL of DM, with external collagenase and dispase (final concentration: 0.25 mg/mL each), was added followed by incubation at 34 ^{o}C in water bath for approximately 10 min with shaking (84 cycles/min) to allow the dissociation of seminiferous tubules. The volume was adjusted to 40 mL with separation media (SM), consisted of DM containing 10 g/L BSA (instead of 1 g/L BSA), inverted several times, and the supernatant, consisting of dissociated cells from all testes of each group, was pooled into a 250-mL tube. This wash with SM was repeated and the supernatant was transferred to the pool of dissociated cells. The remaining sediment, containing aggregated Leydig cells and tubule fragments, was filtered through a 250-µm nylon mesh and the filtrate was added to the tube containing the dissociated cells, followed by two times centrifugation (at 4 ^{o}C, 1200 rpm, 20 min) and resuspension of pellet cells by adding 30 mL of SM.

The samples, containing Leydig cells and large germ cells, were separated by Percoll-gradient centrifugation. For this, the cell pellets were resuspended in an isotonic Percoll solution, consisting of Percoll diluted 1:11 with Percoll Buffer (PB: 95 mL of a 10x Ca and Mg-free HBSS buffered with 0.35 g/L sodium bicarbonate, 4.2 g/L HEPES, 25 mg/L soy trypsin inhibitor, 2.5 g/L BSA, pH 7.2), and centrifuged 40 min at 14,000 rpm, 4 ^{o}C using a Beckman JA-17 rotor. In this type of separation, the cells are partitioned according to their densities and a continuous linear density gradient is generated. The lighter fraction of cells was gently removed and 20 mL (approximately 10-12 x 10^{6} cells) was transferred to a 50-mL centrifuge tube. Percoll was removed by dilution with PB and centrifugation (4 ^{o}C, 250 x g, for 10 min).

After the isolation of Leydig cells described above, semi-purified Leydig cells were resuspended in culture media (CM), prepared with 1 L of Phenol red-free Medium-199 (Gibco 94-0384DK) containing Earle's salts, 2.2 g/L sodium bicarbonate, 1 g/L BSA protease free, 10 mL/L insulin-transferrin-selenium-A 100x, 10 mL/L sodium pyruvate, 10 mL/L non-essential

amino acids 100x, 10 mL/L l-glutamine 100x and 0.24 ml/L gentamicin. Aliquots of 1 mL containing approximately 200,000 Leydig cells were dispensed into a 24-well culture. Three replicates from each animal were incubated at 34 ^{o}C without LH, while other three replicates were incubated for three hours with 10 ng/mL of highly purified ovine LH (NIDDK-oLH-26), the approximate physiological stimulatory concentration [21]. After incubation, the cells were pelleted and a 800-µL aliquot from the supernatant was transferred into a microcentrifuge tube and frozen at -80 ^{o}C until assayed for testosterone quantification by antibody coated radioimmunoassay (RIA) (Testo-US, ALPCO, US), according to the manufacturer's instructions. The lower limit of detection was approximately 0.2 ng/mL and intra- and inter-assay variations were < 5 % and < 10 %, respectively.

2.4.3. Concentration of serum and testicular interstitial fluid testosterone

One to three hours after the last treatment, each male was euthanized by isoflurane anesthesia inhalation followed by decapitation, and the blood was collected from the ruptured cervical vessels into BD collection tubes (BD Vacutainer). The serum obtained by centrifugation (4 ^{o}C, 3000 rpm, 30 min) was frozen at -80 ^{o}C for further analysis of testosterone concentration by RIA. The right testis of each animal was removed, weighed, and the testicular interstitial fluid was collected from a nick in the caudal pole of the testis by centrifugation (4 ^{o}C, 500 rpm, 60 min) and frozen at -80 ^{o}C prior to testosterone assay by RIA (TESTO-US; Cisbio Bioassays). Intra-assay variability minimum detectable limit was 0.2 ng/ml and intra- and inter assay variability were 5 and 10.8 %, respectively.

2.4.4. Fertility after *in utero* artificial insemination (IUI)

In rodents, the spermatogenesis is a highly efficient process that produces an excess of qualitatively normal sperm, compared to humans [47]. Thus, artificial insemination has been

200 considered more sensitive than natural mating to evaluate toxicant-induced alterations in fertility
201 in the rodent [47]. Given that, a cohort of females was synchronized in proestrus 96 h prior to the
202 IUI procedure with 80 μg sc of LHRH agonist (Sigma, #L4513). On the day of IUI, they were
203 paired with sexually-experienced vasectomized males during the dark-phase of photoperiod and
204 the receptive females, selected by the presence of lordosis, were maintained for 1 hour with the
205 same male to stimulate the uterus for IUI.

206 The isolation and insemination of sperm were conducted as previously described [48, 49],
207 with modifications. Briefly, both cauda epididymis were removed from each animal, trimmed
208 free of fat and placed in a 35-mm culture dish containing 2 mL of Medium-199 (Gibco; 11043-
209 023 with Earle's salts and phenol red-free) buffered with 26 mM sodium bicarbonate, 3 mg/L
210 DL-methionine, 0.2% protease-free bovine serum albumin (BSA, Sigma, St Louis, MO), 10 mM
211 sodium pyruvate (Gibco, Grand Island, NY), 1 mM nonessential amino acids (Gibco,Grand
212 Island,NY), 12 mg gentamicin sulfate (Gibco, Grand Island, NY), insulin-transferrin-selenium
213 mixture (Gibco, Grand Island, NY), 200 nM testosterone and 200 nM dihydrotestosterone, pH
214 7.2, with 0.25 mg/ml of bovine lipoprotein (Sigma, St. Louis, MO) freshly added to the medium
215 on the day of each IUI.

216 The proximal cauda was incised with scalpel blade and the sperm were allowed to
217 disperse at 34 ^{o}C, 5% CO_2 for approximately 5 min. A 50-μL aliquot was diluted in 450 mL of
218 fixative (10 % formalin in DPBS with 10% sucrose, pH7.4) and sperm were counted in a
219 Neubauer chamber and a volume containing of 5 x 10^6 sperm (0.1 to 0.2 mL), which results in
220 approximately 75 % fertility in control males [48], was injected in each uterine horn of one
221 female per male through a 18 G iv catheter attached to a 0.5 mL syringe, followed by
222 cauterization immediately after removal of the needle. All inseminations were performed under
223 inhalation of isoflurane anesthesia.

After IUI, the females were maintained one per cage, and nine days later they were euthanized by isoflurane anesthesia followed by decapitation and the uterus and ovaries were collected. The numbers of corpora lutea of pregnancy and implanted embryos were counted to determine the fertility of each male rat, expressed by the percentage of implants/corpora lutea x 100 (efficiency of implantation).

2.4.5. Sperm parameters

Sperm motility and morphology were evaluated in the same sample of sperm dispersion obtained from both cauda epididymis used for the IUI procedure.

2.4.5.1. Sperm motility

Sperm motility analysis was performed as previously described [49]. After a female was inseminated, an aliquot from the same sperm dispersion used for IUI was diluted with in supplemented HBSS medium, carefully inverted several times, placed by capillary action into a 100-µm deep cannula (Vitro Com Inc., Mt. Lakes, NJ) and immediately analyzed using the Tox IVOS HTM-Ident (Hamilton Throne Research, Beverly, MA), a c*omputer-assisted sperm analysis* (CASA) system that identifies sperm heads using DNA-specific dye and fluorescence illumination. For this, images consisting of 30 frames were obtained at 60 frames/sec and a minimum of 200 sperm were analyzed per sample. The analysis of velocity was conducted using the Tox IVOS version 10.8q and no tracks with less than 20 points were accepted.

Progressive sperm tracks (progressive/motile sperm x 100) and beat cross frequency (BCF; number of oscillations across mean trajectory) were analyzed.

2.4.5.2. Sperm morphology

After taking samples for sperm motility analysis, a 50-µL aliquot from the same sperm suspension used for IUI was diluted in 450 mL of 10% formalin in DPBS with 10% sucrose (pH7.4) for further evaluation of sperm morphology. For this, smears were prepared on histological slides and maintained at room temperature for about 30 min to allow the sperm attachment to the bottom of the slides. If the samples started getting dry, they were discarded and another slide was mounted, avoiding the induction of sperm abnormalities. A total of 200 spermatozoa per animal was analyzed under a phase-contrast microscope (400 x magnification) and sperm morphology was classified into three general categories: normal spermatozoa (without abnormalities in sperm head and tail), spermatozoa with morphological abnormalities in the head (without characteristic curvature, pin head or isolated form, i.e. no tail attached) and spermatozoa with morphological abnormalities in the tail (broken or rolled into a spiral) [50]. The presence of the cytoplasmic droplet (CD) was evaluated as a signal of incomplete sperm maturation [51, 52], and its position along the sperm tail was categorized into proximal, medial and distal CD, according to the distance from the sperm head.

2.4.6. Histological procedures

Five animals per group from Cohort 1 were submitted to vascular perfusion fixation via the descending aorta with Dulbecco's phosphate buffered saline (DPBS) followed by Bouin's solution (75% picric acid, 25% formaldehyde, and 5% glacial acetic acid), under isoflurane anesthesia. After 10-15 min of perfusion, the right testes and epididymis were removed and immersed in Bouin's solution. Four hours later, small incisions were made in the tunica albuginea to allow the penetration of the fixative in the testicular parenchyma. On the day following the perfusion (twenty four hours later), the organs were washed with 70 % ethanol saturated with lithium carbonate, which was repeated until the picric acid disappeared.

The whole epididymis was embedded in paraffin, while the right testis was separated into five fragments, from which three intercalated fragments were embedded in paraffin. For histopathological and morphometric analysis, three transversal sections (4 μm) from the testes and longitudinal sections from the epididymis of each animal were obtained and stained with hematoxylin and eosin (HE). The slides from the testes were digitalized and quantitatively analyzed using the Pannoramic Viewer software (3DHISTECH Ltd.), while the epididymis were evaluated qualitatively under a light microscope (Leica DM2500 microscope connected to a Leica DM 2900 camera), using the Leica Application Suite software (LAS; version 4.5.0), according to specific guidelines for toxicological studies [53].

Other transversal sections (4 μm) from the testis were placed into positively charged slides for immunohistochemical analysis.

2.4.6.1. Immunohistochemistry for 3β-HSD

The 3β-hydroxysteroid dehydrogenase (3β-HSD) is an enzyme involved in the biosynthesis of steroid hormones that converts pregnenolone to progesterone. In the testis, the only site of expression of this enzyme is the Leydig cell, where it is detected in the mitochondria [54].

The localization of 3β-HSD was performed by immunofluorescence. For this, the testicular transversal sections were deparaffinized in xylene and hydrated using decreasing concentrations of ethanol and distilled water. After circumscribed with wax pen (H-4000; Vector Laboratories), sections were washed two times in DPBS, incubated with blocking buffer (DPBS containing 3 % protease-free BSA and 0.3% Tween 20) for 1 h at 34 ^{o}C to avoid non-specific reactions, and then incubated overnight at 4 ^{o}C with rabbit anti-3β-HSD receptor antibody (ab167417; Abcam) diluted 1:250 in blocking buffer. After overnight incubation, the sections were washed two times in DPBS and incubated for 1 h at 34 ^{o}C with the secondary antibody

299 donkey anti-rabbit (A21206; Alexa Fluor 488, Life Technologies) diluted 1:100 in DPBS.
300 Following two washes in DPBS and distilled water, the sections were counterstained for 30 min
301 at 34 ^{o}C with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA)
302 diluted 1:200 in ultrapure distilled water, rinsed in distilled water, and coverslips were mounted
303 with Vectashield mounting medium for fluorescence (H-1000; Vector Laboratories, Burlingame,
304 CA, USA). In order to confirm the specific reaction, negative controls were used, excluding the
305 primary antibody incubation.

306 The slides were imaged using a Leica CLSM (TCS-SP1; Heidelberg, Germany) that
307 contained an inverted DMIRBE microscope and an Omnichrome laser emitting at three
308 wavelengths (488, 568, and 647 nm). The images were obtained in a maximum of one week
309 later, to avoid the decrease of the fluorescent staining. Sections from three intercalated fragments
310 of testis, totalizing three sections per animal, were qualitatively evaluated by the intensity of
311 staining.

312

313 **2.4.6.2. Histopathological analysis**

314 Three sections of distanced intercalated fragments of the testis were analyzed in each
315 animal (n = 5/group), and a total of 100 seminiferous tubules per animal were classified as
316 normal (presence of concentric and normally organized germ cell layers in seminiferous
317 epithelium) or abnormal (presence of germ cells and cellular debris in the lumen, multinucleated
318 formation, seminiferous epithelium with acidophilic cells, few germ cell layers, vacuole
319 formation or degeneration in seminiferous epithelium). Interstitial tissue, peritubular myoid cells
320 and Leydig cells morphology as well as the appearance of blood vessels were qualitatively
321 analyzed. Qualitative analysis of epididymal histopathology was also performed by evaluation of
322 each region of the organ according to the epithelium, lumen and interstitial tissue morphological
323 appearance.

2.4.6.3. Testicular morphometric analysis

The analysis of testicular morphometry was performed in 35 sections per animal in the IX stage of spermatogenesis. For this, the diameter of seminiferous tubules was determined by the mean of two different measurements (vertical and horizontal) from each section. The height of germinal epithelium was determined by the mean of two different measurements obtained from opposite regions in the tubules.

2.4.6.4. Assessment of spermatogenesis kinetics

The relative frequency of stages estimates the rate or duration of the spermatogenic process [55]. In order to access the dynamics of the spermatogenesis, the relative frequency of the stages from the seminiferous epithelium cycle was estimated in 100 sections of seminiferous tubules per animal as follows: I-VI (presents two generations of spermatids) and VII-VIII (displays mature spermatozoa located at the edge of the lumen), IX-XIII (presents only one generation of spermatids), XIV (presents secondary spermatocyte), according to Leblond and Clemont [56].

2.4.7. Statistical analysis

For comparison of the results among the experimental groups, One-way Analysis of Variance (ANOVA) followed by Dunnett's test, or Kruskal-Wallis followed by Dunn's test were used to compare parametric or non-parametric values, respectively, and the results were expressed as mean ± SEM or median (Q1-Q2), according to the characteristics of each variable. Chi-square was used to compare proportions of sperm with CD per sperm without CD. A Pearson's coefficient was calculated to investigate the possible correlation between the serum and testicular interstitial fluid levels of testosterone. Differences were considered statistically

348 significant when $p \leq 0.05$. Statistical analyses were performed in the Statistical Analysis System
349 (SAS version 9.1, Inc., Cary, NC).
350
351 **3. RESULTS**
352 **3.1. Body and reproductive organ weights**
353 In both cohorts, the exposure to the drugs for 52 days did not affect the body weight gain
354 (Figure 2). In the same manner, absolute and relative weights of the reproductive organs were
355 not altered by the treatments, investigated in the animals from cohort 2 (Table 1).
356
357 **3.2. Sperm parameters**
358 No effect of treatment was observed in the sperm morphology (Table 2) of animals from
359 Cohort 2. However, there was a significant increase in the index of cytoplasmic droplet (CD)
360 (percentage of sperm with CD per sperm without CD) in the spermatozoa from the epididymis
361 cauda of S and SB-treated groups, compared to the control group (Table 2). No difference was
362 observed in the position of CD.
363 Regarding to sperm motility (Table 2), the animal treated with simvastatin showed
364 reduced beat cross frequency (BCF; rate of crossing the average path trajectory), when compared
365 to the control group.
366
367 **3.3. Serum and IFT testosterone levels and *ex vivo* synthesis of testosterone**
368 All the experimental groups showed decreased levels of testosterone in the serum (Figure
369 3D) and testicular interstitial fluid (TIFT; Figure 3C), when compared to the control group.
370 Moreover, there was a positive correlation between TIFT and serum levels of testosterone
371 (Figure 3E).

372 The basal production of testosterone by the isolated Leydig cells cultured 3 hours in the
373 absence of LH (Figure 3A) was consistently low and comparable among the groups from Cohort
374 1. When the Leydig cells were incubated under the stimulation with a physiological
375 concentration of LH (10 ng/mL) (Figure 3B), all the groups showed an increase in the synthesis
376 of testosterone, and the effect of simvastatin was evidenced by a significant decrease in the
377 levels of testosterone, when compared to the control group.
378
379 **3.4. Histopathological and morphometric analysis**
380 No difference among groups was observed in the testicular morphometry and
381 spermatogenesis kinetics (Table 2) as well as in the testicular morphology in the experiment 1
382 (Figure 4). However, the epididymis of the animals treated with simvastatin alone or associated
383 with bupropion exhibited hyperplasia in the clear cells of the proximal cauda (Figure 5).
384
385 **3.5. Immunohistochemistry**
386 The Leydig cells of all groups from Cohort 1 stained positively with the antibody against
387 the steroidogenic enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD), which was specific to
388 these cells in the testis interstitium (Figure 6). No difference among groups was observed in the
389 staining intensity by qualitative analysis.
390
391 **3.6. Fertility test**
392 In the cohort 2, the fertility of sperm obtained from the proximal cauda epididymis was
393 accessed nine days after the IUI procedure. Significant reduction of fertility was observed in the
394 animals co-exposed to simvastatin and bupropion, evidenced by the decreased efficiency of
395 implantation percentage, when compared to the control group (Figure 7).
396

4. DISCUSSION

Considered a disease, obesity is a health problem associated with several comorbidities, including metabolic syndrome, depression disorder and dyslipidemia, which affects 60 to 70 % of obese patients [1, 4, 6]. Given the limitation of nonpharmacological strategies to reduce body weight and control cholesterol levels in obese patients, and given the strong association between obesity and depression disorder, the use of medicines is a common indicated intervention [3, 57]. Statins, the first line drugs used to treat dyslipidemia, was shown to reduce testosterone in rats and humans [7, 21, 58, 59]. Bupropion, a norepinephrine-dopamine reuptake inhibitor (NDRI) agent used in the treatment of depression disorders, smoking cessation and recently approved in the USA and Europe to be used in association with naltrexone for the treatment of obesity, was shown to increase the contractility of epididymis and impair sperm motility in adult rats [3, 40].

Given the exposure of obese patients to these drugs, the present study aimed to investigate the effects of the co-exposure to simvastatin and bupropion on the reproductive parameters of adult male rats. In this study we report androgen depletion promoted by the isolated or combined exposure to these drugs and impaired sperm motility promoted by the treatment with simvastatin. Moreover, impaired epididymis morphology and increased index of sperm with cytoplasmic droplet were observed in the animals treated with simvastatin alone or associated with bupropion, and the co-exposure to the drugs resulted in impaired fertility.

Despite the approval of bupropion in the treatment of obesity, the present study showed no difference among groups in the body and organ weights of the animals treated for 52 days with simvastatin, bupropion or both drugs. These results corroborate with Cavariani *et al*. [40], in which adult male rats treated with 15 and 30 mg/kg of bupropion for 30 days did not show alterations in the body and organ weights. The absence of alteration on these parameters was also reported by Leite *et al*. [22] in adult male rats exposed to 3 and 10 mg/kg of rosuvastatin during prepuberty.

Testosterone is a hormone derived from cholesterol and biosynthesized by the Leydig cells present in the testicular interstitium [60]. Its biosynthesis involves the activity of steroidogenic enzymes, including several specific cytochrome P450 enzymes (CYPs), hydroxysteroid dehydrogenases (HSDs), and steroid reductases, and starts in the mitochondria of these cells by the conversion of cholesterol to pregnenolone [60]. The 3β-hydroxysteroid dehydrogenase (3β-HSD) is a steroidogenic enzyme detected in the testis, with exclusive expression in the Leydig cells mitochondria that converts pregnenolone into progesterone, which is further metabolized to testosterone by other steroidogenic enzymes [61, 62]. It is known that the normal activity of steroidogenic enzymes is required for the proper testicular steroidogenesis and spermatogenesis [63].

In the present study, analysis of the immunoreactivity for 3β-HSD revealed no difference among groups. However, the animals treated with simvastatin showed decrease in the synthesis of testosterone by the Leydig cells incubated under stimulation of LH, and the levels of testosterone in the serum and testicular interstitial fluid (IFT) were significantly reduced in all experimental groups, compared to the control group. As LH-releasing hormone neurons are innervated by catecholaminergic axons [64], the effect of bupropion on the reuptake of norepinephrine and dopamine may be involved in the reduced androgen levels observed in the animals exposed to this drug, while the reduction observed in the animals treated with simvastatin may be a result of possible reduction in the serum levels of cholesterol and consequent decrease of its availability for the synthesis of testosterone by the Leydig cells.

The epididymis is the organ responsible for sperm maturation and storage [65]. Its function is highly dependent on androgens and influenced by the autonomic innervations [66-68]. The process of sperm maturation occurs by the addition and removal of substances during sperm transit through the epididymis, leading to changes in their morphological and physiological characteristics, allowing the acquisition of motility and fertility capacity [65, 68].

By the time sperm transit to the proximal cauda of epididymis [69, 70], the excess of residual cytoplasm that occasionally remains in the cauda of spermatozoa after spermiation is phagocytosed by the clear cells present in the epididymal wall [70, 71]. These cells also endocytose other luminal debris [65] and several different proteins besides contributing to acidification of the luminal fluid [72].

Studies reported that impairment of the normal function of testes and epididymis observed after exposure to some toxicants was accompanied by the presence of large clear cells filled with lysosomes [72, 73]. In a study that evaluated the ultrastructure of the cauda epididymis of rats treated with *Azadirachta indica*, a medical plant, Ghodesawar *et al*. [73] reported hypertrophy of clear cells, with vacuolized cytoplasm and accumulation of lysosomal bodies, which was associated with the hypoandrogenic status resulted from the treatment. Ghodesawar *et al*. [73] also suggested that clear cells undergo hypertrophy in an attempt to remove the cell debris reaching the epididymal lumen from the testis, including residual bodies, and that these alterations observed in the clear cells may have affected its function, leading to alterations in the composition of epididymal fluid, which in turn may affect the sperm maturation.

It is known that the normal composition of the luminal epididymal environment is required for the proper sperm maturation [74]. In the present study, the epididymis from the animals treated with simvastatin alone or associated with bupropion showed hyperplasia of clear cells in the proximal cauda. These animals also showed a significant increase in the index of cytoplasmic droplet (CD) in the spermatozoa, possibly associated with the alterations observed in the clear cells.

Studies associated the excess of residual cytoplasm on spermatozoa with reduced sperm motility in rodent and human [51, 75]. In the present study we observed impairment of sperm motility in the simvastatin-treated group, evidenced by the reduction in the cross beat frequency

(rate of sperm motion crossing the average path trajectory), which may be explained by the increase d index of CD in the spermatozoa of these animals, associated with the decrease in the levels of testosterone.

In humans, the excess of residual cytoplasm on spermatozoa was also associated with increased sperm with DNA damages resulted from oxidative stress [76], decreased fertility capacity [77], reduced binding of sperm to the zona pellucida [52, 78] and infertility [79, 80]. In the present study, the co-exposure to simvastatin and bupropion resulted in impaired fertility, evidenced by the decreased percentage of implantation efficiency, which may be explained by a combination of the alterations observed in the epididymal morphology, androgen depletion and increased index of CD in these animals.

In conclusion, our results revealed adverse effects of the isolated and combined treatment with simvastatin and bupropion on male reproductive parameters and possible synergism between the drugs, evidenced by the impaired fertility observed after the co-exposure, raising concern of possible impairment of fertility in men exposed to statins and antidepressant drugs, such as bupropion and rosuvastatin, respectively.

To our knowledge, ours is the first study that reports the effects of the combined treatment with hypolipemiant and antidepressant drugs on male reproductive parameters. Additional investigations on sperm parameters and fertility after co-exposure to other statins and antidepressants, and the possible recovery after withdrawal are encouraged.

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698

699 7. CONFLICT OF INTEREST STATEMENT

700 The authors declare that there are no conflicts of interest.

8. LEGENDS OF FIGURES

Figure 1. Schematic experimental design of animals from Cohort 1 (step 1 and 2) and Cohort 2. The oval structures in black represents the testes and the long structure in black represents the epididymis. LCC = Leydig cell culture; TIFT = testicular interstitial fluid testosterone; IUI = intrauterine insemination; IHC = immunohistochemistry; T = testosterone.

Figure 2. Analysis of body weight. Evolution of body weight in the control (C), simvastatin (S), bupropion (B) and simvastatin associated with bupropion (S+B) groups from Cohort 1 (n = 9, n = 10, n = 10, n = 10, respectively) **(A)** and Cohort 2 (n = 10/group) **(B)**. Body weight gain (final weight subtracted from the initial weight) of the animals from Cohort 1 **(C)** and Cohort 2 **(D)**. Values expressed as mean ± S.E.M. ANOVA followed by Dunnett's test; $p > 0.05$.

Figure 3. Analyses of testosterone in the control (C), simvastatin (S), bupropion (B) and simvastatin associated with bupropion (S+B) groups from the Cohort 1 **(A and B)** and Cohort 2 **(C, D and E)**. Levels of testosterone produced by 10^6 isolated Leydig cells incubated 3 hours without **(A)** or with **(B)** 10 ng/mL LH (n = 5/group); Concentration of testicular interstitial fluid testosterone (TIFT) (C, n = 9; S, n = 9; B, n = 8; S+B, n = 9) **(C)**; Serum levels of testosterone (C, n = 8; S, n = 10; B, n = 9; S+B, n = 10) **(D)**; Scatter plot depicting the correlation between TIFT and serum levels of testosterone (C, n = 7; S, n = 9; B, n = 8; S+B, n = 7) **(E)**. Values expressed as mean ± SEM. ANOVA followed by Dunnett's test; $*p < 0.05$, $**p < 0.01$, compared to the control group; $R^2 = 0.7722$ ($p < 0.0001$).

Figure 4. Photomicrographs illustrating testicular transversal sections of the control **(A and E)**, simvastatin **(B and F)**, bupropion **(C and G)** and simvastatin associated with bupropion **(D and H)** groups from Cohort 1. H & E, 100x (**A-D**; scale bar = 200 μm) and 200x (**E-H**, scale bar = 100 μm).

Figure 5. Photomicrography illustrating the epididymal morphology in the experimental groups from the Cohort 1. Note the presence of hyperplasia of clear cells in the proximal cauda of epididymis from the animals treated with simvastatin alone or associated with bupropion (arrows). H & E, 100x, scale bar = 50 μm.

Figure 6. Cellular localization of 3β-hydroxysteroid dehydrogenase (3β-HSD; green), a Leydig cell marker, in sections of testes from control **(A)**, simvastatin **(B)**, bupropion **(C)** and simvastatin associated with bupropion **(D)** groups, from Cohort 1. Negative control (without primary antibody) is inserted in the image A. Localization of 3β-HSD was displayed by immunofluorescent staining for this enzyme and counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue; nuclei marker), showing that this enzyme is localized in the Leydig cell mitochondria. The images are representative results from three replicates, using three different regions of testes and yielded similar results. Qualitative analysis showed no difference of staining intensity among groups. Scale bar = 100 μm.

Figure 7. Fertility accessed by *in utero* artificial insemination in control (C), simvastatin (S), bupropion (B) and simvastatin associated with bupropion (S+B) groups (n = 7, n = 10, n = 9, n = 8, respectively) from Cohort 2. Number of corpora lutea **(A)**, implantation sites **(B)**, and efficiency of implantation (%) **(C)**. Values expressed as mean ± SEM. ANOVA followed by Dunnett's test; $*p < 0.05$, compared to the control group.

Table 1. Final body weight and absolute and relative reproductive organ weights of the animals from Cohort 2.

	Experimental Groups			
Reproductive organs	Control (n=9)	Simvastatin (n=10)	Bupropion (n=10)	Simvastatin + Bupropion (n=10)
Final body weight (g)	534.70 ± 15.84	536.10 ± 14.46	511.30 ± 12.07	511.10 ± 19.74
Absolute organ weights				
Left testis (g)	1.92 ± 0.05	1.94 ± 0.05	1.95 ± 0.07	1.93 ± 0.04
Right testis (g)	1.93 ± 0.04	1.95 ± 0.07	1.96 ± 0.06	1.95 ± 0.03
Left epididymis (mg)	674.40 ± 18.40	672.20 ± 17.14	681.40 ± 19.13	694.20 ± 16.81
Right epididymis (mg)	663.20 ± 13.80	672.80 ± 18.85	699.80 ± 18.85	682.80 ± 15.98
Ventral prostate (mg)	709.10 ± 57.13	762.20 ± 41.26	709.80 ± 40.41	691.00 ± 42.12
Seminal vesicle with fluid (mg)	1324.00 ± 55.17	1412.00 ± 36.50	1469.00 ± 53.87	1330.00 ± 74.50
Relative organ weights				
Left testis (g/100g BW)	0.36 ± 0.01	0.36 ± 0.01	0.38 ± 0.01	0.38 ± 0.01
Right testis (g/100g BW)	0.36 ± 0.01	0.36 ± 0.01	0.38 ± 0.01	0.39 ± 0.01
Left epididymis (mg/100g BW)	126.70 ± 4.16	126.10 ± 4.21	133.60 ± 3.81	137.10 ± 4.95
Right epididymis (mg/100g BW)	124.80 ± 3.87	126.30 ± 4.64	137.30 ± 3.94	135.00 ± 5.11
Ventral prostate (mg/100g BW)	137.80 ± 8.84	143.10 ± 11.00	139.40 ± 6.76	141.10 ± 10.59
Seminal vesicle with fluid (mg/100g BW)	249.10 ± 11.75	265.90 ± 11.77	288.00 ± 15.12	262.60 ± 17.56

BW = body weight. Values expressed as mean ± SEM. ANOVA followed by Dunnett's test; $p > 0.05$, compared to the control group.

Table 2. Testicular morphometric and histopathological analysis in Cohort 1 and sperm parameters and cytoplasmic droplet (CD) in spermatozoa of animals from Cohort 2.

		Experimental groups		
Parameters	Control	Simvastatin	Bupropion	Simvastatin + Bupropion
Testicular morphometry				
Diameter of seminiferous tubule (μm)	335.40 ± 4.91	341.90 ± 3.16	348.00 ± 5.13	340.20 ± 4.99
Height of germinal epithelium (μm)	90.67 ± 0.37	89.73 ± 1.63	90.82 ± 1.46	90.99 ± 1.73
Testicular histopathology				
Normal seminiferous tubule (%)	94.50 (88.00-95.75)	91.00 (86.00-95.50)	90.00 (87.00-92.00)	89.00 (87.00-96.00)
Abnormal seminiferous tubule (%)	5.50 (4.25-12.00)	9.00 (4.50-14.00)	10.00 (8.00-13.00)	11.00 (4.00-13.00)
Spermatogenesis kinetics				
Stages I–VI (%)	46.50 (41.75-54.25)	40.00 (39.50-41.50)	43.00 (42.00-44.00)	44.00 (35.50-45.50)
Stages VII–VIII (%)	25.00 (23.00-26.00)	26.00 (25.00-33.50)	26.00 (25.00-27.50)	29.00 (28.00-30.50)
Stages IX–XIII (%)	25.50 (22.00-26.75)	28.00 (23.50-30.00)	28.00 (23.00-30.00)	21.00 (19.00-31.00)
Stage XIV (%)	5.50 (3.25-7.00)	4.00 (2.50-5.50)	3.00 (2.00-6.50)	6.00 (4.00-6.50)
Sperm morphology				
Normal sperm (%)	99.25 (99.00-99.88)	99.50 (99.00-99.63)	99.25 (98.88-99.63)	98.50 (98.38-99.63)
Abnormal sperm (%)	0.75 (0.12-1.00)	0.50 (0.37-1.00)	0.75 (0.37-1.12)	1.50 (0.37-1.62)
Sperm motility				
Progressive track (%)	46.70 (41.55-49.85)	52.10 (50.40-55.55)	48.15 (44.30-51.80)	44.20 (41.40-46.68)
BCF (Hz)	37.77 ± 0.54	35.72 ± 0.69*	37.63 ± 0.64	38.52 ± 0.75
Cytoplasmic droplet (CD)				
Sperm with CD (%)	90.50 (74.50-91.00)	91.50 (85.25-95.38)	88.50 (73.50-90.25)	90.50 (85.75-94.00)
Sperm without CD (%)	9.50 (9.00-25.50)	8.50 (4.62-14.75)	11.50 (9.75-26.50)	9.50 (6.00-14.25)
Proportion of sperm with/without CD	6.93	9.26***	6.82	10.58***
Proximal CD (%)	0.00 (0.00-1.65)	0.00 (0.00-0.00)	0.00 (0.00-0.70)	0.55 (0.00-0.60)
Medial CD (%)	100.00 (98.35-100.00)	100.00 (100.00-100.00)	100.00 (99.30-100.00)	99.43 (98.87-100.00)
Distal CD (%)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.14)

Differences among groups are indicated by superscripts as follows: a ≠ control, b ≠ rosuvastatin.Values expressed as mean ± SEM were compared using ANOVA followed by Dunnett's test. Values expressed as median (Q3-Q1) were compared using Kruskal-Wallis followed by Dunn's test. Proportions of sperm with/without CD were compared using Chi-square test. $^{*}p < 0.05$ and $^{***}p < 0.001$, compared to the control group.

Figure 1.

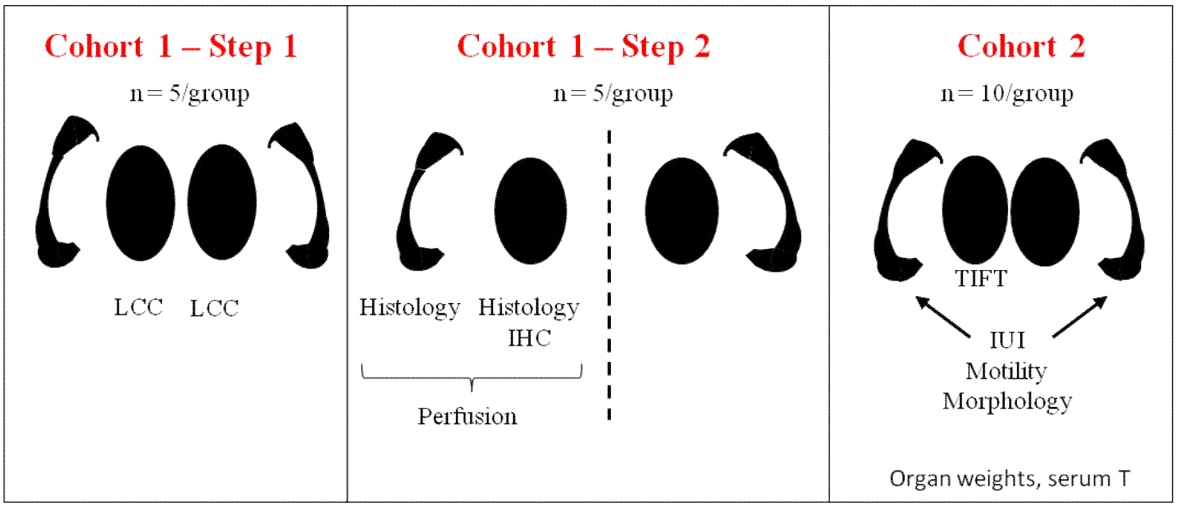

Cohort 1 – Step 1
n = 5/group
LCC LCC
Cohort 1 – Step 2
n = 5/group
Histology Histology
IHC
Perfusion
Cohort 2
n = 10/group
TIFT
IUI
Motility
Morphology
Organ weights, serum T

Figure 2.

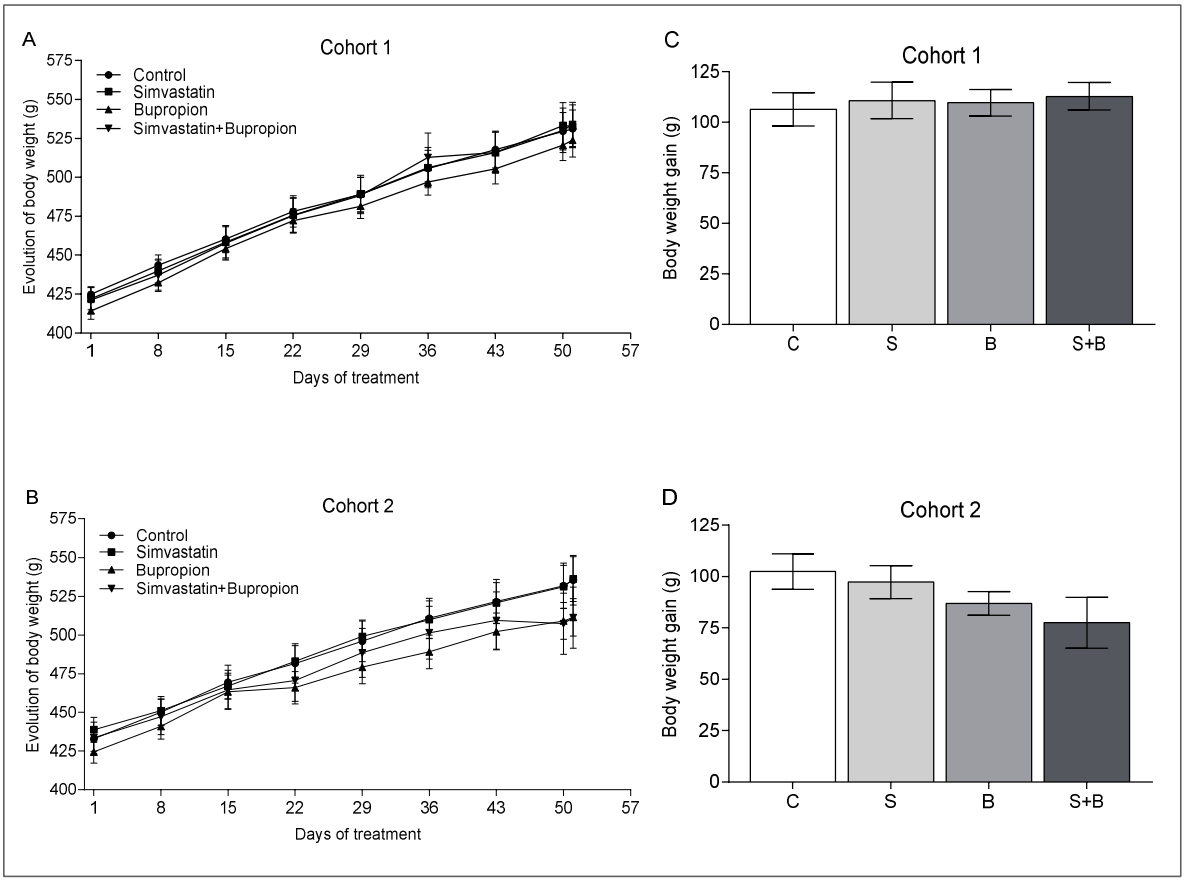
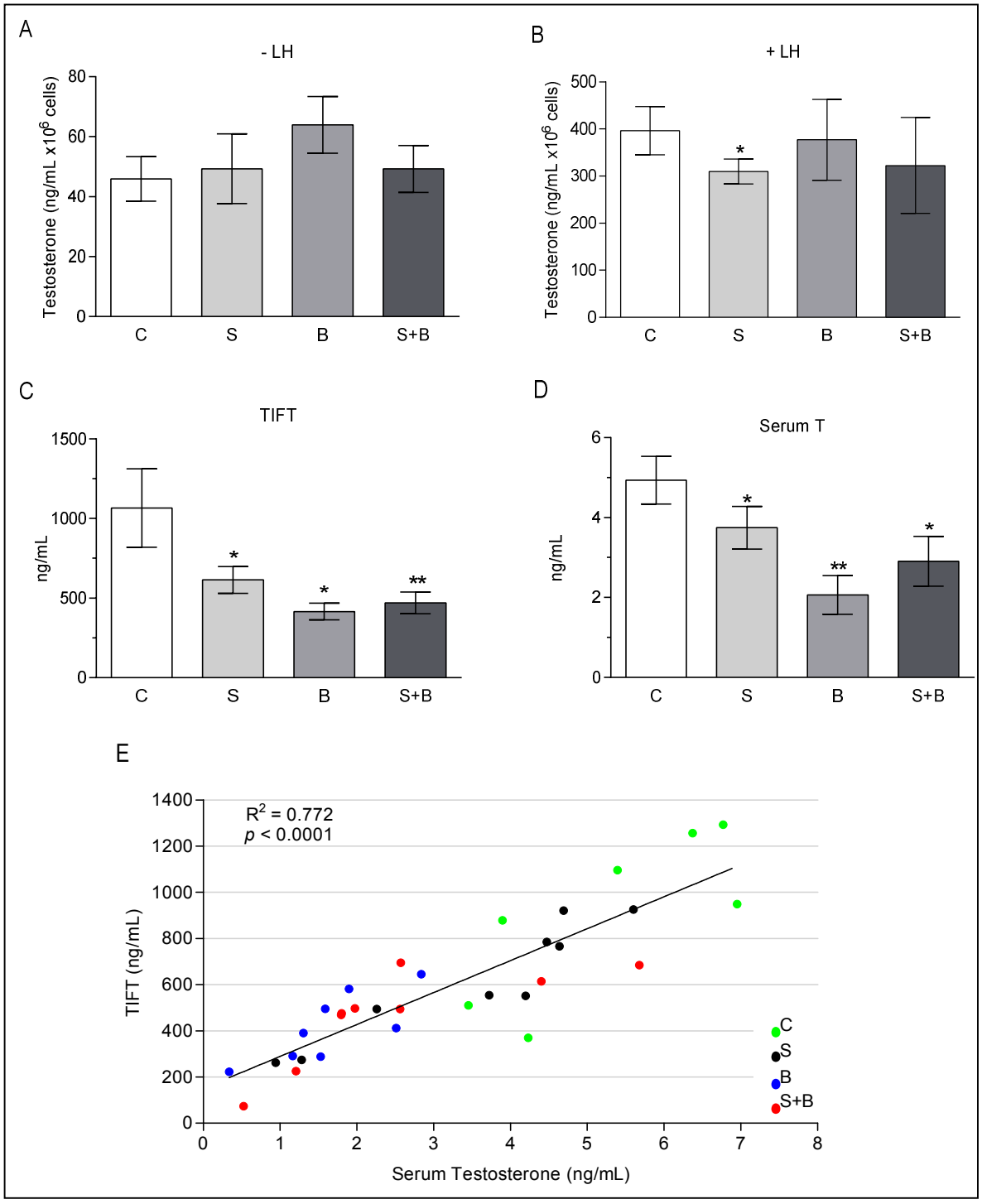

A
Cohort 1
Control
Simvastatin
Bupropion
Simvastatin+Bupropion
Evolution of body weight (g)
575
550
525
500
475
450
425
400
1
8
15
22
29
36
43
50
57
Days of treatment
C
Cohort 1
Body weight gain (g)
125
100
75
50
25
0
C
S
B
S+B
B
Cohort 2
Control
Simvastatin
Bupropion
Simvastatin+Bupropion
Evolution of body weight (g)
575
550
525
500
475
450
425
400
1
8
15
22
29
36
43
50
57
Days of treatment
D
Cohort 2
Body weight gain (g)
125
100
75
50
25
0
C
S
B
S+B

Figure 3.


A
- LH
Testosterone (ng/mL x10^6 cells)
80
60
40
20
0
C
S
B
S+B
B
+ LH
Testosterone (ng/mL x10^6 cells)
500
400
300
200
100
0
*
C
S
B
S+B
C
TIFT
ng/mL
1500
1000
500
0
*
*
**
C
S
B
S+B
D
Serum T
ng/mL
6
4
2
0
*
**
*
C
S
B
S+B
E
R^2 = 0.772
p < 0.0001
TIFT (ng/mL)
1400
1200
1000
800
600
400
200
0
0
1
2
3
4
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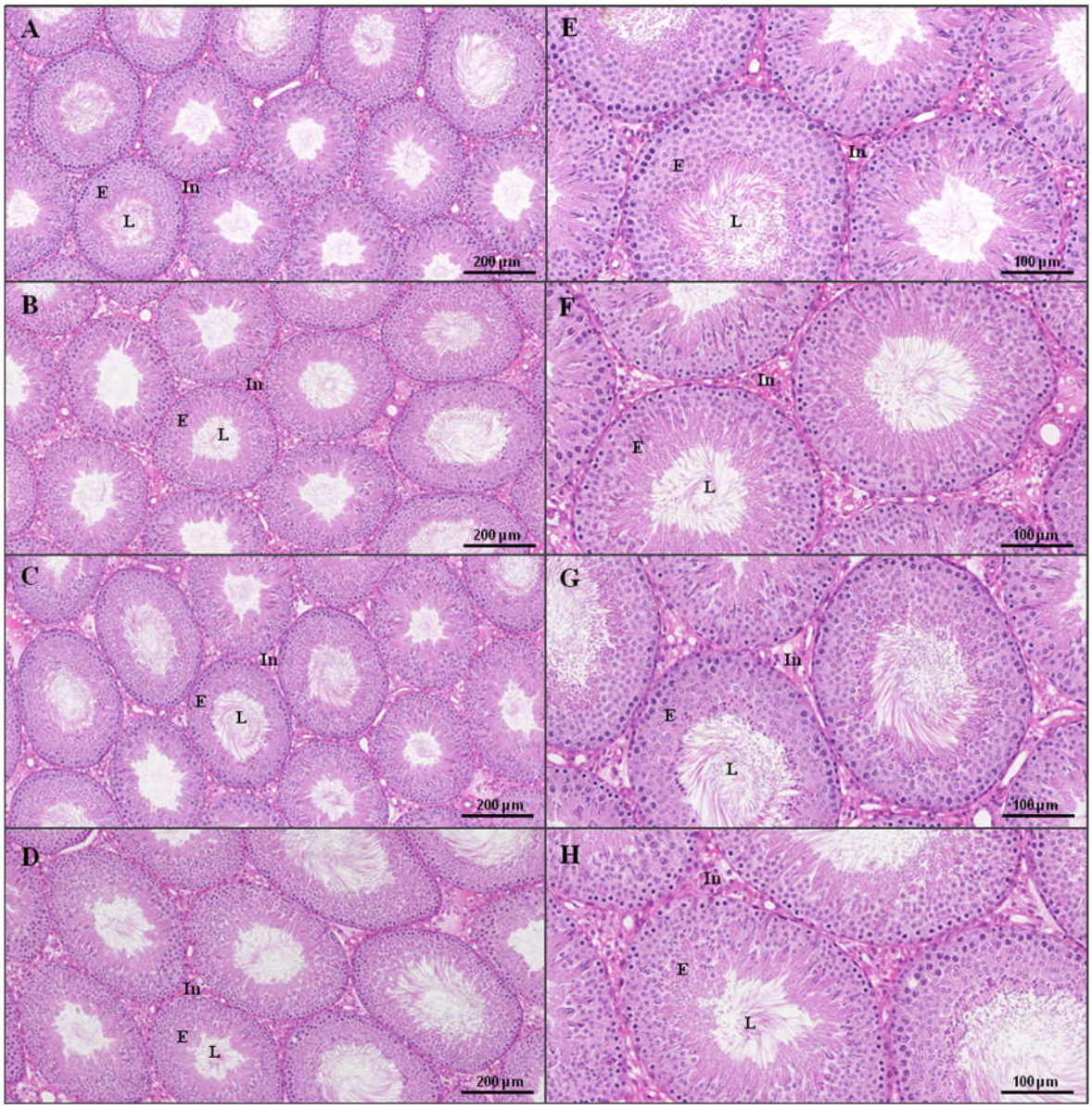
6
7
8
Serum Testosterone (ng/mL)
C
S
B
S+B

Figure 4.


A
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100 µm
B
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L
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F
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100 µm
C
In
E
L
200 µm
G
In
E
L
100 µm
D
In
E
L
200 µm
H
In
E
L
100 µm

Figure 5.

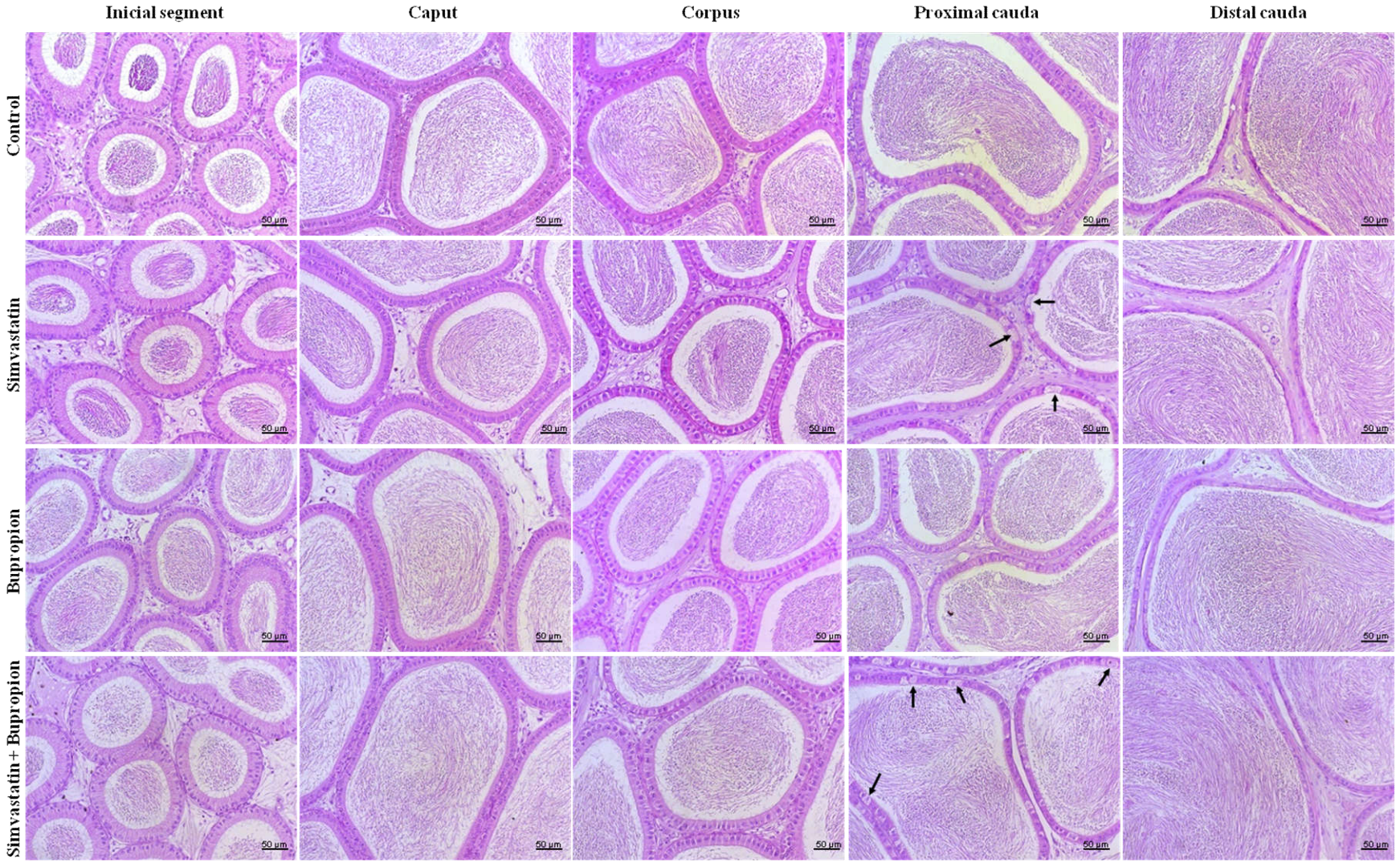

Inicial segment
Caput
Corpus
Proximal cauda
Distal cauda
Control
Simvastatin
Bupropion
Simvastatin + Bupropion
50 µm

Figure 6.

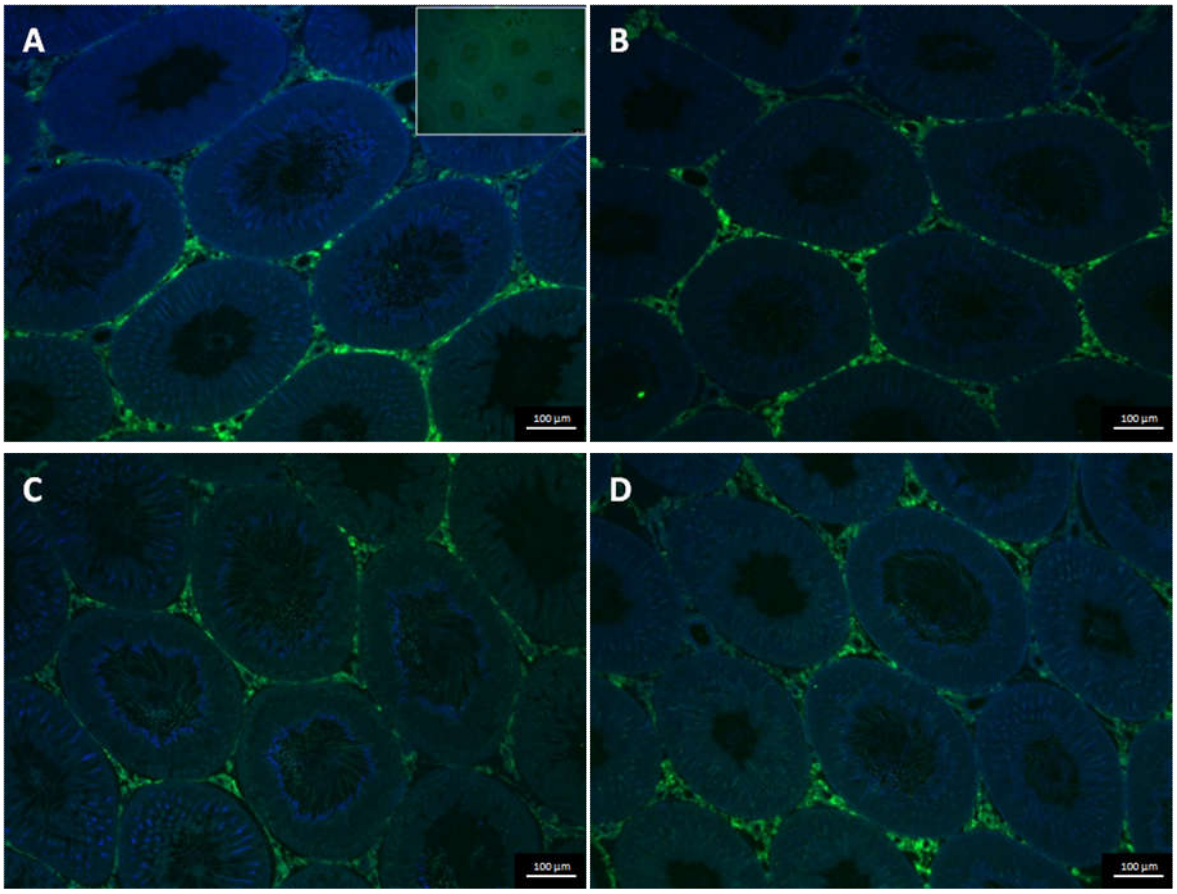
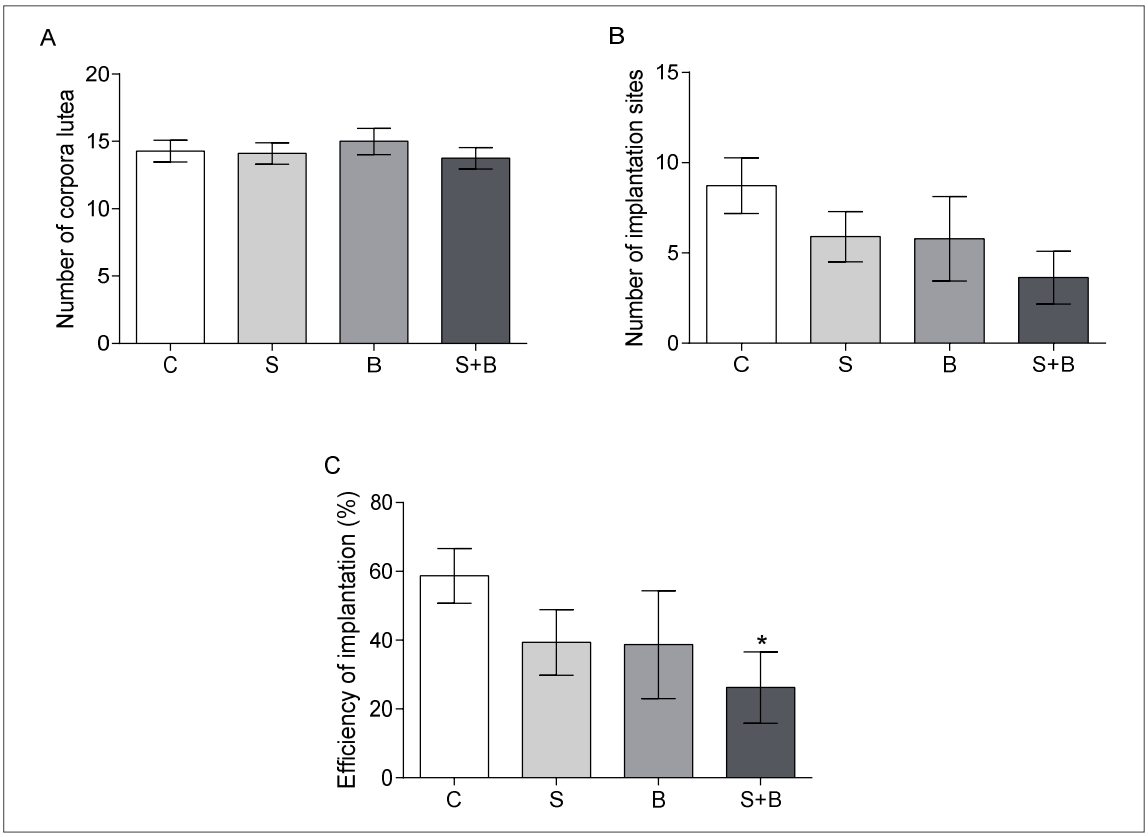


Figure 7.


A
Number of corpora lutea
20
15
10
5
0
C
S
B
S+B
B
Number of implantation sites
15
10
5
0
C
S
B
S+B
C
Efficiency of implantation (%)
80
60
40
20
0
*
C
S
B
S+B

Conclusões

Nossos resultados indicam que, nessas condições experimentais, a exposição isolada ou combinada a estatinas e inibidores de recaptura de neurotransmissores promoveu efeitos adversos sobre parâmetros reprodutivos em ratos adultos, tais como redução do peso corpóreo e de órgãos reprodutores, da concentração sérica e intratesticular de testosterona, da síntese de testosterona pelas células de Leydig, da motilidade espermática, do comportamento sexual e da morfologia epididimária, bem como aumento do índice de espermatozoides com gota citoplasmática. Não foi observado alteração na morfologia testicular e espermárica, bem como na expressão da enzima 3β-HSD, porém, a co-exposição resultou na redução do potencial fértil, indicando possível sinergismo entre os fármacos.

Sabendo-se que roedores apresentam maior eficiência reprodutiva comparada à espécie humana, os resultados obtidos no presente trabalho indicam potencial risco reprodutivo em homens expostos a estatinas e inibidores de recaptura de neurotransmissores durante a idade adulta. Novos estudos são necessários para investigar a atividade de enzimas esteroidogênicas, a contratilidade e reserva endógena de neurotransmissores nos órgãos reprodutores, bem como possíveis efeitos da combinação desses fármacos sobre a prole.

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Apêndices

Esta seção compreende descrições metodológicas, bem como resultados de análises realizadas durante a execução do projeto de pesquisa que deu origem a esta tese, mas que não foram incluídas nos manuscritos, sendo apresentados em forma de apêndices, conforme descrito abaixo.

Apêndice 1

Este apêndice compreende os resultados da avaliação dos pesos absoluto e relativo de órgãos vitais dos animais do Experimento 3 do manuscrito 1, bem como a fertilitdade investigada após a realização de inseminação artificial.

1. Fertility test after *in utero* artificial insemination

Because rodents produce an excess of qualitatively normal sperm, the intrauterine insemination of a fixed number of sperm increases the sensitivity to detect alterations in sperm quality and fertility after exposure to toxicants (Amann, 1986). Therefore, *in utero* artificial insemination was performed as described by Klinefelter *et al*. (Klinefelter et al., 1994) and Kempinas *et al*. (Kempinas et al., 1998). Briefly, the adult female rats were synchronized in estrus by subcutaneous administration of 80 mg of luteinizing releasing hormone agonist (LHRH: Sigma Chemical Co., St. Louis, MO), and after 115 hours they were paired with vasectomized males for 1 hour and the receptive females (that exhibited lordosis) were selected for insemination.

The sperm used for insemination was obtained from the same sperm sample used for the investigation of sperm motility and morphology, as described in the section 2.5.3. After 10-fold dilution, the sperm were counted in a Neubauer chamber and an aliquot containing 5×10^6 perm was injected in each uterine horn. One female was inseminated per male and after twenty days, on GD 20, they were euthanized by CO_2 inhalation followed by decapitation, and the same parameters previously described to assess fertility after natural mating were determined to assess fertility after *in utero* artificial insemination.

2. Vital organ weights

The adult male rats destinated to the investigation of fertility after artificial insemination had the vital organs collected and weighted. The final body weight of these animals was used to calculate the relative weights of these organs.

Results and brief discussion:

Besides no alterations were observed in the fertility assessed after artificial insemination (AI) (Table 1), reduced fertility potential was observed in the co-exposed group after natual mating, as described in the masnucript 1. This impairment of fertility after natural mating can be explained, in part, by the reduced sperm reserves observed in these animals, an interference that is removed in the AI technique, which is performed under the insemination of sperms obtained directly from the epididymis cauda (Klinefelter, 2002).

The final body weight of the animals treated with sibutramine, alone or associated with rosuvastatin, was significantly reduced, compared to control group (Table 2). These animals also showed reduced absolute weight of pituitary, which may be explained by the decrease in the body weight.

The exposure to rosuvastatin, alone or associated with sibutramine, resulted in reduced absolute weight of liver. The co-exposed group also showed increased relative weight of the brain, which can be explained by the decreased body weight, once the brain is a conservative organ (Creasy and Chapin, 2013).

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G.R. Klinefelter, The epididymis, in: B. Robaire, B.T. Hinton (Eds.), The Epididymis – From Molecules to Clinical Practice, Kluwer Academic/Plenum Publisher, New York, 2002, pp. 359–369.

Table 1. Fertility parameters after intrauterine artificial insemination of adult male rats from the experimental groups of experiment 3.

	Experimental Groups			
Parameters	Control (n=12)	Rosuvastatin (n=12)	Sibutramine (n=11)	Rosuvastatin + Sibutramine (n=10)
Pregnant per inseminated females (%)	91.67	100.00	75.00	90.00
Body weight of dams (g)	347.80 (326.20-390.10)	356.29 (333.00-393.90)	362.00 (343.70-405.30)	358.00 (324.90-388.20)
Fetal body weight (g)	3.29 (3.17-3.51)	3.30 (3.15-3.52)	3.12 (2.99-3.29)	3.16 (2.97-3.27)
Uterus weight with fetuses (g)	52.87 (34.95-56.05)	52.69 (40.27-70.87)	42.80 (34.01-57.29)	50.62 (42.00-57.38)
Placental weight (mg)	0.60 (0.53-0.66)	0.61 (0.58-0.63)	0.61 (0.55-0.68)	0.64 (0.55-0.69)
Sex ratio (M/F)	51/40	59/65	40/35	42/36
Corpora lutea				
Total (*N*)	136	166	119	114
N per litter (median (Q3-Q1))	12.00 (10.00-14.00)	13.00 (12.00-14.50)	13.00 (12.00-14.50)	13.00 (11.50-14.00)
Implantation sites				
Total (*N*)	111	135	87	94
N per litter (median (Q3-Q1))	10.00 (9.00-13.00)	11.00 (9.50-13.00)	9.00 (8.50-11.50)	12.00 (8.50-12.50)
Implants per corpora lutea (%)	81.62	81.32	73.11	82.46
Resorptions				
Total (*N*)	20	17	12	16
N per litter (median (Q3-Q1))	2.00 (0.00-3.00)	1.00 (0.00-2.00)	1.00 (1.00-2.00)	1.00 (0.00-2.50)
Per implantation sites (%)	18.02	12.59	13.79	17.02
Live fetuses				
Total (*N*)	91	118	75	78
N per litter (median (Q3-Q1))	10.00 (6.00-10.00)	9.00 (7.50-12.50)	8.00 (6.50-10.00)	10.00 (6.00-11.00)
Per implantation sites (%)	81.99	87.41	86.21	82.98

N, number. Proportions (%) were compared using the Chi-square test. Values expressed as median (Q3-Q1) were compared using Kruskal-Wallis test with an *a posteriori* Dunns's test. $p > 0.05$.

Table 2. Final body weight and absolute and relative vital organ weights of the animals from experiment 3.

	Experimental groups			
Vital organs	Control (n=12)	Rosuvastatin (n=12)	Sibutramine (n=11)	Rosuvastatin + Sibutramine (n=11)
Final body weight (g)	478.90 ± 9.99	450.80 ± 17.23	425.60 ± 14.39[a*]	414.20 ± 6.18[a**]
Absolute weights				
Pituitary (mg)	11.32 ± 0.43	9.81 ± 0.49	8.81 ± 0.49[a**]	9.38 ± 0.50[*]
Liver (g)	15.55 ± 0.60	13.26 ± 0.48[a*]	14.18 ± 0.67	12.80 ± 0.52[a**]
Kidney (g)	1.58 ± 0.04	1.49 ± 0.06	1.49 ± 0.04	1.43 ± 0.05
Adrenal gland (mg)	38.33 ± 3.30	37.31 ± 2.63	32.89 ± 1.49	30.57 ± 0.96
Thyroid (mg)	19.27 ± 0.85	18.84 ± 0.99	19.24 ± 1.02	20.79 ± 1.55
Brain (g)	2.06 ± 0.03	1.99 ± 0.03	1.99 ± 0.04	2.03 ± 0.03
Relative weights				
Pituitary (mg/100g BW)	2.37 ± 0.08	2.21 ± 0.09	2.10 ± 0.09	2.34 ± 0.11
Liver (g/100g BW)	3.23 ± 0.07	3.02 ± 0.04	3.32 ± 0.09[b*]	3.18 ± 0.06
Kidney (g/100g BW)	0.33 ± 0.01	0.33 ± 0.01	0.35 ± 0.01	0.36 ± 0.01
Adrenal gland (mg/100g BW)	7.98 ± 0.63	8.27 ± 0.45	7.75 ± 0.30	7.40 ± 0.47
Thyroid (mg/100g BW)	4.02 ± 0.15	4.23 ± 0.26	4.57 ± 0.31	4.84 ± 0.34
Brain (g/100g BW)	0.43 ± 0.01	0.45 ± 0.01	0.47 ± 0.02	0.49 ± 0.01[a*]

BW = body weight. Differences among groups are indicated by superscripts as follows: a ≠ control, b ≠ rosuvastatin. Values expressed as mean ± SEM. ANOVA followed by Tukey's test. $^{*}p < 0.05$, $^{**}p < 0.01$.

Apêndice 2

Este apêndice compreende as descrições metodológicas e os resultados da investigação da contagem espermática realizada nos testículos e na cabeça e corpo dos epidídimos (Tabela 1) dos animais do Cohort 2 do Manuscrito 2.

Também são apresentadas descrições metodológicas de análise proteômica que está em andamento, para investigar a presença e expressão gênica da proteína SP22 (biomarcador de fertilidade), a partir dos espermatozoides extraídos da cauda epididimária utilizada para a realização do procedimento de inseminação artificial do Cohort 2 do Manuscrito 2. A figura 1 apresenta uma imagem de gel bidimensional representativo do grupo controle, indicando a identificação da proteína SP22.

1. Sperm counts

After the removal of tunica albuginea from the left testis, the parenchyma was weighed and frozen for further determination of testicular spermatid head counts (TSHC) and daily sperm production (DSP), while both epididymis caput/corpus were weighed and frozen for further analysis of sperm counts and transit time through these regions of the epididymis.

In order to determine the TSHC, the testicular parenchyma was thawed at room temperature, homogenized in a polytron for 10 sec with 8 mL of STM (0.9 % saline containing 0.05 % Triton X-100 and 0.01 % Merthiolate), sonicated in an ice bath for 30 sec and vortexed. After a 10-fold dilution with STM, a 200-µL aliquot was placed in an IDENT Stain reaction vial containing a dehydrated pellet of bis benzimide trihydrochloride, immediately vortexed and allowed to sit for at least 5 min (but not more than 1 hour) to allow the cells to absorb the stain. Epididymal caput and corpus were processed together and in the same way described for the testis counts, but diluted to 40 mL with STM and minced with a scissors before a 2-min homogenization.

The samples were placed in both sides of a 20-µm deep Cell-VU chamber and immediately analyzed using the Tox IVOS HTM-Ident (Hamilton Throne Research, Beverly, MA), a computer-assisted sperm analysis (CASA) system that identifies sperm heads using DNA-specific dye and fluorescence illumination. For this, twenty fields from four chambers were counted for each sample, and if variation of counts between the chambers was more than 10 %, the sample was recounted.

In order to calculate the DSP, the TSHC was divided by 6.1, which corresponds to the number of days of the seminiferous cycle in which the spermatids at stage 19 are present in the seminiferous epithelium. The sperm transit time through the epididymal caput and corpus was determined by dividing the number of sperm in these epididymal regions by the DSP.

Table 1. Sperm count in the left testis and in the caput and corpus of both epididymis from the experimental groups of Cohort 2.

Parameters	Experimental Groups			
	Control (n=9)	Simvastatin (n=10)	Bupropion (n=10)	Simvastatin + Bupropion (n=10)
Sperm count in the left testis				
Mature spermatid number ($\times 10^6$/organ)	175.80 ± 8.25	194.60 ± 13.12	186.20 ± 8.27	182.00 ± 5.67
Relative mature spermatid number ($\times 10^6$/g of organ)	91.56 ± 3.52	99.46 ± 4.86	95.54 ± 3.18	94.30 ± 2.15
Daily sperm production ($\times 10^6$/organ/day)	28.81 ± 1.35	31.91 ± 2.15	30.53 ± 1.36	29.84 ± 0.93
Relative daily sperm production ($\times 10^6$/g of organ/day)	15.01 ± 0.58	16.31 ± 0.80	15.66 ± 0.52	15.46 ± 0.35
Sperm count in both epididymal caput/corpus				
Sperm number ($\times 10^6$/organ)	198.40 ± 16.88	201.40 ± 9.42	208.30 ± 12.28	198.90 ± 9.18
Relative sperm number ($\times 10^6$/g of organ)	240.80 ± 15.93	247.60 ± 12.93	254.60 ± 11.85	237.60 ± 11.23
Sperm transit time (days)	6.88 ± 0.47	6.58 ± 0.55	6.89 ± 0.42	6.69 ± 0.31

Values expressed as mean ± SEM. ANOVA followed by Tukey's test; $p > 0.05$.

2. Sperm protein analysis

After taking aliquots of the sperm dispersion for the artificial insemination procedure and evaluation of sperm motility, the remaining sperm were processed for protein analysis as previously described (Klinefelter *et al.*, 2002). Briefly, the sample was transferred from the Petri dish to a microcentrifuge tube and 1 μL of phenylmethylsulfonyl fluoride (PMSF, Sigma, St. Louis, MO; 35 mg/mL DMSO) was freshly added to the sample. After centrifuged at 4 ^{o}C, 1000 rpm, for 10 min, the supernatant was removed and the sample was washed twice by centrifugation (4 ^{o}C, 1000 rpm, 10 min) with a sperm isolation buffer (SIB), which consisted of Hanks' Balanced Salts Solution (HBSS 10X) buffered with 4.2 g/l HEPES and 0.35 g/l NaHCO3 and added 0.9 g/l D-glucose, 100 mM sodium pyruvate, and 0.025 g/l soybean trypsin inhibitor, pH 7.4. After the final wash, the supernatant was stored at -70 ^{o}C for further sperm extraction. In order to extract sperm proteins, the samples were thawed, vortexed and incubated for 1 h at room temperature with 1 mL of sperm membrane solubilization buffer, consisting of 80 mM *n*-octyl-B-glucopyranoside (OBG) in 10 mM Tris, pH 7.2, containing freshly added PMSF. After the final centrifugation (10,000 x g, 5 min), the supernatant was removed and frozen at -70 ^{o}C.

Prior to 2-D gel electrophoresis, the samples were thawed and the extracts were concentrated and desalted in Ultrafree-4 centrifugation filter units (Millipore, Bedford, MA), by two times centrifugation (3750 rpm, 25 min, 4 ^{o}C) with 1 mM Tris buffer (pH 7.2). The sample retained in the filter was transferred to a microcentrifuge tube and protein concentration was determined using a bicinchoninic acid-based protein assay kit (Cat#23235, Thermo Scientific, Rockford, IL, USA). Sample aliquots containing 30 μg proteins were lyophilized.

The samples were incubated for 30 min at room temperature with 45 μL of sample rehydration buffer, consisting of 10.5g urea (denaturating agent), 0.5g chaps, dithiothreitol (DTT; reducing agent), ampholytes (solubilizing agent) and bromophenol blue and proteins were separated by isoelectric focusing in IPG gel strip (ReadyStrip^{TM} IPG Strip, Cat. #163-2014, Bio-

Rad Laboratories, USA). This separation was performed at 750 V for 3.5 h, under a pH gradient. After that, the proteins were separated by their molecular weights at 200 V for 45 min in 14 % acrylamide gels, soaked in 50 % methanol and stained using a silver staining kit (Amersham Biosciences, Upsula, Sweden).

Images were acquired by transmittance on an Ektron 1412 scanner and a Kepler 2D gel analysis system (Large Scale Biology Corp., Rockville, MD) was used for correction of background, spot detection and quantification, which was performed by fitting two-dimensional Gausian distributions to the density distribution of the spot areas (sum of the pixel intensities of each area).

The gels were compared among groups and the spots that showed difference compared to the control group are being excised and subjected to in-gel tryptic digestion and peptide extraction for protein identification by mass spectrometry.

Reference:

G.R. Klinefelter, L.F. Strader, J.D. Suarez, N.L. Roberts, Bromochloroacetic acid exerts qualitative effects on rat sperm: implications for a novel biomarker, Toxicological sciences: an official journal of the Society of Toxicology 68(1) (2002) 164-73.

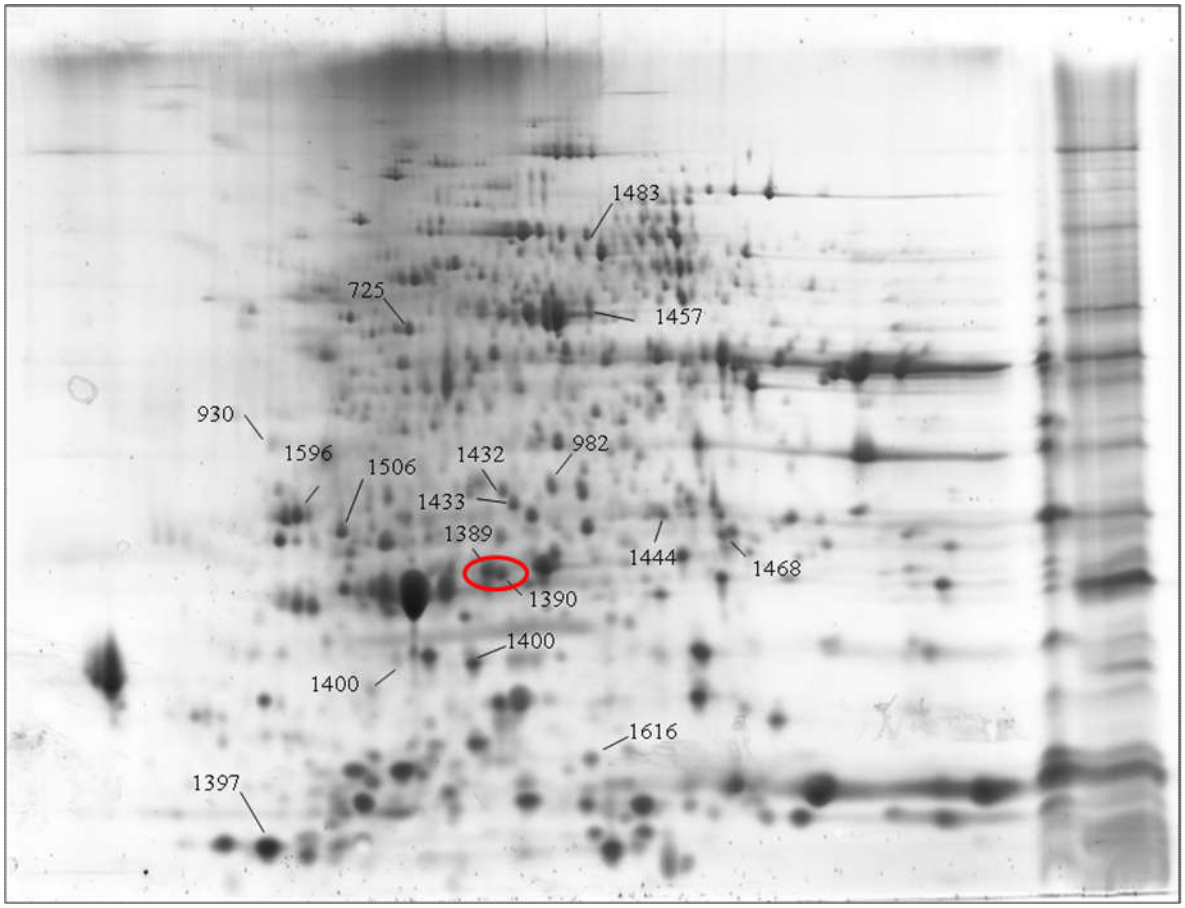

Figure 1. Representative 2D gel image of sperm extracts from the control group, obtained from the aliquots used for artificial inseminaiton in Cohort 2. Spots that were altered, compared to the control group, are indicated by its number. Spots numbers 1389 and 1390, indicated by a red ring, represent the protein SP22, a biomarker of fertility.

Table 1. Spot areas from 2D gels obtained from the same sperm sample used for artificial insemination in the Cohort 2.

		Experimental groups			
Spot number	Protein ID	Control	Simvastatin	Bupropion	Simvastatin + Bupropion
725		12191	11403	10515	8584
930		5539	2055	4541	5042
982		16833	13742	13764	16114
1389		19883	23333	16289	14237
1390	SP22	23425	25968	20151	20966
1397	SP22	102049	105867	107550	73163
1400		34769	24041	27592	33009
1401		7856	4170	5629	6345
1432		6857	6715	5618	5728
1433		10371	11283	8086	10118
1444		9154	8369	7113	7802
1457		7442	8635	6799	6171
1468		5154	4355	1536	3436
1483		2582	3057	2004	2546
1506		12898	12097	9891	10778
1596		11817	6636	7886	8446
1616		3060	4584	1747	1969

ID = identification. Values are expressed as pixels. Values in gray indicate spots that were different, compared to the control group.

Apêndice 3

Este apêndice compreende resultados parciais de experimentos farmacológicos *ex vivo* realizados para investigar os efeitos da rosuvastatina e sibutramina, isoladamente ou em associação, sobre a contratilidade do ducto epididimário após a exposição *in vivo* por 30 e 70 dias. Os animais avaliados neste experimento seguiram o mesmo protocolo de tratamento descrito no Manuscrito 1.

1. Brief description of the experimental design

Pharmacological experiments with the cauda epididymis were performed in order to understand the contractile function of this tissue after treatment of adult male rats with rosuvastatin and sibutramine, isolated or in association, for 30 and 70 days. Treatment was performed as described in the Manuscript 1.

Twenty four hours following the last day of treatment, the animals were euthanized by decapitation and segments (1 cm) from the distal epididymis cauda were obtained, cleaned with flush of nutrient solution and mounted in isometric force transduters in 10-mL organ baths (Figure 1). The tissues were whashed at least two times with nutrient solution and, after 30 min stabilization, 80 mM KCl was added to the organ baths two times, followed by washes and stabilization period of 30 min, in order to evaluate tissue viability. Two protocols were used as described below.

In the first protocol, after the second addition of KCl, a concentration-response curve (CRC) to norepinephrine (NE) was obtained by cumulative addition (10^{-9} to 10^{-3} M) of this agonist to the organ baths, which was taken as control curve. After washes followed by 45 min incubation with 100 nM of desipramine, a norepinephrine transporter (NET) inhibitor, a new CRC to NE was obtained. The tissues were washed again and, 45 min later, a CRC to tyramine (10^{-7} to 10^{-3} M) was obtained (Tyr 1).

In the second protocol, other animals were euthanized and the distal cauda epididymis was mounted and exposed to 80 mM KCl as described above. After the second addition of KCl and stabilization of tissues, a CCR to tyramine without previous exposure of tissues to NE (Tyr 2) was obtained.

The maximum developed tension (E_{max}, in grams) and the tissue sensitivity to agonists (pD_2; $-logEC_{50}$) were determined by non-linear regression analysis and groups were compared using ANOVA followed by Tukey's test in Prism 6.0 software (GraphPad, San Diego, USA).

2. Results and discussion

After 30 days of treatment, NE from the first CRC (in the absence of desipramine) was 3-fold more potent in sibutramine (S) compared to the control (C) group (C = 5.59 ± 0.13 vs S = 6.20 ± 0.08; $p < 0.05$), however, in the presence of the NET inhibitor desipramine, the agonist showed similar potency between these groups (C = 7.23 ± 0.23 vs S = 6.99 ± 0.11, $p > 0.05$) (Figure 2), suggesting a decrease in NET efficiency.

The E_{max} to tyramine, an amine that displaces norepinephrine from neuronal storage vesicles, was lower in S and the co-exposed (R+S) groups than in C, observed in both Tyr 1 (C = 0.85 ± 0.11 vs S = 0.48 ± 0.04 and R+S = 0.42 ± 0.04; $p < 0.05$ and $p < 0.01$, respectively) and Tyr 2 (C = 0.74 ± 0.05 vs S = 0.39 ± 0.05 and R+S = 0.39 ± 0.11; $p < 0.05$) (Figure 2), indicating that the contractile response to endogenous NE is reduced. Interestingly, it is good to note that this effect of tyramine (Tyr1) was observed after two CRC to NE, that was supposed to restore de NE reservoir, supporting the hypothesis that the 30-days treatment with sibutramine impairs NE reuptake. A scheme of this proposed effect is illustrated in Figure 3.

This result does not exclude the hypothesis of a residual effect of sibutramine from the *in vivo* treatment. In order to avoid this influence, a 24-hours interval between the last dose (day 30) and euthanasia (day 31) in the treatment protocol was designed. In addition, in the organ bath assays, the tissues were washed out at least 8 times before the first CRC to NE, enabling the removal of sibutramine of the preparation that could potentially interfere in the results.

In this study, no statistically significant difference was observed between the rosuvastatin and control groups. The co-exposure (rosuvastatin + sibutramine), when statistically different (Figure 2), implied in an effect of sibutramine.

The treatment with sibutramine for 70 days resulted in a depletion of NE stock in the S group (Figure 2), indicated by the reduction of E_{max} to Tyr 2 (C = 0.73 ± 0.02 vs S = 0.40 ± 0.08, $p < 0.01$). In this experimental protocol, no statistical evidence in decreasing NET efficiency was

detected, although there was a trend toward an increase in the potency of NE in animals treated with sibutramine (C = 5.61 ± 0.12 vs S = 6.14 ± 0.20; unpaired t-test, p = 0.067). A scheme of this proposed effect is illustrated in Figure 4.

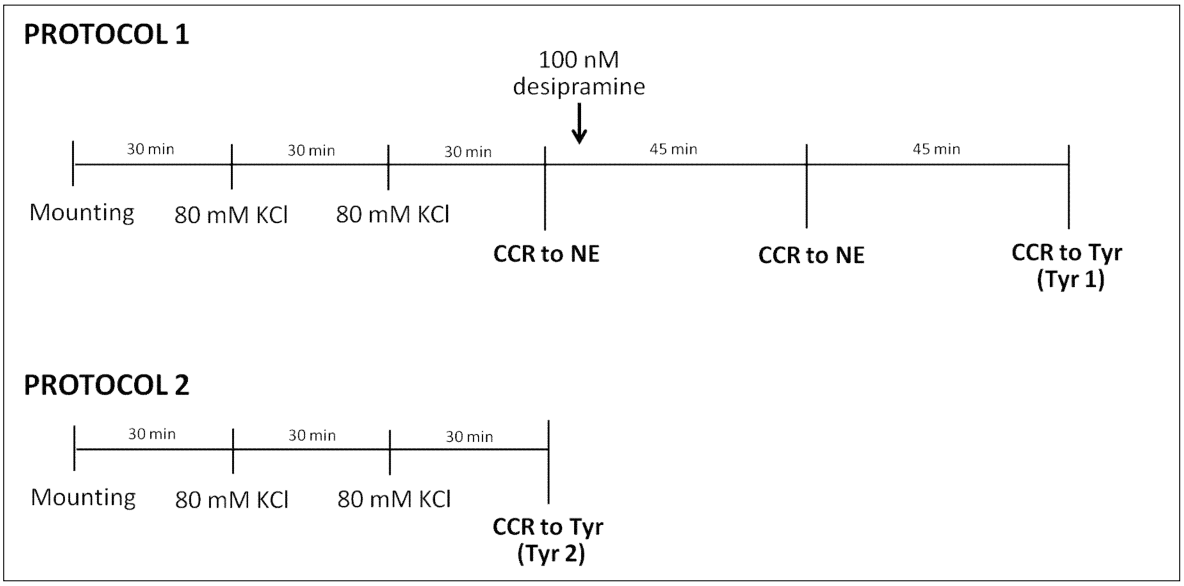

Figure 1. Experimental design. CRC = concentration-response curve; NE = norepinephrine; Tyr = tyramine.

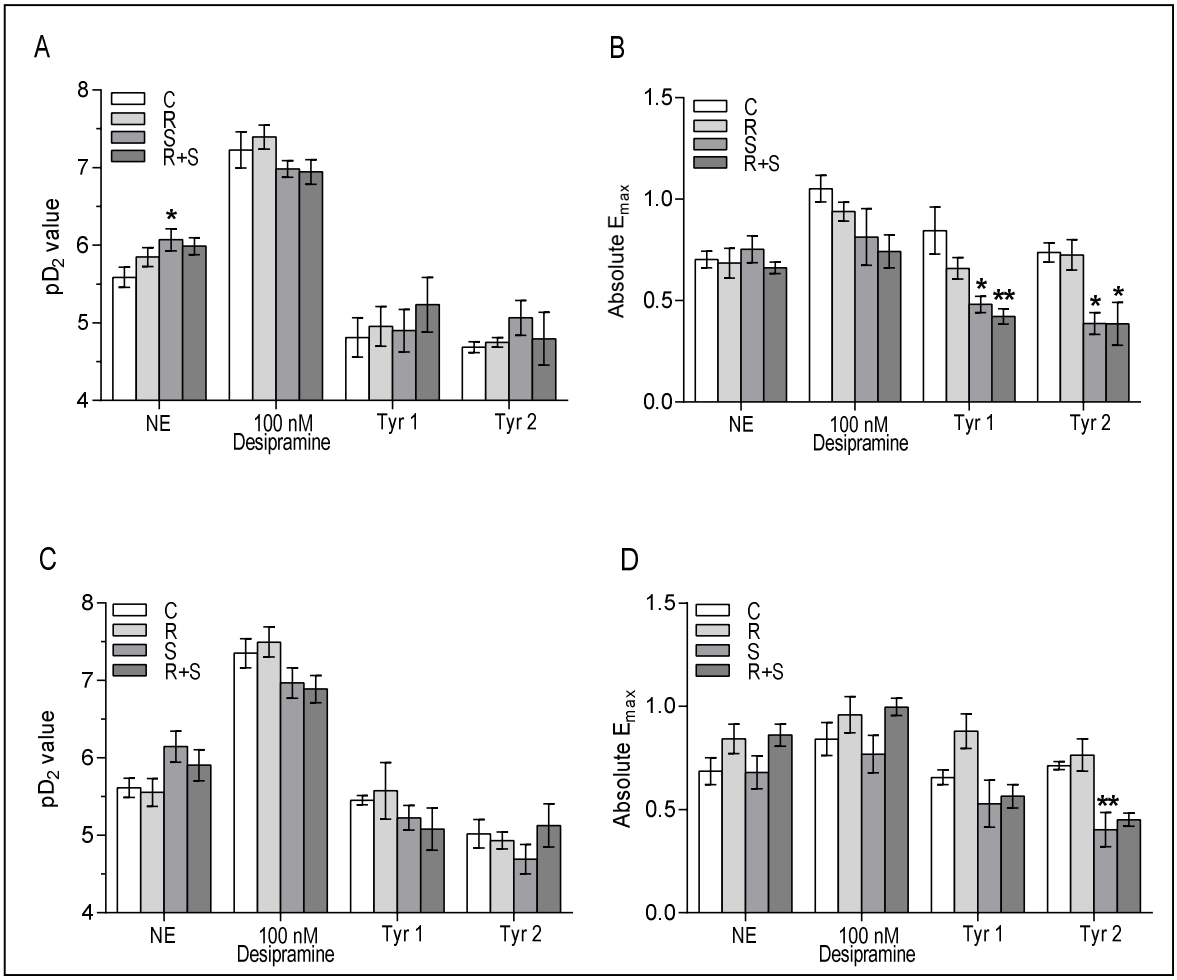

Figure 2. Effect of rosuvastatin and sibutramine (isolated or in association) on pD_2 (A and C) and E_{max} (B and D) of NE and tyramine in cauda epididymis of animals treated for 30 (A and B) and 70 (C and D) days. Concentration-response curve (CRC) to NE (before and after incubation of tissues with desipramine 100 nM) and tyramine (with or without previous CRC to NE; Tyr 1 and 2, respectively). Values are expressed as mean ± SEM. ANOVA followed by Tukey's test. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to the control group.

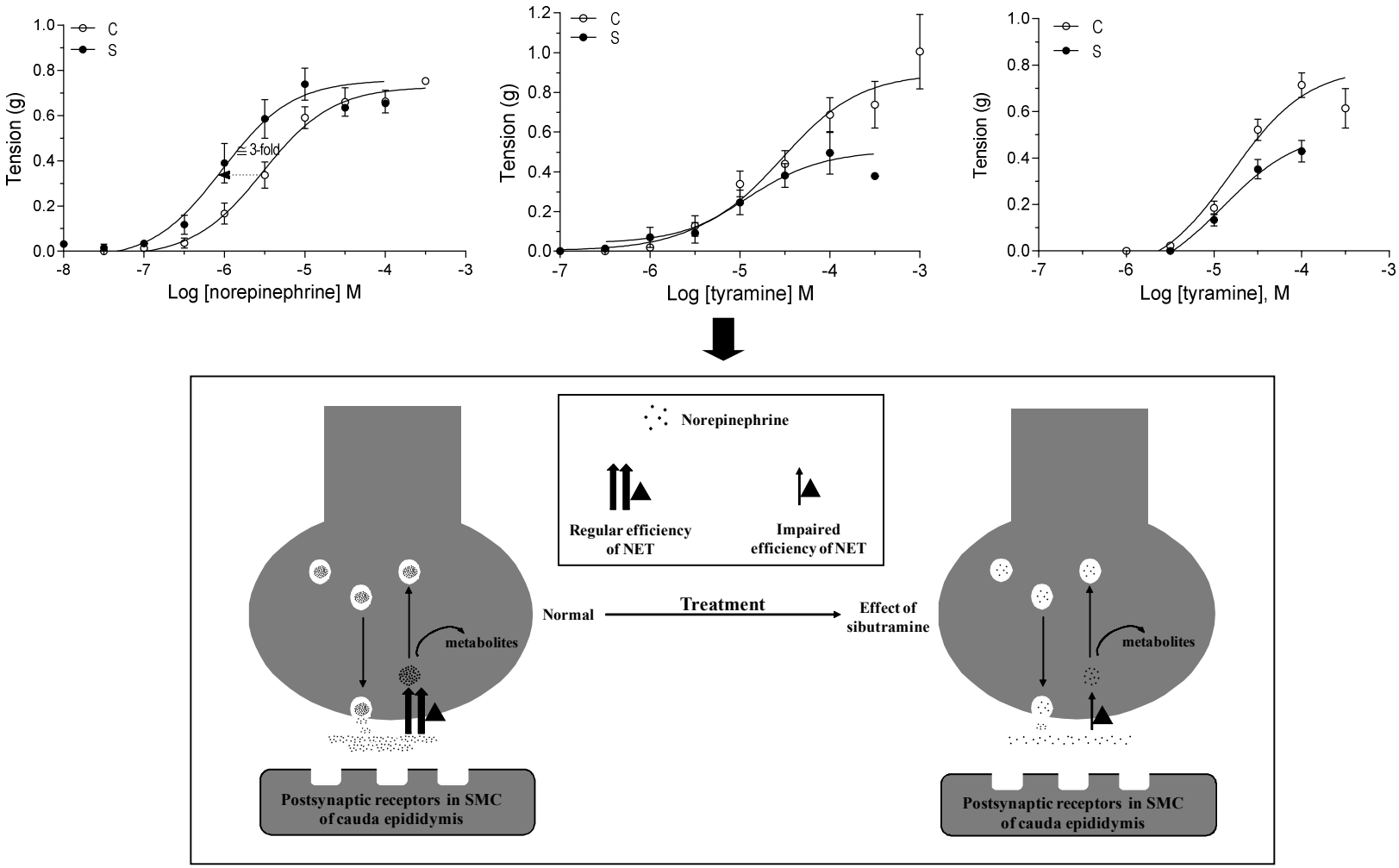

Figure 3. Schematic diagram to depict the proposed effects of sibutramine on the endogenous norepinephrine reservoir and norepinephrine transporter (NET) efficiency after 30 days of treatment. On the left, the scheme represents the regular release of NE from a sympathetic neuron, while the scheme on the right illustrates the effect of sibutramine on NE reservoir and NET efficiency. SMC = smoth muscle cells.

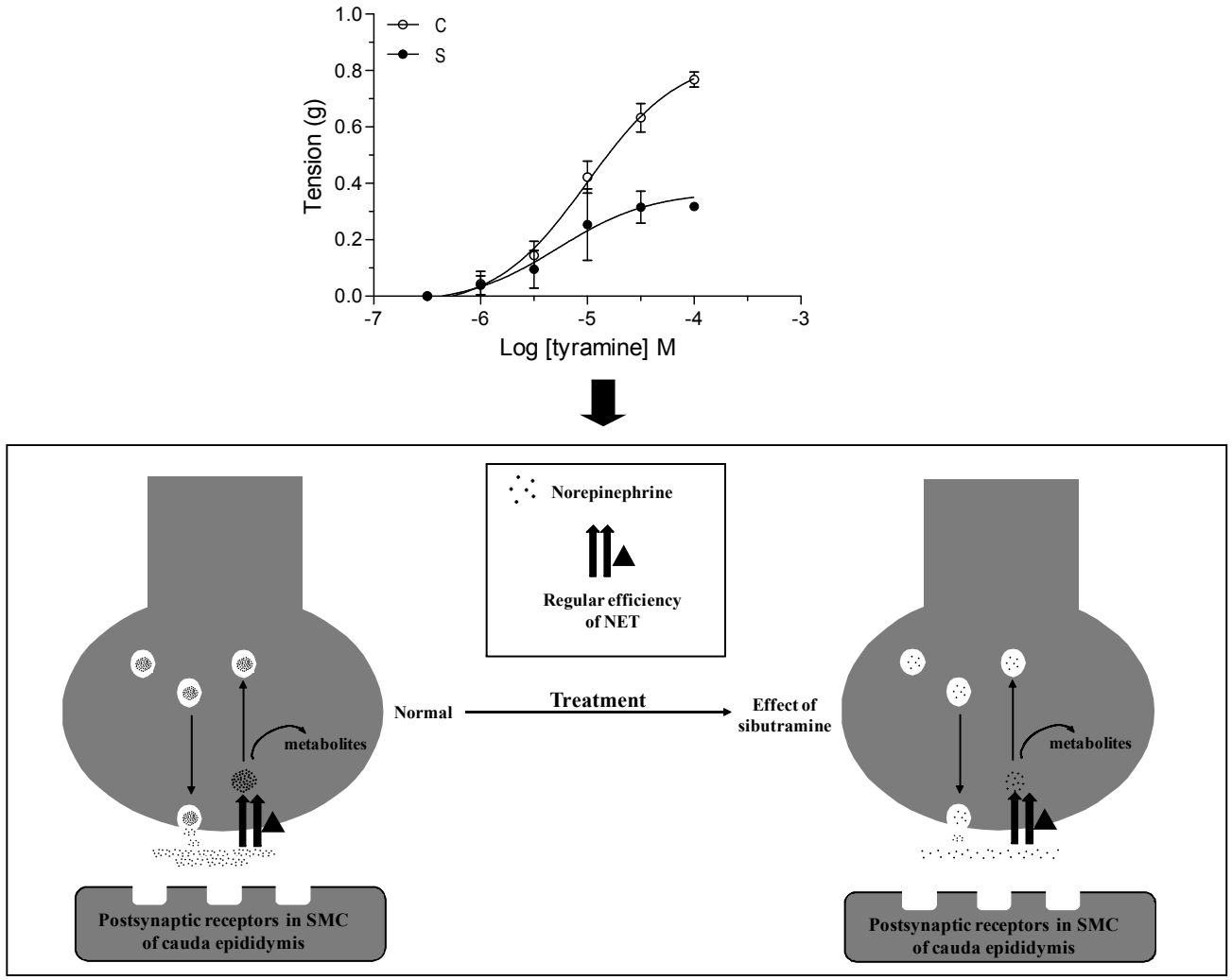

Figure 4. Schematic diagram to depict the proposed effects of sibutramine on the endogenous norepinephrine reservoir and norepinephrine transporter (NET) efficiency after 70 days of treatment. On the left, the scheme represents the regular release of NE from a sympathetic neuron, while the scheme on the right illustrates the effect of sibutramine on NE reservoir with no effect on NET efficiency. SMC = smoth muscle cells.

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Certificado

Certificamos que o Protocolo nº **631-CEUA**, sobre "Efeitos da exposição subcrônica e prolongada à sibutramina e/ou rosuvastatina, sobre parâmetros reprodutivos e fertilidade de ratos machos Wistar adultos", sob a responsabilidade de **Wilma de Grava Kempinas**, está de acordo com a legislação vigente (Lei 11.794/2008 e Decreto 6.899/2009), com as resoluções normativas aplicáveis à luz dos Princípios Éticos na Experimentação Animal elaborados pela Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL/COBEA), e foi aprovado "*Ad referendum*" da ***COMISSÃO DE ÉTICA NO USO DE ANIMAIS*** (CEUA), nesta data.

Botucatu, 14 de julho de 2014.

Prof. Dr. Wellerson Rodrigo Scarano
Presidente da CEUA

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