UNIVERSIDADE ESTADUAL DE CAMPINAS

Instituto de Biologia

JORGE WILLIAN FRANCO DE BARROS

EFEITOS DA EXPOSIÇÃO AO AGENTE HIPOLIPEMIANTE ROSUVASTATINA SOBRE A FUNÇÃO REPRODUTIVA DE RATAS WISTAR

EFFECTS OF THE EXPOSURE TO THE HIPOLIPEMIANT AGENT ROSUVASTATIN ON FEMALE WISTAR RATS REPRODUCTIVE FUNCTION

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Dissertação apresentada ao Instituto de Biologia, da Universidade Estadual de Campinas, como parte dos requisitos exigidos para a obtenção do título de Mestre em Biologia Celular e Estrutural, na Área de Biologia Celular.

Dissertation presented to the Institute of Biology, of the University of Campinas in partial fulfillment of the requirements for the degree of Master in Cellular and Structural Biology, in the area of Cell Biology.

Orientadora: Profa. Dra. Wilma De Grava Kempinas

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Os membros da Comissão Examinadora acima assinaram a Ata de Defesa, que se encontra no processo de vida acadêmica do aluno.

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Provérbio Chinês

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"Quem em cada pouco põe tudo que é, merece ser feliz. E muito."

Tati Bernardi

Resumo

As estatinas correspondem a uma classe de fármacos que atuam de maneira inibitória sobre a enzima HMG-CoA redutase, a qual tem papel crucial na biossíntese do colesterol. Contudo, estudos conduzidos por nosso Laboratório têm mostrado que a exposição à rosuvastatina, uma estatina de última geração, durante a pré-puberdade, está associada com desordens na função reprodutiva de ratos machos. Além disso, dados referentes aos efeitos da exposição à rosuvastatina sobre a esfera reprodutiva feminina são escassos. Assim, o presente trabalho investigou os possíveis efeitos estrogênicos e antiestrogênicos, bem como as alterações reprodutivas resultantes da exposição prolongada de ratas à rosuvastatina. Também foram avaliados os possíveis efeitos que a rosuvastatina pode ter sobre a contratilidade uterina *ex vivo* e *in vitro*. Para os ensaios *in vivo,* ratas Wistar foram alocadas em três diferentes grupos experimentais: controle, tratado com solução salina; tratadas com rosuvastatina nas doses de 3 e 10 mg / Kg / dia. Os tratamentos foram realizados diariamente e por via oral, desde a pré-puberdade, e se encerraram na fase de estro de dois diferentes períodos: após a instalação da puberdade, e na idade adulta. Não foram encontradas alterações quanto à idade de instalação da puberdade, nos níveis hormonais, no peso de órgãos, em parâmetros histológicos ovarianos e uterinos e no desempenho reprodutivo. Por outro lado, a exposição à rosuvastatina está associada com ciclos reprodutivos mais curtos, fêmeas menos receptivas ao acasalamento, além de redução dos pesos e placentário. Para o teste de estrogenicidade *in vivo* realizou-se o teste uterotrófico, com as mesmas doses utilizadas anteriormente, no entanto, nenhum sinal de estrogenicidade ou antiestrogenicidade foi observado. Nos ensaios *ex vivo* (doses de 0, 3 e 10 mg / Kg / dia de rosuvastatina) em *in vitro* (concentrações de 0, 1, 10 e 100 µg / mL de rosuvastatina) com a rosuvastatina sobre a atividade contrátil uterina, realizado também com ratas Wistar, observou-se que este composto é capaz de modular o perfil contrátil do útero tanto em período não-gravídico, quanto em período gravídico. Assim, a exposição à rosuvastatina, nessas condições experimentais, promoveu alguns efeitos deletérios na função reprodutiva e na fisiologia uterina de ratas Wistar, possivelmente por interferência na sinalização hormonal e por efeitos promovidos de forma direta e/ou indireta desta estatina em tecidos reprodutivos desses animais.

Abstract

Statins are a class of drugs that act inhibiting the enzyme HMG-CoA reductase, which plays a crucial role in cholesterol biosynthesis. However, studies conducted by our laboratory have shown that exposure to rosuvastatin, a last generation statin, during pre-puberty, is associated with disorders in the reproductive function of male rats. In addition, data regarding the effects of rosuvastatin exposure on the female reproductive sphere are scarce. Thus, the present study investigated the possible estrogenic and antiestrogenic effects, as well as reproductive alterations resulting from prolonged exposure of female rats to rosuvastatin. The possible *ex vivo* and *in vitro* effects that rosuvastatin have on uterine contractility were also evaluated. For *in vivo* assays, female Wistar rats were allocated into three different experimental groups: control group, treated with saline solution; and two groups treated with rosuvastatin at doses of 3 and 10 mg / kg / day. The treatments were performed daily and orally, since pre-puberty, and ended in the estrus phase of two different periods: after the onset of puberty, and in adulthood. No changes were found regarding the age of puberty onset, reproductive hormone levels, organ weights, ovarian and uterine histological parameters and reproductive performance. On the other hand, exposure to rosuvastatin is associated with shorter reproductive cycles, females less receptive to mating, and reduced hypophysary and placental weights. For the *in vivo* estrogenicity assessment, the uterotrophic assay was performed with the same doses previously used, however, no sign of estrogenicity or antiestrogenicity was observed. In *ex vivo* (rosuvastatin at doses of 0, 3 and 10 mg / kg / day) and *in vitro* (rosuvastatin at concentrations of 0, 1, 10 and 100 µg / mL) assays with rosuvastatin on uterine contractile activity, also performed with Wistar rats, it was observed that this compound is capable to modulate the contractile profile of the uterus in both non-gravid and gravid periods. Thus, exposure to rosuvastatin, in these experimental conditions, promoted some deleterious effects on reproductive function and uterine physiology of Wistar rats, possibly due to interference with hormonal signaling, and directly and / or indirectly effects of this statin on reproductive tissues of these animals.

Sumário

Introdução

Desenvolvimento e fisiologia reprodutiva feminina

Em mamíferos, o desenvolvimento inicial do sistema genital ocorre durante o período intrauterino. Como resultado da fecundação, tem-se a determinação do sexo cromossômico do indivíduo em formação, o qual dá início a uma cascata de eventos que levam à diferenciação sexual. O desenvolvimento e a diferenciação do sistema genital feminino iniciam-se durante a vida intrauterina e seguem até o período pós-natal (Vue et al., 2018). Esse sistema é constituído por um par de ovários, local onde são produzidos e maturados os gametas femininos; por um par de tubas uterinas, pelo útero, pela cérvice e pela vagina (Standring, 2010), conforme esquematizado em humanos e roedores, o modelo experimental mais clássico para estudos em fisiologia da reprodução (Maeda et al., 2000), na figura 1.

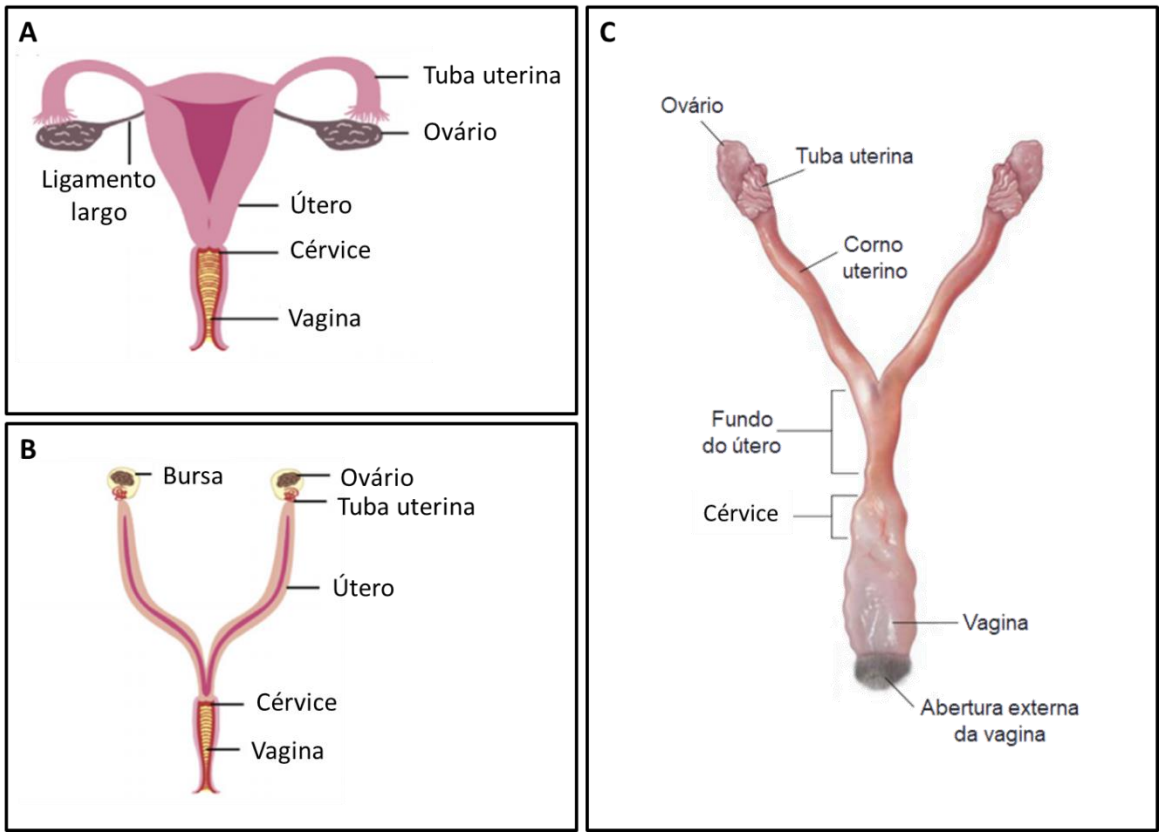

Figura 1. Organização anatômica do sistema genital feminino em humanos (A – vista anterior) e em roedores (B e C – vista ventral), constituído por um par de ovários, um par de tubas uterinas, o útero, a cérvice e a vagina. Adaptado de Vue et al. (2018) e Boyd et al. (2012).

Em humanos, a diferenciação sexual se inicia ao final da quinta semana de desenvolvimento (Schoenwolf et al., 2016), enquanto que em roedores, este processo tem início por volta do nono dia gestacional (Sarraj e Drummond, 2012), com a migração e proliferação de células germinativas primordiais até chegarem à região das futuras gônadas (crista gonadal), as quais formam-se adjacente aos rins mesonéfricos. Ao colonizarem as gônadas em formação, as células germinativas primordiais passam por sucessivas divisões mitóticas e permanecem próximas umas às outras, o que leva à formação de ninhos de células germinativas. Em roedores, por volta do 14º dia gestacional, as divisões mitóticas dessas células se encerram e logo inicia-se o processo de meiose, o qual permanece estacionada na fase de prófase I, e só tem sua retomada após o nascimento, com a puberdade (Figura 2) (Sarraj e Drummond, 2012).

Com as células germinativas também associam-se células somáticas derivadas do epitélio celomático e do mesênquima da crista gonadal, as quais contribuem para a formação do folículos ovarianos. Estas duas populações celulares são responsáveis pela formação das células da granulosa e células da teca, respectivamente (Schoenwolf et al., 2016).

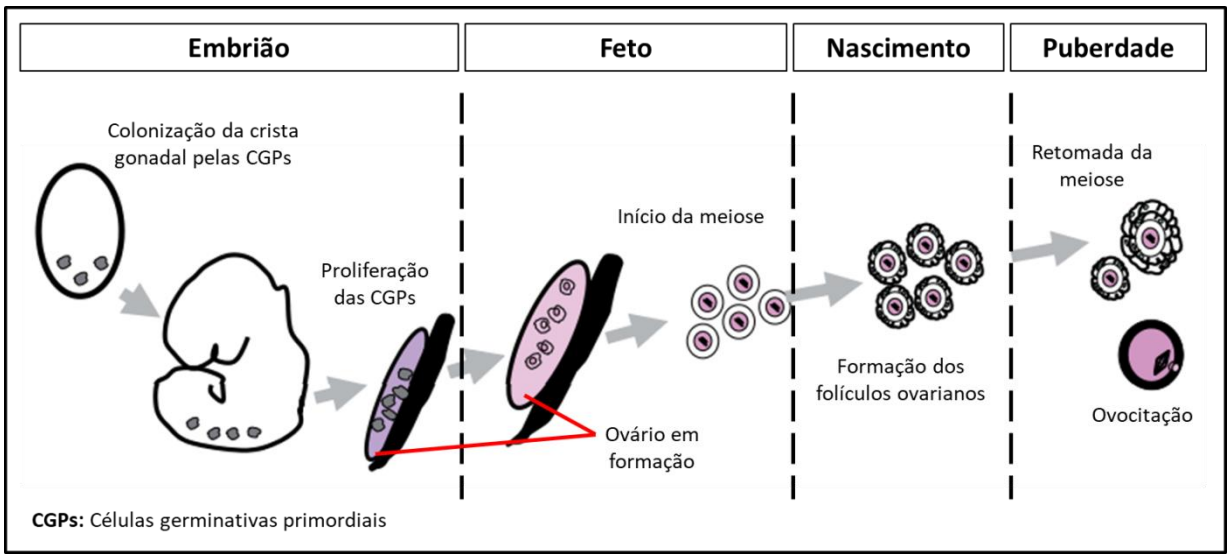

Figura 2. Modelo esquemático da formação dos folículos, no interior dos ovários. Adaptado de Sarraj e Drummond (2012).

Um par de ductos paramesonéfricos (também conhecidos com ductos de Müller) desenvolve-se na altura dos rins mesonéfricos, lateralmente aos ductos mesonéfricos (Schoenwolf et al., 2016; Vue et al., 2018). A partir da diferenciação dos ductos paramesonéfricos ocorre a formação das tubas uterinas, do útero, da cérvice e da parte superior da vagina (Figura 3) (Vue et al., 2018).

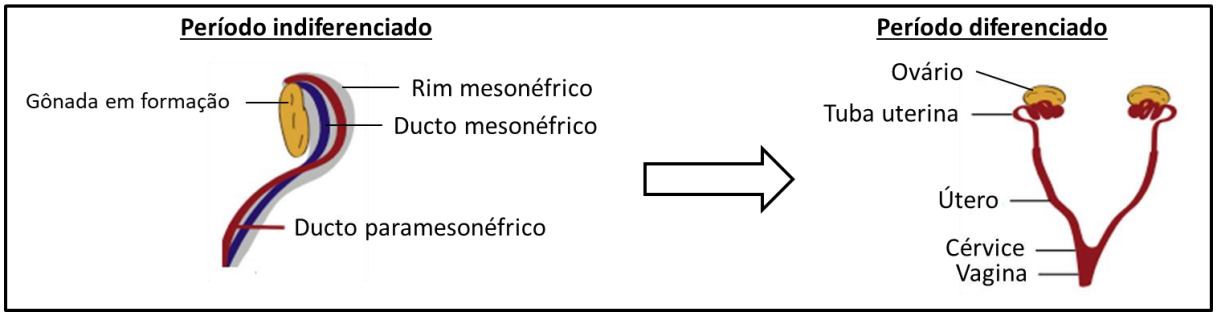

Figura 3. Modelo esquemático da diferenciação do ducto paramesonéfrico, em roedores, nos órgãos constituintes do sistema genital feminino. Adaptado de Vue et al. (2018).

Após o nascimento, o desenvolvimento reprodutivo inicial ocorre independentemente da ação de hormônios sexuais, uma vez que o eixo hipotalâmico-hipofisário-ovariano (HHO) encontra-se quiescente. Nos ovários, o desenvolvimento folicular inicial ocorre sem a necessidade de influências dos hormônios hipofisário gonadotróficos FSH (hormônio folículo-estimulante) e LH (hormônio luteinizante). Desta forma, os folículos primordiais recém-formados, contendo o ovócito primário circundado por células foliculares pavimentosas, chegam ao estágio de folículos primários e, em seguida, de folículos secundários (Picut et al., 2015) por influência de fatores produzidos pelas próprias células foliculares que circundam o ovócito (Ojeda e Skinner, 2006).

O desenvolvimento e a histoarquitetura uterina também se completam no período pós-natal. Na morfogênese uterina estabelecem-se dois compartimentos funcionais, são eles o endométrio e o miométrio, os quais são envoltos pelo perimétrio (Figura 4) (Vue et al., 2018). O endométrio é constituído por duas populações de células epiteliais, sendo as células do epitélio luminal e as células do epitélio glandular. Além disso, também apresenta dois compartimentos estromais, nos quais se encontram vasos sanguíneos e células do sistema imunológico (Boyd et al., 2012; Vue et al., 2018). O miométrio corresponde à camada muscular da parede uterina, a qual é constituída por musculatura lisa organizada em duas subcamadas, no caso de roedores: uma subcamada circular interna e a outra longitudinal externa (Boyd et al., 2012; Vue et al., 2018).

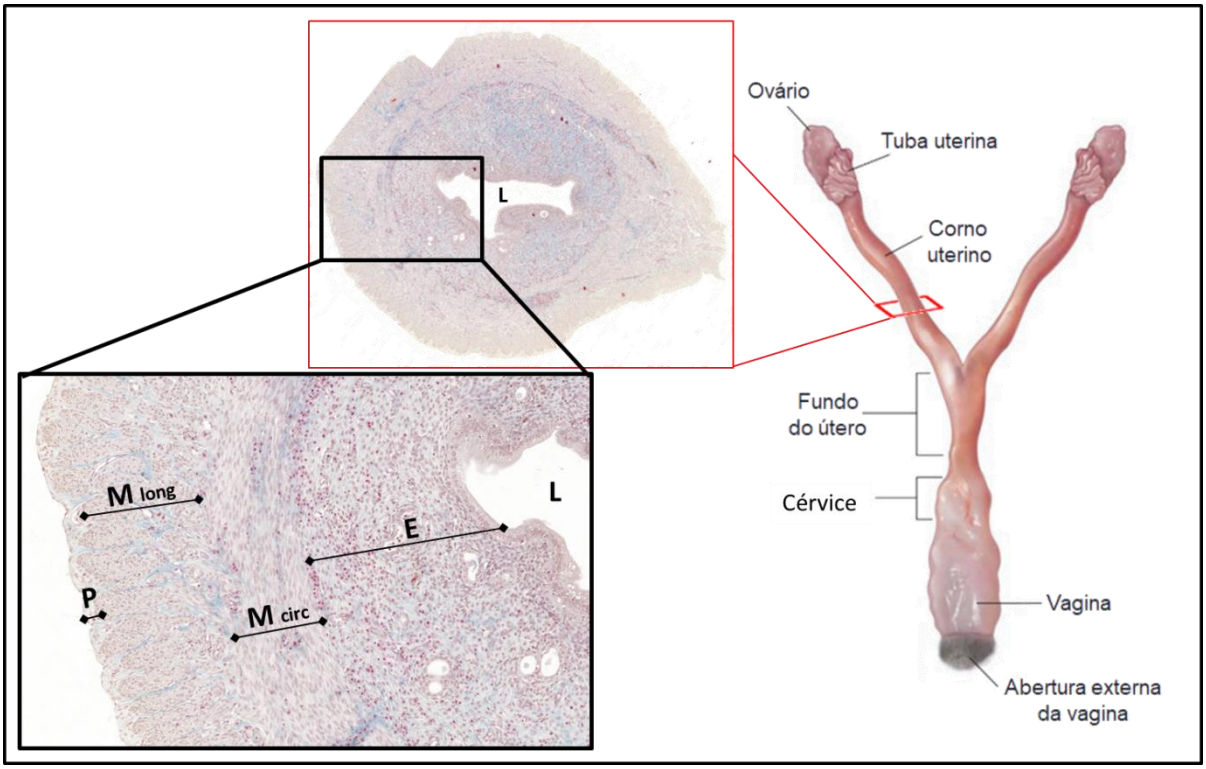

Figura 4. Aspecto histológico do útero de roedor e de suas camadas, em corte transversal. L: Luz; E: Endométrio; M circ: Camada circular do miométrio; M long: Camada longitudinal do miométrio; P: Perimétrio. Modelo esquemático à direita adaptado de Boyd et al. (2012); Representações histológicas produzidas e coradas com eosina e hematoxilina, pelo Laboratório ReproTox.

Durante a pré-puberdade, o eixo HHO começa a exercer sua atividade, com a secreção do hormônio liberador de gonadotrofina (GnRH) pelo hipotálamo, que estimula a adeno-hipófise a liberar FSH e LH. As gônadas gradualmente se tornam mais sensíveis aos estímulos dessas duas gonadotrofinas, e têm um aumento considerável na sua taxa de crescimento, passando a liberar hormônios esteroides na corrente sanguínea, eventos que culminam na instalação da puberdade (Rosenfield et al., 2015).

São estabelecidos cinco diferentes estágios do desenvolvimento pós-natal em roedores, comparadas com o humano, sendo elas a fase neonatal, infantil, juvenil, peri-puberal e púbere (Picut et al., 2014; 2015). As respectivas idades correspondentes entre ratos e humanos, bem como modificações morfológicas observadas nos ovários e no útero de roedores são apresentados na tabela 1.

Tabela 1. Estágios do desenvolvimento pós-natal em roedores.

Estágio	Rato	Humano	Ovário	Útero
Neonatal	DPN 0 – 7	0 a 28 dias	-Apoptose de ovogônias; -Predomínio de folículos primordiais no córtex; -Folículos primários e secundários na medula.	-Quiescente.
Infantil	DPN 8 – 20	1 a 23 meses	-Expansão dos folículos secundários; -Surgimento da zona pelúcida; -Desenvolvimento dos primeiros folículos terciários; -Presença de folículos atípicos;	-Quiescente; -Presença de epitélio glandular cuboide no endométrio.
Juvenil	DPN 21 – 32	2 a 12 anos	-Apoptose de células da granulosa; -Ondas foliculares em atresia na medula; -Crescimento de folículos terciários.	-Quiescente; -Presença de infiltrado leucocitário; -Expansão do epitélio glandular cuboide do endométrio.
Peri-púbere	DPN 33 – 37	??? *	-Folículos pré-ovulatórios já são observados; -Corpos lúteos ausentes; -Folículos atrésicos em número reduzido.	-Poucas figuras mitóticas são vistas no epitélio glandular do endométrio.
Púbere	DPN 38 – 46	12 a 16 anos	-Corpos lúteos recém-formados já presentes;	-Expansão do epitélio glandular cuboide do endométrio. -Poucas figuras mitóticas e de vacuolização são vistas no epitélio glandular do endométrio; -Alterações cíclicas no endométrio e miométrio.

*Estágio ainda não definido para o ser humano. Informações adaptadas de Picut et al. (2014; 2015).

Com a chegada à puberdade, a capacidade reprodutiva é estabelecida, evento que envolve alterações complexas de caráter morfológico, fisiológico, comportamental e psicológico no indivíduo do sexo feminino, com destaque ao desenvolvimento ovariano e ao desenvolvimento de características relacionadas à maturidade sexual (Castellano et al., 2018). Em roedores, a chegada à puberdade é observada por volta dos 30-40 dias de idade nas fêmeas (Maeda et al., 2000), por meio de sinais físicos externos, como a completa canalização da vagina, conhecida como abertura vaginal; e a observação de células epiteliais queratinizadas nessa região, caracterizando-se o primeiro estro e a primeira ovocitação (Ojeda e Skinner, 2006; Castellano et al., 2018).

A partir da puberdade ocorre o início da capacidade reprodutiva, com a geração de descendentes, que pode ocorrer múltiplas vezes ao longo da vida de um indivíduo, e encerra-se na senescência reprodutiva, período conhecido como menopausa, em fêmeas (Christian, 2007). A capacidade reprodutiva em mamíferos ocorre de forma cíclica, por meio de ciclos menstruais, no caso dos primatas, ou ciclos estrais em roedores e nos demais mamíferos eutérios não primatas (Boyd et al., 2012).

A ciclicidade reprodutiva envolve uma sequência de eventos que ocorrem nos ovários, no útero e sistemicamente nas fêmeas, guiadas pelas variações hormonais a cada ciclo. Os eventos cíclicos que ocorrem nos ovários estão relacionados com a maturação ovocitária, a foliculogênese, a ovocitação e a formação do corpo lúteo (Christian, 2007). Já os eventos que ocorrem no útero ciclicamente envolvem principalmente o desenvolvimento e a regressão do endométrio e de suas glândulas (Boyd et al., 2012).

Ainda com relação às alterações que ocorrem a cada ciclo reprodutivo, o crescimento do útero e a atividade contrátil promovida pelo miométrio estão sob constante controle hormonal. Tal atividade é de grande relevância para a fertilidade feminina, uma vez que ela está associada com a condução e a promoção do encontro dos gametas, a condução do pré-embrião até o local de implantação, a manutenção do desenvolvimento da prole, e a expulsão do concepto durante o parto (Spencer at al., 2005; Abbas et al., 2019).

Colesterol e suas implicações

Trabalhos têm associado os hábitos de vida, a administração de diferentes classes de medicamentos e o contato com moléculas presentes no ambiente com prejuízos à capacidade

reprodutiva feminina, por meio de estudos em diferentes modelos experimentais. Por exemplo, estudos que relacionam a obesidade com a função reprodutiva indicam que esta doença está associada com disfunções ovulatórias, aumento na incidência de perdas gestacionais, e maior ocorrência de morbidades gestacionais, como a pré-eclâmpsia, diabetes gestacional e parto prematuro (Legro, 2017).

Nas últimas décadas, uma molécula orgânica de natureza lipídica vem ganhando atenção de diversos pesquisadores e centros de pesquisa: o colesterol. Com exceção à membrana mitocondrial interna, esta molécula de caráter anfipático pode ser encontrada em todas as membranas biológicas das células animais, e é responsável por reduzir a fluidez desta estrutura celular. Além de sua função estrutural nas membranas, o colesterol também é precursor de sais biliares, da vitamina D e hormônios esteroides, como aqueles produzidos pela glândula adrenal e pelas gônadas (Carvalho e Recco-Pimentel, 2013; Wulp et al., 2013).

A biossíntese do colesterol ocorre a partir de seu precursor, a molécula de acetil-CoA. Durante sua complexa via metabólica, dezoito moléculas de acetil-CoA são utilizadas para constituir uma molécula de colesterol (Wulp et al., 2013). Uma das enzimas de grande importância neste processo biossintético é a 3-hidroxi-3-metilglutaril-CoA redutase (HMG-CoA redutase). Esta enzima atua em uma etapa muito precoce e limitante da síntese do colesterol, na qual o HMG-CoA é convertido em mevalonato (Wulp et al., 2013; DeLucia et al., 2014).

De modo geral, o colesterol pode ser sintetizado pelo nosso organismo ou ingerido por meio do consumo de alimentos de origem animal (Morzycki, 2014). No organismo, o colesterol é transportado pelo sangue na forma de lipoproteínas, e estas apresentam variações quanto a sua densidade e composição (Wulp et al., 2013). Dentre as classes de lipoproteínas, as que apresentam menor densidade, como as partículas de LDL-colesterol (Low-density lipoprotein) são transportadas para as paredes dos vasos sanguíneos, onde são oxidadas pelas células endoteliais e macrófagos (Rang et al., 2008). Por meio de diversas reações de oxidação, ocorre a liberação de subprodutos de natureza tóxica, os quais têm sido associados ao estabelecimento e progressão de doenças crônicas, como aterosclerose e desordens cardiovasculares (Morzycki, 2014; McEvoy et al., 2017), processos neurodegenerativos, diabetes e falência renal (Morzycki, 2014).

A elevação do colesterol no organismo dos indivíduos, uma condição enquadrada dentro das dislipidemias ou hiperlipidemias (DeLucia et al., 2014), tem sido associada aos

hábitos alimentares precários e ao sedentarismo, problema que tem se estabelecido cada vez mais cedo na população (Ross, 2016). Estudos em 2008 mostram que das 17,3 milhões de mortes relacionadas com problemas cardiovasculares notificadas por todo o mundo, 15% delas foram causadas por hipercolesterolemia (McEvoy et al., 2017). Além disso, estima-se que uma fração considerável das crianças e adolescentes com idade entre 8 e 17 anos, principalmente meninas, apresentem as concentrações lipídicas no organismo elevadas (Colesterol total ≥ 200mg/dL) (Kit et al., 2015; Ross, 2016).

Estratégias farmacológicas são empregadas com o objetivo de controlar ou reduzir os níveis de colesterol de indivíduos com hipercolesterolemia, como é o caso dos fibratos, dos inibidores da absorção de colesterol, do ácido nicotínico e seus derivados, de derivados de óleos de peixes, e das estatinas (Rang et al., 2008; DeLucia et al., 2014).

Estatinas: Aspectos gerais

As estatinas, fármacos de maior destaque no controle dos níveis de colesterol (Patel e Kothari, 2016), atuam de maneira inibitória sobre a enzima HMG-CoA redutase, e acabam por impedir a biossíntese desta molécula no organismo (Figura 5) (Rang et al., 2008; DeLucia et al., 2014). Estes fármacos, autorizados para comercialização, podem ser classificados em dois diferentes grupos: as estatinas derivadas de fermentação, como a sinvastatina e a pravastatina, e as estatinas sintéticas, como a atorvastatina, a fluvastatina e a rosuvastatina (Grover et al., 2014). Destas, a estatina de menor potência é a fluvastatina, ao passo que, as de maior potência são a atorvastatina e a rosuvastatina (Golan et al., 2009).

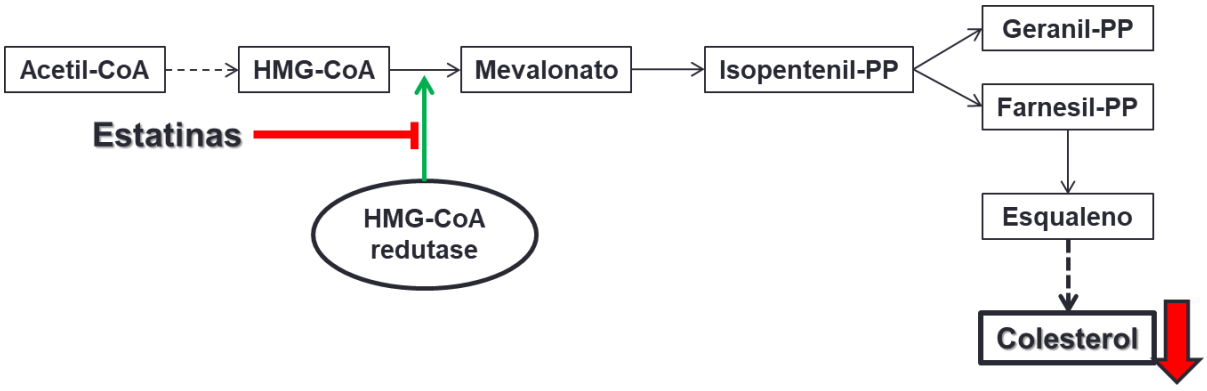

Figura 5. Mecanismo de ação das estatinas, de forma inibitória sobre a enzima HMG-CoA redutase, de modo a reduzir biossíntese do colesterol.

Os efeitos das estatinas não têm se limitado apenas à prevenção do risco de doenças cardiovasculares pela redução das concentrações de colesterol no organismo, como se acreditava (Girardi, 2014). Parte dos efeitos benéficos associados à administração desta classe de fármacos tem sido atribuída aos seus efeitos pleiotrópicos, os quais independem da inibição da biossíntese do colesterol, como a proteção endotelial, as propriedades antioxidante e anti-inflamatória, a redução na resposta trombogênica, os efeitos pró-angiogênicos (Girardi, 2014; Rohilla et al., 2016) e imunomoduladores (Ferri e Corsini, 2014).

Por conta de seus efeitos pleiotrópicos, as estatinas passaram a ser consideradas efetivas no tratamento de outras condições patológicas, não necessariamente relacionadas ao perfil lipídico do paciente, tais como artrite reumatoide, tromboembolismo venoso, doenças hepáticas e síndrome do ovário policístico (Ferri e Corsini, 2014).

Rosuvastatina

Dentre as estatinas, a rosuvastatina (Figura 4), é uma das mais recentes da classe (Olsson et al., 2002), comercializada nas doses de 5mg, 10mg, 20mg e 40mg para administração oral, e tem mostrado melhores resultados na redução das concentrações de LDL-colesterol em comparação a outras estatinas, como a atorvastatina ou a sinvastatina, as quais necessitam de doses maiores para se obter resultados semelhantes (Cortese et al., 2016).

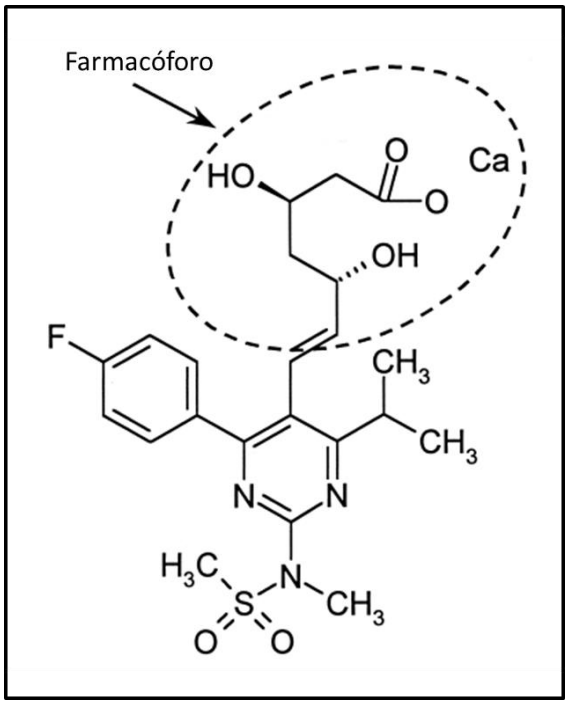

Figura 6. Estrutura química da rosuvastatina, na qual evidencia-se o farmacóforo que confere a atividade biológica mais conhecida das estatinas. Adaptado de McTaggart et al. (2001).

Assim como as outras estatinas, a rosuvastatina se liga à enzima HMG-CoA redutase por meio de seu grupo funcional (farmacóforo) constituído por um ácido heptanoico (McTaggart et al., 2001; Cortese et al., 2016). Desta forma, por competição pelo sítio catalítico da enzima, a rosuvastatina e as outras estatinas impedem que a HMG-CoA seja convertida em mevalonato e, consequentemente, impede a biossíntese do colesterol (Holdgate et al., 2003).

Efeitos das estatinas sobre a função reprodutiva

Com relação aos efeitos das estatinas sobre a função reprodutiva, estudos têm mostrado diferentes resultados sobre a sua utilização. Em machos, a partir de estudos conduzidos por nosso Laboratório, a exposição de ratos à rosuvastatin, nas doses de 3 e 10 mg / Kg / dia, desde a pré-puberdade, está associada a consequências para a saúde reprodutiva, como atraso da instalação da puberdade, prejuízos à qualidade espermática, redução nas concentrações androgênicas e danos ao DNA espermático (Leite et al., 2014; 2017a,b). Além disso, o prejuízo na função reprodutiva de ratos expostos à rosuvastatina durante a peri-puberdade se evidencia ainda mais pela sinalização hormonal alterada, avaliada pelo padrão de imunomarcação de receptores androgênicos e estrogênicos, e aumento em marcadores de estresse oxidativo e de células germinativas mortas nos testículos destes animais (Leite et al., 2018a).

Nosso grupo de pesquisa também investigou os efeitos da exposição à rosuvastatina em ratos durante a idade adulta, e o tratamento diário com 5 mg / kg dessa estatina é capaz de afetar a frequência ejaculatória, bem como a morfologia epididimária sem afetar os níveis de testosterona dos animais (Silva et al., 2020).

Em estudo *in vitro*, associando-se estatinas em cultura de células de Leydig de ratos, nota-se a inibição da síntese de testosterona (Klinefelter et al., 2014). Um trabalho clínico, com pacientes que faziam o uso de atorvastatina, mostrou relação entre o uso da estatina com a queda nas concentrações séricas de deidroepiandrosterona, um esteroide androgênico precursor da testosterona e de estrógenos (Dogru et al., 2008). Entretanto, neste mesmo estudo, parte dos pacientes que inicialmente apresentavam problemas relacionados à disfunção erétil e baixa libido, posteriormente apresentaram melhora nestes parâmetros (Dogru et al., 2008).

Dados mais recentes publicados por nosso Laboratório indicam que a exposição à rosuvastatina em ratos durante a peri-puberdade tem potencial para alterar parâmetros reprodutivos de forma transgeracional, já que a prole masculina desses ratos apresenta aumento na fragmentação de DNA espermático, depleção androgênica, alterações estruturais nos testículos e epidídimos, além de prejuízos à qualidade espermática (Leite et al., 2018b). A prole feminina, por sua vez, também mostra alterações na função reprodutiva, evidenciada pela diminuição da contagem de corpos lúteos nos ovários, além de alterações histomorfométricas no epitélio luminal uterino e desregulação dos níveis de LH durante a puberdade (Leite et al., 2018c).

Já os estudos que abordam a relação direta entre o uso de estatinas e a reprodução feminina mostram que a exposição *in vitro* de células da teca ovariana de ratas à mevastatina, é associada à inibição da proliferação destas células, além da redução na síntese de testosterona e progesterona por elas (Izquierdo et al., 2004). Outro estudo, conduzido por Guldvang e colaboradores (2015), mostrou que tanto a sinvastatina, uma pró-droga, quanto seu metabólito ativo, apresenta potencial para impactar o eixo hipotalâmico-hipofisário-gonadal de fêmeas, de modo a reduzir as concentrações de hormônio folículo estimulante (FSH) e progesterona, dois hormônios de suma importância para a reprodução feminina.

Embora estudos clínicos sobre a associação do uso de estatinas e a função gonadal e reprodutiva de mulheres adultas não indicarem disfunções relevantes derivadas dessa terapia (Ali et al., 2014), o uso de estatinas, até o momento, não é recomendado para mulheres que estejam tentando engravidar, gestantes e lactantes (ANVISA, 2010; Zarek et al., 2013; Karalis et al., 2016), sendo medicamentos considerados pela Food and Drug Administration (FDA) como de classe X, cujos riscos de malefícios para o desenvolvimento da prole aparentam ser maiores do que seu potencial benefício (Zarek et al., 2013). No caso da rosuvastatina, não existem estudos de associação entre o uso dessa estatina durante a gestação e prejuízos ao desenvolvimento embrionário/fetal em humanos. Porém, conforme informativo do FDA, em modelos experimentais de roedores, a exposição a doses elevadas de rosuvastatina durante a prenhez (10 vezes maior que as doses humanas), pode levar à redução nas taxas de sobrevida e no peso dos filhotes, em especial das fêmeas, além de atrasar o processo de ossificação (FDA, 2010).

Mesmo com os relatos anteriores de efeitos adversos das estatinas sobre a função reprodutiva em diferentes modelos e delineamentos experimentais, essa classe farmacológica

ainda vem sendo explorada como um mecanismo adjuvante, melhorando parâmetros reprodutivos e fisiológicos em diferentes condições patológicas. Em ratos machos induzidos à diabetes, o tratamento com rosuvastatina foi capaz de melhorar danos reprodutivos trazidos por essa disfunção, atuando de forma anti-inflamatória, antioxidante e anti-apoptótica nos testículos (Heeba e Hamza, 2015). Outro exemplo, em ratas prenhes induzidas à hipertensão gestacional, de modo a mimetizar a pré-eclâmpsia, o tratamento com pravastatina foi responsável por prevenir a elevação da pressão sanguínea e o desequilíbrio de fatores angiogênicos placentários, além de impedir a restrição de crescimento fetal, problemas decorrentes da pré-eclâmpsia (Chimini et al., 2018).

É importante ressaltar que estudos sobre os efeitos da exposição à rosuvastatina e as consequências, sejam elas positivas ou negativas, sobre o sistema genital feminino permanecem escassos. Além disso, a partir do fato de que esses fármacos têm sido utilizados cada vez mais cedo pela população por conta dos hábitos sedentários e maus hábitos alimentares, de modo a reduzir as concentrações de colesterol totais (Kit et al., 2015; Ross, 2016), temos que levar em consideração que a faixa etária infanto-juvenil trata-se de um período crítico do desenvolvimento pós-natal, com o estabelecimento da puberdade (Sultan et al., 2017). Essa analogia se torna ainda mais preocupante, uma vez que a puberdade é influenciada por fatores neuroendócrinos, como os hormônios esteroides, dos quais destacam-se a testosterona, o estrógeno e a progesterona, derivados do colesterol.

Desta forma, existe a necessidade de se compreender os possíveis efeitos que a rosuvastatina pode trazer para o desenvolvimento reprodutivo e para a fertilidade feminina, para que, a partir de um modelo experimental, possam ser extraídos os possíveis efeitos translacionais e suas implicações para a saúde reprodutiva do ser humano.

Justificativa

Este trabalho justifica-se pela crescente aplicação da rosuvastatina em indivíduos cada vez mais jovens e em diferentes condições patológicas não ligadas necessariamente ao perfil lipídico alterado, utilizando-se de seus efeitos pleiotrópicos, cujos mecanismos de ação permanecem pouco conhecidos e pela escassez de estudos que abordem os efeitos dessa estatina sobre a reprodução feminina.

Além disso, com base em estudos anteriores, os quais indicam prejuízos à função reprodutiva após a exposição a estatinas em diferentes modelos experimentais, têm-se a necessidade de se investigar os riscos que a rosuvastatina pode trazer à reprodução feminina, uma vez que todo o desenvolvimento e a fisiologia reprodutiva feminina são orquestrados por eventos e fatores altamente complexos, nos quais incluem-se os hormônios esteroides, derivados da molécula de colesterol, cuja biossíntese é inibida por ação das estatinas.

Objetivos

Objetivo Geral

O objetivo deste trabalho foi investigar os possíveis efeitos da exposição prolongada ao agente hipolipemiante rosuvastatina, desde a pré-puberdade até a idade adulta, sobre parâmetros reprodutivos de ratas Wistar, bem como avaliar os possíveis efeitos de desregulação endócrina e de ação da rosuvastatina sobre a motilidade uterina.

Objetivos Específicos

➔ Avaliar se a rosuvastatina pode promover alterações nos pesos corporais e de órgãos reprodutivos, no eixo hipotalâmico-hipofisário-ovariano, na histofisiologia de ovários e útero, na ciclicidade reprodutiva, no comportamento sexual e no desempenho reprodutivo de ratas Wistar.

➔ Investigar se os efeitos da rosuvastatina podem ser mediados por interações com vias estrogênicas ou antiestrogências.

➔ Avaliar os efeitos da exposição *in vivo* e *in vitro* da rosuvastatina sobre a atividade contrátil uterina, tanto em período não-gravídico quanto em período gravídico.

Capítulo 1

O trabalho desenvolvido com avalições de perfil contrátil uterino e estrogenicidade/antiestrogenicidade associada à exposição à rosuvastatina, deu origem ao manuscrito "*Alterations in the uterine contractility profile and in vivo assessment of (anti)estrogenic effects mediated by rosuvastatin in Wistar rats*", a ser submetido para publicação no periódico *Toxicology and Applied Pharmacology* (Fator de impacto: 3,585).

Alterations in the uterine contractility profile and *in vivo* assessment of (anti)estrogenic effects mediated by rosuvastatin in Wistar rats

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Abstract

Statins are HMG-CoA reductase inhibitor drugs that lead to serum cholesterol lowering effects. Rosuvastatin, a last generation statin, shows better results in reducing cholesterol concentrations when compared to other highly prescribed statins. Recent studies of our group reported that rosuvastatin impairs reproductive function in rats possibly by disrupting reproductive endocrine axis. In this study, we evaluated whether rosuvastatin presents estrogenic or antiestrogenic effects, by *in vivo* uterotrophic assay in rats, and we investigated the direct effect of this drug upon rat uterine tissue contractility *ex vivo* and *in vitro* both in non-gravid and gravid periods. Rosuvastatin exposure *in vivo* exposure at doses of 0 (control), 3 and 10 mg / kg / day was not associated with estrogenic and antiestrogenic effects on uterine tissue. However, *in vivo* (doses of 0, 3 and 10 mg / kg / day) and *in vitro* (concentrations of 0, 1, 10 and 100 µg / mL) exposures to this drug is related to alterations in uterine basal contraction pattern. Also, *in vivo* and *in vitro* rosuvastatin exposures potentially modulate the action of uterine contraction inducer agents. Thus, rosuvastatin can affect uterine physiology not necessarily by an endocrine mechanism related to the estrogen signaling, but possibly by direct and/or indirect tissue interactions, requiring further studies upon the precise mechanism of action of this drug in female reproductive function.

Keywords: Statin; Uterine Activity; Estrogenicity; Reproductive Toxicology.

1. Introduction

Statins correspond to a class of drugs in which the main mechanism of action is the competitive inhibition of the enzyme 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase and consequently inhibit cellular cholesterol biosynthesis (Schachter, 2005). Thus, statins are the drugs of choice for treatment of hypercholesterolemia (McTaggart et al., 2001; Schachter, 2005). Additionally, the use of this class of drugs is rising on the treatment of many pathological conditions because of the beneficial effects promoted by statins, through secondary pathways other than lipid-modifying action already known (Cortese et al., 2016; Ferri and Corsini, 2014). These pleiotropic effects are associated with endothelial protection, anti-inflammatory and antioxidant effects, and prevention or treatment of several cardiovascular and metabolic diseases (Cortese et al., 2016).

Rosuvastatin is a member of the statin class and has shown a higher inhibitory potential on HMG-CoA reductase when compared to other well-known statins, such as simvastatin and atorvastatin (McTaggart et al., 2001). This compound presents hydrophilic property and its effects as a HMG-CoA reductase inhibitor is selectively exerted on hepatocytes (Olsson et al., 2002). Given its properties, rosuvastatin is thought to be limited to penetrate extrahepatic tissues and consequently with low risks to cause adverse effects (Calza, 2009).

Recently, studies conducted by our group, with rosuvastatin exposure during peri-pubertal period in male rats, revealed that this drug leads to reproductive impairments in adulthood. Rosuvastatin, at doses of 3 and 10 mg / kg, is able to delay pubertal development, reduce sperm quality, impair spermatogenesis and alter hormonal signaling (Leite et al., 2019, 2018b, 2014). Also, peri-pubertal rosuvastatin exposure in male rats might affect reproductive function in an intergeneration manner, by damaging ovarian and uterine histophysiology of female offspring (Leite et al., 2018a).

Our group additionally investigated the effects of rosuvastatin exposure in rats during adulthood, and found that chronicle daily treatment with 5 mg / kg of this statin is able to affects male sexual behavior and epididymis morphology without affecting testosterone levels (E Silva et al., 2020).

Almost half of the population submitted to statin therapy is composed by women (Lewey et al., 2013), nevertheless studies regarding the effects of rosuvastatin and other statins on the female reproductive function remains inconclusive. While *in vivo* and *in vitro* studies show that statins affect sex hormones synthesis and signaling (Guldvang et al., 2015; Izquierdo et al., 2004; Klinefelter et al., 2014), clinical studies did not find evidences that support the negative impact of statins therapy on reproductive parameters of female patients (Ali et al., 2015; Lavie et al., 2013). Furthermore, the use of statins during pregnancy is still contraindicated once the possible gestational and developmental complications promoted by these drugs are poorly known (Karalis et al., 2016).

The uterus is an essential organ for female fertility and its development and function is under hormonal control, especially of estrogens (Condon et al., 2020; Spencer et al., 2005). It is known that exposure to endocrine disruptors at different stages of life are associated with abnormal function of uterus and might lead to infertility and/or reduced reproductive performance (Guerra et al., 2013; Spencer et al., 2005).

Together, these data raise the doubt whether rosuvastatin might act as an endocrine disruptor or act directly in different tissues, potentially impairing reproductive function. Thus, the present study aimed to investigate the potential effects of rosuvastatin as an estrogenic and/or antiestrogenic agent. Also, we evaluated the effects of *in vivo* and *in vitro* exposure of uterine tissue to rosuvastatin and its effects upon the myometrial contractility both in non-gravid and gravid rats.

2. Material and Methods

2.1. Animals

Adult male and female Wistar rats (80 days old) were obtained from the Central Biotherium, São Paulo State University (UNESP), *campus* Botucatu/SP, Brazil, and maintained under controlled conditions (12h of light/12h of darkness; average temperature of 23 °C) on the Small Mammal Biotherium of Morphology Department, at the UNESP, Institute of Biosciences of Botucatu, with food and water *ad libitum*. The animals were kept according to the Ethical Principles for Animal Experimentation, adopted by the Brazilian College of Animal Experimentation. The project was filed under protocol number 1089 with the Ethics Committee on Animal Experimentation of the UNESP Institute of Biosciences, in Botucatu.

The mating of these animals in order to obtain pregnant females and the pups was performed during the dark period of the light/dark cycle, with 2 females being placed in the male cage. The gestational day (GD) 0 was determined by the presence of spermatozoa in vaginal smears of females in estrus. Pregnant females were then kept in individual cages. After birth, the number of pups per litter was reduced to 8, balancing 3 male and 5 female pups at postnatal day (PND) 1.

2.2. Uterotrophic assay: *In vivo* evaluation of (anti)estrogenic potential of rosuvastatin

At weaning, on PND 21, female pups were randomly distributed among six experimental groups (one pup per litter for each group; n = 7/group), keeping the same body weight mean for each group. Primarily, experimental groups were treated with saline (Control); or rosuvastatin (purchased at a commercial pharmacy, Farmácia Cruz Vermelha, Botucatu/SP, Brazil) at two different doses: 3 or 10 mg / Kg / day diluted in saline. Additionaly, the experimental groups received the treatment associated or not with 0.4mg /

Kg / day of estradiol benzoate (β-estradiol 3-benzoate, Sigma, St. Louis, Missouri, USA), diluted in corn oil. Groups that did not receive estradiol benzoate were treated with corn oil. The treatment was performed daily and by oral administration (gavage) from PND 21 to PND 23.

On PND 24, female rats were weighted and then euthanized by decapitation following narcosis in CO_2. Weight of uterus with fluid was recorded, and then this organ was punctured by a thin needle to withdraw the fluid and weighted again. Ovaries and liver were also collected and weighted. Organ weights were divided by the body weight and multiplied by 100, in order to determine the relative organ weights (%).

The doses of rosuvastatin chosen for this study are based on the lowest and higher doses applied in human therapy for children (Leite et al., 2019, 2018b), adapted for rodents considering their body surface area, as proposed by Reagan-Shaw et al. (2008). The estradiol dose was chosen based on Andrade et al. (2002) and Guerra et al. (2016), which corresponds to a dose sufficient to promote uterine growth in rats.

2.3. *Ex vivo* pharmacological reactivity of uterine fragments

Soon after the weaning, on PND 21, female pups were randomly distributed among three experimental groups (n = 10/group): control group, which received treatment with saline; and rosuvastatin groups, which received the statin at doses of 3 or 10 mg / Kg / day diluted in saline. The treatment was performed daily and by oral administration since PND 22, and finished on the first estrus after PND 75, at adulthood.

Half of the animals of each group was euthanized during the estrus phase, and the uterus was collected for the pharmacological reactivity assay of non-gravid uterus. The other animals were mated with non-treated male rats during the dark period of the light/dark cycle, in order to obtain pregnant rats. The pregnant rats were then kept in individual cages until GD

20, when they were euthanized and the gravid uterus was collected. The right uterine horn from non-gravid and gravid females of experimental groups was isolated, trimmed free of fat and transversal fragments of 5mm were obtained from its medial portion. For gravid uterus, fetuses and placentas were removed, and the uterine fragments contained one implantation site for this assay.

Tissues were mounted in 10mL organ baths in a modified Tyrode's solution (composition in mM: NaCl 274.0; KCl 11.27; CaCl2 3.6; Glucose 11.1; NaHCO3 29.76; NaH2PO4 0.83) at 37°C under 1g resting tension and allowed a 30 min equilibration period. After the resting period, tissues were repeatedly challenged with KCl 80mM every 30min until two reproducible contractions were obtained. Again, the tissues were allowed to rest during 30min and the basal contractility was recorded after the stabilization.

After the resting period, tissues were challenged with 100μL of carbachol 10^{-3}M (a selective agonist for muscarinic receptors) and the tissue response was recorded during 7min. Then the tissues were washed and allowed to rest for 30min. The same procedures were performed with 100μL of norepinephrine 10^{-3}M (a selective agonist for adrenergic receptors) associated with 100μL of propranolol 10^{-5}M (a selective antagonist for β-adrenergic receptors). The contractility profile was evaluated by the area under the curve (AUC) and frequency of contractions (modified from Borges et al., 2017).

2.4. *In vitro* pharmacological reactivity of non-gravid uterine fragments

Six non-treated and nulliparous adult female rats (85-90 days of age; one female from each litter) were euthanized during estrus phase of reproductive cycle, and the uteri was collected and trimmed free of fat. One uterine ring of approximately 5mm of height was isolated from the medial part of each uterine horn.

Each uterine ring was mounted in 10mL organ baths (Ch) in a modified Tyrode's solution at 37°C, under 1g resting tension and allowed a 30min equilibration period, as follows: Ch1 – uterine ring obtained from right uterine horn; Ch2 – uterine ring obtained from left uterine horn. After the resting period, the tissues were repeatedly challenged with KCl 80mM every 30min until two reproducible contractions were obtained in order to observe the tissue viability.

After the resting period, the uterine rings in each Ch were incubated for 45min with rosuvastatin diluted in distilled water and dimethylsulfoxide (DMSO, 0.2%), at different concentrations: 0 (Control), 1, 10, and 100 μg / mL. The concentrations were chosen based on Moussa et al. (2018) that found the maximum concentration of 10μg/mL in rat plasma after oral treatment with rosuvastatin at dose of 1mg/Kg. Herein this concentration was extrapolated to 10× lower and higher values.

After the incubation period, tissues in Ch1 were challenged with 100μL of carbachol 10^{-3}M and the tissue response was recorded during 7min. Then tissues were washed and allowed to rest for 15min, and the next incubation with rosuvastatin was performed in the Ch. The same procedures were performed in Ch2 with 100μL of norepinephrine 10^{-3}M, associated with 100μL of propranolol 10^{-5}M. The contractility profile of the uterine tissues was evaluated by the AUC and frequency of contractions.

2.5. *In vitro* pharmacological reactivity of gravid uterine fragments

Other six non-treated and nulliparous adult female rats were mated with non-treated male rats during the dark period of the light/dark cycle, in order to obtain pregnant rats. The pregnant rats were then kept in individual cages until GD 20, when they were euthanized and the gravid uterus were collected and trimmed free of fat, fetuses and placentas. Two uterine rings were isolated from the medial part of the right uterine horn, and one uterine ring was

isolated from the left uterine horn. Each uterine ring contained one implantation site for this assay.

Similarly to non-gravid uterine rings, the tissues were mounted in 10mL organ bath, in a modified Tyrode's solution, and viability of the tissues was tested with KCl 80mM. The uterine rings were also allocated among the organ baths (Ch) as follow: Ch1 and Ch2 – uterine rings obtained from right uterine horn; Ch3 – uterine ring obtained from left uterine horn.

After the resting period, the uterine rings in each Ch were incubated for 45min with rosuvastatin diluted in distilled water and dimethylsulfoxide (DMSO, 0.2%), at different concentrations: 0 (Control), 1, 10, and 100 μg / mL. Following the incubation period, tissues in Ch1 were challenged with 100μL of prostaglandin E2 10^{-5}M and the tissue response was recorded during 7min. Then tissues were washed and allowed to rest for 15min, and the next incubation with rosuvastatin was performed in the Ch. The same procedures were performed in Ch2 with 100μL of carbachol 10^{-3}M; and in Ch3 with 100μL of norepinephrine 10^{-3}M associated with 100μL propranolol 10^{-5}M. The contractility profile of the uterine tissues was evaluated by the AUC and frequency of contractions.

2.6. Statistical analysis

Data are presented as mean ± standard error of mean (S.E.M.). The results were compared among groups by One-way ANOVA followed by Tukey's test, for parametric variables. Differences were considered statistically significant when $p \leq 0.05$. Statistical analyses were performed using the software GraphPad Prism (version 6.0).

3. Results

Assessment of possible estrogenic effects associated with rosuvastatin exposure did not show any alteration on uterine weight of groups treated with this statin when compared with control group, as evidenced by estradiol positive control (Figure 1-A). Antiestrogenic effects were also not seen after the association of rosuvastatin and estradiol, as shown by uterine weight (Figure 1-B). Moreover, weights of ovaries and liver did not show alterations among experimental groups (Table 1).

Long-term treatment with rosuvastatin presented alterations in the uterine contractility profile. Basal contractility of non-gravid uterus was increased by the *in vivo* exposure to the highest dose of rosuvastatin, as showed by the increase in the AUC and in the frequency of contractions. On the other hand, basal contractions of gravid uterus were not affected by this statin (Figure 2).

The uterine tissues of non-gravid and treated animals did not show alterations in the contractile activity when challenged with carbachol or norepinephrine. However, the pattern of contractions of gravid uterus was affected by the treatment when the tissues were challenged with the same agonists. Contractions induced by carbachol were decreased in the animals exposed *in vivo* to both doses of rosuvastatin, as showed by the reduced AUC. Despite that, contractions induced by norepinephrine did not show to be affected by the treatment with rosuvastatin (Figure 3).

Uterine pattern of contraction assessed *in vitro* showed alterations in association with crescent concentrations of rosuvastatin. Concentrations at 10 and 100 μg/mL of this statin reduced the frequency of basal contractions in both non-gravid and in gravid uterine tissues (Figure 4) without affecting the AUC.

Non-gravid uterine rings did not present alterations in the contractility profile when challenged with a sympathetic neurotransmitter associated with crescent rosuvastatin

concentrations. However, uterine tissues challenged with parasympathetic neurotransmitter, after incubation with rosuvastatin, showed changes in the contractility pattern of the tissues (Figure 5). Contractility induced by carbachol presented a reduction in the frequency of peaks, at concentration of 10 and 100 μg/mL of statin, without affecting AUC of the uterine tissues.

In gravid uterine rings the contractions induced by prostaglandin E2 caused reduction on the frequency of peaks (p = 0.0784), when associated with the higher concentration of rosuvastatin, despite AUC did not being altered (Figure 5). Additionally, contractility, induced by norepinephrine, showed that rosuvastatin at all concentrations is capable to reduce the frequency of contraction, without affecting AUC. However, uterine tissues challenged with carbachol after incubation with rosuvastatin did not show changes in the contractility pattern.

4. Discussion

Despite the widely known benefic effects of statin therapy not only for lipid disorders, this class of drugs is associated with several side effects, such as myopathies, hepatotoxicity and neuropathy (Grover et al., 2014). We reported recently that rosuvastatin, a last generation statin (McTaggart et al., 2001), is also associated with impaired reproductive function in male rats after peri-pubertal exposure to this compound (Leite et al., 2019, 2018b, 2018a, 2014), which suggests a possible endocrine disruption promoted by this statin.

In this study, we investigated the effects of rosuvastatin directly on uterine tissues via uterotrophic assay and pharmacological reactivity both *ex vivo* and *in vitro*. Uterus physiology is under hormonal control, promoted mainly by estrogen and progesterone (Abbas et al., 2019). Both hormones have contrary roles on uterine contractility profile: estrogen is associated with the increase on myometrial activity, while progesterone depresses the excitability of smooth muscle cells of myometrium (Abbas et al., 2019). Thus, uterine tissue evaluation is of great relevance to elucidate whether rosuvastatin can impair female reproductive function through endocrine or other distinct mechanisms.

There are few *in vivo* and *in vitro* evidences showing the effects of rosuvastatin and other statins on sex steroid hormones synthesis and signaling. These studies demonstrate that mevastatin is able to reduce testosterone and progesterone production by theca cells *in vitro* (Izquierdo et al., 2004), and simvastatin reduces plasma levels of progesterone in female rats (Guldvang et al., 2015).

In vivo assessment of possible estrogenic and/or antiestrogenic effects of rosuvastatin on uterine and other tissues of female rats did not corroborate the idea of endocrine disruption of this compound, at least not by an estrogen-related pathway. On the other hand, Leite et al. (2018a) showed histological alterations on uterus of female rats whose fathers were exposed to rosuvastatin. In this case the alterations found are supposed to be caused by a decrease on

estrogen signaling. A study suggests that statins and estrogen might act in a synergistic way, since both present similar secondary mechanisms of action (Das, 2002), however in our experimental approach, these considerations were not observed.

Regarding the uterine physiology, myometrial contraction plays crucial roles to female fertility, once it is involved with transport of gametes, embryo implantation and nutrition, and parturition (Taylor and Gomel, 2008). Uterine dysmotility whose contractility pattern presents to be decreased, hyperactive or asynchronous is related to infertility, implantation failure and many other pathological conditions, such as endometriosis and adenomyosis (Dodds et al., 2015; Kunz and Leyendecker, 2002).

Pharmacological reactivity of uterus assay provides a reliable tool to investigate whether a compound can affect myometrial pattern of contraction both *ex vivo* and *in vitro*. Thus, it allows the classification of a compound as a possible excitatory or inhibitory molecule on uterine tissue (Arrowsmith et al., 2018). In our experiment, uterine contractility patterns were assessed both in non-gravid period, during estrus phase, and in gravid period, on GD 20. During estrus phase, the ovulatory period, myometrial contractility presents phasic and stronger pattern of contractions (Dodds et al., 2015; Gravina et al., 2014). By the end of pregnancy, myometrial contractions increase, in order to promote the parturition (Abbas et al., 2019).

Evaluation of basal contractility of uterus associated with crescent concentrations of rosuvastatin *in vitro* shows that this drug is able to reduce the frequency of contractions in both periods, while *ex vivo* experiments demonstrate that chronical exposure to this drugs is related to an increase in basal contractility of uterus during estrus phase. Myometrial contraction is a result of liberation of calcium from intracellular stores and extracellular fluid in the cytosol of smooth muscle cells (Abbas et al., 2019; Gravina et al., 2014). Our results

suggest that rosuvastatin might delay the increase of cytosolic calcium, and consequently reduce the frequency of peaks.

It is also relevant to emphasize that one of the main adverse effects after a chronic statins therapy is myopathies, once statins are able to induce apoptosis in smooth muscle cells (du Souich et al., 2017) and impair muscle mitochondrial metabolism (Sirvent et al., 2012). Together with a possible alteration in hypothalamic-hypophyseal-ovarian axis, this data may explain the observed effects of rosuvastatin upon basal contractility of *in vivo* exposed non-gravid uterus.

The uterus is supplied by autonomic nervous system neurons (Mónica Brauer and Smith, 2015), and presents smooth muscle cells that contract spontaneously and rhythmically throughout estrous cycle and gestation, which guarantees the proper maintenance of the reproductive functions. Also, by the end of gestational period, prostaglandins are responsible for the augment of intracellular calcium, which increases the myometrial contractions for parturition (Abbas et al., 2019).

Interestingly, in our experiments, the challenge of uterine tissues with sympathetic and parasympathetic neurotransmitters after *in vivo* treatment or incubation with rosuvastatin, showed different effects of this drug on the different periods evaluated. Contractions induced by parasympathetic neurotransmitters revealed that *in vitro* rosuvastatin reduces the frequency of contractions during estrus phase. However, during late pregnancy, this drug *in vitro* did not seem to affect contraction pattern mediated by parasympathetic agonist, while *in vivo* treatment is associated with a decreased AUC. On the other way, contractions induced by sympathetic neurotransmitters did not affect the contraction pattern at non-gravid period both in *ex vivo* and *in vitro* assays. But on gravid period, *in vitro* rosuvastatin reduced the frequency of contractions induced by norepinephrine.

Regarding the hormonal signaling effects, estradiol is known as a regulator of uterine autonomic neuroplasticity in uterus, especially upon sympathetic innervation (Mónica Brauer and Smith, 2015), but in this experimental model, during estrus phase, rosuvastatin did not seem to alter estrogen and its regulatory effects on uterine sympathetic innervation and function.

During estrus phase, the major innervation pattern corresponds to parasympathetic neurons (Gnanamanickam and Llewellyn-Smith, 2011); while at late pregnancy there is a decrease in both sympathetic and parasympathetic innervation (Mónica Brauer and Smith, 2015). There are few studies associating the effects of statins upon autonomic innervation. In a clinical study with patients treated with atorvastatin, the heart sympathetic activity was decreased, and heart parasympathetic activity was increased by this statin (Doğru et al., 2008). Thus it is possible that rosuvastatin might modulate autonomic activity of uterus according to the density of neurons in the tissue in each reproductive phase.

In our study, prostaglandin-induced contractions on gravid uterus after incubation with rosuvastatin did not show statistical differences. However, it was seen in this experimental approach that the highest concentrations of rosuvastatin lead to a reduction on the frequency of contractions of the tissues. It is known that rosuvastatin present anti-inflammatory effects (Cortese et al., 2016; Dolkart et al., 2015; Ferri and Corsini, 2014), and might affect the signaling pathway of prostaglandins. In the myometrium, contractions induced by prostaglandin are mediated by interactions with the receptor EP3 (Shu et al., 2017), thus, it is possible that rosuvastatin might interact with this receptor and lead to a delay in the activity of prostaglandin and consequently, on peaks of contraction.

This study provides information about the effects of rosuvastatin upon the rat uterus. Uterine physiology can be affected by this statin not by estrogenic or antiestrogenic pathway, but by direct and/or indirect effects on the tissue, as observed with both *in vivo* and *in vitro*

assays. Also, it is relevant to note that rosuvastatin possibly interacts with different pathways which regulate myometrial contraction throughout different female reproductive phases. Thus, the direct effects promoted by rosuvastatin in uterus should be analyzed with more details in order to elucidate whether this drug is able to impair female fertility or might be a future adjuvant in the treatment of uterine pathological conditions that affect female reproductive health.

5. Declaration of Interest

The authors declare that there is no conflict of interests.

6. Acknowledgments

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Tables

Table 1. Final body and organ weights of female rats submitted to uterotrophic assay.

Assessment of estrogenic effects				
Parameter	**Experimental Groups (n = 7/group)**			
	Control	**3 mg/Kg**	**10 mg/Kg**	**Estradiol**
Final body weight (g)	58.51 ± 2.22	58.10 ± 1.89	56.69 ± 2.16	57.79 ± 1.26
Ovaries (mg)	36.46 ± 2.99	36.31 ± 1.49	33.31 ± 1.38	35.60 ± 1.45
Liver (g)	3.00 ± 0.10	2.63 ± 0.11	2.72 ± 0.12	2.96 ± 0.15
Assessment of antiestrogenic effects				
Parameter	**Experimental Groups (n = 7/group)**			
	Control	**Estradiol**	**Estradiol + 3 mg/Kg**	**Estradiol + 10 mg/Kg**
Final body weight (g)	58.51 ± 2.22	57.79 ± 1.26	56.37 ± 1.80	55.74 ± 2.05
Ovaries (mg)	36.46 ± 2.99	35.60 ± 1.45	35.29 ± 2.75	33.40 ± 1.47
Liver (g)	3.00 ± 0.10	2.96 ± 0.15	2.79 ± 0.03	2.85 ± 0.18

Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. $p > 0.05$ compared to control group.

Figure legends

Figure 1. Evaluation of (A) estrogenic and (B) antiestrogenic effects of rosuvastatin in rats by uterotrophic assay. Uterine weights with fluid (n = 7/group). Values expressed as mean ± S.E.M. Values expressed in percentage of final body weight (BW). Different letters indicate $p \leq 0.0001$ compared to control group. ANOVA followed by Tukey's test.

Figure 2. Basal contractility of non-gravid uterus (n = 5/group), during estrus phase, and gravid uterus (n = 4-5/group), at late pregnancy, after *in vivo* treatment with rosuvastatin or vehicle. Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. $*p \leq 0.05$, $**p \leq 0.01$ compared to control group.

Figure 3. Contractile response of non-gravid uterus (n = 5/group), during estrus phase, and gravid uterus (n = 4-5/group), at late pregnancy, after *in vivo* treatment with rosuvastatin or vehicle. Tissues were challenged to contract in the presence of carbachol and norepinephrine associated with propranolol. Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. $*p \leq 0.05$ compared to control group.

Figure 4. Basal contractility of non-gravid uterus (n = 6), during estrus phase, and gravid uterus (n = 6), at late pregnancy, after *in vitro* treatment with increasing rosuvastatin concentrations. Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. $*p \leq 0.05$, $**p \leq 0.01$, $****p \leq 0.0001$ compared to control group (0μg/mL).

Figure 5. Contractile response of non-gravid uterus (n = 6), during estrus phase, and gravid uterus (n = 6), at late pregnancy, after *in vitro* treatment with increasing rosuvastatin concentrations. Tissues were challenged to contract in the presence of carbachol,

norepinephrine associated with propranolol, and prostaglandin E2. Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. *$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.0001$ compared to control group (0µg/mL).

Figures

Figure 1.

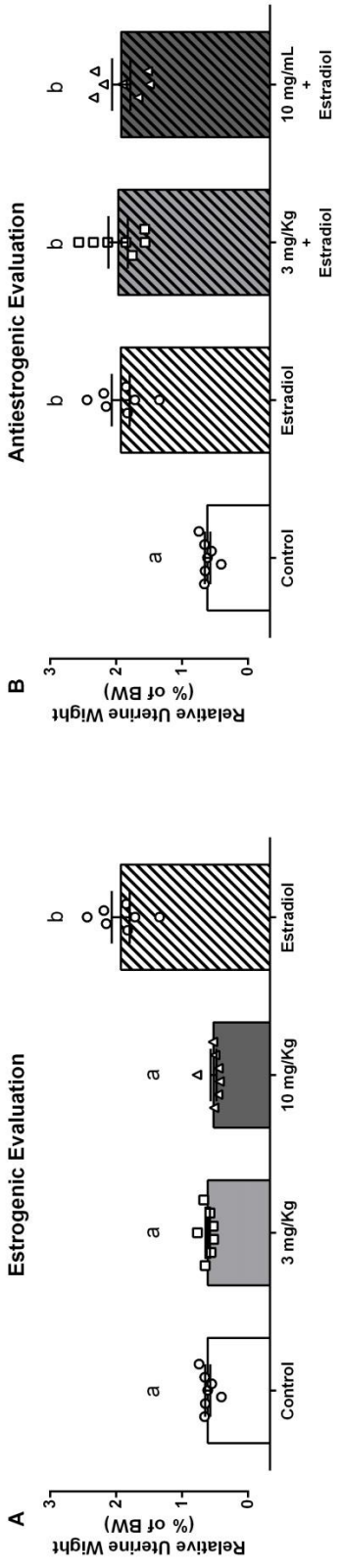

Figure 2.

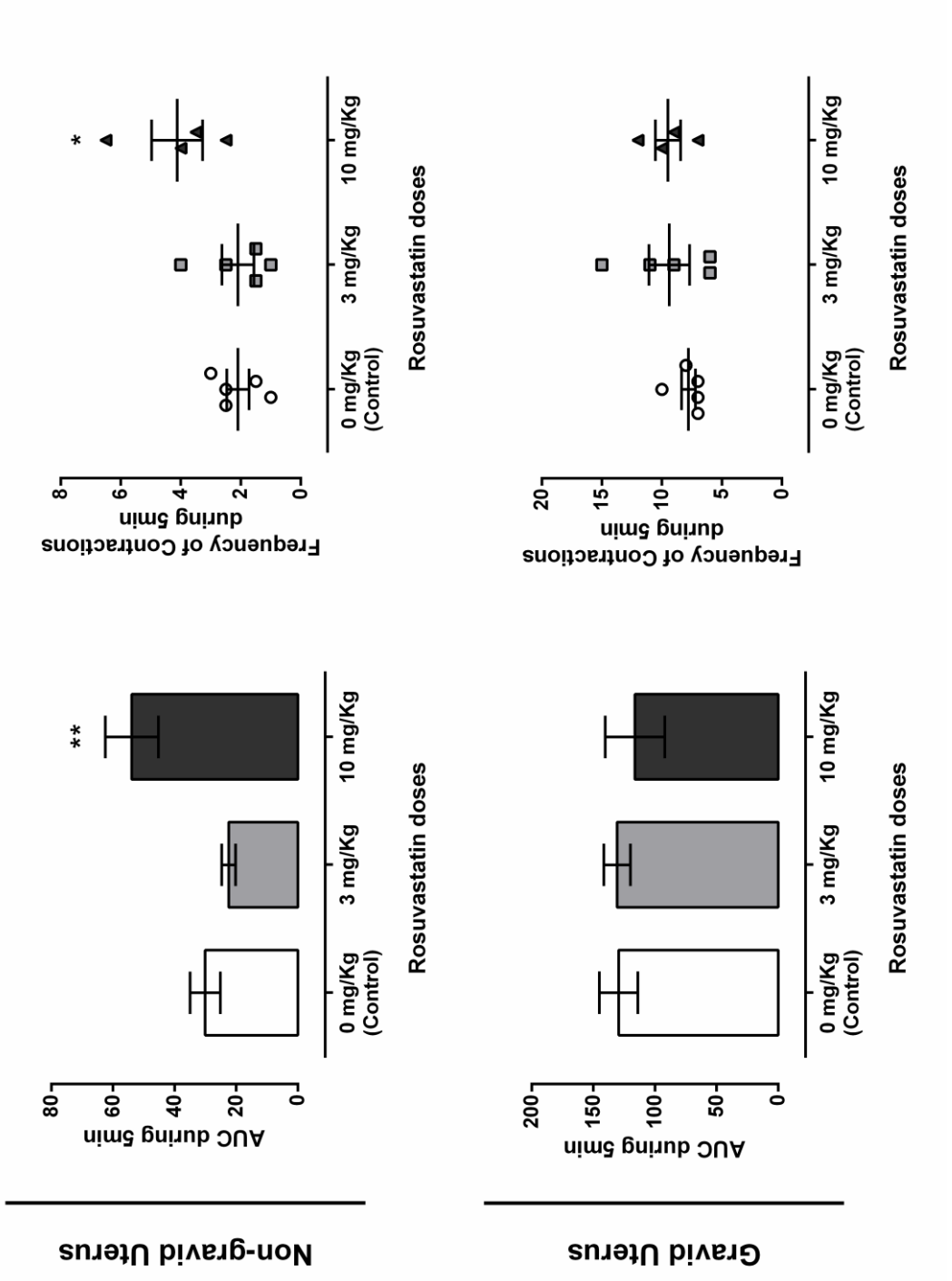
Non-gravid Uterus
Gravid Uterus
AUC during 5min
Frequency of Contractions during 5min
0 mg/Kg (Control)
3 mg/Kg
10 mg/Kg
Rosuvastatin doses
**
*

Figure 3.

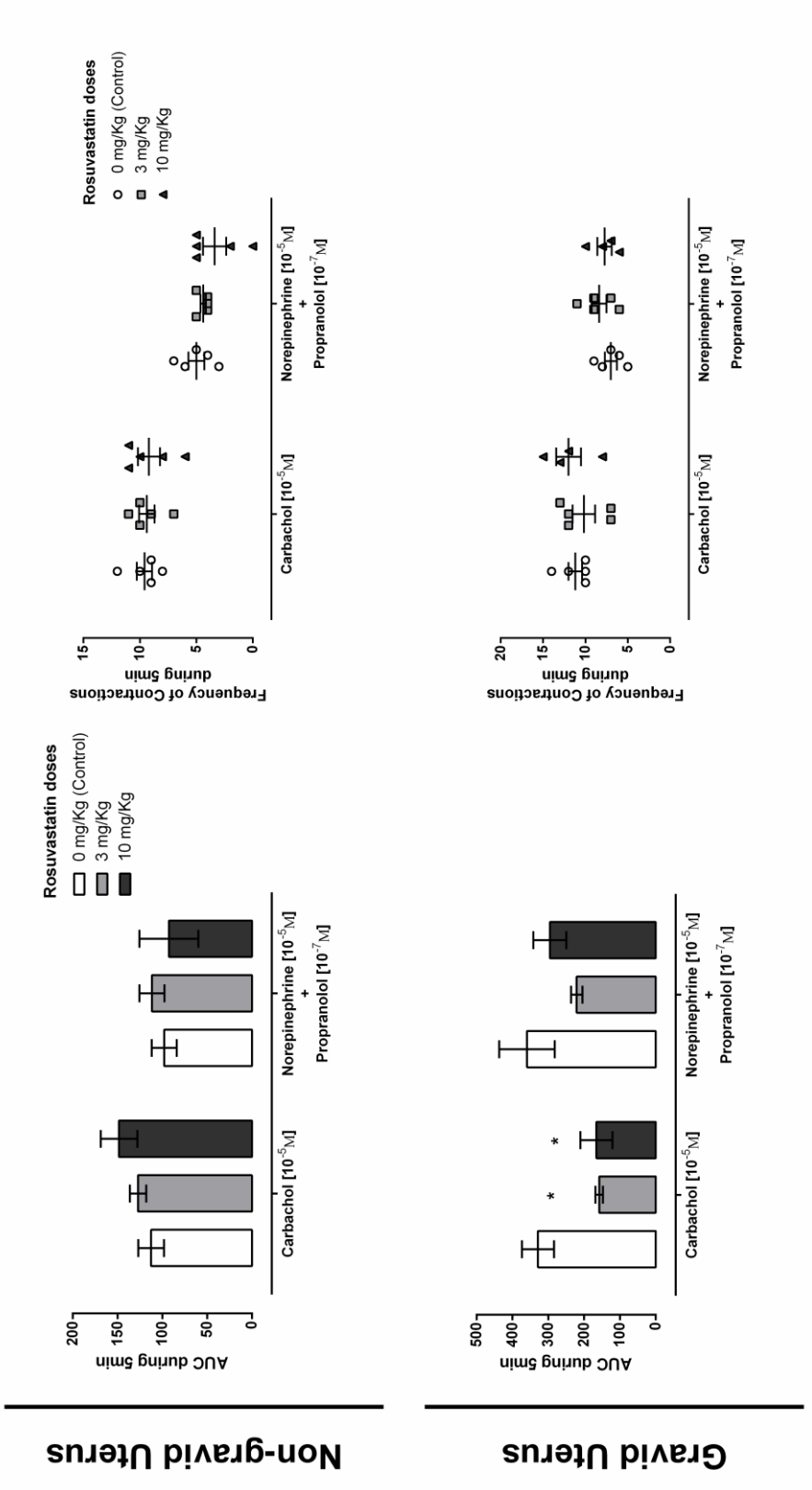
Non-gravid Uterus
Gravid Uterus
Rosuvastatin doses
0 mg/Kg (Control)
3 mg/Kg
10 mg/Kg
AUC during 5min
Frequency of Contractions during 5min
Carbachol [10^{-5}M]
Norepinephrine [10^{-5}M] + Propranolol [10^{-7}M]
*

Figure 4.

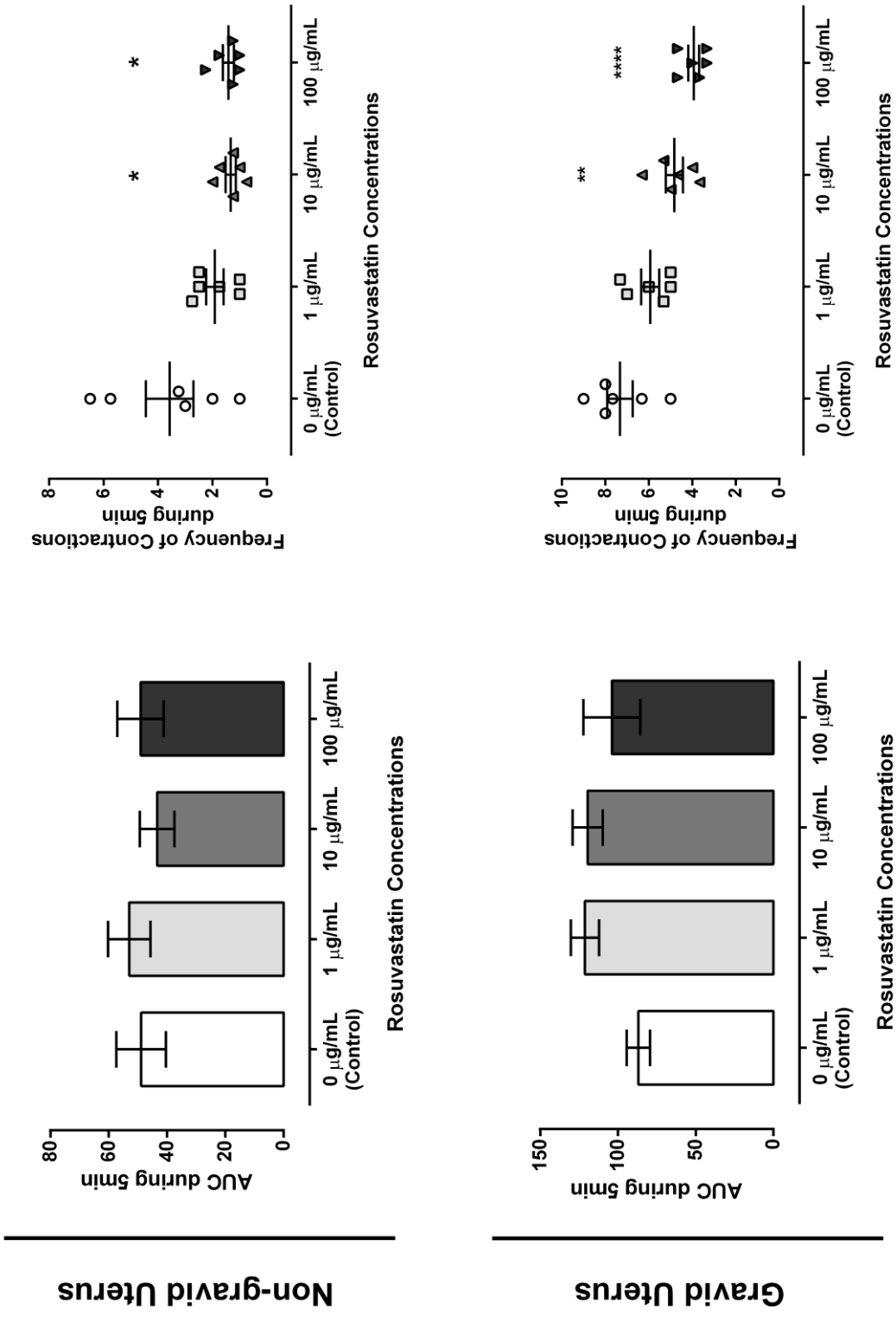
Non-gravid Uterus
Gravid Uterus
AUC during 5min
Frequency of Contractions during 5min
Rosuvastatin Concentrations
0 μg/mL (Control)
1 μg/mL
10 μg/mL
100 μg/mL
*
**
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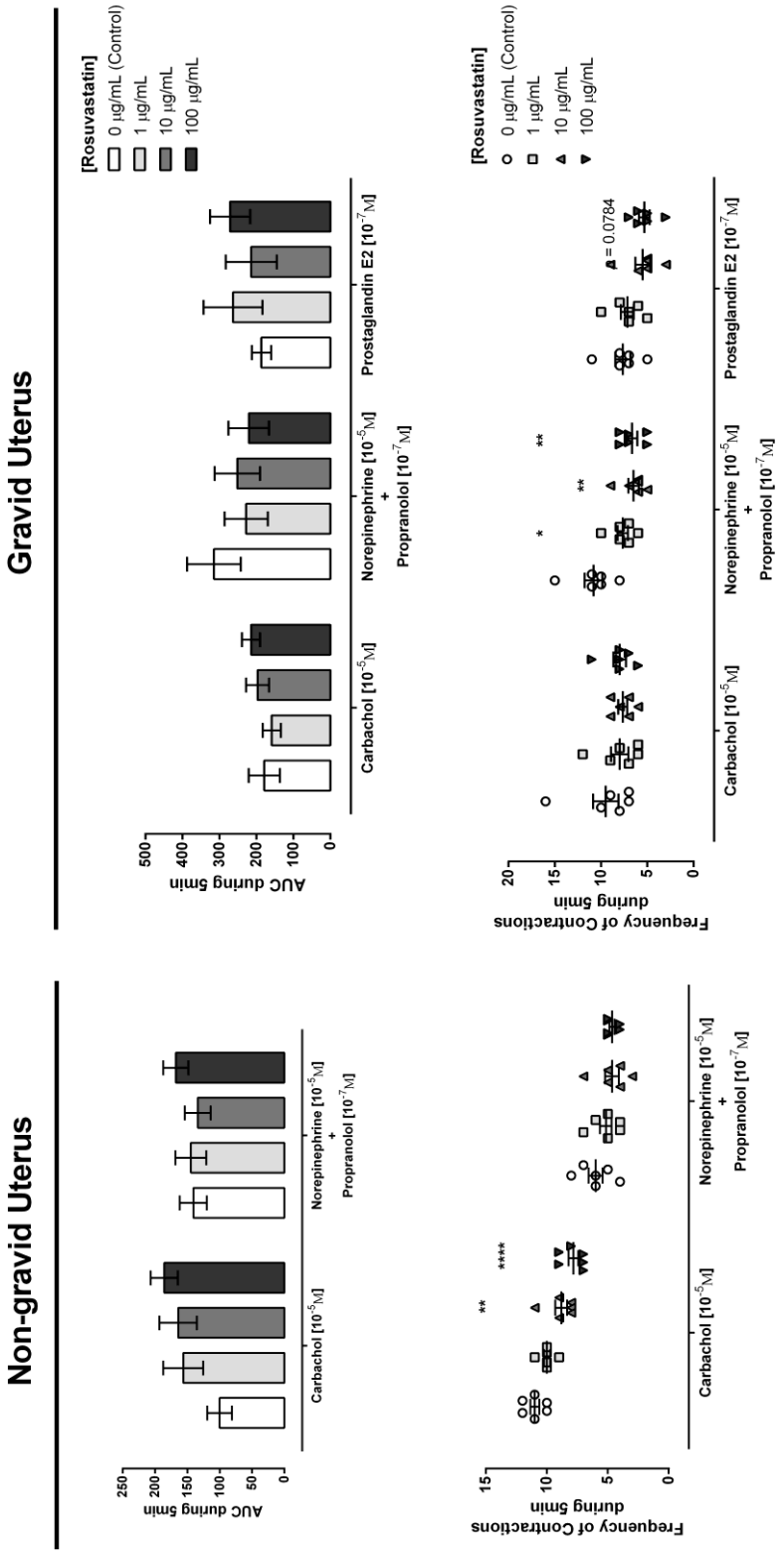
Figure 5.


Non-gravid Uterus
Gravid Uterus
AUC during 5min
Frequency of Contractions during 5min
[Rosuvastatin]
0 µg/mL (Control)
1 µg/mL
10 µg/mL
100 µg/mL
Carbachol [10-5M]
Norepinephrine [10-5M] + Propranolol [10-7M]
Prostaglandin E2 [10-7M]
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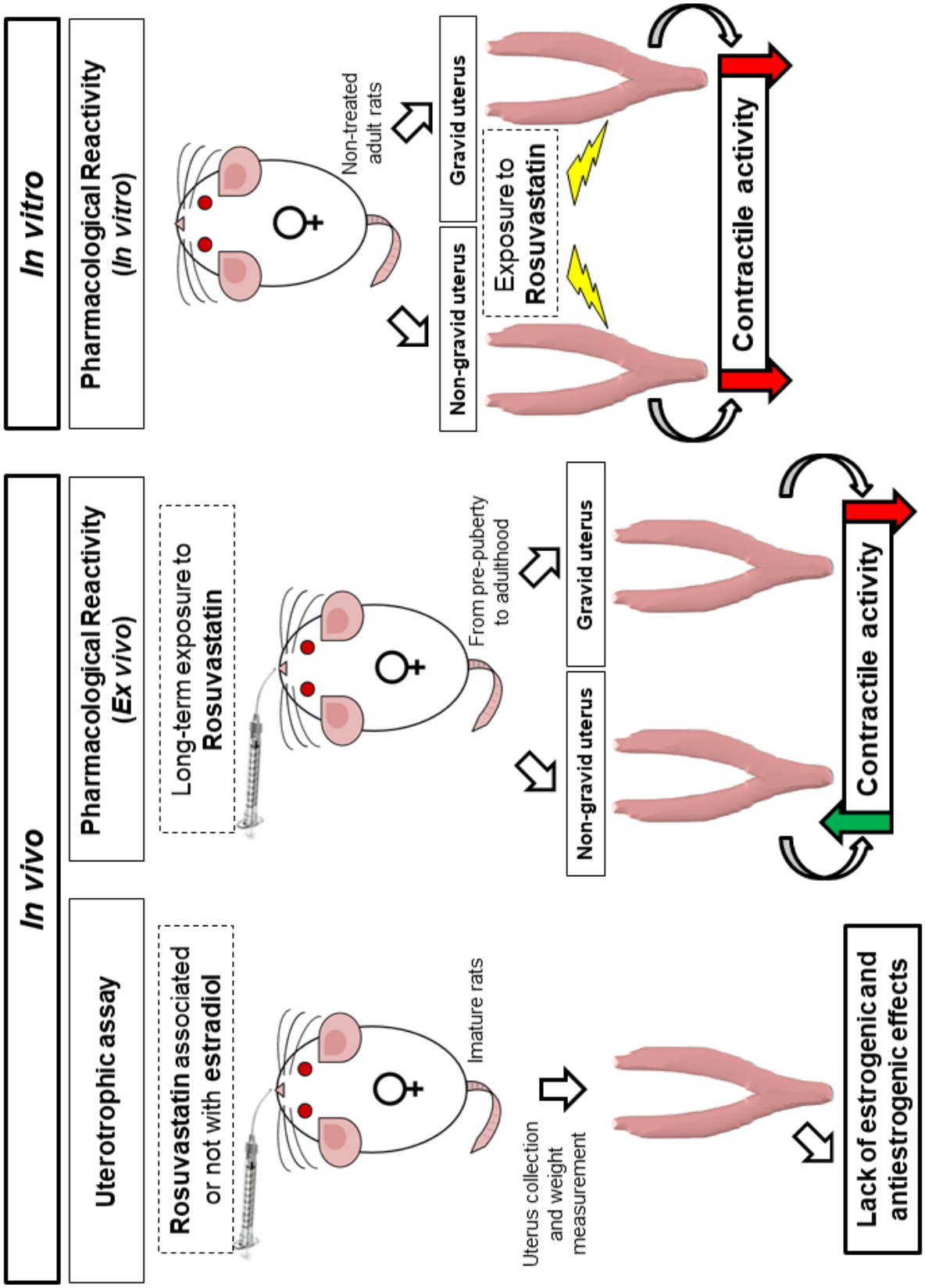
*
**
= 0.0784

Graphical Abstract



Capítulo 2

O trabalho desenvolvido com a exposição aguda e crônica de ratas Wistar à rosuvastatina, desde a pré-puberdade, deu origem ao manuscrito "*Short- and long-term effects on reproductive parameters of female Wistar rats after exposure to rosuvastatin since pre-puberty*", a ser submetido para publicação no periódico *Life Sciences* (Fator de impacto: 3,448).

Short- and long-term effects on reproductive parameters of female Wistar rats after exposure to rosuvastatin since pre-puberty

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Highlights

- Rosuvastatin is a lipid lowering drug that inhibits cholesterol biosynthesis.
- Exposure of female rats to rosuvastatin since pre-puberty leads to reproductive disorders.
- No alterations were found in puberty timing, reproductive hormone levels, ovarian and uterine histological endpoints and reproductive performance.
- Estrous cycle, sexual behavior, and hypophysis and placental development were affected by rosuvastatin exposure.

Abstract

Statins are a class of drugs which acts mainly with lipid lowering effects. Rosuvastatin is a last generation statin and has shown better results in reducing cholesterol concentrations when compared to other statins. Recent studies suggest that rosuvastatin may act as an endocrine disruptor, once potentially damages hormonal axis and, consequently reproductive development and function of male rats. However, the effects of rosuvastatin exposure in rat female reproductive parameters remain unknown. In this study female rats exposed to rosuvastatin at dose of 10 mg / Kg / day since pre-puberty exhibited shorter estrous cycles, altered sexual behavior, decreased serum prolactin level, and alterations on hypophysis and placental development, parameters highly influenced by hormonal signaling. On the other hand, pubertal onset, reproductive hormone levels, fertility and histological parameters of ovary, uterus and placenta were not altered by the exposure to the statin. Thus, rosuvastatin exposure, in these experimental conditions, promoted some deleterious effects on the reproductive function of female rats, possibly damaging the hormonal axis signaling, suggesting its potential as an endocrine disruptor.

Keywords: Rosuvastatin; Statin; Female Reproduction; Endocrine Disruptor.

1. Introduction

Puberty is the period when the animals become capable of reproducing sexually for the first time. During this time, genital organs mature and secondary sex characteristics develops both in male and female animals [1]. Puberty is considered a critical period of development, once requires the action of different endocrine factors which are crucial to promote an adequate development of body systems [2].

During pre-puberty, the hypothalamic-hypophysis-gonadal axis is activated, and secretes gonadotrophin releasing hormone (GnRH) by the hypothalamus, which stimulates the adenohypophysis to release follicle-stimulating hormones (FSH) and luteinizing hormones (LH). The gonads gradually become more sensitive to the stimuli of these two gonadotrophins, and have a considerable increase in their growth rate, releasing steroid hormones into the bloodstream. These events culminate in the onset of puberty [3].

Different substances may act in these physiological events and disrupt the normal reproductive development during this period. These substances are known as “endocrine disrupters”, defined as an exogenous substance that alters the endocrine system functions and leads to adverse health effects to the organism [4].

Statins are a class of drugs which the main mechanism of action is the inhibition of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, a limiting enzyme in the cholesterol biosynthesis, consequently reducing lipid levels in the organism [5]. Also, part of the beneficial effects associated with the administration of statins has been attributed to its pleiotropic effects, that are independent of inhibition of cholesterol biosynthesis, which include endothelial protection, antioxidant and anti-inflammatory properties, reduction in thrombogenic response, pro-angiogenic [6,7] and immunomodulatory effects [8].

Recently, statins use has increased, once people have poor eating habits and sedentary lifestyle, mainly during childhood and early adulthood, in order to reduce cholesterol levels [9,10].

Among the statins, rosuvastatin is one of the most recent in the class [11], and has shown better results in reducing LDL cholesterol concentrations when compared to other statins [12].

Previous studies with rosuvastatin exposure during peri-pubertal period in rodent experimental model revealed that this drug leads to reproductive impairments in male rats. Rosuvastatin is able to delay pubertal development, reduce sperm quality, impair spermatogenesis and alter hormonal signaling [13–15]. Also, rosuvastatin exposure in male rats might affect reproductive function in an intergeneration manner, by damaging ovarian and uterine histophysiology of female offspring [16].

These data indicate that rosuvastatin may act as an endocrine disruptor during the peri-puberal window of exposure, and could impair reproductive development and function. However, there are no data regarding direct rosuvastatin or other statins exposure in female peri-puberal period and its effects to female reproductive parameters. Thus, this study aimed to investigate the effects of rosuvastatin exposure, since pre-puberty, in pubertal development and reproductive function of female Wistar rats.

2. Material and Methods

2.1. Animals

Adult male and female Wistar rats (80 days old) were obtained from the Central Biotherium, São Paulo State University (UNESP), *campus* Botucatu/SP, Brazil, and maintained under controlled conditions (12h of light/12h of darkness; average temperature of 23 °C) on the Small Mammal Biotherium of Morphology Department, at the UNESP, Institute of Biosciences of Botucatu, with food and water *ad libitum*. The animals were kept according to the Ethical Principles for Animal Experimentation, adopted by the Brazilian College of Animal Experimentation. The project was filed under number 1089 with the Ethics Committee on Animal Experimentation of the UNESP Institute of Biosciences, in Botucatu.

The mating of these animals in order to obtain pregnant females and the pups, was performed during the dark period of the cycle, with 2 females being placed in the male cage. The gestational day (GD) 0 was determined by the presence of spermatozoa in vaginal smears of estrus females. These were then kept in individual cages. After birth, number of pups per litter was reduced to 8, balancing 3 male and 5 female pups at postnatal day (PND) 1.

2.2. Experimental design

At weaning, on PND 21, female pups were pseudo randomly distributed among three experimental groups, keeping the same body weight mean for each group: control group, which received treatment with saline; and rosuvastatin groups, which received the statin in doses of 3 or 10 mg / Kg / day diluted in saline. The treatment was performed daily and by oral administration since PND 22, and finished on the first estrus after PND 42 or 75. During treatment period, body weight of rats was measured weekly. A brief and visual description of the experimental procedures performed in this study is presented in the figure 1.

The doses of rosuvastatin chosen for this study are based on the lowest and higher doses of rosuvastatin applied in human therapy for children [14–16], adapted for rodents considering their body surface area, as proposed for Reagan-Shaw et al. [17].

In order to investigate the effects of rosuvastatin exposure on female reproductive function, the period of treatment is based on the use of statins that becomes earlier in the child and adolescent population because of the risk of lipid abnormalities [10,18].

2.3. External physical signs of puberty onset

Starting on PND 30, female rats were evaluated daily for complete vaginal opening. Then, rats were weighted and daily checked for the occurrence of first estrus by assessing vaginal fluid, characterized for the presence of cornified epithelial cells [19]. Vaginal fluid was collected with a micropipette, that inserts 10μL of saline in rats vagina, collecting it back. Fluid was deposited in a slide and analyzed under light microscopy. Both procedures, vaginal opening and detection of first estrus, were performed for determine the age of puberty onset in female rats [20].

2.4. Estrous cyclicity

On PND 60, the animals were evaluated daily for 15 consecutive days for estrous cyclicity based on cellular composition of vaginal fluid. This procedure was used to determine the length of cycle, as well as of each phase of the cycle (proestrus, estrus, metaestrus and diestrus). The proestrus phase consists of predominance of nucleated epithelial cells; estrus phase is characterized for the presence of cornified epithelial cells; metaestrus present nucleated and cornified epithelial cells, and leukocytes; diestrus consists of predominance of leukocytes [19].

2.5. Euthanasia of rats and organ collection

Rats from each experimental group were euthanized during estrus phase in two different ages: at PND 42, right after onset of puberty, as established for U. S. Environmental Protection Agency [21], and at PND 75, during adulthood. Initially, animals were weighted and then euthanized by narcosis in CO_2. Blood samples for hormonal dosages were obtained by collecting blood from inferior vena cava. Toxicological target (hypophysis, thyroid, liver, adrenal and kidney) and reproductive (ovaries and uterus) organ were collected and weighted. Left uterine horn and ovary were used for histological evaluation.

2.6. Serum reproductive hormone levels

Blood samples were centrifuged at 2500rpm for 10min at 4ºC and serum was obtained for hormonal dosages. Serum levels of FSH, LH, prolactin, progesterone and testosterone were measured using a double-antibody radioimmunoassay kit supplied by MP Biomedicals.

2.7. Copulatory behavior and reproductive performance

During the first estrus after PND 75, female rats from experimental groups were submitted to the sexual behavior test. After detection of estrus phase, female rats were put into cages of sexually experienced male rats, then allowed 10 mounts on the females and presence of lordosis was registered. Results were expressed as the lordosis quotient (number of lordosis/10 mounts × 100) [22]. All procedures were performed during de dark phase of light/dark cycle, and females were used only once.

After the sexual behavior test, the females were maintained with the males for additional 8 hours. Finished this period, rats were separated in individual cages and vaginal smears were collected for detection of spermatozoa, and then established the gestational day (GD) 0. On GD 20, female were weighted and euthanized by CO_2. Gravid uterus and ovaries

were collected, number of corpora lutea were determined, implantation sites, resorptions, live fetuses, and weights of fetus and placentas were recorded. From these results the following parameters were calculated: Gestational rate: number of pregnant females/number of inseminated females × 100; Fertility potential (efficiency of implantation): implantation sites/corpora lutea × 100; Rate of pre-implantation loss: (number of corpora lutea – number of implantations/number of corpora lutea) × 100; Rate of post-implantation loss: (number of implantations – number of live fetuses)/number of implantations × 100; and Sex ratio: number of female fetuses/number of male fetuses [23,24].

2.8. Histological procedures

Left uterine horn and ovary, during the estrus phase, and placenta of fetuses on GD 20 were collected and immersed in Bouin's fixative solution, histologically processed and included in Paraplast®. Then, three sections of each organ were cut at a thickness of 5μm, with an interval of 50μm, and placed into silanized slides and stained with hematoxylin and eosin (H & E). The analysis on the ovary was performed by the observation of the histological aspect of this organ and by counting the number of corpora lutea and follicles on the different stages of the follicular development, as described by Talsness et al. [25] and Guerra et al. [26]. On the uterine sections, the height of perimetrium, myometrium, endometrium and luminal epithelium was measured, as described by Silva et al. [27]. Placental tissues were evaluated in its general histological aspect and the height of basal zone was measured in five different places of each section. Histological analyses were conducted under light microscopy, with the softwares Leica QWin 3 and ImageJ 1.48.

2.9. Immunohistochemistry for Proliferating Cell Nuclear Antigen (PCNA)

Immunohistochemistry assay for Proliferating Cell Nuclear Antigen (PCNA) was performed based on Borges et al. [28] and Barros, et al. [29], with modifications. Initially, the placenta were sectioned at a thickness of 5μm and placed on silanized slides. Then, the sections were dewaxed with xylol, hydrated with decreasing concentrations of alcohol, and washed with phosphate buffered saline (PBS - pH 7.4). Antigenic recovery was performed with citrate buffer (pH 6.0) for 10min in a microwave. After this step, the sections were incubated for 15min with hydrogen peroxide (3.5%) and PBS, for blocking the endogenous peroxidase. In the next step, the sections were incubated for 30min with Bovine Serum Albumin (BSA 3%) diluted in PBS, and then washed with PBS to be incubated overnight with the primary antibody anti-PCNA (PCNA PC10: sc-56, Monoclonal, Santa Cruz Biotechnology, CA, USA – 1:100). After the incubation period, the sections were washed again with PBS and incubated for 1h with the secondary antibody (Goat Anti-Mouse peroxidase-labeled IgG, Catalog No. 474-1806, KPL Antibody – 1:200). Then, after further washing with PBS, the cuts were submitted, for 4min, to the diaminobenzidine (DAB) associated with hydrogen peroxide. After the reaction, the sections were washed with water and counterstained with hematoxylin. At the end of the procedure, the sections were dehydrated with increasing concentrations of alcohol and then immersed in xylol. The sections were covered with coverslips and analyzed under light microscope, coupled to a digital camera and a computer containing the software Leica Q-win (version 3). Immunostaining for PCNA on the placental tissues were evaluated specially in the basal zone, in a qualitatively way, according with immunostaining intensity, and classified as “absent”, “weak”, “moderate” or “strong”.

2.10. Statistical analysis

Data are presented as mean ± standard error of mean (S.E.M.), median and interquartile range or percentage. The results were compared among groups by ANOVA followed by Tukey's test, for parametric variables, and by Kruskal-Wallis followed by Dunn's test, for nonparametric variables. Differences were considered statistically significant when $p \leq 0.05$. Statistical analyses were performed using the software GraphPad Prism (version 6.0).

3. Results

3.1. Short-term effects of exposure to rosuvastatin since pre-puberty

In this study, rosuvastatin exposure was not able to alter age of puberty onset, here analyzed by the vaginal opening followed by detection of first estrus (Figure 2). Additionally, on PND 42, the animals exposed to rosuvastatin did not exhibit alterations in body weight (Figure 3) and weight of reproductive organs (Table 1). However, there was an increase in liver weight and decrease in hypophysis weight, (Table 1) in the animals treated with 10 mg/Kg, compared to control group. On PND 42, serum levels of FSH, LH, prolactin, progesterone and testosterone were similar among experimental groups (Figure 4). Also, ovarian follicles counting, uterine morphometries and general histological aspect of both organs were not affected at this age by the exposure to rosuvastatin (Figure 5).

3.2. Long-term effects of exposure to rosuvastatin since pre-puberty

Reproductive cyclicity of animals from experimental groups were not altered by exposure to rosuvastatin, except animals treated with rosuvastatin at doses of 10mg/Kg, which showed shorter estrous cycles than control group (Figure 6). Despite that, on PND 75, weight of body (Figure 3) and organs (Table 1) were similar among experimental groups. At this age, serum levels of FSH, LH, progesterone and testosterone were similar among experimental groups (Figure 4). However, serum levels of prolactin were reduced in animals exposed to the higher dose of rosuvastatin. Histological evaluation of ovary and uterus at this age did not show any sign of alterations associated with the treatment with this statin (Figure 7).

Sexual behavior test showed that animals exposed to rosuvastatin at doses of 10mg/Kg ware less receptive to male mounting than control animals. For this test, 9 female from 10mg/Kg group were used during estrus phase, however 4 of these animals (44.4%) were not receptive to male mounting. The other 5 females (55.6%) were receptive during the test

[lordosis quotient 90.0% (75.0 - 100.0)], as did all the females from the control [n = 8; lordosis quotient 100.0% (90.0 – 100.0)] and 3mg/Kg [n = 9; lordosis quotient 90.0% (75.0 – 100.0)] groups (Kruskal-Wallis test, followed by Dunn's test. $p = 0.2612$).

Reproductive performance assessed on GD 20 showed similar results among groups (Table 2). However, placental weight was decreased on animals exposed to the highest dose of rosuvastatin, when compared to control group. Additionally, histological evaluation of placental tissues and morphometry of the basal zone did not show evidences of alterations induced by rosuvastatin exposure (Figure 8). In the same way, immunohistochemistry for PCNA in this transitory organ showed an immunostaining pattern similar among experimental groups.

4. Discussion

Cholesterol is a molecule of great relevance in reproductive physiology, because of its importance as a precursor of sex steroid hormone synthesis. Statins, a class of drugs that inhibits biosynthesis of cholesterol, may alter the normal reproductive development, during pubertal period, and function during adulthood.

Besides the beneficial effects of statins therapy, there are some adverse effects that should be considered before starting the treatment, such as myopathy, nephrotoxicity, neurologic manifestations, proinflammatory and immunogenic actions, and hepatotoxicity [30].

In this study, rosuvastatin exposure showed no evident signal of toxicity in target organs for toxicology, based on their weight. However, during pubertal period, on PND 42, liver weight was increased by rosuvastatin treatment in the higher dose. Statins present some hepatotoxicity potential, by increasing apoptosis in hepatocytes and oxidative stress in the liver [31], but in this study, the increased weight of liver might be associated with its adaptation to the rosuvastatin exposure, because alterations in this parameter were not found after a long-term exposure, on adulthood. Ahmadi et al. [32] shows that rosuvastatin exposure in rats is related to a compensatory mechanism in cholesterol biosynthesis and metabolism in the liver and extrahepatic tissues, which enforces the idea of adaptive response of the liver to the statin.

On PND 42, female rats exposed to rosuvastatin at dose of 10mg/Kg, also presented reduced hypophysis weight, data not found during adulthood. Statin exposure might affect hypophysis function, once it has potential to inhibit releasing of follicle-stimulating hormone (FSH) [33]. However, whether rosuvastatin can directly or indirectly impair hypophysis physiology and consequently its weight, it is not possible to confirm in this study, once serum gonadotropin hormone levels were not affected by the treatment. Another possibility is that

decreased hypophysis weight might be related to decreased estrogen stimulation [34], since rosuvastatin is able to alter estradiol releasing and signaling in male rats [14,15]. It is also important to note that hypophysis weight was not decreased in adult rats exposed to rosuvastatin, but their serum prolactin levels were reduced by the treatment. Thus it is fair to think that rosuvastatin might interact with lactotroph cells in the anterior hypophysis and disrupt its development and function. Additionally, once that in rodents, estrogen signaling is the major stimulatory factor for prolactin gene expression [35], it is possible that rosuvastatin exerts its effects upon estrogen releasing and signaling.

During puberty, hypothalamic-hypophysis-ovarian axis becomes active and is crucial to the occurrence of normal morphological, physiological, behavioral and psychological changes in the female [36]. This period is also considered a biological sensor for abnormalities derived from genetic and environmental interactions during pre- and postnatal development [37]. External markers of pubertal development are relevant to investigate the time of puberty onset. Vaginal opening and first occurrence of estrus in rats are indicators of puberty onset, derived from an increased level of estrogen in blood [38]. These events are also associated with the first ovulation on female rats [37].

Rosuvastatin exposure since pre-puberty delays onset of puberty in male rats as shows by Leite et al. [13]. However, in this study this statin did not alter the timing of puberty in female rats, as well as did not affect the serum levels of reproductive hormones, ovarian follicular dynamics and uterine morphology during puberty. Despite these data indicate that rosuvastatin might lead to gender-specific alterations on pubertal development, absence of estradiol levels measurements increase the difficulty of evaluation and understanding of rosuvastatin effects during this period.

Assessment of estrous cyclicity is a way to evaluate integrity of the hypothalamic-hypophysis-ovarian axis and consequently female reproductive function [39]. In this study,

estrous cycle was altered by rosuvastatin exposure, once animals treated with the highest dose of the statin presented shorter cycles than control group. On the other hand, during adulthood, serum levels of FSH, LH, progesterone and testosterone were not affected by the treatment with rosuvastatin, as well as ovarian and uterine histological endpoints and reproductive organ weights. Guerra et al. [26,40] showed that reproductive function can be affected not necessarily by the alterations in serum levels of steroid hormones, but by their signaling pathways mediated by cell receptors, which expression pattern can be altered in different cells and tissues. Thus, impaired reproductive cyclicity of female rats exposed to rosuvastatin might derive of an abnormal hormonal axis signaling.

Alterations in hormonal signaling caused by rosuvastatin exposure can also impair sexual behavior, once females presented disrupted reproductive cycles. Furthermore, another factor that could be influencing reproductive behavior in female rats treated with the highest dose of rosuvastatin is the uterine physiology, which is highly dependent of steroid hormones [41]. Studies with ovariectomized rats show that impaired hormone binding capacity in the uterine tissue is related to inhibitory effects in female receptivity [42], which explains the absence of lordosis quotient in part of the female rats as observed in this study.

A recent study from our Lab showed that reproductive parameters such as sexual behavior and epididymal morphology were affected after a long-term exposure to rosuvastatin with no effect on serum testosterone levels [43], which corroborates the idea that rosuvastatin might disrupts hormonal signaling through interactions with steroid receptors.

In this study, treatment with rosuvastatin in female rats started at pre-pubertal period ending at adulthood, before confirmation of pregnancy, due to the fact that statins therapy is not indicate during gestational period [44]. By the end of the gestational period, no evident signals of maternal toxicity and fertility impairment of rosuvastatin exposure were found, as well as any evidence of fetal growth restriction or impairment to offspring intrauterine

development. However, placental weight of animals exposed to the highest dose of this statin was reduced compared to control group. Studies of rosuvastatin exposure in male rats show that post-implantation loss are increased by this statin due to decreased sperm quality [45], however other study of Dostal et al. [46] with atorvastatin, another statin, did not show evidence of impairment in both male and female rats fertility. Considering this, it is fair to suppose that rosuvastatin might lead to fertility impairments in a gender-specific manner.

Placenta corresponds to the maternal-fetal interface for exchange of substances. In rats, the placenta becomes completely functional at midgestation and grows continuously up to last few days before parturition, when placental weight stays relatively stable [47]. Alterations in the placental weight, might compromise the fetal development, being associated with fetal reprograming effects [48].

Histologically, placenta is composed by four distinct parts; two of them constitute the fetal components while the other two corresponds to the maternal components of placenta. Maternal components of placenta correspond to the decidua and metrial gland, both derived from the endometrium. Fetal components of placenta are the labyrinth zone, where maternal and fetal exchanges occur; and the basal zone (also known as Trophospongium), formed by three different types of trophoblastic cells: the spongiotrophoblast cells, the glycogen cells and the trophoblast giant cells. These trophoblast giant cells exert important roles for maintenance of pregnancy, such as endocrine function and releasing of factors and molecules that promote local and systemic physiological maternal adaptations throughout pregnancy [47,49].

In this study, placental histology of rosuvastatin-exposed animals was not altered. Even cell proliferation status, assessed by immunostaining with PCNA marker did not show any signs of placental tissue impairment, especially on the basal zone. Despite the absence of alterations in fetus weight, further investigations are necessary to conclude whether

rosuvastatin has potential to promote fetal reprograming through placental damaging related to apoptosis or to other cell mechanisms.

In conclusion, the present study suggests that the exposure of female rats to rosuvastatin, since pre-puberty, acted, at some extent, as an endocrine disruptor, disrupting hormonal axis signaling, and consequently reproductive function. Additional studies on the effects of this statin on female reproductive development and function are encouraged.

5. Declaration of Interest

The authors declare that there is no conflict of interests.

6. Acknowledgments

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Tables

Table 1. Body and organ weights of female rats during estrus phase on postnatal days 42 and 75.

Parameters	**Experimental Groups**		
Postnatal Day 42 (n = 6-7/group)	**Control**	**3 mg/Kg**	**10 mg/Kg**
Final body weight (g)	144.6 ± 6.2	151.2 ± 6.3	142.4 ± 5.1
Relative organ weights			
Ovaries (mg/100g)	38.06 ± 3.8	45.07 ± 7.0	45.33 ± 4.8
Uterus with fluid (mg/100g)	187.8 ± 35.9	187.0 ± 19.1	170.8 ± 8.4
Hypophysis (mg/100g)	4.21 ± 0.2	4.73 ± 0.7	2.67 ± 0.4 *
Thyroid (mg/100g)	9.30 ± 1.4	8.48 ± 0.6	7.04 ± 0.7
Liver (g/100g)	5.07 ± 0.07	5.38 ± 0.11	5.73 ± 0.14 **
Adrenals (mg/100g)	36.35 ± 4.3	33.12 ± 4.2	34.32 ± 1.3
Kidneys (g/100g)	1.01 ± 0.03	1.06 ± 0.01	1.06 ± 0.02
Postnatal Day 75 (n = 7/group)	**Control**	**3 mg/Kg**	**10 mg/Kg**
Final body weight (g)	222.4 ± 8.7	215.3 ± 5.1	230.1 ± 8.6
Relative organ weights			
Ovaries (mg/100g)	42.02 ± 4.1	38.76 ± 4.8	42.55 ± 1.9
Uterus with fluid (mg/100g)	218.3 ± 38.8	218.7 ± 30.0	243.5 ± 40.7
Hypophysis (mg/100g)	5.99 ± 0.5	5.59 ± 0.4	6.02 ± 0.9
Thyroid (mg/100g)	8.07 ± 1.2	7.43 ± 0.5	7.99 ± 0.7
Liver (g/100g)	4.20 ± 0.07	4.11 ± 0.07	4.32 ± 0.08
Adrenals (mg/100g)	45.33 ± 2.3	42.99 ± 0.6	46.82 ± 2.2
Kidneys (g/100g)	0.88 ± 0.02	0.89 ± 0.03	0.92 ± 0.02

Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. *$p \leq 0.05$, **$p < 0.01$ compared to control group.

Table 2. Reproductive performance and fertility test assessed on gestational day 20.

Parameters	Experimental Groups		
	Control (n = 9)	**3 mg/Kg** (n = 8)	**10 mg/Kg** (n = 5)
Gestational rate (%)	88.8	75.0	80.0
[2]Fertility potential (%)	96.88 (92.98 - 100.00)	96.16 (87.98 - 100.00)	96.16 (90.58 - 100.00)
[1]Number of Implantations	12.7 ± 0.7	12.5 ± 0.8	10.7 ± 0.6
[1]Number of corpora lutea	13.5 ± 0.8	13.3 ± 0.4	11.2 ± 0.6
[1]Number of fetuses	12.2 ± 0.6	12.2 ± 0.6	10.7 ± 0.6
[1]Number of resorptions	0.5 ± 0.3	0.3 ± 0.3	0.0 ± 0.0
[2]Pre-implantation loss (%)	3.1 (0.0 - 7.0)	3.8 (0.0 - 12.0)	3.8 (0.0 - 9.4)
[2]Post-implantation loss (%)	0.0 (0.0 - 6.8)	0.0 (0.0 - 3.3)	0.0 (0.0 - 0.0)
[1]Gravid uterus weight (g)	71.8 ± 4.5	70.0 ± 3.9	63.3 ± 2.7
[1]Fetal weight (g)	4.05 ± 0.10	3.89 ± 0.14	4.05 ± 0.09
[1]Placental weight (g)	0.66 ± 0.02	0.62 ± 0.03	0.56 ± 0.02*
[1]Sex ratio (F:M)	0.91 ± 0.1	1.12 ± 0.3	0.71 ± 0.2

Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. *$p \leq 0.05$ compared to control group. [2]Values expressed as median and interquartile intervals. Kruskal-Wallis test, followed by Dunn's test. $p > 0.05$.

Figure legends

Figure 1. Experimental design of the study.

Figure 2. Evaluation of external signs of puberty onset. (A) Ages of vaginal opening and first estrus (n = 25/group) and (B) body weight at these ages. Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. $p > 0.05$.

Figure 3. Evolution of body weight of female rats euthanized on postnatal days 42 or 75, after treatment with rosuvastatin or vehicle (n = 6-7/group). Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. $p > 0.05$.

Figure 4. Serum hormonal levels of female rats, during estrus phase on PND 42 (n = 6-7/group) and 75 (n = 7/group). Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. $p > 0.05$. PND (Postnatal Day).

Figure 5. Histological evaluation of ovary and uterus of female rats, during the estrus phase on postnatal day 42. (A-C) Histological aspect of ovaries. (D-F) Histological aspect of uterus. (G) Follicular counting on ovarian sections (n = 5-6/group). Values expressed as mean ± S.E.M. Kruskal-Wallis test, followed by Dunn's test. $p > 0.05$. (H) Histomorphometric measurements of uterine layers (n = 6-7/group). Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. $p > 0.05$.

Figure 6. Evaluation of estrous cycle during 15 consecutive days (n = 17-18/group). Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. *$p \leq 0.05$ compared to control group.

Figure 7. Histological evaluation of ovary and uterus of female rats, during the estrus phase on postnatal day 75. (A-C) Histological aspect of ovaries. (D-F) Histological aspect of uterus. (G) Follicular counting on ovarian sections (n = 5-6/group). Values expressed as mean ± S.E.M. Kruskal-Wallis test, followed by Dunn's test. $p > 0.05$. (H) Histomorphometric measurements of uterine layers (n = 7/group). Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. $p > 0.05$.

Figure 8. Histological evaluation of placenta of pregnant female rats on gestational day 20. (A-C) Histological aspect of placentas. (D-F) Immunostaining for Proliferating Cell Nuclear Antigen (PCNA) on the cells of placenta's basal zone. (G) Histological organization of cell on the basal zone. (H) Histomorphometric measurement of basal zone (n = 4-5/group). Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. $p > 0.05$.

Figure 1.

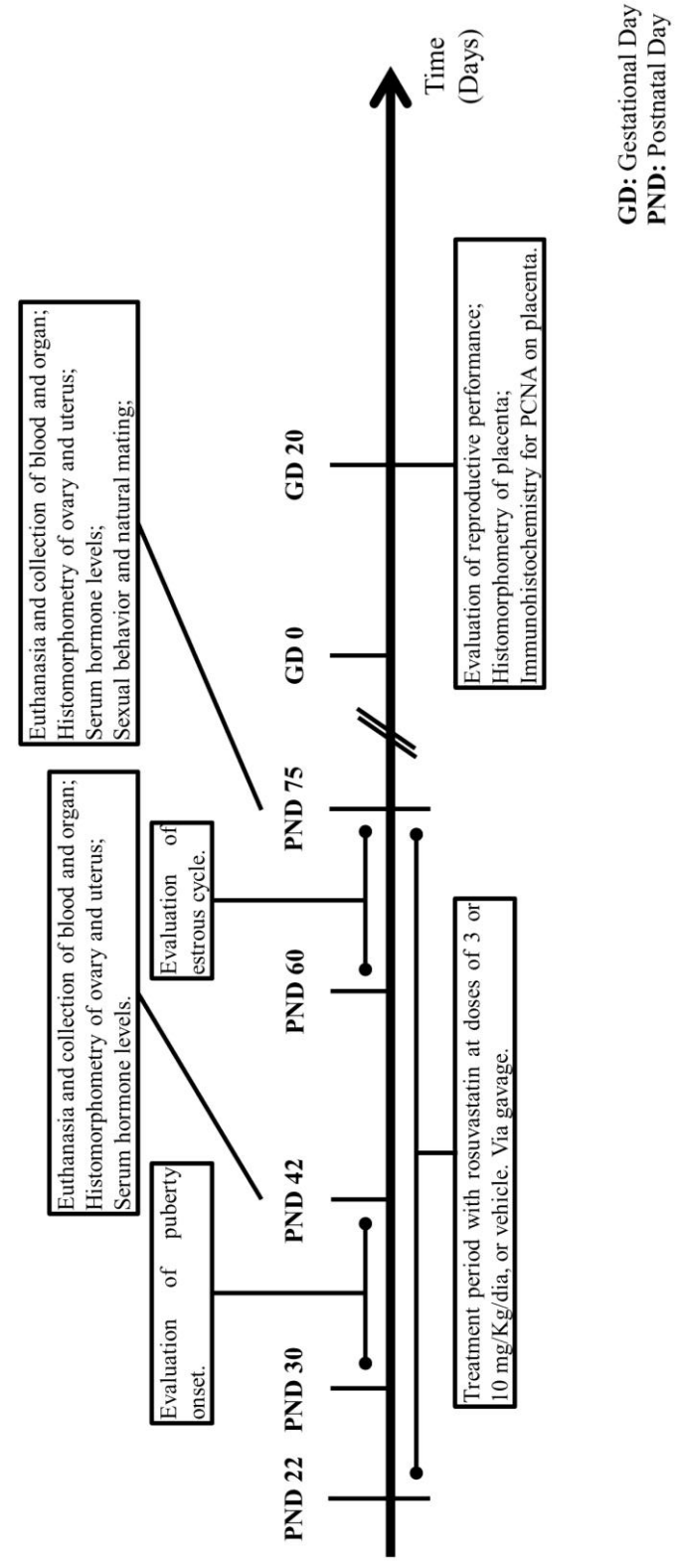

PND 22
PND 30
PND 42
PND 60
PND 75
GD 0
GD 20
Time (Days)
Evaluation of puberty onset.
Euthanasia and collection of blood and organ; Histomorphometry of ovary and uterus; Serum hormone levels.
Evaluation of estrous cycle.
Euthanasia and collection of blood and organ; Histomorphometry of ovary and uterus; Serum hormone levels; Sexual behavior and natural mating;
Treatment period with rosuvastatin at doses of 3 or 10 mg/Kg/dia, or vehicle. Via gavage.
Evaluation of reproductive performance; Histomorphometry of placenta; Immunohistochemistry for PCNA on placenta.
GD: Gestational Day
PND: Postnatal Day

Figure 2.

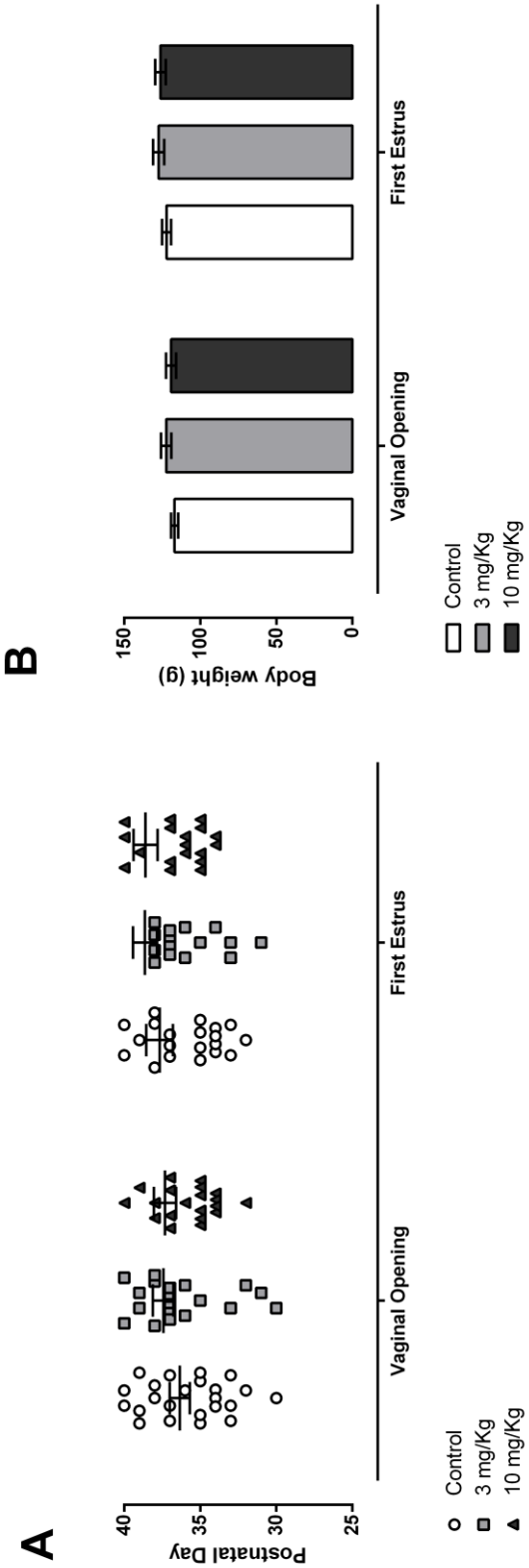

A
Postnatal Day
40
35
30
25
Vaginal Opening
First Estrus
Control
3 mg/Kg
10 mg/Kg
B
Body weight (g)
150
100
50
0
Vaginal Opening
First Estrus
Control
3 mg/Kg
10 mg/Kg

Figure 3.

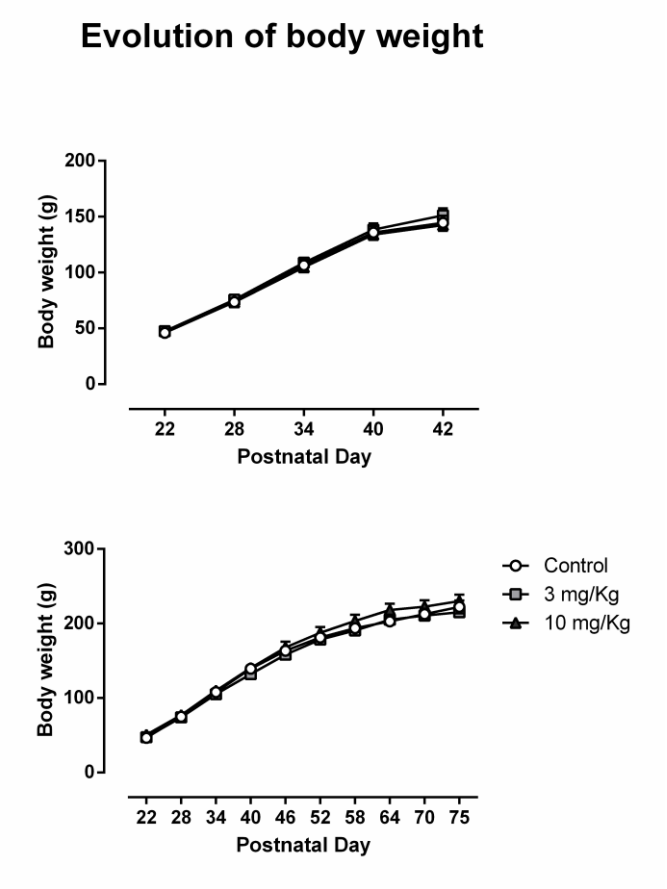

Evolution of body weight
Body weight (g)
200
150
100
50
0
22
28
34
40
42
Postnatal Day
Body weight (g)
300
200
100
0
22 28 34 40 46 52 58 64 70 75
Postnatal Day
Control
3 mg/Kg
10 mg/Kg

Figure 4.

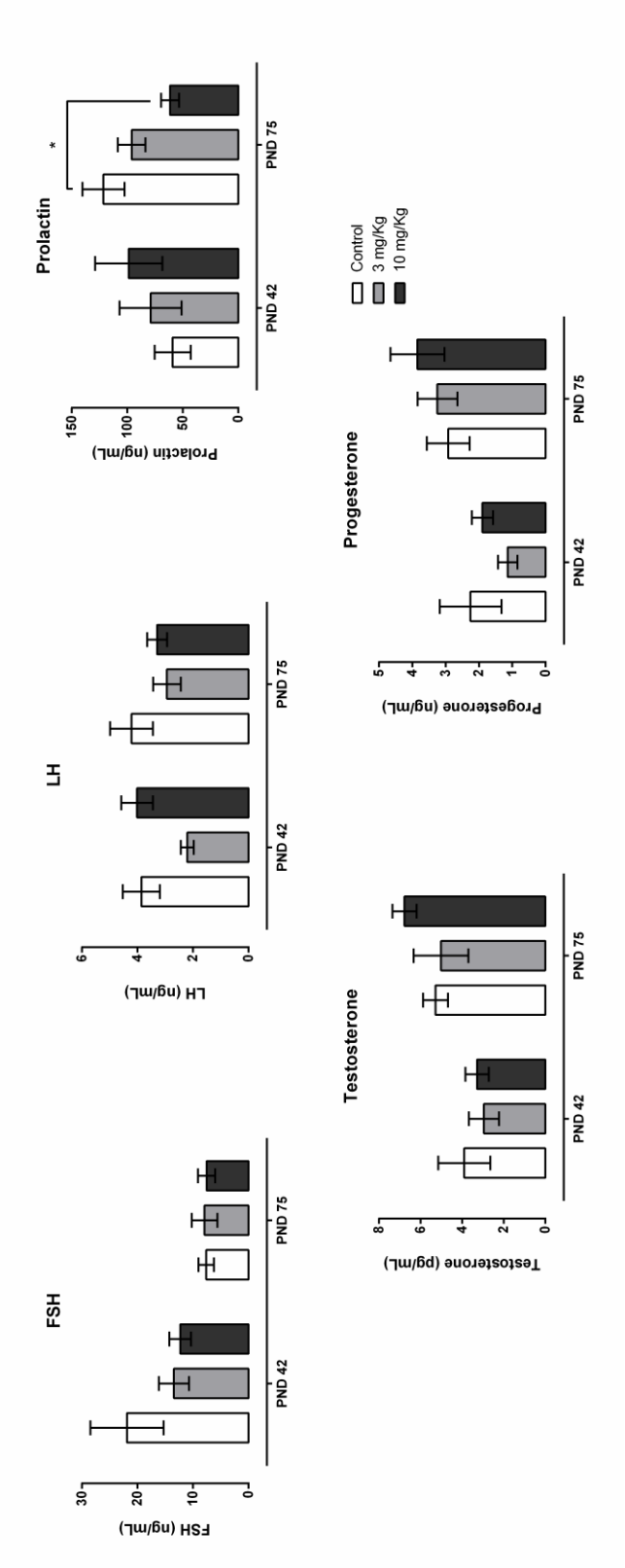

Prolactin
Prolactin (ng/mL)
150
100
50
0
*
PND 42
PND 75
Control
3 mg/Kg
10 mg/Kg
LH
LH (ng/mL)
6
4
2
0
PND 42
PND 75
Progesterone
Progesterone (ng/mL)
5
4
3
2
1
0
PND 42
PND 75
FSH
FSH (ng/mL)
30
20
10
0
PND 42
PND 75
Testosterone
Testosterone (pg/mL)
8
6
4
2
0
PND 42
PND 75

Figure 5.

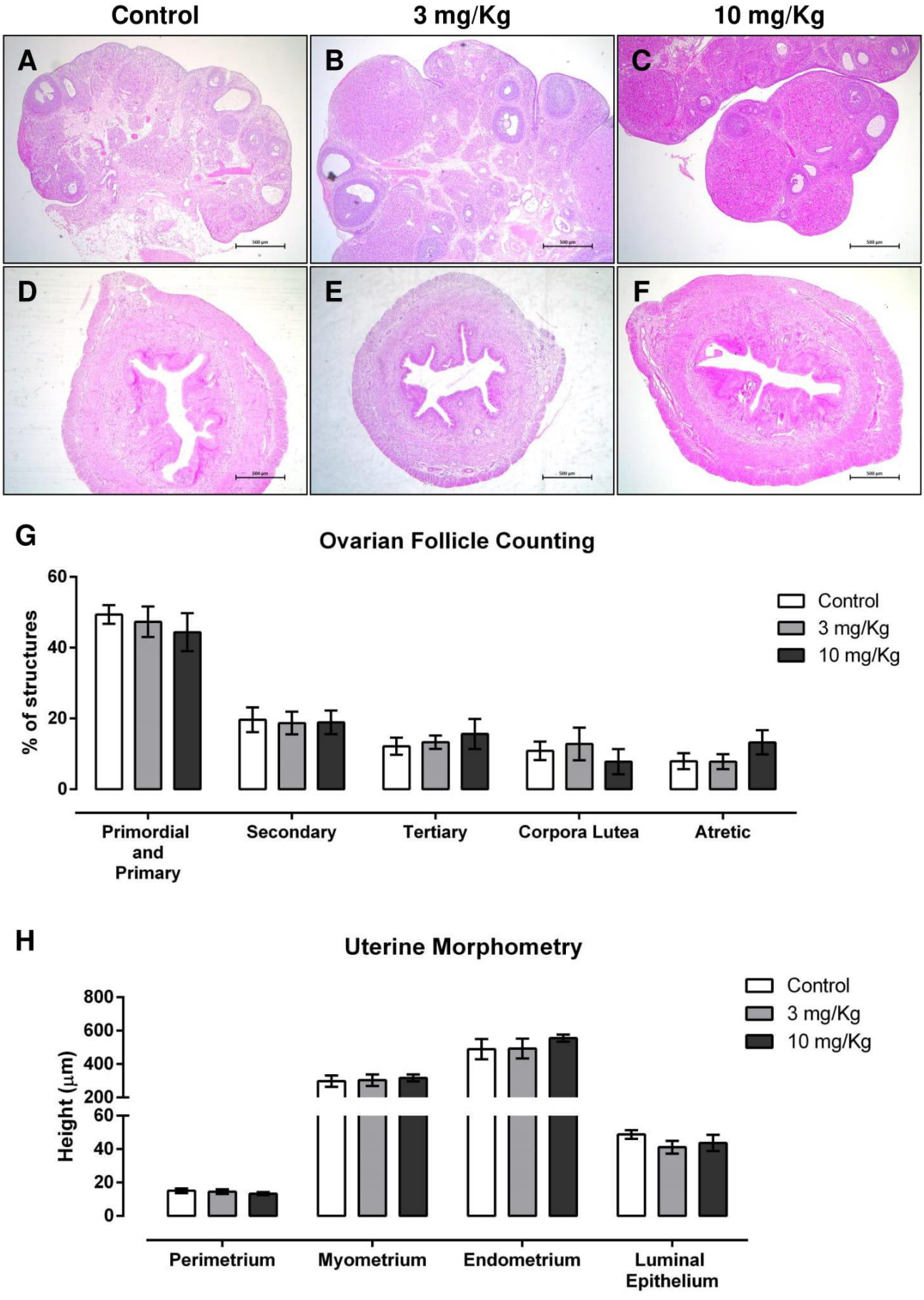

Control
3 mg/Kg
10 mg/Kg
A
B
C
D
E
F
G
Ovarian Follicle Counting
% of structures
60
40
20
0
Control
3 mg/Kg
10 mg/Kg
Primordial and Primary
Secondary
Tertiary
Corpora Lutea
Atretic
H
Uterine Morphometry
Height (µm)
800
600
400
200
60
40
20
0
Control
3 mg/Kg
10 mg/Kg
Perimetrium
Myometrium
Endometrium
Luminal Epithelium

Figure 6.

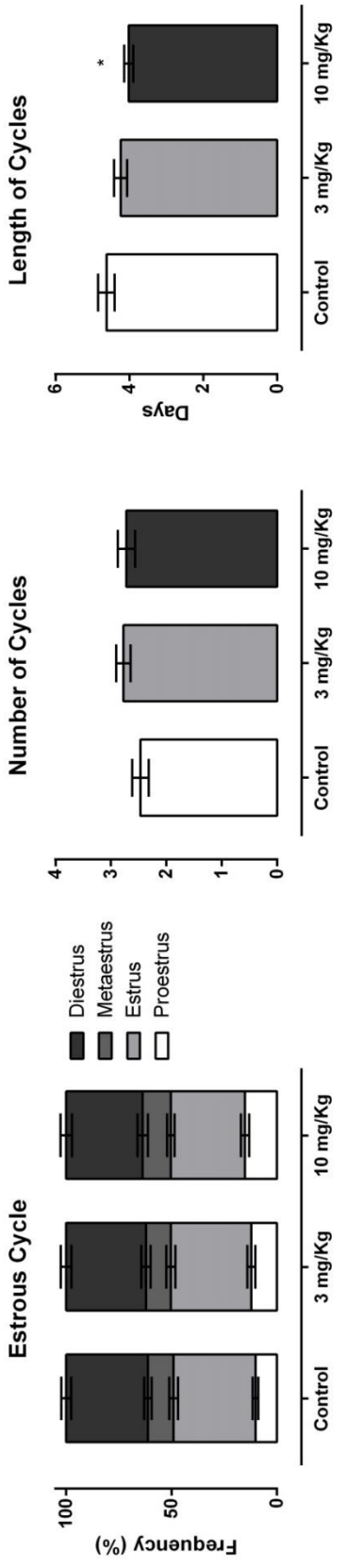
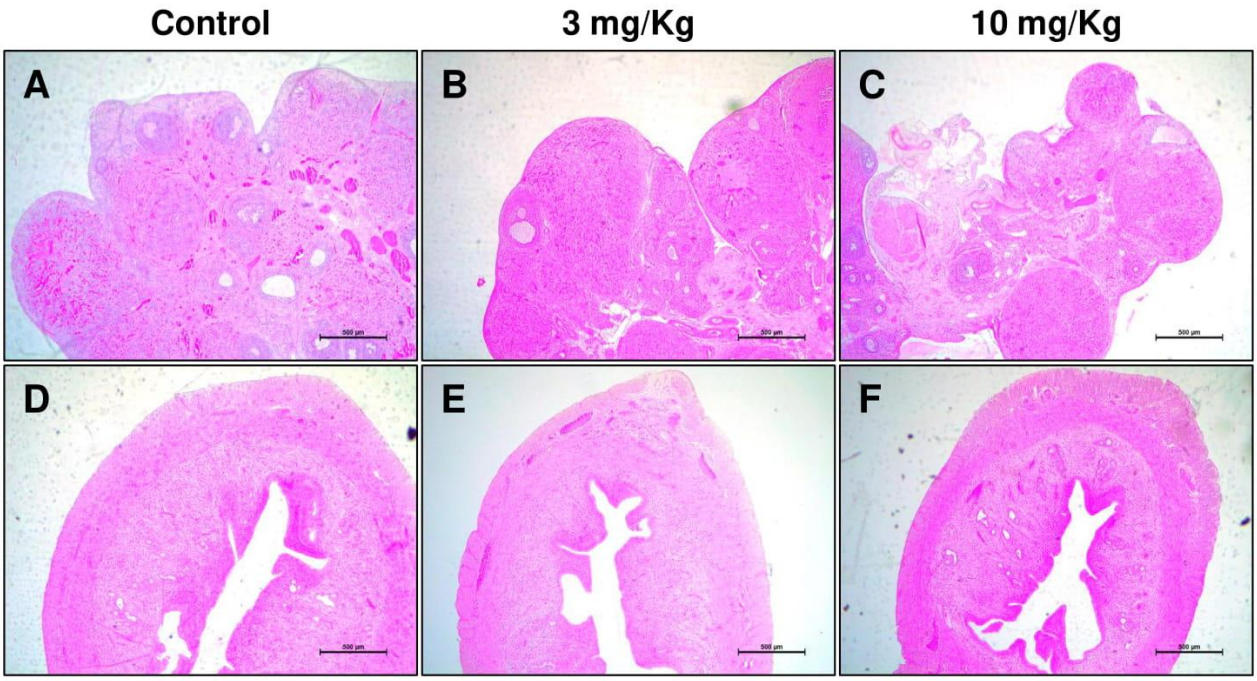
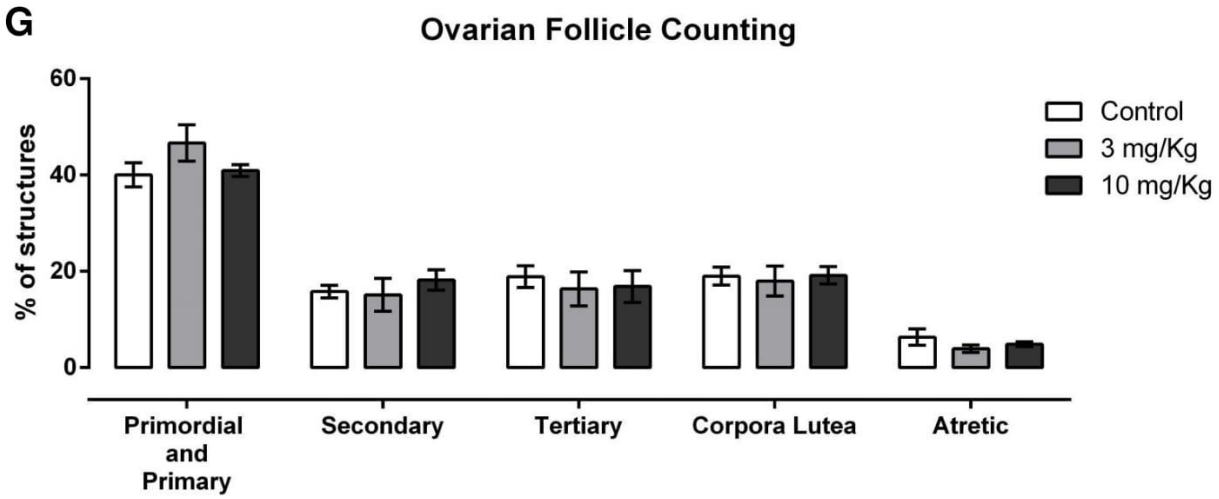

Estrous Cycle
Frequency (%)
100
50
0
Control
3 mg/Kg
10 mg/Kg
Diestrus
Metaestrus
Estrus
Proestrus
Number of Cycles
4
3
2
1
0
Control
3 mg/Kg
10 mg/Kg
Length of Cycles
Days
6
4
2
0
*
Control
3 mg/Kg
10 mg/Kg

Figure 7.


Control
3 mg/Kg
10 mg/Kg
A
B
C
D
E
F


G
Ovarian Follicle Counting
% of structures
60
40
20
0
Control
3 mg/Kg
10 mg/Kg
Primordial and Primary
Secondary
Tertiary
Corpora Lutea
Atretic

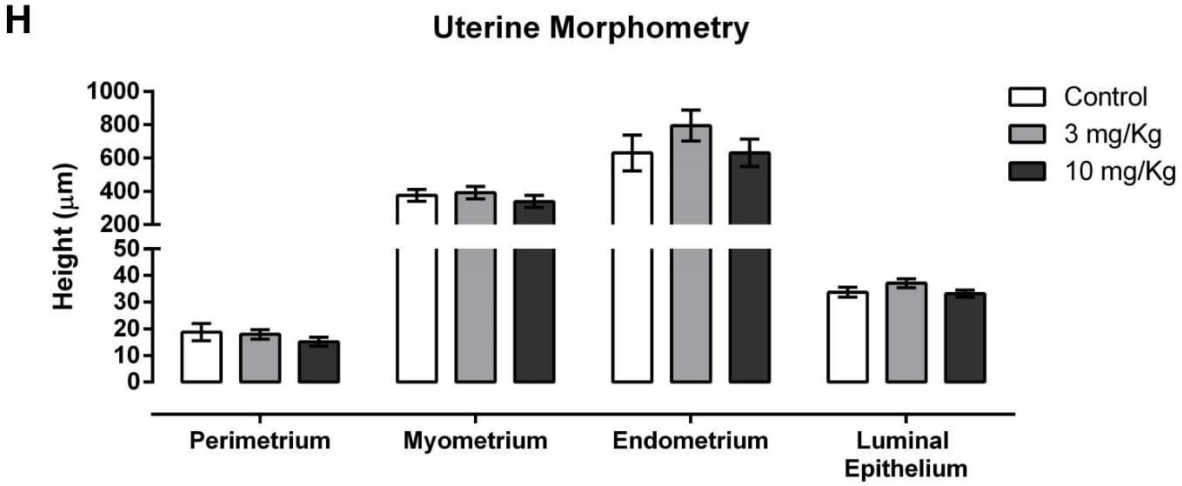
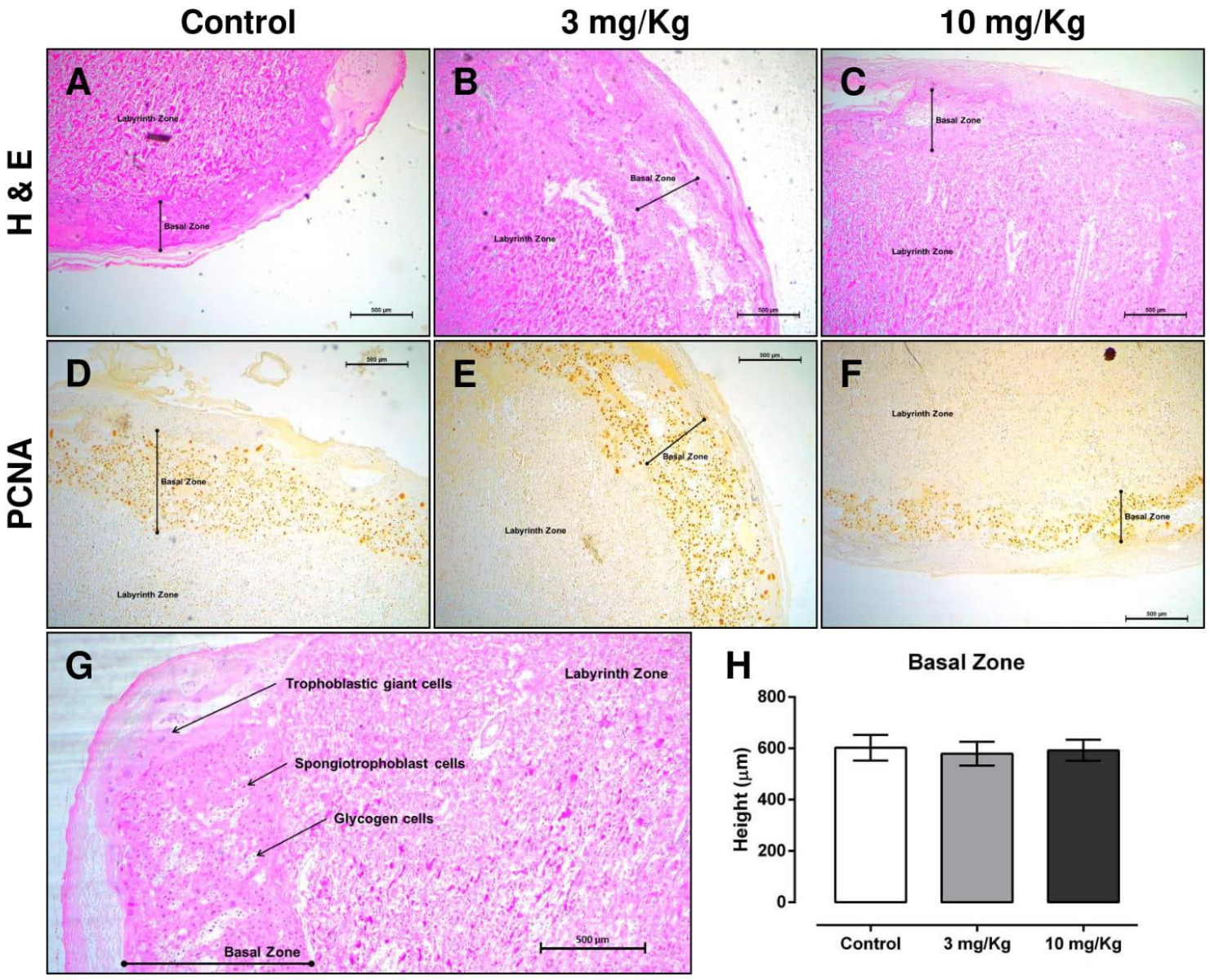

H
Uterine Morphometry
Height (μm)
1000
800
600
400
200
50
40
30
20
10
0
Control
3 mg/Kg
10 mg/Kg
Perimetrium
Myometrium
Endometrium
Luminal Epithelium

Figura 8.


Control
3 mg/Kg
10 mg/Kg
H & E
PCNA
A
B
C
D
E
F
G
H
Labyrinth Zone
Basal Zone
Trophoblastic giant cells
Spongiotrophoblast cells
Glycogen cells
500 µm
Basal Zone
Height (µm)
800
600
400
200
0
Control
3 mg/Kg
10 mg/Kg

Conclusão

Com base nos resultados obtidos em ratas Wistar, pode-se concluir que a exposição à rosuvastatina não foi diretamente relacionada a mecanismos de ação associados às vias estrogênicas ou antiestrogênicas. Por outro lado, o tratamento desde a pré-puberdade, com essa estatina alterou a função reprodutiva, o que indica possíveis efeitos ligados à produção e à sinalização estrogênica. Além disso, os mecanismos de ação da rosuvastatina também podem estar relacionados à modulação da atividade contrátil uterina.

Desta forma, os resultados aqui obtidos permitem uma maior compreensão dos efeitos da rosuvastatina sobre a função reprodutiva feminina e levantam o questionamento sobre a translação dos efeitos dessa e de outras estatinas para a saúde reprodutiva do ser humano.

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Apêndice

Parâmetros avaliados durante a prenhez

Em caráter adicional, foram realizadas algumas avaliações com as fêmeas tratadas da pré-puberdade (DPN 22) até a idade adulta (DPN 75), durante o período gestacional. As avaliações realizadas foram o acompanhamento da evolução do peso corpóreo; o ganho de peso materno líquido, descontado do peso do útero gravídico; a pesagem de órgãos vitais e reprodutores ao final da prenhez (DG 20); e a dosagem dos níveis séricos de prolactina, progesterona, testosterona e prolactina no DG 20.

Resultados obtidos

O tratamento com rosuvastatina desde a pré-puberdade até a idade adulta não está relacionado com alterações na evolução do peso corporal, durante a gestação, bem como no ganho de peso bruto, ao final da prenhez (Figura 1). Além disso, o peso corpóreo final e o ganho de peso líquido (desconsidera-se o peso do útero gravídico, incluindo tecido uterino + fetos + placenta) não se mostram afetado pela exposição à rosuvastatina (Figura 2). O peso de órgãos vitais e reprodutores também não se mostram alterados pelo tratamento crônico com rosuvastatina (Tabela 1). Adicionalmente, também não foram evidenciadas alterações nos níveis séricos de hormônios como a progesterona, a testosterona e a prolactina (Figura 3).

Desta forma, evidencia-se que a exposição prolongada à rosuvastatina não está associada a efeitos toxicológicos gerais para ratas não expostas diretamente durante a gestação. Porém é importante ressaltar que a investigação de parâmetros toxicológicos adicionais ainda se faz necessária para se concluir sobre os efeitos diretos e indiretos da rosuvastatina sobre a saúde materna.

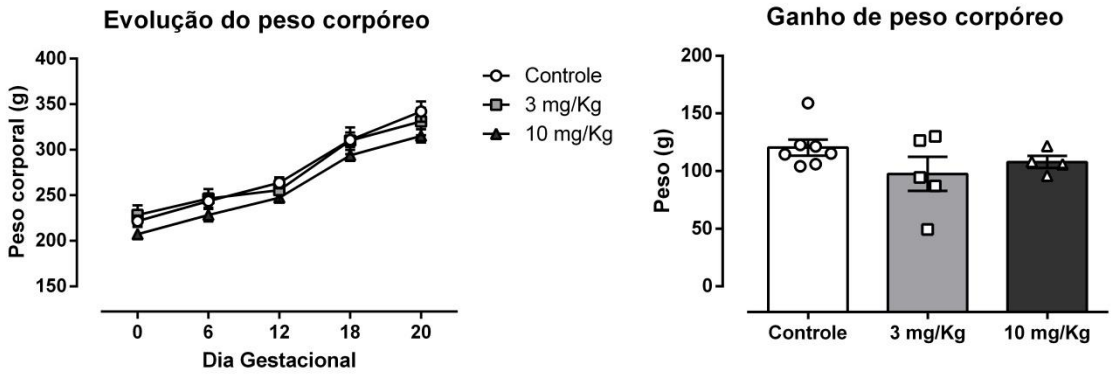

Figura 1. Avaliação da evolução do peso corpóreo e do ganho de peso ao final da gestação de ratas prenhes, tratadas desde a pré-puberdade até a idade adulta com rosuvastatina ou salina (n = 4-7/grupo). Valores expressos em média ± E.P.M. Teste de ANOVA seguida pelo teste de Tukey. $p > 0,05$.

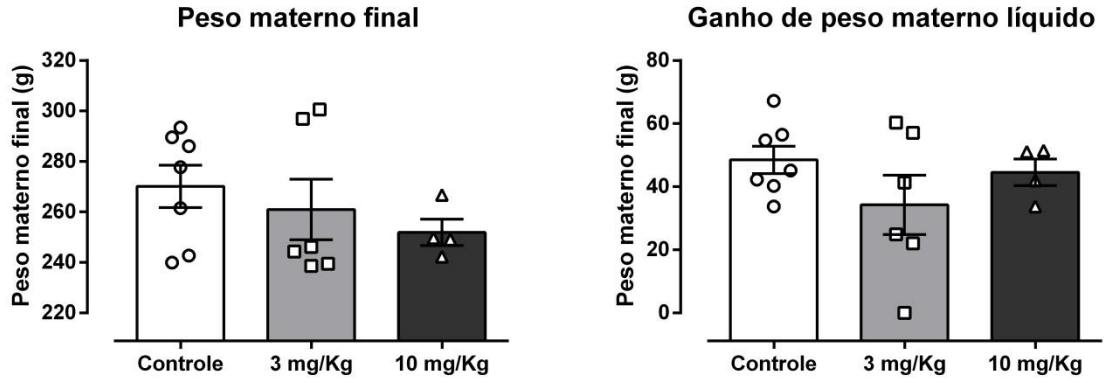

Figura 2. Avaliação do peso materno final e do ganho de peso líquido (desconsiderando-se o peso do útero gravídico), ao final da gestação de ratas prenhes, tratadas desde a pré-puberdade até a idade adulta com rosuvastatina ou salina (n = 4-7/grupo). Valores expressos em média ± E.P.M. Teste de ANOVA seguida pelo teste de Tukey. $p > 0,05$.

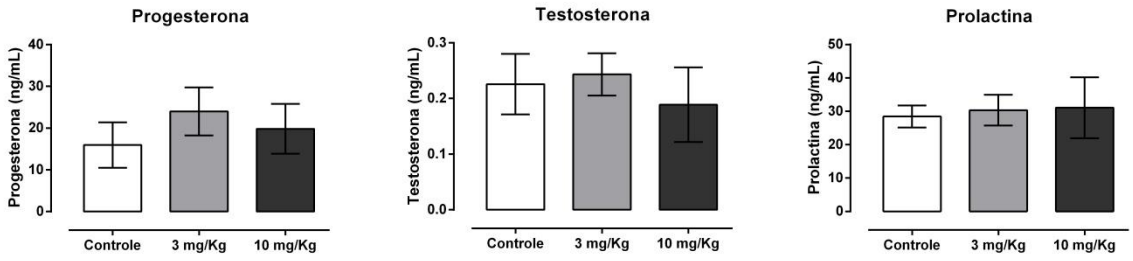

Figura 3. Níveis séricos de progesterona, testosterona e prolactina ao final da gestação, de ratas prenhes, tratadas desde a pré-puberdade até a idade adulta com rosuvastatina ou salina (n = 4-7/grupo). Valores expressos em média ± E.P.M. Teste de ANOVA seguida pelo teste de Tukey. $p > 0,05$.

Tabela 1. Efeitos da exposição à rosuvastatina desde a pré-puberdade até a idade adulta, sobre o peso corporal e de órgãos, aos 20 dias de prenhez.

Parâmetro	**Grupos experimentais**		
	Controle (n = 7)	**3 mg/Kg** (n = 6)	**10 mg/Kg** (n = 4)
Peso corpóreo final (g)	342,0 ± 11,1	331,1 ± 15,0	315,3 ± 7,3
Pesos relativos			
Ovários (mg/100g)	103,0 ± 9,8	109,7 ± 6,4	106,8 ± 15,9
Útero gravídico (g/100g)	20,96 ± 0,9	21,16 ± 0,6	20,07 ± 0,5
Hipófise (mg/100g)	11,08 ± 1,4	13,02 ± 1,1	11,85 ± 1,0
Tireoide (mg/100g)	16,62 ± 2,0	20,03 ± 3,2	17,23 ± 2,6
Fígado (g/100g)	15,04 ± 0,6	15,27 ± 0,8	14,28 ± 0,3
Adrenais (mg/100g)	101,2 ± 5,4	117,9 ± 7,4	109,4 ± 7,7
Rins (g/100g)	1,95 ± 0,08	1,92 ± 0,09	1,81 ± 0,05

Valores expressos em média ± E.P.M. Teste de ANOVA seguida pelo teste de Tukey. $p > 0,05$.

Anexos

Anexo I - Certificado de aprovação da pesquisa pela Comissão de Bioética

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

Instituto de Biociências de Botucatu - Unesp

Certificado

Certificamos que o projeto intitulado "Efeitos da exposição ao agente hipolipemiante rosuvastatina sobre a função reprodutiva de ratas Wistar", Protocolo nº **1089-CEUA**, sob a responsabilidade de **Wilma De Grava Kempinas** e colaboração de **Jorge Willian Franco de Barros**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 9 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela ***COMISSÃO DE ÉTICA NO USO DE ANIMAIS*** (CEUA), nesta data.

Finalidade:	( ) Ensino	(x) Pesquisa Científica
Vigência do Projeto:	Início: 1/6/2018	Término: 29/2/2020
Espécie/linhagem:	*Rato wistar*	
Nº de animais:	*136*	
Peso:	*60-280g*	Idade: 21-110 dias
Sexo:	*Macho e fêmea*	
Origem	*Biotério Central da Unesp – Câmpus de Botucatu/SP*	

Botucatu, 4 de maio de 2018.

Comissão de Ética no Uso de Animais IBB-Unesp

Prof. Dr. Bruno Cesar Schimming
Presidente da CEUA

Instituto de Biociências – Diretoria Técnica Acadêmica
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Anexo II - Declaração de que a dissertação ou tese não infringe os dispositivos da lei nº 9610/98, nem o direito autoral de qualquer editora

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As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **EFEITOS DA EXPOSIÇÃO AO AGENTE HIPOLIPEMIANTE ROSUVASTATINA SOBRE A FUNÇÃO REPRODUTIVA DE RATAS WISTAR**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 09 de abril de 2020.

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Nome do(a) autor(a): **Jorge Willian Franco de Barros**
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