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

DESENVOLVIMENTO REPRODUTIVO DE RATOS MACHOS EXPOSTOS AO AGENTE HIPOLIPEMIANTE ROSUVASTATINA

GABRIEL ADAN ARAÚJO LEITE

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

Profa. Dra. Wilma De Grava Kempinas

BOTUCATU – SP
2014

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

UNIVERSIDADE ESTADUAL PAULISTA

"Julio de Mesquita Filho"

INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

DESENVOLVIMENTO REPRODUTIVO DE RATOS MACHOS EXPOSTOS AO AGENTE HIPOLIPEMIANTE ROSUVASTATINA

GABRIEL ADAN ARAÚJO LEITE

PROFA. DRA. WILMA DE GRAVA KEMPINAS

Dissertação apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Mestre no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração *Biologia Celular, Estrutural e Funcional*

Profa. Dra. Wilma De Grava Kempinas

BOTUCATU – SP
2014

Instituto de Biociências

unesp

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

PG-BGA

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. DE AQUIS. E TRATAMENTO DA INFORM.
DIVISÃO DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSEMEIRE APARECIDA VICENTE - CRB 8/5661

Leite, Gabriel Adan Araújo.
Desenvolvimento reprodutivo de ratos machos expostos ao agente hipolipemiante rosuvastatina / Gabriel Adan Araújo Leite. – Botucatu, 2014.

Dissertação (Mestrado) – Universidade Estadual Paulista, Instituto de Biociências de Botucatu
Orientadora: Wilma De Grava Kempinas
Assunto CAPES: 20600003

1. Hipolipemiantes 2. Hiperlipidemia 3. Enzimas 4. Reprodução 5. Toxicologia - Modelos animais.

Palavras-chave: Hipolipemiantes; Reprodução; Rosuvastatina; Toxicologia.

Dedicatória

"Dedico este trabalho aos meus pais Vera Aparecida de Araújo Leite e Milton Carlos Ferreira Leite, por todo amor e carinho recebidos, e pelo apoio e incentivo sempre constantes para que este objetivo fosse alcançado."

Agradecimentos

Agradeço a Deus, por sempre estar ao meu lado me ajudando e amparando em todos os momentos de minha vida

Agradeço a minha orientadora Profa. Dra. Wilma De Grava Kempinas, pela oportunidade que me deu de tornar esse sonho realidade e por ser exemplo de dedicação e orientação.

Agradeço a todos os meus familiares, que direta ou indiretamente torceram para que esse objetivo fosse conquistado e que sempre me deram uma palavra de incentivo e apoio quando precisei.

Agradeço em especial à minha mãe, que sempre esteve comigo, mesmo que distante fisicamente, mas sempre me dando força para continuar e concluir meus objetivos e sonhos.

Agradeço ao Laboratório ReproTox, por toda ajuda e aprendizado adquirido ao longo desses dois anos.

Agradeço aos meus amigos Danilo, Douglas e Vinícius, pela amizade, por estarem sempre ao meu lado e pelo incentivo nessa importante etapa da minha vida.

Agradeço à Marciana e a Marina, pela amizade, companheirismo e por fazerem de meus dias mais alegres em Botucatu.

Agradeço à Profa. Dra. Isabel Cristina Cherici Camargo, porque um dia acreditou no meu potencial e me deu a oportunidade de começar a fazer pesquisa.

Agradeço a todos os professores que de alguma maneira contribuíram para o que sou hoje.

Agradeço aos funcionários da Pós-graduação, toda ajuda cedida e por sempre querer ajudar.

Agradeço à secretária do departamento de Morfologia, Luciana, por toda ajuda cedida.

Agradeço à Dra. Janete Aparecida Anselmo-Franci do Laboratório de Estomatologia e Fisiologia da USP - Ribeirão Preto, pela realização das dosagens hormonais.

Agradeço à Profa. Dra. Patrícia Fernanda Felipe Pinheiro e à Profa. Dra. Raquel Domeniconi, pelo auxílio e colaboração na realização da metodologia de imunohistoquímica.

Agradeço à FAPESP (2011/15065-5) pelo apoio financeiro concedido na forma de bolsa para este estudo.

Agradeço aos ratos, porque sem eles a realização deste estudo não seria possível.

Agradeço a todos que de alguma forma contribuíram para a realização deste trabalho.

SUMÁRIO

Resumo..11

Abstract..14

1. INTRODUÇÃO..16

1.1. Obesidade e dislipidemias...17

1.2. Origem, caracterização e prevalência das dislipidemias..................................18

1.3. Tratamento para as dislipidemias..20

1.4. Mecanismos de ação das estatinas...22

1.5. Diferenças entre as estatinas..23

1.6. Efeitos pleiotrópicos das estatinas...24

1.7. Efeitos adversos das estatinas...25

1.8. Rosuvastatina: uma estatina de última geração..26

1.9. Morte Celular e estatinas...27

1.10. Estatinas, gestação e lactação...30

1.11. Estatinas e reprodução..31

1.12. Puberdade e exposição aos desreguladores endócrinos..................................32

2. CAPÍTULO I..34

2.1. Abstract..36

2.2. Introduction...37

2.3. Material and Methods 38

2.4. Results..44

2.5. Discussion..46

2.6. Conclusion...49

2.7. Conflict of interest statement....49

2.8. Acknowledgments....49

2.9. References....50

2.10. Legends of figures....54

2.11. Tables and figures....56

3. CAPÍTULO II....70

3.1. Abstract....72

3.2. Introduction....73

3.3. Material and Methods 74

3.4. Results....81

3.5. Discussion....83

3.6. Acknowledgments....86

3.7. Disclosure summary....87

3.8. References....87

3.9. Legends of figures....91

3.10. Tables and figures....92

4. CONCLUSÃO....100

5. REFERÊNCIAS BIBLIOGRÁFICAS....102

Resumo

As dislipidemias têm sido frequentemente encontradas nas crianças e adolescentes devido a obesidade, maus hábitos alimentares e a falta de exercícios físicos. A rosuvastatina atua como inibidor da enzima HMG-CoA redutase e pode ser indicada para a prevenção de doenças cardiovasculares e para o tratamento das dislipidemias, devido a sua grande eficiência na redução das concentrações plasmáticas do colesterol sérico. Este estudo pretendeu investigar o desenvolvimento sexual inicial e os possíveis efeitos reprodutivos adversos na maturidade sexual decorrentes da exposição de ratos juvenis à rosuvastatina na pré-puberdade. Foram formados três grupos aleatoriamente com ratos recém-desmamados (n= 20/por grupo): grupo controle, que recebeu solução salina 0.9%, e os grupos tratados com 3 ou 10 mg/Kg de rosuvastatina por dia via gavagem, desde o dia pós-natal (DPN) 21 até a instalação da puberdade. Parte dos animais de cada grupo (n=10/por grupo) foi eutanasiada no DPN55 e os animais remanescentes foram mantidos até a maturidade sexual e foram eutanasiados no DPN110. No DPN55 foram avaliados as concentrações hormonais, histologia testicular e epididimária e expressão de receptores de andrógenos (AR) no testículo e epidídimo. Na maturidade sexual foram avaliados o comportamento sexual, concentrações hormonais, produção, morfologia e motilidade dos espermatozóides, além da histologia do testículo e epidídimo e imunohistoquímica para AR no testículo. Nos grupos tratados com rosuvastatina, os resultados demonstraram uma tendência para a diminuição das concentrações de testosterona, mas abaixo do nível de significância, bem como houve atraso na idade da instalação da puberdade e no desenvolvimento epididimário. Houve ainda alterações testiculares que podem estar relacionadas com o atraso na puberdade e a diminuição da testosterona. Na maturidade sexual, os animais que foram expostos à rosuvastatina exibiram diminuição das concentrações séricas dos hormônio folículo-estimulante (FSH), hormônio luteinizante (LH) e testosterona, atraso na latência para a primeira intromissão peniana, alterações patológicas no testículo e epidídimo e diminuição da qualidade espermática. Conclui-se que a administração de rosuvastatina à ratos juvenis não só atrasou a puberdade e o

desenvolvimento epididimário, mas também alterou o eixo hipotalâmico-hipofisário-gonadal, provocou diminuição dos níveis séricos de testosterona, prejudicou a qualidade espermática, o comportamento sexual e a morfologia testicular e epididimária na maturidade sexual.

Abstract

Dyslipidemias are frequently found in children due to obesity, bad eating habits and the lack of physical exercises. Rosuvastatin acts as an HMG-CoA reductase inhibitor and has been indicated to prevent cardiovascular diseases and to treat dyslipidemias due to its higher efficiency to reduce serum cholesterol concentrations. This study aimed to investigate initial sexual development and the possible reproductive adverse effects on sexual maturity due to juvenile male rats exposure to rosuvastatin during prepuberty. Three groups were formed with newly weaned rats (n= 20/per group): control, whose rats received saline solution 0.9%, rosuvastatin at doses of 3 or 10 mg/Kg daily by gavage, since post-natal day (PND) 21 until puberty onset. Part of each group (n=10/per group) was euthanized on PND55 and the remaining rats (n=10/per group) were maintained until sexual maturity and were euthanized on PND110. On PND55, we analyzed the hormonal concentrations, testicular and epididymal histology and the expression of androgen receptors (AR) on testis and epididymis. During sexual maturity, the parameters evaluated were sexual behavior, hormonal concentrations, sperm production, morphology and motility, besides testicular and epididymal histology and immunohistochemistry for AR. In the rosuvastatin-treated groups, the results demonstrated a trend towards a decrease in testosterone concentration, but below the significance level, as well as delays in both the age of puberty onset and in epididymal development. There were also testicular alterations that might be related to delayed puberty and decrease of serum testosterone. In the adulthood, the rosuvastatin-treated groups showed diminution in follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone concentrations, delay in the latency to the first penis intromission, pathologic alterations on testis and epididymis and decreased sperm quality. In conclusion, rosuvastatin administration to juvenile rats not only delayed puberty onset and epididymal development, but also altered hypothalamic-pituitary-gonadal axis, provoked diminution of serum testosterone concentrations, impaired sperm quality, sexual behavior and testicular and epididymal morphology at adulthood.

Introdução

1.1. Obesidade e dislipidemias

A obesidade é um dos grandes problemas emergentes de saúde pública que tem afetado adultos, crianças e adolescentes, e se apresenta como o transtorno metabólico e nutricional com maior destaque nos países desenvolvidos, afetando também os países subdesenvolvidos (Jiménez & Ferre 2011).

A etiologia da obesidade está relacionada a fatores genéticos, alterações endócrinas associadas a deficiência de hormônio do crescimento (GH), hipotireoidismo, fatores ambientais e sócio-culturais que incluem a má alimentação, o aumento da ingestão calórica e o sedentarismo, e fatores pré-natais que podem predispor à obesidade, dos quais se destacam a privação nutricional intra-uterina, a insuficiência placentária e o retardo no crescimento fetal (Seth & Sharma 2013).

Em consequência do aumento do número de obesos, as dislipidemias têm sido um achado frequente e se manifestado cada vez mais cedo na população, sendo ainda mais comum na faixa pediátrica, principalmente em crianças e adolescentes que apresentam obesidade variando de leve a moderada, o que acarreta em níveis aumentados de LDL-colesterol e VLDL-colesterol, diminuição do HDL-colesterol sérico (Jiménez & Ferre 2011). e aumento dos níveis séricos de triglicerídeos (Kwiterovich 2008).

A obesidade pediátrica, além da íntima relação com as dislipidemias, têm sido relacionada com o risco aumentado de doenças cardiovasculares, resistência a insulina, hipertensão, aceleração do processo aterosclerótico (McGill *et al.* 2002; Klop *et al.* 2013) diabetes mellitus do tipo 2, síndromes metabólicas, disfunções reprodutivas como a síndrome do ovário policístico, problemas associados ao sono e ao sistema gastrointestinal e alterações psicológicas, incluindo a depressão (Seth & Sharma 2013).

As dislipidemias podem ser desencadeadas na infância ou mais tardiamente devido ao estilo de vida inapropriado, que incluem o sedentarismo, a má alimentação e outros fatores relacionados ao estilo de vida (Izar *et al.* 2011).

1.2. Origem, caracterização e prevalência das dislipidemias

O colesterol, formado através dos isoprenóides, é um componente essencial da estrutura da membrana plasmática, é precursor dos hormônios esteroides, da vitamina D e dos sais biliares e tem grande importância na mielinização e no crescimento encefálico (Ceballos *et al.* 2008).

Além disso, o colesterol está relacionado com a instalação da puberdade e a regulação endócrina, devido a sua contribuição na síntese de hormônios esteroides, tais como andrógenos, estrógenos e progestógenos, mineralocorticóides e glicocorticóides (Ceballos *et al.* 2008).

As dislipidemias podem ter causa genética, serem desencadeadas por fatores ambientais ou mesmo surgir da associação causal de origem genética e ambiental (Campo & Carvalho 2007). O aumento do colesterol total e dos níveis de LDL-colesterol é muito comum em casos de hipercolesterolemia familiar monogênica e é encontrado em 1 a cada 500 pessoas, sendo que é herdado por padrão autossômico dominante (Cook & Kavey 2011; Jiménez & Ferre 2011).

A obesidade tem sido um dos fatores que tem aumentado a incidência das dislipidemias, no entanto, nem todas os casos de dislipidemias estão diretamente associados à obesidade, tais como os casos de hipercolesterolemia familiar que apresentam causa genética (Cook & Kavey 2011).

As dislipidemias são classificadas de acordo com o tipo de lipídio que se encontra alterado no sangue, e desta forma destacam-se quatro principais disfunções: hipercolesterolemia isolada, (somente os níveis séricos de colesterol estão aumentados) hipertrigliceridemia isolada (somente os triglicerídeos séricos estão aumentados), dislipidemia mista (correspondente a associação de hipercolesterolemia e hipertrigliceridemia) e a redução do HDL-colesterol, que

pode ainda estar associada ao aumento do LDL-colesterol e/ou aumento de triglicerídeos (SBC 2007).

Em crianças e adolescentes, os níveis de colesterol total devem ser menores do que 170 mg/dL, o LDL-colesterol menor que 110 mg/dL, o HDL-colesterol maior que 45 mg/dL e os triglicerídeos séricos devem ser menores que 75 mg/dL para crianças com até 9 anos de idade e menores que 90 mg/dL para crianças a partir de 10 anos até o começo da idade adulta, ou seja, até os 19 anos de idade (Cook & Kavey 2011).

Nos EUA, o tratamento medicamentoso para as dislipidemias segue as normas da Academia Americana de Pediatria, que recomenda que o uso de agentes hipolipemiantes seja destinado a crianças e adolescentes que apresentam LDL-colesterol maior que 190 mg/dL, desde que não haja outros fatores de risco (Cook & Kavey 2011).

Em caso de fatores de risco como obesidade, hipertensão, fumo ou histórico de doença cardiovascular familiar, o uso de medicamentos é recomendado a partir de 160 mg/dL de LDL-colesterol sérico desde que se apresente pelo menos dois desses fatores de risco, e em caso de diabetes mellitus, o nível sérico de LDL-colesterol máximo permitido é de 130 mg/dL antes de se partir para o tratamento hipolipemiante (Cook & Kavey 2011).

Alguns sinais clínicos são levados em consideração para a caracterização das dislipidemias, tais como: xantomas tuberosos tendíneos e xantomas eruptivos, especialmente nas hipercolesterolemias, deposições lipídicas em órgãos linfoides, opacificação da córnea e alterações retinianas, esta última mais comumente encontrada em casos de hipertrigliceridemia (Izar *et al.* 2011).

Nas crianças a partir dos 10 anos, recomenda-se a determinação dos níveis de colesterol total e do perfil lipídico em jejum, sendo que nas situações em que os pais ou avós apresentam caso de aterosclerose precoce, parentes de primeiro grau apresentam valores de colesterol e triglicerídeos acima dos níveis normais ou história positiva de pancreatite aguda, recomenda-se a

realização do perfil lipídico das crianças ainda mais cedo, devendo ser realizado entre os 2 e 10 anos de idade (Giuliano & Caramelli 2008).

A hipercolesterolemia é o tipo de dislipidemia onde o colesterol total e o LDL-colesterol encontram aumentados no plasma, sendo que para crianças e adolescentes a prevalência deste tipo de dislipidemia varia no mundo entre 2,9 e 33%, quando considerados os níveis séricos de colesterol total acima de 200 mg/dL (Giuliano & Caramelli 2008).

No Brasil, o panorama das hipercolesterolemias em crianças e adolescentes está entre 28 e 40%, quando o critério adotado para o aumento dos níveis de colesterol excede os 170 mg/dL (Giuliano & Caramelli 2008).

1.3. Tratamento para as dislipidemias

O tratamento farmacológico para as dislipidemias pode ser realizado através das seguintes classes de medicamentos com diferentes mecanismos de ação: estatinas, inibidores de absorção intestinal do colesterol, ácido nicotínico, ácidos graxos ômega-3 e os fibratos, derivados do ácido fíbrico (Izar *et al.* 2011), sendo que destes, as estatinas são os fármacos que têm maior destaque, devido a sua maior redução das concentrações plasmáticas de colesterol total (Jiménez & Ferre 2011).

A primeira estatina utilizada com potencial de redução dos níveis de colesterol total foi a mevastatina, a qual foi inicialmente isolada como produto metabólico de culturas de *Penicillium citrinium* e demonstrou afinidade superior pelo sítio enzimático da HMG-CoA redutase (Liao & Laufs 2005). Em seguida, a lovastatina que apresentou estrutura química semelhante a mevastatina, foi isolada da cultura de fungos das espécies *Aspergillus terreus* e *Monascus ruber* e demonstrou ação inibitória superior em relação a inibição observada pela mevastatina (Liao & Laufs 2005).

Posteriormente, a mevastatina foi retirada do mercado devido a toxicidade hepática observada em ratos, e neste contexto, surgiram as estatinas sintéticas, tais como a atorvastatina e a fluvastatina, e mais recentemente foi lançada a rosuvastatina, que pertence a nova geração de estatinas sintéticas (Liao & Laufs 2005).

Com relação aos inibidores da absorção intestinal do colesterol, destaca-se a ezetimiba que interage com a proteína transmembrana Niemann-Pick C1 Like1 (NPC1L1) que é um transportador de colesterol da membrana do enterócito na região do jejuno e portanto, diminui a captação do colesterol proveniente da alimentação via NPC1L1, reduzindo em 20% o LDL-colesterol sérico, mas não promove efeitos sobre os níveis de trigicerídeos e o HDL-colesterol séricos (Davis *et al.* 2004; Klop *et al.* 2013).

O ácido nicotínico por sua vez, inibe a lipólise dos adipócitos, o que resulta em níveis diminuídos de ácidos graxos livres, redução na síntese de VLDL-colesterol, diminuição no catabolismo do HDL-colesterol e nas concentrações de triglicerídios (15-35%), além de promover discreto aumento do HDL-colesterol (10-25%) (Klop *et al.* 2013).

Os ácidos graxos ômega-3 atuam diminuindo a síntese hepática e acumulação de triglicerídeos, levando à redução plasmática de 25 a 30% dos mesmos, através da diminuição da secreção de VLDL-colesterol (Klop *et al.* 2013).

Além disso, os ácidos graxos ômega-3 têm demonstrado aumento na conversão de VLDL-colesterol para IDL-colesterol, que pode ser um fator benéfico se forem associados aos ácidos graxos ômega-3 outra classe de medicamentos como as estatinas, por exemplo, o que aumentaria o catabolismo das lipoproteínas de muito baixa até intermediária densidade (Chan *et al.* 2002).

A última classe de medicamentos utilizados como agentes hipolipemiantes são os fibratos, os quais são agonistas dos receptores ativados de proliferação dos peroxissomos α

(PPAR- α) e regulam genes relacionados ao metabolismo lipídico através da transcrição gênica (Klop *et al.* 2013).

Os fibratos são indicados em casos de hipertrigliceridemia e reduzem os triglicerídeos em aproximadamente 30%, aumentam o HDL-colesterol em 9% e reduzem o LDL-colesterol em 8% (Rubenfire *et al.* 2010; Klop *et al.* 2013).

Além do tratamento medicamentoso para as dislipidemias é recomendado que seja realizada uma dieta apropriada e associada às mudanças no estilo de vida, tais como a realização de atividade física periodicamente e a abolição de práticas sedentárias comuns em nossa sociedade (Ostlund 2002).

Em crianças obesas que apresentam dislipidemias, a perda de peso e a prática de exercícios físicos periódicos tem auxiliado na diminuição dos níveis de triglicerídeos, no aumento do HDL-colesterol e na melhora do perfil lipídico total (Kang *et al.* 2002; Woo *et al.* 2004). Além disso, a diminuição na ingestão de carboidratos por adolescentes tem auxiliado também na redução de triglicerídeos (Sondike *et al.* 2003).

1.4. Mecanismos de ação das estatinas

As estatinas são inibidores da enzima 3-hidroxi-3-metilglutaril coenzima A redutase (HMG-CoA redutase), que é uma enzima limitante para a biossíntese do colesterol (Istvan & Deisenhofer 2001; Jiménez & Ferre 2011) e portanto, as estatinas são responsáveis por diminuir o colesterol total, reduzindo principalmente o LDL-colesterol (Istvan & Deisenhofer 2001; Tandon *et al.* 2005; Endres 2006).

O mecanismo de ação dessas drogas envolve a inibição da enzima HMG-CoA redutase no fígado, sendo esta inibição reversível e competitiva com o substrato HMG-CoA, o que resulta no aumento de receptores LDL-colesterol no hepatócito e aumenta o metabolismo de LDL-colesterol (Istvan & Deisenhofer 2001; Fonseca 2005).

As estatinas ao inibirem a enzima HMG-CoA redutase impedem a conversão da HMG-CoA em mevalonato, e consequentemente diminuem a formação do colesterol endógeno. Estes fármacos também inibem a síntese de outros compostos importantes, tais como os isoprenóides intermediários, dos quais se destacam o geranilgeranilpirofosfato (GGPP) e farnesilpirofosfato (FPP) (Istvan 2003; Adam & Laufs 2008).

1.5. Diferenças entre as estatinas

Estão disponíveis para comercialização no Brasil seis estatinas: fluvastatina, lovastatina, sinvastatina, atorvastatina, pravastatina e rosuvastatina (SBC 2007). Estes fármacos diferem entre si com relação às suas propriedades farmacológicas, tais como: afinidade pelo sítio ativo da enzima HMG-CoA redutase, taxa de entrada nos hepatócitos e em outros tecidos, disponibilidade do fármaco na circulação sistêmica para captação por tecidos extra-hepáticos e mecanismos de transformação metabólica (McTaggart 2003).

Algumas estatinas, tais como a fluvastatina, sinvastatina, lovastatina e pravastatina exercem seu maior efeito na redução do LDL-colesterol quando administradas a noite, sendo que, se administradas no período da manhã apresentam menor redução nos níveis de LDL-colesterol. Deste modo, estas estatinas apresentam sua farmacocinética relacionada ao período em que são administradas, sendo tempo e dose dependentes (Martin *et al.* 2002).

A rosuvastatina tem demonstrado capacidade similar na redução dos níveis de LDL-colesterol quando administrada em diferentes períodos do dia, diferentemente das outras estatinas, ou seja, a farmacocinética da rosuvastatina independe do seu horário de administração, e esta característica está diretamente relacionada com o seu tempo de meia-vida relativamente longo (Martin *et al.* 2002).

Em crianças e adolescentes que utilizam as estatinas para o tratamento das dislipidemais tem-se observado uma redução do LDL- colesterol que varia de 17 a 45%, aumento no HDL-

colesterol entre 1 e 11% e redução de triglicerídeos de até 17%, dependendo da estatina utilizada (Jiménez & Ferre 2011).

1.6. Efeitos pleiotrópicos das estatinas

Os efeitos pleiotrópicos das estatinas representam os efeitos benéficos promovidos por elas que independem do colesterol, e geralmente estão associados a inibição dos isoprenóides intermediários da via do mevalonato, tais como o GGPP e o FPP, os quais são metabólitos necessários para a síntese do colesterol (Adam & Laufs 2008).

Os isoprenóides intermediários apresentam grande papel sinalizador e importante função associada à determinação da forma celular, motilidade, secreção e proliferação celular(Adam & Laufs 2008).

As estatinas são indicadas para o tratamento primário e secundário de doença arterial coronária em pacientes que apresentam dislipidemias, e têm sido os fármacos de escolha para o tratamento de aterosclerose, pois apresentam duas vantagens, além de diminuírem as concentrações do colesterol sérico, estas drogas atuam na proteção do endotélio vascular contra a oxidação do LDL, promovendo deste modo, a redução no estresse oxidativo e na inflamação (Tandon *et al.* 2005; Endres 2006).

Estes fármacos atuam desempenhando outras funções, tais como a estabilização da placa aterosclerótica, melhora da função endotelial, maior biodisponibilidade de óxido nítrico e a inibição de ações imunomoduladoras, o que contribui para a prevenção de doenças cardiovasculares e diminuição da incidência de infarto (Tandon *et al.* 2005; Ludman *et al.* 2009).

Os inibidores da HMG-CoA redutase têm sido indicados como potenciais anti-inflamatórios, imunomoduladores e protetores vasculares para o tratamento de doenças cerebrais como a doença de Alzheimer, esclerose múltipla e depressão, (Corder *et al.* 1993; Campo &

Carvalho 2007) além de possuírem potencial para o tratamento da osteoporose, devido aos seus efeitos osteogênicos (Campo & Carvalho 2007).

As estatinas possuem também grande potencial de inibição de proliferação e indução de apoptose em células tumorais, sendo que em modelos animais já foram observados efeitos antitumorais contra melanoma, carcinoma mamário, fibrosarcoma, adenocarcinoma pancreático, glioma, neuroblastoma e linfoma, onde foram evidenciados o retardamento do crescimento tumoral e a inibição de processos metastáticos (Feleszko *et al.* 1998).

Estudos pré-clínicos indicam que as estatinas têm sido capazes de potencializar os efeitos antitumorais das citocinas e de quimioterápicos como a cisplatina, 5-fluorouracil, e doxorubicina (Feleszko *et al.* 1998).

As estatinas têm sido capazes de inibir a proliferação de linhagens de células normais, entretanto os efeitos antiproliferativos parecem ser mais potentes em células tumorais do que nas linhagens de células normais já testadas, que incluem fibras musculares lisas e estriadas, células endoteliais, fibroblastos, pneumócitos e linfócitos B (Campo & Carvalho 2007).

1.7. Efeitos adversos das estatinas

Apesar do amplo espectro para a utilização das estatinas, há relatos de que elas causem efeitos colaterais como alterações hepáticas e miopatias, que compreendem as mialgias, miosite e rabdomiólise, onde o grande problema acarretado pela rabdomiólise é a insuficiência renal aguda (Istvan & Deisenhofer 2001; Rosenson 2004). Outros efeitos adversos já foram relatados, tais como: diarreia, dores abdominais e dispepsias, elevação de enzimas hepáticas e cefaleias (Ceballos *et al.* 2008).

Em crianças especificamente, têm sido relatado que as estatinas apresentam outros efeitos colaterais como diarreia, dores abdominais, prurido e transtornos do sono, porém os efeitos adversos promovidos pelo uso das estatinas não parecem ser maiores nas crianças do que nos adultos (Jiménez & Ferre 2011).

Além disso, constatou-se também que doses mais elevadas de cada estatina podem levar ao aumento do risco de miopatias sendo, portanto dose-dependentes, e o risco de toxicidade relacionado ao uso das estatinas pode aumentar consideravelmente com a associação de fármacos que tenham potencial de interação farmacocinética, como ocorre no caso de tratamento concomitante de genfibrozila (fibrato) com rosuvastatina ou sinvastatina, levando ao aumento de risco de rabdomiólise (Gotto 2006).

1.8. Rosuvastatina: uma estatina de última geração

Em comparação com as outras estatinas comercializadas no Brasil, a rosuvastatina (rosuvastatina cálcica) apresenta várias vantagens farmacológicas, dispondo de características únicas de ligação com a enzima HMG-CoA redutase e efeitos inibitórios superiores em relação às outras estatinas (Holdgate *et al.* 2003; McTaggart 2003).

Além disso, este medicamento é altamente hidrofílico e apresenta seletividade por hepatócitos, o que sugere menor toxicidade para as fibras musculares esqueléticas devido ao baixo potencial de inibição do colesterol nestes tipos de fibras musculares, uma vez que alterações musculares têm sido relatadas como o principal efeito adverso das estatinas (McTaggart 2003).

A rosuvastatina é uma estatina de última geração e foi a última aprovada pelas instituições regulatórias em 2001, sendo a estatina que apresenta mais afinidade e interação com o sítio enzimático da HMG-CoA redutase (McTaggart 2003). O fármaco tem demonstrado, a longo prazo, eficácia, tolerabilidade e segurança em pacientes que apresentam hipercolesterolemia (Ballantyne *et al.* 2004), hipertrigliceridemia (Hunninghake *et al.* 2004) e hiperlipidemia mista (Caslake *et al.* 2003).

A rosuvastatina é a estatina que mais reduz os níveis séricos de LDL-colesterol (Fonseca 2005) e os níveis de colesterol total quando comparada com as outras estatinas na mesma dose

utilizada diariamente, indicando que para a dose de 10 mg/dia ocorre diminuição de 43% do colesterol total e para a dose de 40mg/dia os níveis de colesterol total alcançam uma redução de 46% a 53% (Vaughan & Gotto 2004; Penning-van Beest *et al.* 2007).

O fármaco em questão reduz ainda os níveis séricos de triglicerídeos em 10% na dose de 10 mg/dia e alcança redução de 35% para a dose de 40 mg/dia, e além disso, contribui para o aumento de HDL-colesterol em 8% na dose de 10 mg/dia e 14% quando a dose considerada é de 40 mg/dia, (Vaughan & Gotto 2004) sendo que não apresenta riscos maiores de miopatia, rabdomiólise, insuficiência renal e hepatopatias agudas em relação às outras estatinas, o que a torna ainda mais vantajosa (Goettsch *et al.* 2006; Garcia-Rodriguez *et al.* 2008).

Finalmente, com relação ao tratamento da hipercolesterolemia e morbidades associadas, tem sido demonstrado que as doses indicadas para tratamento com a rosuvastatina variam de 5 a 40 mg/dia e a dose indicada para o início do tratamento é de 10 mg/dia, (Tiwari 2006).

Considerando as estatinas disponíveis no mercado, a rosuvastatina é a estatina que possui maior tempo de meia-vida para sua eliminação (20 horas), quando comparado com a atorvastatina (15 horas), pravastatina, fluvastatina e sinvastatina (1-3 horas) (McTaggart 2003).

1.9. Morte celular e estatinas

O acúmulo de danos frente a agentes estressores internos e externos que incluem o estresse oxidativo, a disfunção das telomerases e os danos no DNA podem levar a deterioração de componentes celulares, dano na função celular e a perda da homeostase tecidual, de modo que os danos celulares quando acumulados podem aumentar o risco das células adquirirem modificações no DNA, podendo consequentemente conduzir à formação de tumores (Vicencio *et al.* 2008).

Como mecanismo de preservação do organismo, as células danificadas tendem a entrar em senescência, ou seja, em parada permanente do ciclo celular, ou em morte celular programada por apoptose ou autofagia (Vicencio *et al.* 2008).

A apoptose é o tipo de morte celular mais descrito na literatura e envolve a atuação de proteases e hidrolases que degradam rapidamente todas as estruturas no interior da célula, e neste caso, a membrana plasmática permanece intacta sem que haja extravazamento do conteúdo celular para as células vizinhas (Galluzzi *et al.* 2007).

As características morfológicas que definem a apoptose são a condensação da cromatina, a fragmentação nuclear, a contração celular, a formação de corpos apoptóticos e a acidificação citoplasmática (Lagadic-Gossmann *et al.* 2004; Galluzzi *et al.* 2007).

A autofagia é um processo lento e espacialmente restrito pelo qual os componentes da própria célula são entregues aos lisossomos para degradação, e é por este mecanismo que a célula realiza a eliminação de organelas degradadas e supérfluas, bem como a eliminação de proteínas anormais e agregados proteicos (Galluzzi *et al.* 2007; Vicencio *et al.* 2008).

O mecanismo de autofagia mais amplamente descrito é a macroautofagia, mecanismo pelo qual as organelas e porções celulares são envolvidos por estruturas que possuem membrana dupla, denominadas autofagossomos, e que ao se fundir com os lisossomos originam outras estruturas denominadas autolisossomos, que possuem membrana única (Galluzzi *et al.* 2007; Vicencio *et al.* 2008). No interior dos autolisossomos os componentes são degradados por enzimas hidrolíticas e em seguida, os compostos resultantes retornam para o citoplasma e ficam disponíveis para reações metabólicas (Galluzzi *et al.* 2007; Vicencio *et al.* 2008).

Normalmente a autofagia desempenha um papel importante na sobrevivência e na homeostase celular em períodos de estresse e deficiência nutricional em casos de privação de fatores de crescimento, entretanto as células com intensa autofagia podem entrar em processo de

morte celular devido ao fato de ter que lidar com excessivos fatores de estresse (Ouyang *et al.* 2012).

Vários estudos têm demonstrado a ação de indução da morte celular em linhagens celulares normais e tumorais promovido pelas estatinas (Tanaka *et al.* 2000; Lee & Kim 2013; Qi *et al.* 2013). Os efeitos antineoplásicos promovidos pelas estatinas em muitos tipos de tumores ocorre através da indução da morte celular e da parada do ciclo celular de maneira independente da diminuição do colesterol, sendo esses efeitos mais associados a diminuição dos isoprenóides intermediários (Lee & Kim 2013; Qi *et al.* 2013).

Em linhagens celulares de linfoma, as estatinas (atorvastatina, fluvastatina e sinvastatina) exibiram aumento na fragmentação do DNA e a ativação de membros pró-apoptóticos como as caspases-3 e o Bax e suprimiu a ação da molécula anti-apoptótica Bcl-2, provavelmente devido ao aumento nas espécies reativas de oxigênio e inibição de compostos da via do mevalonato, tais como o GGPP, FPP e próprio mevalonato (Qi *et al.* 2013).

Em outro estudo realizado com linhagens de câncer de mama observou que a administração de sinvastatina induziu à apoptose através da ativação das vias pró-apoptóticas e da redução de GGPP e do mevalonato (Gopalan *et al.* 2013).

Em culturas primárias de neurônios corticais fetais de ratos foi observado que a inibição da cascata do mevalonato pode induzir a morte neuronal por apoptose devido a expressão de Bax e indução da p53, uma vez que o GGPP é um metabólito importante para a proteção neuronal e atua impedindo que essas células entrem no processo de morte celular programada (Tanaka *et al.* 2000).

As estatinas podem induzir à autofagia algumas linhagens tumorais, tais como as células de rabdomiosarcoma e células de câncer prostático (PC3), e levar consequentemente à morte celular autofágica, provavelmente devido a inibição da síntese do GGPP (Araki *et al.* 2012; Zhang *et al.* 2013).

Outros estudos indicam que as estatinas também podem promover autofagia em células normais, tais como em cardiomiócitos quando em cultura e *in vivo*, em linhagens de fibras musculares lisas, cultura de fibroblastos das vias aéreas humanas e em células mesenquimais de pulmão humano *in vitro* (Zhang *et al.* 2013).

O mecanismo mais bem estabelecido que demonstra a indução de morte celular autofágica pelas estatinas está relacionado com a ativação do AMPK (AMP dependente de proteína quinase) que reprime a atividade do mTOR (alvo da rapamicina em mamíferos), o qual ativa os genes relacionados a autofagia e leva ao processo autofágico, e deste modo, sob estresse e processo de autofagia contínuos, a célula tende a entrar em morte celular autofágica (Zhang *et al.* 2013).

1.10. Estatinas, gestação e lactação

A Food and Drug Administration (FDA) classifica as estatinas como categoria X com relação ao risco para a gestação, ou seja, possuem alto risco de promover malformações fetais, e estes medicamentos são por este motivo contraindicados para a gestante, porém alguns estudos sugerem que o risco fetal é baixo para o caso de exposição inadvertida (Hosokawa *et al.* 2003).

As estatinas têm a capacidade de atravessar a membrana placentária e interferir com o metabolismo fetal, já que os produtos do metabolismo do colesterol são essenciais para a formação da membrana plasmática, a síntese de hormônios esteroides e para a morfogênese fetal (Edison & Muenke 2004; Pollack *et al.* 2005)

A diminuição na biossíntese dos metabólitos da via do mevalonato pode interferir com o desenvolvimento embrionário de várias maneiras, podendo alterar o fluxo do desenvolvimento cardíaco e a angiogênese, a indução e o crescimento do sistema nervoso central, a sobrevivência de células da crista neural, a proliferação e diferenciação cartilaginosa e a função das células de Sertoli e das células de Leydig (Edison & Muenke 2004).

Com relação ao uso das estatinas durante a lactação, não há relatos na literatura da segurança para o lactente e, portanto o uso das estatinas durante a lactação é contra-indicado, pois há a possibilidade de que algumas estatinas sejam transmitidas através do leite materno ao lactente (Edison & Muenke 2004).

1.11. Estatinas e reprodução

Na esfera reprodutiva foi observado que o tratamento com a sinvastatina na dose de 20 mg/dia em homens adultos no período de 3, 6 e 12 meses promoveu diminuição moderada dos níveis de testosterona livre, mas sem afetar a função testicular (Azzarito *et al.* 1996).

Outro estudo demonstrou que homens que faziam uso de pravastatina na dose de 40 mg/dia não tiveram alterados as concentrações séricas dos seguintes hormônios: testosterona, estradiol, cortisol, sulfato de dehidroepiandrosterona, hormônio folículo estimulante (FSH) e hormônio luteinizante (LH) (Bohm *et al.* 2004).

Em estudos realizados em homens hipercolesterolêmicos que faziam uso das estatinas, foi demonstrado que a administração desses agentes hipolipemiantes pode contribuir para melhora na função erétil, a qual está relacionada a diminuição do perfil lipídico sérico e a restauração da função endotelial, (Dogru *et al.* 2008), além de diminuir as concentrações séricas do antígeno específico prostático ao longo do tempo (Cyrus-David *et al.* 2005).

Outros estudos realizados em animais de experimentação, tais como ratos que receberam 0, 20, 100 e 175 mg/kg de atorvastatina, via gavagem durante 11 semanas, e cachorros que receberam 0, 10, 40 e 120 mg/kg, via gavagem por 104 semanas não demonstraram alterações em vários parâmetros reprodutivos tais como: morfologia, motilidade e contagens espermáticas, histologia de testículo e epidídimo e fertilidade por acasalamento natural (Dostal *et al.* 1996; Dostal *et al.* 2001).

Não há relatos na literatura de trabalhos que avaliem os efeitos reprodutivos imediatos, tais como, o tempo de instalação da puberdade e o desenvolvimento da função reprodutiva masculina e as repercussões reprodutivas na maturidade sexual provenientes da exposição à rosuvastatina durante a infância e adolescência.

1.12. Puberdade e exposição aos desreguladores endócrinos

A exposição a possíveis desreguladores endócrinos na infância e adolescência, ou seja, no período da peripuberdade, pode alterar o crescimento e a distribuição da gordura corporal ainda neste período, bem como promover alterações de comportamento e aumentar o risco de câncer na vida adulta (Mantovani & Fucic 2013).

Os desreguladores endócrinos que tendem a atrasar a instalação da puberdade masculina têm sido associados com a ocorrência de síndrome metabólica, osteoporose e fraturas ósseas na vida adulta (Bianco 2012).

Durante a peripuberdade a espermatogênese e a esteroidogênese ainda não estão completamente estabelecidas, o que torna o sistema genital masculino mais susceptível a ação de agentes químicos (Johnson *et al.* 1997; Favareto *et al.* 2011), assim muitas destas substâncias diminuem a função androgênica e podem atrasar o desenvolvimento do sistema genital masculino, sendo os efeitos adversos promovidos por esses agentes tóxicos dependentes do tempo de exposição a esses compostos (Blystone *et al.* 2007).

As crianças e adolescentes estão expostos a vários agentes químicos que podem alterar o tempo de instalação da puberdade e o desenvolvimento e aquisição da função reprodutiva, logo essa problemática é de grande importância para a toxicologia reprodutiva (Stoker *et al.* 2000), uma vez que alterações na puberdade podem inclusive afetar a função reprodutiva na maturidade sexual (Perobelli *et al.* 2012; Mantovani & Fucic 2013; Perobelli *et al.* 2013).

Considerando-se o uso de substâncias hipolipemiantes por crianças e a falta de estudos sobre os possíveis efeitos tóxicos para a reprodução masculina, propomos o presente estudo experimental em que ratos jovens serão expostos à rosuvastatina a partir do desmame até a instalação da puberdade, avaliando-se os parâmetros reprodutivos imediatos e tardios, ou seja, na maturidade sexual. A escolha da "janela" de exposição se justifica pela tentativa de mimetizar a situação humana em que algumas crianças necessitam da droga durante a infância e adolescência, e a partir do momento em que se tornam adultos, adquirem consciência do problema e passam a controlar os índices de colesterol a partir de hábitos alimentares saudáveis e exercícios físicos.

Capítulo I

Artigo aceito para publicação no periódico "Reproductive Toxicology"

Delayed reproductive development in pubertal male rats exposed to the hypolipemiant agent rosuvastatin since prepuberty

Gabriel Adan Araújo Leite[a,*], Josiane de Lima Rosa[a], Marciana Sanabria[a], Marília Martins Cavariani[a], Janete Aparecida Anselmo Franci[b], Patrícia Fernanda Felipe Pinheiro[c], Wilma De Grava Kempinas[d]

[a] Graduate Program in General and Applied Biology, Institute of Biosciences, UNESP –Univ. Estadual Paulista, Botucatu, São Paulo, Brazil.

[b] Department of Morphology, Stomatology and Physiology, School of Dentistry, USP – University of São Paulo, Ribeirão Preto, São Paulo, Brazil.

[c] Department of Anatomy, Institute of Biosciences, UNESP–Univ. Estadual Paulista, Botucatu, São Paulo, Brazil.

[d] Department of Morphology, Institute of Biosciences, UNESP–Univ. Estadual Paulista, Botucatu, São Paulo, Brazil.

* Corresponding author at: Departamento de Morfologia, Instituto de Biociências, UNESP, Caixa Postal 510, 18618-970 Botucatu, SP, Brazil. Tel.: +55 14 3880-0476. E-mail address: gabriel_adan_1990@hotmail.com (G.A.A. Leite).

Abstract

Dyslipidemias are frequently found in children due to obesity, bad eating habits and the lack of physical exercises. Rosuvastatin acts as an HMG-CoA reductase inhibitor, decreasing total cholesterol and triglycerides. This study aimed to investigate initial sexual development and morphological aspect of the testis and epididymis in juvenile rats exposed to rosuvastatin since pre-puberty. Three groups were formed with newly weaned rats: control, whose rats received saline solution 0.9%, rosuvastatin at doses of 3 or 10 mg/Kg daily by gavage, since post-natal day 21 until puberty onset. In the rosuvastatin-treated groups, the results demonstrated a trend towards a decrease in testosterone concentration, but below the significance level, as well as delays in both the age of puberty onset and in epididymal development. There were also testicular alterations that might be related to delayed puberty and decrease of serum testosterone. In conclusion, rosuvastatin administration to juvenile rats not only delayed puberty onset and epididymal development, but also impaired testicular and epididymal morphology.

Keywords: male reproduction, puberty, rosuvastatin, androgen receptors, epididymis

1. Introduction

Dyslipidemias are dysfunctions in serum levels of lipids and consist of increased triglycerides, decreased HDL-cholesterol and / or increased levels of LDL-cholesterol [1]. The lipid dysfunctions are intrinsically related to childhood obesity, which can lead to hypertension, insulin resistance and acceleration of the atherosclerotic process [2, 3].

Statins, best-selling drugs, are medications widely utilized to diminish total cholesterol, especially LDL-cholesterol and act inhibiting the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) [4-6]. These drugs, upon inhibiting HMG-CoA reductase, prevent the conversion of HMG-CoA into mevalonate and, consequently, diminish the formation of endogenous cholesterol. Furthermore, they inhibit the synthesis of other important compounds, such as the intermediate isoprenoids, prominent among which are geranylgeranylpyrophosphate (GGPP) and farnesylpyrophosphate (FPP) [7, 8].

Statins, besides being used as hypolipemiant agents, possess the following pleiotropic properties: high potential for inhibiting the proliferation and induction of apoptosis in tumor cells [9] , improving endothelial function, increasing bioavailability of nitric oxide and the inhibiting immunomodulatory actions, which contribute to the prevention of cardiovascular diseases and diminution of infarction incidence [5, 10].

Rosuvastatin, the newest statin on the market, presents several pharmacological advantages, featuring unique characteristics of bonding with the enzyme HMG-CoA reductase and inhibitory effects superior to those of other statins [11-13], besides promoting the diminution of serum triglyceride levels [14].

In addition to drug treatment for dyslipidemias, it is recommended that the patient adopt an appropriate diet and associated lifestyle changes, including periodic physical activity and the abolition of the sedentary practices common in the current society [15].

Puberty is a critical period during which important morphological alterations and neuroendocrine interactions occur in mammals [16] and it requires androgenic action for the development of the male genital system [17]. It commences with an increase in the GnRH pulses in the hypothalamus and the release of FSH and LH by the pituitary, leading to the stimulation of secretion of steroidal hormones by the gonads and consequently enabling the acquisition of secondary sexual characteristics and reproductive capacity. In boys, testicular enlargement and thinning of the scrotum have been reported as the primary signals of puberty onset [18].

During peripuberty, the spermatogenesis and steroidogenesis are not yet completely established, which makes the male genital system more susceptible to the action of chemical agents [19, 20]. Many of these substances diminish the androgenic function and can delay the development of the male genital system, with effects being dependent on the time of exposure to a determinate toxic agent [21].

Exposure to chemical agents that can alter reproductive function during this period is highly important to reproductive toxicology [16], given that changes in puberty can also alter reproductive function during sexual maturity [22-24].

Considering the use of hypolipemiant substances by children and the lack of studies on the possible effects of statins on reproductive development, the present study aimed to evaluate the immediate reproductive parameters of pre-pubertal rats exposed to rosuvastatin from weaning until the puberty onset, by mimicking the human situation in which some children require statins during childhood and adolescence.

2. Material and Methods

2.1. Animals

2.1.1. Obtention of pregnant females and reduction of litters

Adult male (90 days of age) and female (60 days of age) nonpathogenic free Wistar rats were supplied from Central Biotherium of State University of São Paulo (UNESP), Botucatu/SP and maintained in the Small Mammal Biotherium of the Morphology Department at the UNESP Biosciences Institute, Botucatu.

Animals were housed in polypropylene cages (43 cm × 30 cm × 15 cm) with laboratory grade pine shavings as bedding. Rats were maintained under controlled temperature (23 ± 1°C) and lighting conditions (12:12-h photoperiod) and the health of the animals was monitored along the experiment. Standard rodent chow (Purina Labina, Agribrands do Brasil Ltda, Paulínia/SP, Brazil) and filtered tap water were provided *ad libitum*.

Two nulliparous female rats were mated with one male, during the dark portion of the lighting cycle, and the day of sperm detection in the vaginal smear of female rats in estrus was considered day zero of gestation (gestational day 0 – GD 0). Pregnant and lactating rats were maintained in individual cages.

After birth, the number of pups per litter was culled to eight on post-natal day (PND) 4 to balance male and female pups (4 each per litter). Litters with fewer than eight pups were not entered into the experimental protocol and were euthanized.

2.1.2. Experimental design

After weaning (PND 21), male pups were distributed in three experimental groups (n=10 per group, with one or two pups per litter for each group), that received vehicle (saline solution 0,9%), 3 or 10 mg/Kg/day of rosuvastatin diluted in saline solution 0,9% administered by gavage since PND 21 until puberty onset, which was indicated by complete preputial separation (variable age for each animal). The doses of 5 mg/day to 40 mg/day of rosuvastatin are available to be utilized by humans to decrease total cholesterol and LDL-cholesterol [14], thus the doses

used in this study were choose based on body surface area correction from children available doses of rosuvastatin to pup rats equivalent doses [25]. During the exposure period, the animals were monitored in relation to the indications of distress, such as the presence of bristling hair, and whether they were ingesting food and water. Individual pup weights were recorded during the exposure period.

The experimental protocol followed the Ethical Principles in Animal Research of the Brazil College of Animal Experimentation and was approved by the Bioscience Institute/UNESP Ethics Committee for Animal Experimentation (protocol no. 336-CEUA).

In order to evaluate the possible immediate reproductive effects after statin exposure, male rats were euthanized at peripuberty (PND 55), when all animals presented complete preputial separation, to obtain the final body weight, reproductive and vital organ weights, hormonal serum concentrations, testicular and epididymal histopathological analyses, testicular morphometric analysis and immunohistochemistry for androgen receptors (AR) on testis and epididimys.

2.2. External physical sign of puberty onset

Preputial separation is an indicative of puberty onset, and was investigated since PND 30, based on manual retraction of prepuce [26], and classified as incomplete and complete preputial separation. In this sense, partial retraction of penile glans characterized the incomplete preputial separation and total retraction of penile glans was indicative of complete preputial separation.

2.3. Euthanasia, body weight and organ weights

On PND 55, male rats were weighed, euthanized following narcosis by CO_2 asphyxiation and thereafter, blood was collected by hepatic portal vein, between 9:00 and 11:30 a.m. The left testis, epididymis and vas deferens, seminal gland (full and empty, without the

coagulating gland) and ventral prostate, from all rats were collected and weighed. The liver, kidney, adrenal, thyroid and pituitary were also collected and weighed. Female pups were treated with rosuvastatin at prepubertal period and utilized to evaluate the possible effects of this hypolipemiant agent on the female reproductive system in a posterior study.

2.4. Serum hormonal levels

Serum was obtained by centrifugation (2400 rpm, 20 minutes, 4ºC) in a refrigerated device and was frozen at –20ºC until the moment of hormonal determination. Testosterone, FSH and LH were determined by double-antibody radioimmunoassay. Testosterone concentrations were measured by Testosterone Maia kit (Biochem Immuno System, Allentown, PA) and LH e FSH used specified kits supplied by National Institute of Arthritis, Diabetes and Kidney Diseases (NIADDK). All samples were measured in the same assay to avoid the inter-assay errors. Intra-assay variabilities were 3.4% for LH, 2.8% for FSH, and 4% for testosterone.

2.5. Histological procedures

Left testis and epididymis were fixed in Bouin's fluid, embedded in Paraplast® and sectionated in 4µm (transversal sections of testis and longitudinal sections of epididymis). Sections were stained with hematoxylin and eosin (HE) to evaluate testicular and epididymal morphology under light microscopy. Sections destined for immunohistochemistry were obtained in silanized slides. The evaluation was performed in blind assay and the figures were obtained using a light microscopy Zeiss model Scope A1-Axio, coupled to the image digitizer system Axio Vision, version 4.7.2.

Seminiferous tubules cross-sections were randomly chosen in three non-serial testicular sections per animal obtained with a distance of 50 µm among them, totaling 200 tubules evaluated per animal. Seminiferous tubules were classified as: normal (presence of concentric and normally organized germ cell layers in seminiferous epithelium) or abnormal

(presence of germ cells and cellular debris in the lumen, multinucleated formation, seminiferous epithelium with acidophilic cells, few germ cell layers, vacuole formation or degeneration in seminiferous epithelium). Interstitial tissue and peritubular myoid cells histopathological analysis were qualitative and the interstitial analysis aimed to assess Leydig cells morphology and the appearance of blood vessels. Epididymal histopathological analysis was also qualitative, evaluating each region of the organ according to the epithelium, lumen and interstitial tissue morphological appearance.

2.6. Testicular morphometric evaluation

2.6.1. Maturation degree of seminiferous epithelium

In order to evaluate the maturation degree of the seminiferous epithelium, 100 cross-sections of seminiferous tubules per animal (n = 10 per group), were evaluated randomly, using the adapted method of assigning values according to the type of mature germ cell most numerous in the tubular epithelium: degree 1: spermatocytes I or II; degree 2: young spermatids with rounded nucleus (stage 1 to 8 of spermiogenesis); degree 3: spermatids in maturation phase, with ovoid or elongated nucleus (stage 9 to 14 of spermiogenesis); degree 4: spermatids in maturation phase, with elongated nucleus (stage 15 to 18 of spermiogenesis); degree 5: mature spermatids (stage 19 of spermiogenesis) in small quantity; degree 6: mature spermatids (stage 19 of spermiogenesis) in average amount; degree 7: mature spermatids (stage 19 of spermiogenesis) in larger amount. The number of seminiferous tubules in each degree was multiplied by its degree, and then the values were added and divided by 100, resulting in the “average degree” [27].

2.6.2. Nuclear area and volume of Leydig cells

Cariometric analysis were performed in 50 nuclei of Leydig cells per animal (n=10), chosen randomly with circular or elliptical shape [28] [24]. Major (D) and minor (d) diameter of

the Leydig cell nuclei were obtained using a light microscopy Zeiss model Scope A1-Axio, coupled to the image digitizer system Axio Vision, version 4.7.2. Thereafter, the medium diameter (M) was calculated using the formula M = (D . d)/2 and nuclear area (A) and volume (V) were obtained with the formulas $A = \pi . 1/4 . M^2$ and $V = \pi . 1/6 . M^3$, respectively [29].

2.7. Immunohistochemistry for androgen receptors (AR) on testis and epididymis

Histological sections were allocated in silanized slides, dewaxed using toluene, hydrated using decreasing concentrations of ethanol and washed in phosphate buffer (PBS), followed by antigen recovery with citrate buffer (0.01M, pH 6.0) in a pressure cooker submitted to high temperatures for 40 minutes. After these steps, the slides were incubated in hydrogen peroxide in 3% of methanol during 15 minutes. Thus, the sections were incubated overnight with primary antibody anti-androgen receptor (Clone SC-816 - AR N-20 from SANTA CRUZ BIOTECHINOLOGY INC.®). After incubating, the slides were washed with PBS buffer and during 1 hour, at room temperature, the sections were incubated with secondary antibody (Biotinylated Sheap Anti-Rabbit Immunoglobulins - DAKO CYT. INC. Ò). After this step, the slides were washed with PBS buffer and submitted to avidin-biotin-peroxidase solution (StreptABComplex DAKO CYT. INC. Ò) for 45 minutes. Thereafter, the slides were washed with PBS buffer and submitted to diaminobenzidine (DAB) (di-amino-benzidine-SigmaÒ) for 5 minutes, then, the slides were washed in tap water and counterstained with Harris hematoxylin. During this technique, we obtained negative and positive controls.

2.8. Daily sperm production per testis

Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) were enumerated as described previously [30], with adaptations as following: the right testis, decapsulated and weighed soon after collection, were homogenized in 5 mL of NaCl 0.9%

containing Triton X 100 0.5%, followed by sonication for 30 seconds. After a 10-fold dilution a sample was transferred to Neubauer chambers (4 fields per animal), proceeding an enumeration of mature spermatids. To calculate the daily sperm production (DSP), the number of spermatids at stage 19 was divided by 6.1, which is the number of days of the seminiferous cycle in which these spermatids are present in the seminiferous epithelium.

2.9. Statistical analysis

For comparison of the results among the experimental groups, ANOVA or Kruskal-Wallis were performed, followed by Tukey or Dunns test, respectively, according to the characteristics of each variable. Differences were considered significant when $p<0.05$. Statistical analyses were performed on GraphPad Prism (version 5.00).

3. Results

3.1. Puberty onset, hormone concentrations and daily sperm production

The rosuvastatin treatment provoked a delay in the age of preputial separation (complete and incomplete) in relation to the control group, infering a delay in puberty onset (Fig. 1).

The concentrations of FSH and LH in treated groups were similar to the control animals. On the other hand, there was a decrease in the testosterone concentrations in both treated groups (3 mg/Kg = 68.3% and 10 mg/Kg = 36.9%, compared with the control group). Although these results were not statistically significant, there was a trend below the significance level ($p = 0.078$) (Fig. 2).

The absolute and relative numbers of mature spermatids per testis and daily sperm production were decreased at peripubertal period in the rosuvastatin-treated groups, indicating a delay in the establishment of spermatogenesis (Table 1).

3.2. Histopathological and morphometric analyses

The frequency of abnormal seminiferous tubules increased in the groups treated with rosuvastatin (Table 2). The most frequent alterations were seminiferous tubules with acidophilic spermatogonias and spermatocytes, especially in tubules in stages IX - XIV (Fig. 3). No alterations were observed in the peritubular cells, in the morphology of Leydig cells and in the appearance of blood vessels. On the other hand, the morphometric measurements in the testis were not altered by the treatment (Table 3).

The epithelium of the epididymal proximal cauda was less differentiated and thicker (cribriform aspect) in the animals exposed to rosuvastatin (Fig. 4). Moreover, this same Figure suggests that the sperm reserves and the diameter of the epididymal duct were lower in the cauda epididymis of the treated groups.

The initial segment, caput and corpus epididymidis were comparable among groups (Fig. 4). The epididymal interstitium was not affected by rosuvastatin exposure (Fig. 4).

3.3. Immunohistochemistry for androgen receptors

Androgen receptor expression in Sertoli cells of treated animals at both rosuvastatin doses was lower in stages IX-XIII seminiferous tubules, in comparison with the control group (Table 4 and Fig. 5). The expression of androgen receptors in peritubular myoid cells and in Leydig cells was similar in the different experimental groups (Fig. 5).

The AR immunostaining in the epididymis epithelial cells was weaker in the corpus and cauda of the epididymis of the rosuvastatin-exposed animals (Table 5 and Fig. 6). In the interstitium and smooth muscle cells, the AR immunostaining pattern was comparable among groups in all epididymal regions (Fig. 6).

3.4. Evolution of body weight and organ weights

There were diminutions in both body weight gain and the final weight in animals treated with rosuvastatin, when compared to the control group, but the differences were not statistically significant (Fig. 7 and Table 6).

The absolute weights of reproductive organs (testis, epididymis, vas deferens, prostate and the full and empty seminal gland) (Table 6) and vital organs (pituitary, adrenal, thyroid, liver and kidney) were not found to be altered as a function of the experimental treatments (Table 7). The relative weights of the organs also did not differ statistically among the experimental groups (Table 6 and 7).

4. Discussion

This study is based on a therapeutic use of rosuvastatin to manage the cardiovascular risk in childhood obesity. Although we have used normal, non-obese juvenile rats, our study simulates the human exposure to rosuvastatin, since some experts are suggesting that more people should be taking advantage of the prescription medication [31].

In the present study we investigated whether exposure of pre-pubertal rats to the hypolipemiant agent rosuvastatin might interfere with reproductive development in Wistar rats, focusing on the puberty onset and the postnatal development of the testis and epididymis. The results showed that treating young rats with this statin provoked androgenic depletion, delayed puberty onset, and brought structural damage to the epididymis and testis.

Preputial separation represents a physical marker for puberty onset and is highly dependent on androgens. Thus, delays in preputial separation can demonstrate antiandrogenic action of toxic agents [32]. In the present study, puberty onset was delayed in the experimental groups that were treated with rosuvastatin at the doses of 3 and 10 mg/Kg/day.

Cholesterol is the precursor of steroid hormones and, when its blood levels are reduced, indirectly diminishes the levels of testosterone secreted by the Leydig cells, since cholesterol is required for testosterone synthesis. Testosterone serum concentrations showed a trend below the significance level to be reduced in the treated-groups, with respective diminutions in serum testosterone of 68.3% and 36.9%, in the groups treated with statin at doses of 3 and 10 mg/Kg/day, respectively. Although serum lipids were not profiled in this study, the diminution of testosterone concentrations enables to infer that cholesterol levels were decreased in the groups treated with rosuvastatin.

In the present study, the rosuvastatin-treated animals presented a higher frequency of abnormal seminiferous tubules, which displayed acidophilic cells, indicative of cell death. This result may be attributable to the diminution of testosterone and intermediate isoprenoids, and to the reduced expression of androgenic receptors by the Sertoli cells. Studies have reported that the depletion of intermediate isoprenoids leads to an augmentation of reactive oxygen species [33] and to cell death, either by apoptosis[33-35] or autophagy [36, 37]. The development of meiotic cells is dependent on proteins secreted by Sertoli cells, under androgenic stimulation [38, 39]. In the presence of androgenic depletion, there may be oxidative stress, DNA damage and increase in the apoptosis frequency in seminiferous tubules, especially the death of spermatocytes [38, 40].

During peripuberty, the daily sperm production increases rapidly and only reaches to the total capacity about PND75 [22, 30], thus until this period spermatogenesis is not yet completely established [20]. In the present study, decreased daily sperm production may be related to the decreased testosterone concentrations and cell death, observed on germ cell lineage that can compromise the total number of sperm.

The androgen receptors act as ligand-dependent transcription factors that regulate function of important genes for male development in puberty and fertility [41]. The lower AR

expression in Sertoli cells in stages IX-XIII coincided with the increase of acidophilic cells in these same stages. Androgenic depletion, allied with the lesser AR expression, may have contributed to the death of germ-line cells.

Furthermore, statins, by inhibiting HMG-CoA reductase, impede the conversion of HMG-CoA into mevalonate. Studies have reported that the metabolites of the mevalonate cascade are involved in other biological processes besides cell death, namely: proliferation, differentiation, cellular maturation and the maintenance of cellular functions [8, 37]. In this manner, the metabolites of the mevalonate pathway play an important signaling role associated with these biological processes [34, 42].

The postnatal differentiation of epididymal epithelium occurs together with an increase of AR expression along the epididymis [43] while androgenic depletion is directly related to diminution of AR expression in the epididymis, indicating compromise in the androgenic action mechanisms [23, 44].

In the present study, there was a delay in the epididymal development of the rosuvastatin-exposed animals, such that the proximal and distal cauda were still undifferentiated. The delay in differentiation of the epididymal duct might be related to the diminished testosterone levels and lower androgen receptor expression in the epithelium of the still undifferentiated regions, since androgens and AR expression drive the differentiation of androgen-dependent organs, such as the epididymis.

The rosuvastatin-exposed rats presented lower body weight in relation to the control group. Other studies demonstrate that statins can serve as coadjuvants for the reduction of body weight [45] and suggest that the administration of rosuvastatin diminishes the acceleration of body weight gain in mice treated with a cholesterol-rich diet, therefore reducing the final body weight [46]. Administration of rosuvastatin to pre-pubertal rats did not provoke significant

alterations in absolute or relative weight of reproductive and vital organs, indicating that rosuvastatin did not induce systemic toxicity.

Recent studies have reported that delays in puberty onset and in epididymal differentiation can have repercussions during sexual maturity [22-24], compromising the male genital system and fertility, and increasing cancer risk during adulthood [24]. The long term reproductive effects of prepubertal rosuvastatin exposure are currently under investigation by our research group.

5. Conclusion

The exposure to rosuvastatin during pre-puberty delayed epididymal differentiation, provoked alteration of testicular morphology, reduction of daily sperm production and diminution of the expression of androgen receptors in the testis and epididymis of rats. These alterations were related to delayed puberty onset, as well as to a trend towards a decrease in testosterone concentration in the rosuvastatin-treated groups. We concluded that juvenile exposure of rats to rovustatin is a possible risk for effects to male reproductive development.

6. Conflict of interest statement

The authors declare that there are no conflicts of interest.

7. Acknowledgments

The authors are grateful to São Paulo Research Foundation (FAPESP) for its financial support as part of Masters Scholarship (Grant #011/15065-5) and to José Eduardo Bozano, from the Department of Morphology, Institute of Biosciences, UNESP, Botucatu/SP – Brazil, for excellent technical assistance.

8. References

[1] Klop B, Elte JW, Cabezas MC. Dyslipidemia in obesity: mechanisms and potential targets. Nutrients. 2013;5:1218-40.

[2] Cook S, Kavey RE. Dyslipidemia and pediatric obesity. Pediatric clinics of North America. 2011;58:1363-73, ix.

[3] Jiménez EG, Ferre JA. Estatinas: Características y efectos sobre el control lipídico en el niño y adolescente obeso. Rev Clin Med Fam 2011;4:69-75.

[4] Istvan ES, Deisenhofer J. Structural mechanism for statin inhibition of HMG-CoA reductase. Science. 2001;292:1160-4.

[5] Tandon V, Bano G, Khajuria V, Parihar A, Gupta S. Pleiotropic effects of statins. Int J Pharmacol. 2005;37:77-85.

[6] Endres M. Statins: potential new indications in inflammatory conditions. Atherosclerosis Supplements. 2006;7:31-5.

[7] Istvan E. Statin inhibition of HMG-CoA reductase: a 3-dimensional view. Atherosclerosis Supplements. 2003;4:3-8.

[8] Adam O, Laufs U. Antioxidative effects of statins. Archives of toxicology. 2008;82:885-92.

[9] Feleszko W, Zagozdzon R, Golab J, Jakobisiak M. Potentiated antitumour effects of cisplatin and lovastatin against MmB16 melanoma in mice. European journal of cancer. 1998;34:406-11.

[10] Ludman A, Venugopal V, Yellon DM, Hausenloy DJ. Statins and cardioprotection--more than just lipid lowering? Pharmacology & therapeutics. 2009;122:30-43.

[11] Olsson AG, McTaggart F, Raza A. Rosuvastatin: a highly effective new HMG-CoA reductase inhibitor. Cardiovascular drug reviews. 2002;20:303-28.

[12] McTaggart F. Comparative pharmacology of rosuvastatin. Atherosclerosis Supplements. 2003;4:9-14.

[13] Holdgate GA, Ward WH, McTaggart F. Molecular mechanism for inhibition of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase by rosuvastatin. Biochemical Society transactions. 2003;31:528-31.

[14] Vaughan CJ, Gotto AM, Jr. Update on statins: 2003. Circulation. 2004;110:886-92.

[15] Ostlund RE, Jr. Phytosterols in human nutrition. Annual review of nutrition. 2002;22:533-49.

[16] Stoker TE, Laws SC, Guidici DL, Cooper RL. The effect of atrazine on puberty in male Wistar rats: An evaluation in the protocol for the assessment of pubertal development and thyroid function. Toxicol Sci. 2000;58:50-9.

[17] Blystone CR, Lambright CS, Furr J, Wilson VS, Gray LE, Jr. Iprodione delays male rat pubertal development, reduces serum testosterone levels, and decreases ex vivo testicular testosterone production. Toxicol Lett. 2007;174:74-81.

[18] Fisher MM, Eugster EA. What is in our environment that effects puberty? 2013.

[19] Johnson L, Welsh THJ, Wilker CE. Anatomy and physiology of the male reproductive system and potential targets of toxicants. In: Boekleheide K, Chapin RE, Hoyer PB, Harris C, editors. Comprehensive Toxicology. 1 ed. New York: Pergamon; 1997. p. 5-61.

[20] Favareto AP, Fernandez CD, da Silva DA, Anselmo-Franci JA, Kempinas Wde G. Persistent impairment of testicular histology and sperm motility in adult rats treated with Cisplatin at peri-puberty. Basic & clinical pharmacology & toxicology. 2011;109:85-96.

[21] Blystone CR, Furr J, Lambright CS, Howdeshell KL, Ryan BC, Wilson VS, et al. Prochloraz inhibits testosterone production at dosages below those that affect androgen-dependent organ weights or the onset of puberty in the male Sprague Dawley rat. Toxicol Sci. 2007;97:65-74.

[22] Perobelli JE, Alves TR, de Toledo FC, Fernandez CD, Anselmo-Franci JA, Klinefelter GR, et al. Impairment on sperm quality and fertility of adult rats after antiandrogen exposure during prepuberty. Reproductive toxicology. 2012;33:308-15.

[23] Perobelli JE, Patrao MT, Fernandez CD, Sanabria M, Klinefelter GR, Avellar MC, et al. Androgen deprivation from pre-puberty to peripuberty interferes in proteins expression in pubertal and adult rat epididymis. Reproductive toxicology. 2013;38:65-71.

[24] Mantovani A, Fucic A. Puberty dysregulation and increased risk of disease in adult life: Possible modes of action. Reproductive Toxicology2013.

[25] Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2008;22:659-61.

[26] Hood RD. Developmental and reproductive toxicology: a practical approach. In: Parker RM, editor. Testing for reproductive toxicity. New York: CRC Press; 2006. p. 472-4.

[27] Toledo FC, Perobelli JE, Pedrosa FP, Anselmo-Franci JA, Kempinas WD. In utero protein restriction causes growth delay and alters sperm parameters in adult male rats. Reproductive biology and endocrinology : RB&E. 2011;9:94.

[28] Fichna P, Malendowicz LK. A karyometric and stereologic study of the effects of gonadotrophin and testosterone on the interstitial gland of the testis of intact and endoxan treated rats. Cell and tissue research. 1975;164:411-24.

[29] Cury CA, Azoubel R, Batigalia F. Bladder drainage and glandular epithelial morphometry of the prostate in benign prostatic hyperplasia with severe symptoms. International braz j urol : official journal of the Brazilian Society of Urology. 2006;32:211-5.

[30] Robb GW, Amann RP, Killian GJ. Daily sperm production and epididymal sperm reserves of pubertal and adult rats. Journal of reproduction and fertility. 1978;54:103-7.

[31] Park A. Statins have few side effects, but should more people be taking them? Health and family. http://healthland.time.com/2013/07/10/statins-have-few-side-effects-but-should-more-people-be-taking-them/: Time; 2013.

[32] Monosson E, Kelce WR, Lambright C, Ostby J, Gray LE, Jr. Peripubertal exposure to the antiandrogenic fungicide, vinclozolin, delays puberty, inhibits the development of androgen-dependent tissues, and alters androgen receptor function in the male rat. Toxicology and industrial health. 1999;15:65-79.

[33] Qi XF, Zheng L, Lee KJ, Kim DH, Kim CS, Cai DQ, et al. HMG-CoA reductase inhibitors induce apoptosis of lymphoma cells by promoting ROS generation and regulating Akt, Erk and p38 signals via suppression of mevalonate pathway. Cell Death Dis. 2013;4.

[34] Tanaka T, Tatsuno I, Uchida D, Moroo I, Morio H, Nakamura S, et al. Geranylgeranyl-pyrophosphate, an isoprenoid of mevalonate cascade, is a critical compound for rat primary cultured cortical neurons to protect the cell death induced by 3-hydroxy-3-methylglutaryl-CoA reductase inhibition. Journal of Neuroscience. 2000;20:2852-9.

[35] Marcuzzi A, Zanin V, Piscianz E, Tricarico PM, Vuch J, Girardelli M, et al. Lovastatin-induced apoptosis is modulated by geranylgeraniol in a neuroblastoma cell line. International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience. 2012;30:451-6.

[36] Araki M, Maeda M, Motojima K. Hydrophobic statins induce autophagy and cell death in human rhabdomyosarcoma cells by depleting geranylgeranyl diphosphate. Eur J Pharmacol. 2012;674:95-103.

[37] Zhang J, Yang ZZ, Xie L, Xu L, Xu D, Liu XF. Statins, autophagy and cancer metastasis. Int J Biochem Cell B. 2013;45:745-52.

[38] De Gendt K, Swinnen JV, Saunders PT, Schoonjans L, Dewerchin M, Devos A, et al. A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. Proceedings of the National Academy of Sciences of the United States of America. 2004;101:1327-32.

[39] Stanton PG, Sluka P, Foo CF, Stephens AN, Smith AI, McLachlan RI, et al. Proteomic changes in rat spermatogenesis in response to in vivo androgen manipulation; impact on meiotic cells. PloS one. 2012;7:e41718.

[40] Lue YH, Hikim AP, Swerdloff RS, Im P, Taing KS, Bui T, et al. Single exposure to heat induces stage-specific germ cell apoptosis in rats: role of intratesticular testosterone on stage specificity. Endocrinology. 1999;140:1709-17.

[41] Collins LL, Lee HJ, Chen YT, Chang M, Hsu HY, Yeh S, et al. The androgen receptor in spermatogenesis. Cytogenetic and genome research. 2003;103:299-301.

[42] Casey PJ, Seabra MC. Protein prenyltransferases. The Journal of biological chemistry. 1996;271:5289-92.

[43] You L, Sar M. Androgen receptor expression in the testes and epididymides of prenatal and postnatal Sprague-Dawley rats. Endocrine. 1998;9:253-61.

[44] Ezer N, Robaire B. Androgenic regulationof the structure and function of the epididymis In: Robaire B, Hinton BT, editors. The epididymis - from molecules to clinical practice. New York: Kluwer Academic/Plenum Publisher; 2002. p. 297-316.

[45] Salem NA, Assaf N, Ahmed HH. Pleiotropic effects of rimonabant and simvastatin on obesity associated multiple metabolic risk factors in rats. European review for medical and pharmacological sciences. 2012;16:797-807.

[46] Famer D, Crisby M. Rosuvastatin reduces gliosis and the accelerated weight gain observed in WT and ApoE-/- mice exposed to a high cholesterol diet. Neuroscience letters. 2007;419:68-73.

Legends of figures

Fig. 1. Age of incomplete (white bar) and complete preputial separation (gray bar). Values expressed as mean ± SEM. ANOVA followed by Tukey's test. $p < 0.0001$.

Fig. 2. Serum FSH, LH and testosterone levels (ng/ml) in peripubertal male rats at 55 days of age. Values expressed as mean ± SEM. ANOVA followed by Tukey's test. FSH ($p = 0.0888$), LH ($p = 0.3304$) and testosterone ($p = 0.0781$).

Fig. 3. Histopathological analysis of the seminiferous epithelium of rats on PND55. Photomicrograph of testicular sections from control (1A–3A), 3mg (1B–3B) or 10mg of rosuvastatin (1C–3C). Observe the presence of acidophilic cells (arrows) that include spermatogonias (2B and 2C) and spermatocytes (3B and 3C) in the seminiferous epithelium. Hematoxiline and Eosin (HE). Scale bar = 100µm in 1A–1C; 50µm in 2A–2C and 3A–3C.

Fig. 4. Histopathological analysis of the epididymis of rats on PND55. Photomicrograph of epididymal sections from control (1A–5A), 3mg (1B–5B) or 10mg of rosuvastatin (1C–5C). Inicial segment (1A–1C), caput (2A–2C), corpus (3A–3C), proximal cauda (4A–4C) and distal cauda (5A–5C). Observe the presence of stratified and undifferentieted epithelium (arrows, 4B and 4C). Note an apparent smaller diameter in the epididymal duct and lower sperm storage in the cauda of rosuvastatin-treated groups. (asterisks, 5B and 5C). Hematoxiline and Eosin (HE). Scale bar = 100µm.

Fig. 5. Immunostaining of AR in pubertal rat testis from groups exposed to saline solution (control group, $n = 5$ animals), or to rosuvastatin at doses of 3 mg/kg ($n = 5$ animals) or 10 mg/kg ($n = 5$ animals). Note the differential nuclear staining at AR along the stages. Observe a decrease of staining at Sertoli cells on stages IX-XIII in the rosuvastatin-treated groups (arrows). Scale bar = 10µm.

Fig. 6. Immunostaining of AR in pubertal rat epididymis from groups exposed to saline solution (control group, $n = 5$ animals), or to rosuvastatin at doses of 3 mg/kg ($n = 5$ animals) or 10 mg/kg ($n = 5$ animals). IS = Initial Segment. Observe a decrease of staining at epididymal epithelium in the corpus and cauda in the rosuvastatin-treated groups (arrows). Scale bar = 50μm.

Fig. 7. Body weight evolution along the treatment. ANOVA followed by Tukey's test. $p > 0.05$.

Table 1.

Sperm counts on pubertal testis from control and rosuvastatin-treated groups on PND55.

Sperm Counts	Experimental groups		
	Control (n=9)	**3mg/Kg (n=10)**	**10mg/Kg (n=10)**
Mature spermatid number (10^6/testis)	109.90 ± 6.23 a	93.01 ± 3.79 b	90.38 ± 2.95 b
Mature spermatid number (10^6/g testis)	109.40 ± 6.00 a	91.79 ± 4.88 b	88.09 ± 2.51 b
Daily sperm production (10^6/testis/day)	18.01 ± 1.02 a	15.25 ± 0.62 b	14.75 ± 0.49 b
Relative daily sperm production (10^6/g testis/day)	17.94 ± 0.98 a	15.05 ± 0.80 b	14.51 ± 0.41 b

Values expressed as mean ± SEM. ANOVA followed by Tukey test. Different letters indicate statistically significant differences among the groups ($p < 0.05$).

Table 2.

Testicular histology evaluation of rats from control and rosuvastatin-treated groups on PND 55.

	Experimental groups		
	Control (n=9)	**3mg/Kg (n=10)**	**10mg/Kg (n=10)**
Normal seminiferous tubules	95.25 (94.13 - 96.75) a	89.25 (85.88 - 91.75) b	89.00 (86.38 - 90.88) b
Seminiferous tubules with acidophilic cells	4.00 (2.00 - 5.00) a	9.00 (7.25 - 11.50) b	9.25 (8.13 - 12.38) b

Values expressed as median and interquartile intervals. Kruskal-Wallis followed by Dunn test. Different letters indicate statistically significant differences among the groups ($p < 0.05$).

Table 3.

Testicular morphometry of rats from control and rosuvastatin-treated groups on PND 55.

	Experimental groups		
	Control (n=9)	**3mg/Kg (n=10)**	**10mg/Kg (n=10)**
Degree of maturation of the germinal epithelium	4.38 ± 0.04	4.32 ± 0.03	4.31 ± 0.03
Nuclear area of the Leydig cells (μm^2)	29.76 ± 0.57	29.22 + 0.67	28.97 + 0.78
Nuclear volume of the Leydig cells (μm^3)	122.3 ± 3.57	119.4 ± 4.08	114.6 ± 4.02

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

Table 4.

Summary of the results from testicular AR immunohistochemistry analysis of rosuvastatin-treated groups when compared to control group.

	Puberty	
AR expression in Sertoli Cells	**3mg/Kg (n=5)**	**10mg/Kg (n=5)**
Stages I-VI	n.o.e.	n.o.e.
Stages VII-VIII	n.o.e.	n.o.e.
Stages IX-XIII	↓	↓
Stage XIV	n.o.e.	n.o.e.

n.o.e: no observed effects - similar to control group; ↓: decrease of staining when compared to control group.

Table 5.

Summary of the results from epididymal AR immunohistochemistry analysis of rosuvastatin-treated groups when compared to control group.

Epididymal Epithelium	Puberty	
	3mg/Kg (n=5)	**10mg/Kg (n=5)**
Proximal Initial Segment	n.o.e.	n.o.e.
Distal Initial Segment	n.o.e.	n.o.e.
Caput	n.o.e.	n.o.e.
Corpus	↓	↓
Proximal Cauda	↓	↓
Distal Cauda	↓	↓

n.o.e.: no observed effects - similar to control group; ↓ : decrease of staining when compared to control group.

Table 6.

Final body weight and absolute and relative reproductive organ weights from control and rosuvastatin-treated groups.

	Experimental groups		
	Control (n=9)	**3mg/Kg (n=10)**	**10mg/Kg (n=10)**
Final body weight (g)	278.40 ± 7.40	252.60 ± 14.20	254.50 ± 12.60
Testis (g)	1.36 ± 0.03	1.27 ± 0.03	1.31 ± 0.04
Testis (g/100g BW)	0.49 ± 0.01	0.51 ± 0.02	0.52 ± 0.03
Epididymis (mg)	259.60 ± 11.00	243.20 ±12.30	239.60 ± 8.50
Epididymis (mg/100g BW)	93.56 ± 4.15	97.05 ± 2.90	96.25 ± 5.53
Vas deferens (mg)	60.38 ± 3.37	56.92 ± 3.46	56.79 ± 2.98
Vas deferens (mg/100g BW)	21.88 ± 1.48	22.99 ± 1.55	22.84 ± 1.61
Prostate (mg)	170.00 ± 11.10	161.70 ± 11.82	150.30 ± 9.13
Prostate (mg/100g BW)	61.49 ± 4.30	65.18 ± 5.03	59.52 ± 2.68
Seminal vesicle full (mg)	381.20 ± 20.08	347.10 ± 35.73	301.30 ± 24.88
Seminal vesicle full (mg/100g BW)	137.50 ± 7.65	136.20 ± 11.44	118.50 ± 6.92
Seminal vesicle empty (mg)	188.70 ± 15.40	168.70 ± 15.23	152.80 ± 15.52
Seminal vesicle empty (mg/100g BW)	68.65 ± 6.76	66.86 ± 4.47	59.94 ± 4.54

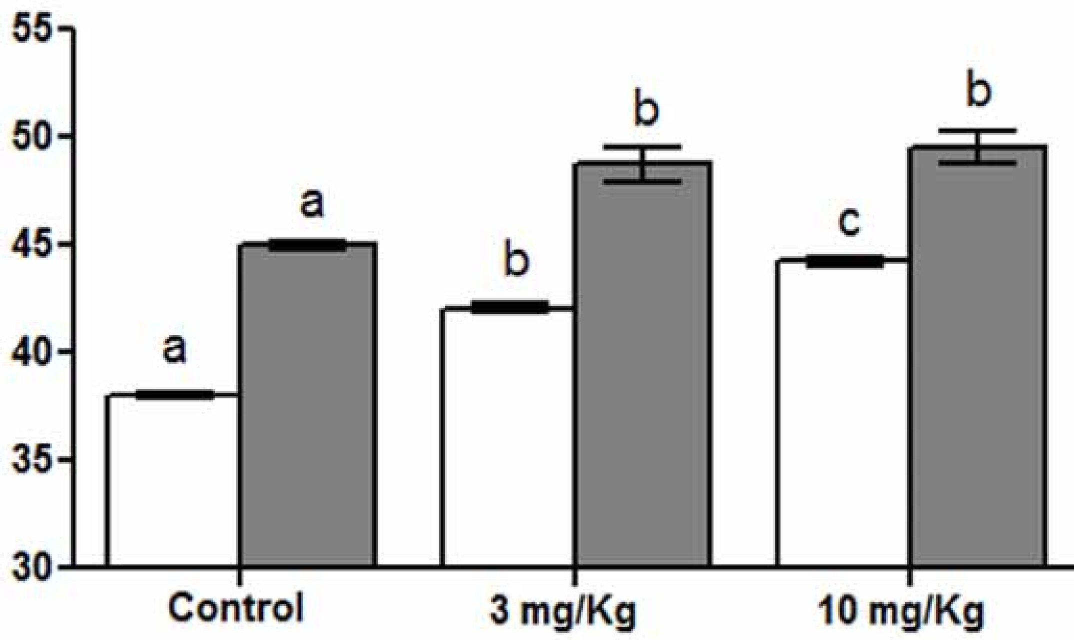
Values expressed as mean ± SEM, $p > 0.05$. ANOVA followed by Tukey test. BW = body weight.

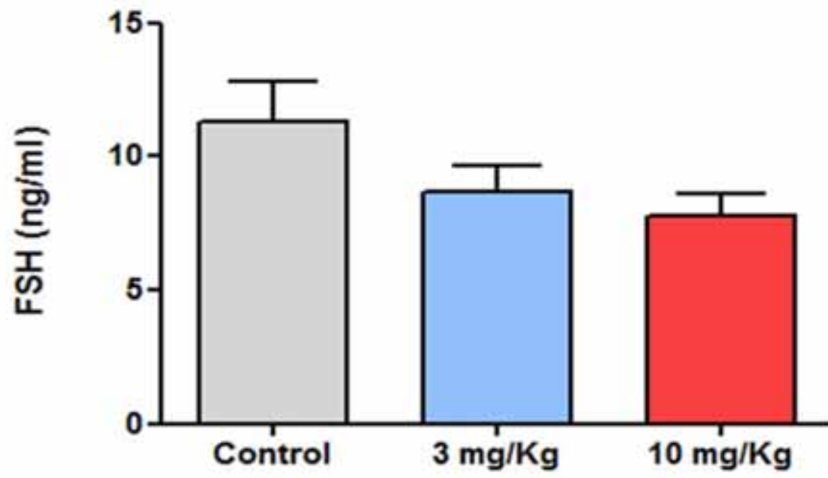
Table 7.

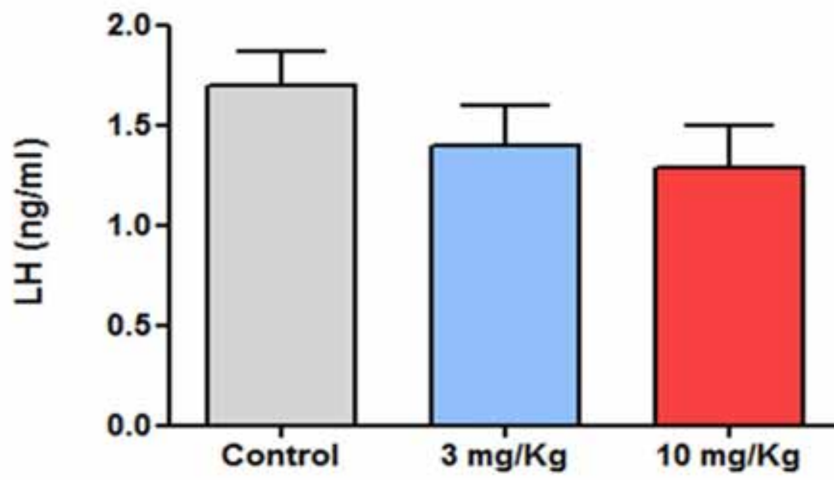
Absolute and relative vital organ weights from control and rosuvastatin-treated groups.

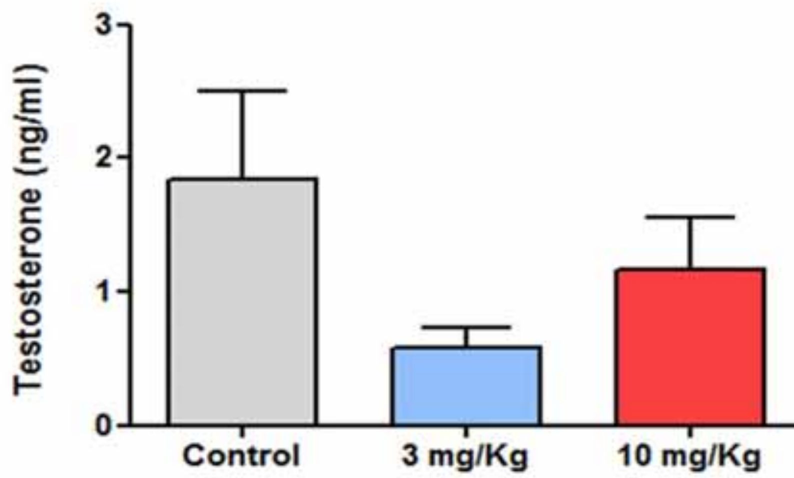
	Experimental groups		
	Control (n=9)	**3mg/Kg (n=10)**	**10mg/Kg (n=10)**
Pituitary (mg)	9.44 ± 0.32	8.15 ± 0.42	8.20 ± 0.51
Pituitary (mg/100g BW)	3.40 ± 0.12	3.27 ± 0.14	3.25 ± 0.16
Adrenal (mg)	31.01 ± 1.56	31.60 ± 2.52	26.67 ± 1.53
Adrenal (mg/100g BW)	11.15 ± 0.52	12.70 ± 0.95	10.77 ± 0.89
Thyroid (mg)	24.29 ± 1.12	22.14 ± 1.48	20.37 ±1.27
Thyroid (mg/100g BW)	8.76 ± 0.43	8.94 ± 0.68	8.11 ± 0.46
Liver (g)	15.72 ± 0.55	13.81 ± 0.66	14.42 ± 0.89
Liver (g/100g BW)	5.65 ± 0.13	5.50 ± 0.12	5.63 ± 0.13
Kidney (g)	1.35 ± 0.02	1.20 ± 0.05	1.23 ± 0.06
Kidney (g/100g BW)	0.49 ± 0.01	0.48 ± 0.01	0.48 ± 0.01

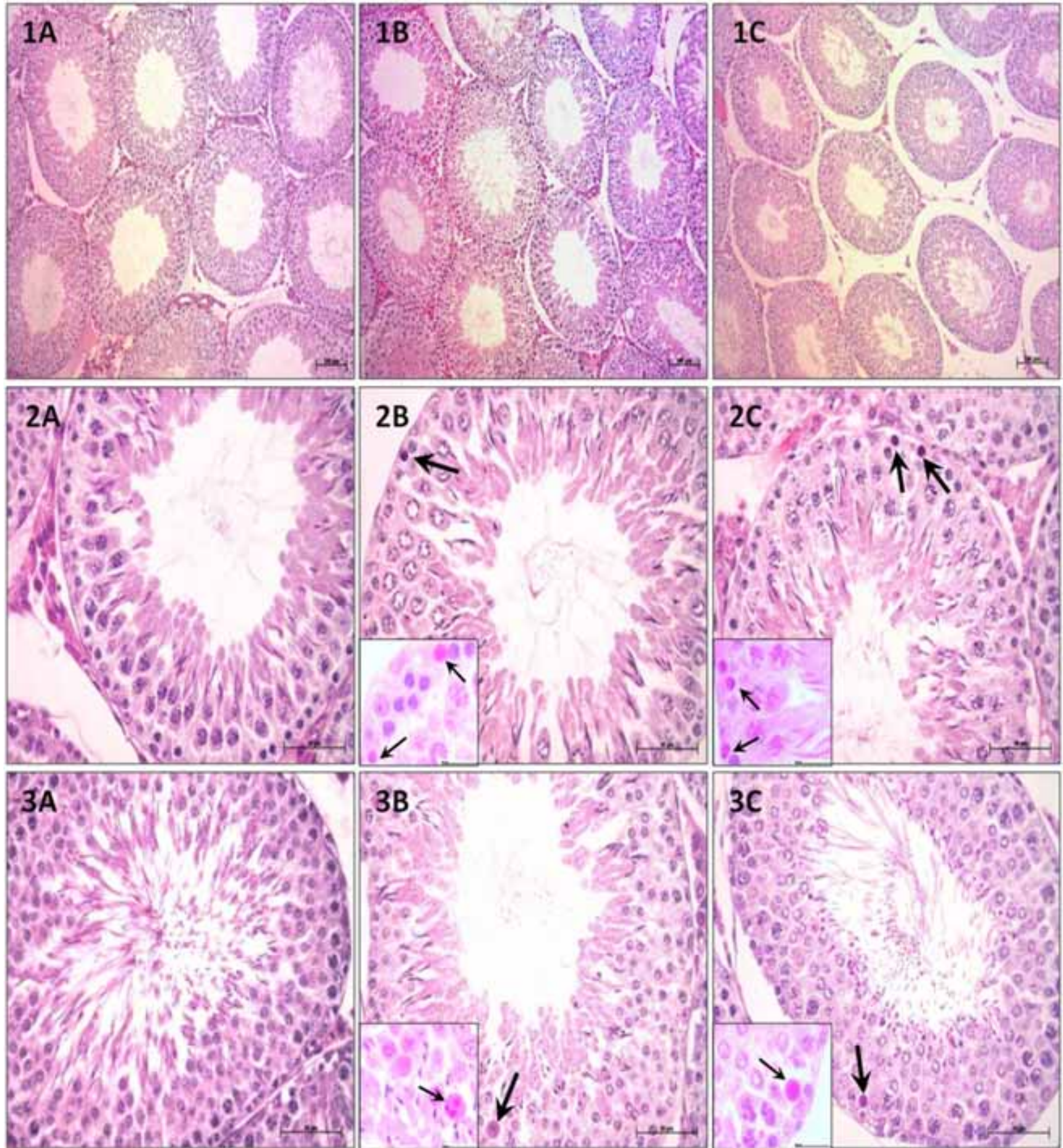
Values expressed as mean ± SEM, $p > 0.05$. ANOVA followed by Tukey test. BW = body weight.

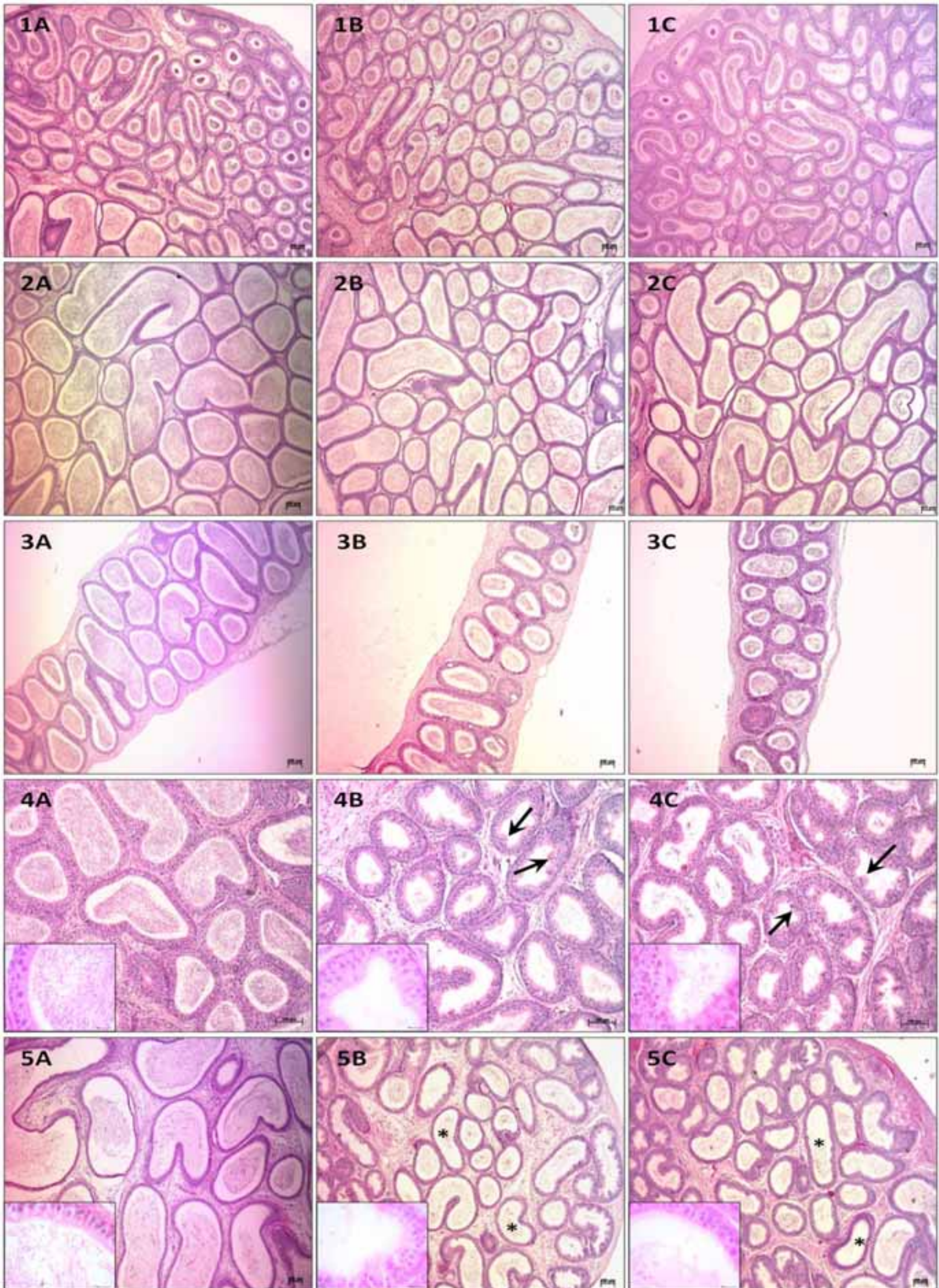

55
50
45
40
35
30
a
a
b
b
c
b
Control
3 mg/Kg
10 mg/Kg

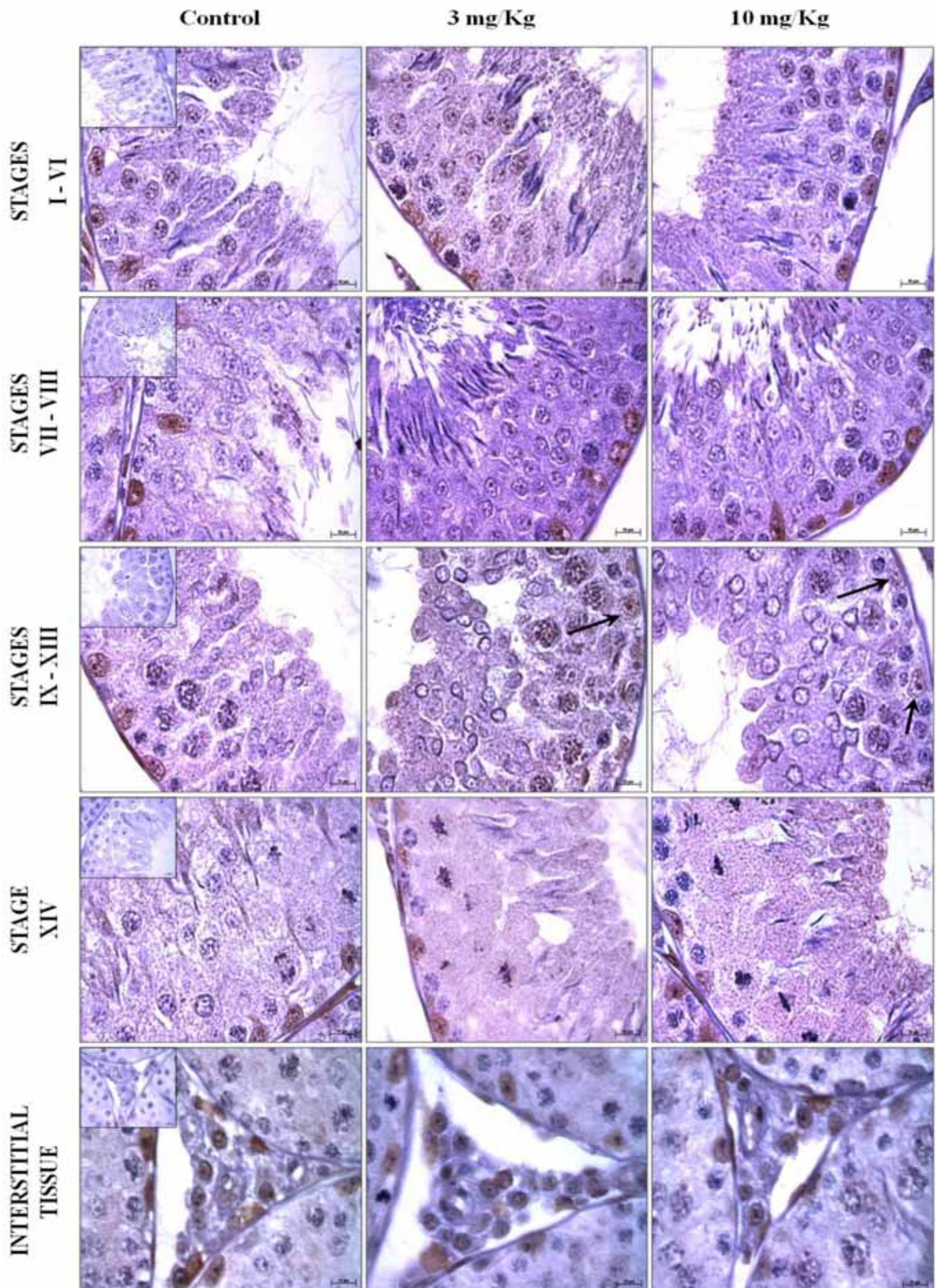

FSH (ng/ml)
15
10
5
0
Control
3 mg/Kg
10 mg/Kg

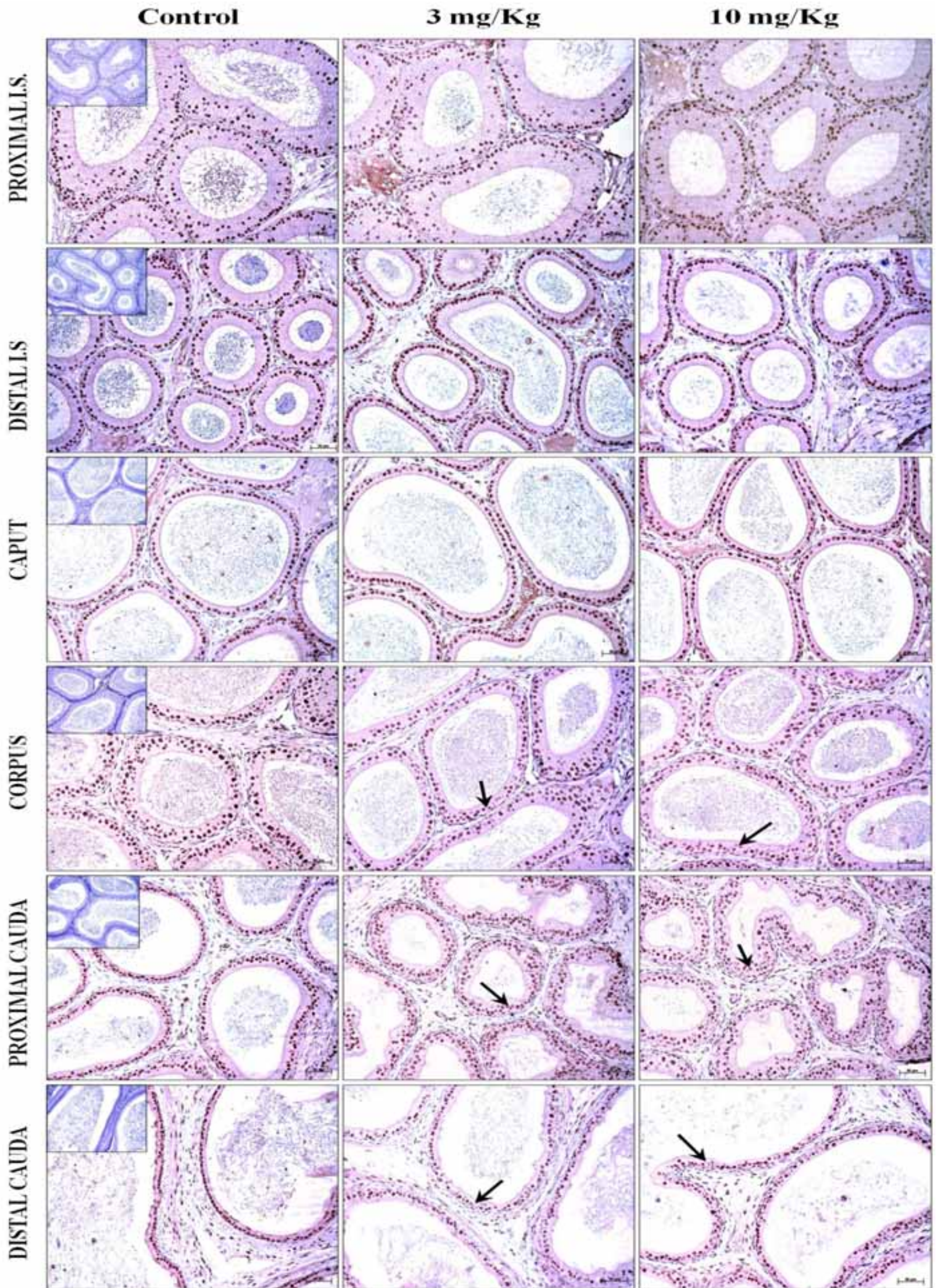

LH (ng/ml)
2.0
1.5
1.0
0.5
0.0
Control
3 mg/Kg
10 mg/Kg


Testosterone (ng/ml)
3
2
1
0
Control
3 mg/Kg
10 mg/Kg

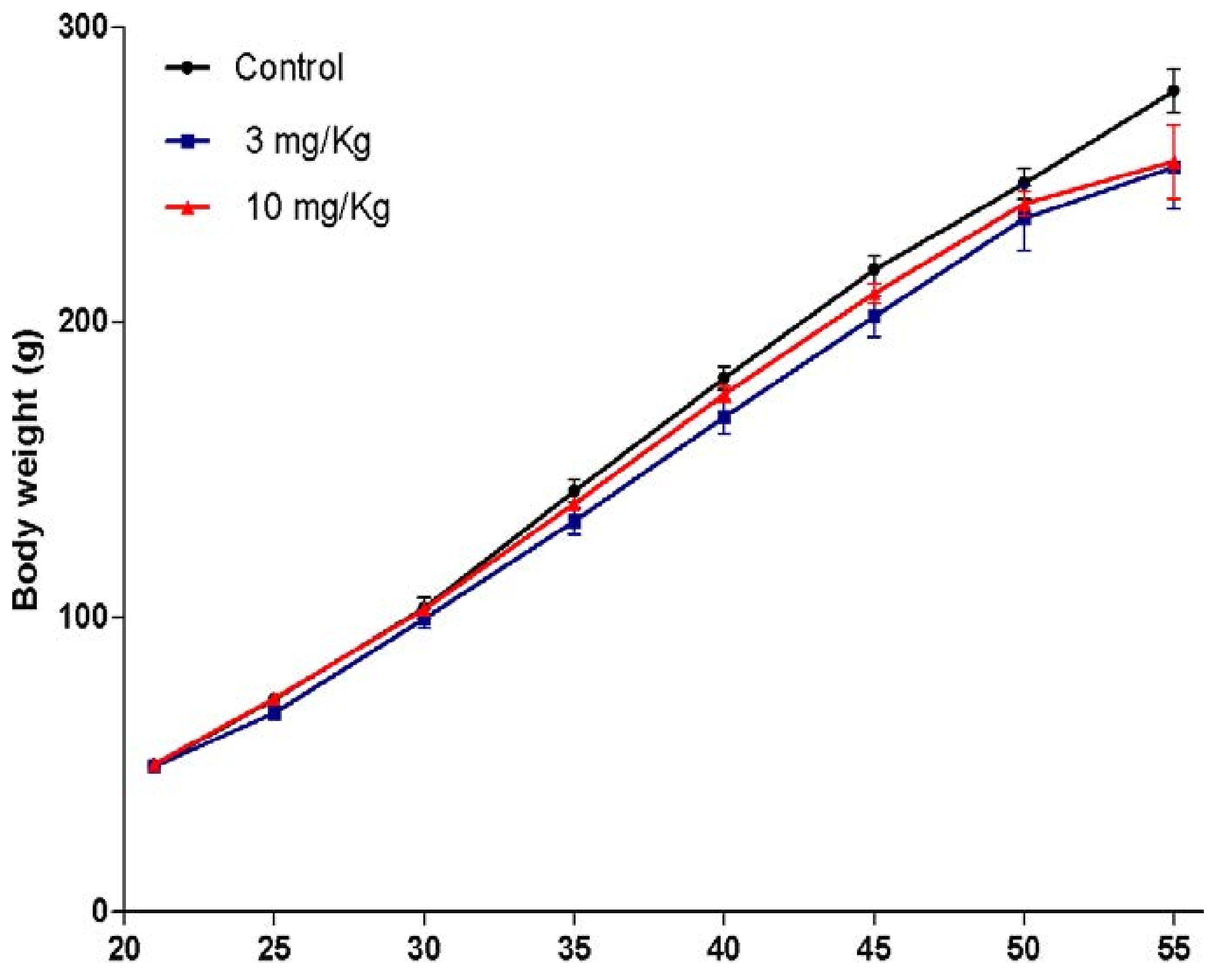

1A
1B
1C
2A
2B
2C
3A
3B
3C


1A
1B
1C
2A
2B
2C
3A
3B
3C
4A
4B
4C
5A
5B
5C
*
*
*
*


Control
3 mg/Kg
10 mg/Kg
STAGES I - VI
STAGES VII - VIII
STAGES IX - XIII
STAGE XIV
INTERSTITIAL TISSUE



Control
3 mg/Kg
10 mg/Kg
PROXIMAL I.S.
DISTAL I.S
CAPUT
CORPUS
PROXIMAL CAUDA
DISTAL CAUDA


Body weight (g)
300
200
100
0
20
25
30
35
40
45
50
55
Control
3 mg/Kg
10 mg/Kg

Capítulo II

Artigo a ser submetido para publicação

no periódico "Endocrinology"

Long-term adverse reproductive effects in male rats exposed to rosuvastatin during prepuberty

Gabriel Adan Araújo Leite[a]*, Marciana Sanabria[a], Marília Martins Cavariani[a], Patrícia Fernanda Felipe Pinheiro[b], Wilma De Grava Kempinas[c]

[a] Graduate Program in General and Applied Biology, Institute of Biosciences, UNESP –Univ. Estadual Paulista, Botucatu, São Paulo, Brazil.

[b] Department of Anatomy, Institute of Biosciences, UNESP–Univ. Estadual Paulista, Botucatu, São Paulo, Brazil.

[c] Department of Morphology, Institute of Biosciences, UNESP–Univ. Estadual Paulista, Botucatu, São Paulo, Brazil.

* Corresponding author at: Departamento de Morfologia, Instituto de Biociências, UNESP, Caixa Postal 510, 18618-970 Botucatu, SP, Brazil. Tel.: +55 14 3811 6264.

E-mail address: gabriel_adan_1990@hotmail.com (G.A.A. Leite).

Abstract

The increase of obesity, bad eating habits and the lack of physical exercises are intrinsically related to dyslipidemias. Rosuvastatin acts as an HMG-CoA reductase inhibitor and has been indicated to prevent cardiovascular diseases and to treat dyslipidemias due to its higher efficiency to reduce serum cholesterol concentrations. This study aimed to evaluate the possible reproductive adverse effects on sexual maturity due to juvenile male rats exposure to rosuvastatin during prepuberty. Three groups were randomly formed with newly weaned rats: control, whose rats received saline solution 0.9% and rosuvastatin at doses of 3 or 10 mg/Kg daily by gavage, since post-natal day (PND) 21 until puberty onset. Male rats were maintained until sexual maturity and they were euthanized on PND110. In the rosuvastatin-treated groups, the results showed diminution in follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone concentrations, delayed latency to the first penis intromission, pathologic alterations in the testis and epididymis and decreased sperm quality. In conclusion, exposure to rosuvastatin at prepuberty provokes long term adverse reproductive effects in male rats.

Introduction

Dyslipidemias have been frequently found and manifested earlier in children due to the increase of childhood and adolescence obese population. These dysfunctions are common in children and adolescents that have mild or moderate obesity, which may lead to increased levels of LDL-cholesterol and VLDL-cholesterol, decreased levels of HDL-cholesterol (1) and augmentation of serum levels of triglycerides (2).

Dysfunctions on lipid profile may be triggered at childhood or later due to genetic factors (1,3) or due to inappropriate life style, that include sedentary, bad eating habits and other factors related to life style (4).

Statins inhibits the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), which is a limiting enzyme for cholesterol biosynthesis (1,5) thus, statins are responsible to diminish total cholesterol, mainly decreasing LDL-cholesterol (5-7).

These medications are indicated to treat patients that have coronary artery disease and associated dyslipidemia, and have been the drug chosen to treat atherosclerosis, because it exhibits two advantages, such as the diminution of serum cholesterol and protection for vascular endothelium (6,7).

Rosuvastatin calcium is the last statin aproved by the regulatory institutions in 2001 and demonstrates higher afinity and interaction with enzymatic site of HMG-CoA reductase (8). In comparison with the other statins, rosuvastatin shows pharmacological advantages, such as its exclusive binding characteristics with the enzyme HMG-CoA redutase and its superior inhibitory effects when compared to the other statins (8-12).

Puberty is a critic period considered as an important parameter for reproductive health, because it represents the transitional phase from childhood to adult reproductive stage (13,14). Moreover during puberty occurs neuroendocrine interactions and important morphological alterations for mammals (13,14).

Exposure to endocrine disruptors at childhood and adolescence, in other words, during prepuberty, may alter the growth and distribution of body fat during this period, as well as, it may promote behavior alterations and increase the risk of cancer at adulthood (15).

During puberty, children are exposed to chemical agents that mat alter puberty timing and the development and acquisition of reproductive function, thus this situation is very important for reproductive toxicology (14), since these alterations during puberty may even affect reproduction on sexual maturity (15-17).

Previous study in our laboratory suggests that administration of rosuvastatin to prepubertal male rats provoked immediate reproductive damage, such as delayed puberty onset and delayed development of epididymis, besides alterations on testicular and epididymal morphology (18).

Considering the utilization of hypolipemiant agents by children at prepuberty and the indication by some experts that more people should be taking advantage of the prescription medication (19), the present study aimed to evaluate possible long term reproductive effects at adulthood due to prepubertal exposure to rosuvastatin, using male rats as experimental model.

Materials and Methods

Obtention of pregnant females and reduction of litters

Adult male (90 days of age) and female (60 days of age) Wistar rats were supplied from Central Biotherium of State University of São Paulo (UNESP), Botucatu/SP and maintained in the Small Mammal Biotherium of the Morphology Department at the UNESP Biosciences Institute, Botucatu. Animals were housed in polypropylene cages (43 cm × 30 cm × 15 cm) with laboratory grade pine shavings as bedding. Rats were maintained under controlled temperature (23 ± 1°C) and lighting conditions (12:12-h photoperiod). Standard rodent chow (Purina Labina, Agribrands do Brasil Ltda, Paulínia/SP, Brazil) and filtered tap water were provided *ad libitum*.

Two nulliparous female rats were mated with one male, during the dark portion of the lighting cycle, and the day of sperm detection in the vaginal smear of female rats in estrus was considered day zero of gestation (gestational day 0 – GD 0). Pregnant and lactating rats were maintained in individual cages. After birth, the number of pups per litter was culled to eight on post-natal day (PND) 4 to balance male and female pups (4 each per litter). Litters with fewer than eight pups were not entered into the experimental protocol and were euthanized.

Experimental design

After weaning (PND 21), male pups were distributed into three experimental groups (n=10 per group, with one or two pups per litter for each group), that received vehicle (saline solution 0,9%), 3 or 10 mg/Kg/day of rosuvastatin diluted in saline solution 0,9% administered by gavage since PND 21 until puberty onset, which was indicated by complete preputial separation (variable age for each animal). The doses of 5 mg/day to 40 mg/day of rosuvastatin are available to be utilized by humans to decrease total cholesterol and LDL-cholesterol (11), thus the doses used in this study were choose based on body surface area correction from children available doses of rosuvastatin to pup rats equivalent doses (20).

In this study, we used normal, non-obese juvenile rats and simulate the human exposure to rosuvastatin, since some experts are suggesting that more people should be taking advantage of the prescription medication (19).

The experimental protocol followed the Ethical Principles in Animal Research of the Brazil College of Animal Experimentation and was approved by the Bioscience Institute/UNESP Ethics Committee for Animal Experimentation (protocol no. 336-CEUA).

In order to evaluate the possible reproductive long term effects due to prepubertal statin exposure, male rats were euthanized at the sexual maturity (PND 110), ten days after the evaluation of sexual behavior (PND 100), when all animals have already re-established the

epididymal sperm storage to obtain the final body weight, reproductive and vital organ weights, hormonal dosages, sperm parameters, testicular and epididymal histopathological analysis, testicular morphometric analysis and immunohistochemistry for androgen receptors (AR) on testis.

Evaluation of sexual behavior

Male rats were placed individually in polycarbonate crystal cages, measuring 44 x 31 x 16 cm, five minutes before the introduction of one adult female in natural proestrus or estrus (sexually receptive) determined by vaginal smear. Behavioral testing was conducted in the dark period of the cycle between 8:00 and 12:00 am in a separate room under dim red illumination. If the male rat did not mount within the next 10 minutes, the rat would have just another chance to perform the sexual behavior on the next day, as a second chance. The following measures were recorded (21): mount and intromission latencies, defined as the times from introduction of the female in the cage to the first mount and intromission, respectively; intromission frequency, the number of intromissions preceding the first ejaculation; ejaculation latency, the time from introduction of the female in the cage to the first ejaculation; intromission latency post-ejaculation, the time to the first intromission after the first ejaculation; intromission frequency post-ejaculation, the number of intromissions after the first ejaculation; and total number of ejaculations. If the male did not mount in the first 10 minutes following the introduction of one adult female in the box, it was considered sexually inactive.

Euthanasia, body weight and organ weights

On PND 110, male rats were weighted and thereafter, submitted to narcosis in a box of CO_2, following blood collection by hepatic portal vein, between 9:00 and 11:30 a.m. Reproductive organs, such as: left testis, epididymis and vas deferens, seminal gland (full and

empty, without the coagulating gland) and ventral prostate, from all rats were collected and weighted. Vital organs that demonstrate very important role for toxicological parameters, such as liver, kidney, adrenal, thyroid and pituitary were also collected and weighed.

Serum hormonal levels

Serum was obtained by centrifugation (2400 rpm, 20 minutes, 4ºC) in a refrigerated device and was frozen at –20ºC until the moment of hormonal determination. Testosterone, FSH and LH were determined by double-antibody radioimmunoassay. Testosterone concentrations were measured by Testosterone Maia kit (Biochem Immuno System, Allentown, PA) and LH e FSH used specified kits supplied by National Institute of Arthritis, Diabetes and Kidney Diseases (NIADDK). All samples were measured in the same assay to avoid the inter-assay errors. Intra-assay variability were 3.4% for LH, 2.8% for FSH, and 4% for testosterone.

Sperm motility

Sperm motility was evaluated right after euthanasia. The right epididymal cauda was collected, semen was extracted and a sample was dilluted in 2 mL of the HTF modified medium (Spectrun 90126); a 10µL aliquot was transferred to a Mackler chamber. Under a light microscope (20x magnification), 100 spermatozoa were analyzed and classified as: type A, motile with regular and fast progressive movement; type B, motile with non-progressive movement or type C, immotile. Sperm motility was expressed as the percentage of total sperm (16).

Sperm morphology

Semen was extracted from the right epididymal cauda of all rats, diluted in HTF modified medium and a sample was obtained and added 1.0 mL of saline formol. For the

analysis, smears were prepared on histological slides that were left to dry for 90 minutes and observed in a phase-contrast microscope (400X magnification); 200 spermatozoa were analyzed per animal (22). Morphological abnormalities were classified into general categories pertaining to head morphology (without curvature, without characteristic curvature, pin head or isolated form, i.e., no tail attached) and tail morphology (broken, isolated, i.e., no head attached, or rolled into a spiral). Moreover, the presence and position (proximal, medial or distal) of the cytoplasmic droplet were evaluated in the same sperm (23).

Sperm counts in the testis

Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) were enumerated as described previously (24), with adaptations as following: the right testes, decapsulated and weighed soon after collection, were homogenized in 5 mL of NaCl 0.9% containing Triton X 100 0.5%, followed by sonication for 30 seconds. After a 10-fold dilution a sample was transferred to Neubauer chambers (4 fields per animal), proceeding an enumeration of mature spermatids. To calculate the daily sperm production (DSP), the number of spermatids at stage 19 was divided by 6.1, which is the number of days of the seminiferous cycle in which these spermatids are present in the seminiferous epithelium. To obtain the number of mature spermatids per gram of testis and the relative DSP, the number of mature spermatids and the DSP were divided by the weight of the testicular parenchyma.

Histological procedures and histopathological evaluation

Left testis and epididymis were fixed in Bouin's fluid, embedded in Paraplast® and sectionated in 4µm (transversal sections of testis and longitudinal sections of epididymis). Sections were stained with hematoxylin and eosin (HE) to evaluate testicular and epididymal morphology under light microscopy. Sections destined for immunohistochemistry were obtained

in silanized slides. The evaluation was performed in blind assay and the figures were obtained using a light microscopy Zeiss model Scope A1-Axio, coupled to the image digitizer system Axio Vision, version 4.7.2.

In the testis, seminiferous tubules cross-sections were randomly chosen in three non-serial sections per animal obtained with a distance of 50 µm among them, totaling 200 tubules evaluated per animal. Seminiferous tubules were classified as: normal (presence of concentric and normally organized germ cell layers in seminiferous epithelium) or abnormal (presence of germ cells and cellular debris in the lumen, multinucleated formation, seminiferous epithelium with acidophilic cells, few germ cell layers, vacuole formation or degeneration in seminiferous epithelium). Interstitial tissue and peritubular myoid cells histopathological analysis were qualitative and the interstitial analysis aimed to assess Leydig cells morphology and the appearance of blood vessels. Epididymal histopathological analysis was qualitative, evaluating each region of the organ according to the epithelium, lumen and interstitial tissue morphological appearance.

Testicular and epididymal morphometric evaluation

Tubular diameters, tubular and luminal area of the seminiferous tubules and epithelial height of the seminiferous epithelium, were measured using a light microscopy Zeiss model Scope A1-Axio, coupled to the image digitizer system Axio Vision, version 4.7.2. For this, 10 random testicular cross-sections (stage IX of the seminiferous epithelium cycle) per animal (n = 9 or 10 animals⁄group) were examined blindly at x200 magnification. To assess spermatogenesis kinetics, one hundred random tubular sections per animal (n = 9 or 10 animals⁄ group) in three non-consecutive testis cross-sections were classified into four categories: stages I–VI, VII–VIII, IX–XIII and XIV of the seminiferous epithelium cycle (25), under a light microscope (Zeiss, Axiostar plus, Oberkochen, Germany) at x200 magnification. Besides, epididymal stereology was

performed in photomicrographs of the different regions of the epididymis, which were obtained using a light microscopy Zeiss model Scope A1-Axio, coupled to the image digitizer system Axio Vision, version 4.7.2 and evaluated utilizing the program Image Pro Plus.

Immunohistochemistry for AR on testis

Histological sections were allocated in silanized slides, dewaxed using toluene, hydrated using decreasing concentrations of ethanol and washed in phosphate buffer (PBS), followed by antigen recovery with citrate buffer (0.01M, pH 6.0) in a pressure cooker submitted to high temperatures for 40 minutes. After these steps, the slides were incubated in hydrogen peroxide in 3% of methanol during 15 minutes. Thus, the sections were incubated overnight with primary antibody anti-androgen receptor (Clone SC-816 - AR N-20 from Santa Cruz Biotechnology Inc.®). After incubating, the slides were washed with PBS buffer and during 1 hour, at room temperature, the sections were incubated with secondary antibody (Biotinylated Sheep Anti-Rabbit Immunoglobulins - DAKO Cyt. Inc. Ò). After this step, the slides were washed with PBS buffer and submitted to avidin-biotin-peroxidase solution (StreptABComplex DAKO Cyt. Inc. Ò) for 45 minutes. Thereafter, the slides were washed with PBS buffer and submitted to diaminobenzidine (DAB) (di-amino-benzidine- Sigma Ò) for 5 minutes, then, the slides were washed in tap water, dehydrated in 70%, 90% and 100% ethanol and counterstained with Harris hematoxylin. Coverslips were mounted on the slides with Permount. Negative controls were performed without primary antibody. All analysis were carried out in using a light microscopy Zeiss model Scope A1-Axio, coupled to the image digitizer system Axio Vision, version 4.7

Statistical analysis

For comparison of the results among the experimental groups, ANOVA or Kruskall Wallis folllowed by "a posteriori" test as Tukey or Dunns, respectively, were performed

according to the characteristics of each variable. Differences were considered significant when $p<0.05$. Statistical analysis were conducted on GraphPad Prism (version 5.00).

Results

Decreased daily sperm production, diminution of sperm motility and increase of sperm abnormalities

Rats in both rosuvastatin doses exhibited decrease in the number of progressive motile sperm and increase of the rate of non-progressive and imotile sperm, when compared to the control group ($p<0.05$) (Fig. 1). Moreover, there was diminution of the normal sperm, with increase of tail and head sperm abnormalities, such as isolated sperm tail and head ($p>0.05$) (Table 1). In addition, the total number of mature spermatids per testis, daily sperm production, the number of mature spermatids per gram of testis and relative sperm production were reduced in the animals that received rosuvastatin at the dose of 3mg/Kg of rosuvastatin during prepuberty (Table 2).

Histopathological and morphometric alterations and reduction of AR staining on adult testis induced by prepubertal exposure to rosuvastatin

Histopathological analysis showed a decrease in the percentage of normal seminiferous tubules ($p<0.05$), followed by an aumengtation in the number of tubules with acidophilic germ cells and/or germ cells with fragmented cromatin ($p<0.05$) (Table 1), which are characteristics of cell death and occured mainly on stages XI-XIV (Fig. 2). Interstitial tissue and peritubular cells exhibited similar morphology among the groups.

Testicular morphometric analysis demonstrated lower height of the seminiferous epithelium in the group exposed to rosuvastatin at the dose of 3mg/Kg ($p<0.05$), however, no alterations were observed on epithelium height at the dose of 10mg/Kg of rosuvastatin ($p>0.05$)

in comparison with the control group (Table 2). Tubular area and diameter were not altered due to the experimental treatment ($p>0.05$) (Table 2). Spermatogenesis kinetics was altered at adulthood and occurred an increase in the frequency of seminiferous tubules in stages I-VI, followed by the diminution in the frequency of seminiferous tubules in stages VII-VIII and in stage XIV in the animals that received 3 or 10mg/Kg of rosuvastatin from weaning until puberty onset (Table 1).

Immunohistochemistry for androgen receptors on testis revealed decreased staining in the Sertoli cells at stages I-VI and IX-XIII (Fig. 3). The Leydig cells androgen receptor staining were similar among the groups (Fig. 3).

Epididymal histopathological alterations during sexual maturity due to prepubertal exposure to rosuvastatin

There was increase in the frequency of leukocyte infiltrates in the epididymis interstitial tissue, whereas 70% of the rats treated with 3mg/Kg and 80% of the rats that received 10mg/Kg of rosuvastatin presented this histopathological alteration in the interstitium, when compared to the control group, in which 20% of the rats showed this morphological alteration (Fig. 2). Moreover, there was hypertrophy on clear cells in the proximal epididymal cauda at both doses (Fig. 2).

Epididymis epithelium morphology was similar among the groups throughout initial segment, caput, corpus and distal cauda. In addition, all epididymal regions were occupied with sperm inside the lumen.

Diminution of serum hormonal concentrations and delayed first intromission on male sexual behavior

Hormonal serum concentrations demonstrated reduction on serum FSH, LH and testosterone concentrations in the animals that received rosuvastatin at both doses during prepurberty ($p<0.05$) (Fig. 4).

Sexual behavior analyses showed that 80% of animals in each group performed at least the behavior of mount during evaluation of sexual behavior (n=8/group). The latency to the first mount and ejaculation were not statistically significant ($p>0.05$) (Table 3). The latency to the first intromission demonstrated a delayed beginning to the first intromission in the rosuvastatin-treated groups, but below the significance level ($p = 0.0719$), with delay of 180,62% at the dose of 3mg/Kg and 181,33% at the dose of 10mg/Kg when compared to the control group. The other parameters assessed on sexual behavior, such as the total of mounts and intromissions until the first ejaculation, number of post-ejaculatory intromissions and number of ejaculations were not affected due to prepubertal exposure to rosuvastatin ($p>0.05$) (Table 3).

Assessment of the final body weight and reproductive and vital organ weights

Body weight did not show significant differences among the experimental groups ($p = 0.1181$), although there was discrete diminution on final body weight in the rosuvastatin-treated groups during prepuberty (Table 4). Reproductive (right testis, epididymis and vas deferens, ventral prostate and full and empty seminal gland without coagulating gland) and vital organ weights (pituitary, adrenal, thyroid, liver and kidney) were not affected by the experimental treatment with rosuvastatin ($p>0,05$) (Table 4).

Discussion

The present study aimed to investigate whether the exposure to rosuvastatin since prepuberty may compromise sperm quality and male reproduction at adulthood, since there were delay on puberty onset and diminution on testosterone serum concentrations in this period, as

reported in previous study (18). Moreover, this study enables to understand how hypolipemiant agents administration and other compounds that decrease testosterone concentrations may interfere with male reproductive capability during sexual maturity.

Decreased sperm count is a marker for toxic effects on male reproduction and is related to the diminution of male fertility (26). In this study, the total number of mature spermatids in the testis and daily sperm production were decreased in the rats that received 3 mg/Kg of rosuvastatin since pré-puberty. The diminution of FSH and testosterone concentrations may have contributed to the decreased daily sperm production, because these hormones have an important role in spermatogenesis, such as the qualitative and quantitative maintenance of sperm production, respectively (27). Besides, in a previous study for us performed, there were delay on pubert onset and in spermatogenesis installation in the rosuvastatin-treated groups (18) and during adult life occurred persistent diminution on sperm production in the rosuvastatin-exposed animals.

Sperm motility is another important parameter for male fertility and is a target for many toxic agents, thus, diminished percentage of progressively motile sperm provides information about the action of toxic agents on epididymis and during sperm maturation (26,28,29). The rosuvastatin-treated animals exhibited lower progressive motile sperm percentage, with an increase on non-progressive and immotile sperm percentage in comparison with control group, demonstrating that the delay on epididymal post-natal development induced by rosuvastatin exposure (18) promoted a toxic action on epididymis and on processes of sperm maturation.

The assessment of sperm morphology collected from epididymal cauda have been widely used to detect morphological alterations that occurred during spermatogenesis due to the exposure to a toxic agent (23). Besides sperm morphology provides information about spermatogenesis, it is normally followed by other toxic effects, such as diminution of sperm counts and sperm motility and testicular histopathological alterations (23). In the present work,

there was increase on tail and head sperm abnormalities, that suggests toxic effects of rosuvastatin in spermatogenesis since prepubertal exposure.

In addition, there was a dysregulation in spermatogenesis kinetics with an augmentation of the percentage of seminiferous tubules on stages I-VI (pre-spermiation stage) and a diminution on spermiating tubules, namely stages VII-VIII in the rosuvastatin-exposed rats. The diminution of spermiating tubules is associated with the decreased sperm production demonstrated in this work.

Several studies have reported cell death induced by statin exposure due to decreased levels of intermediate isoprenoids in cell cultures and *in vivo* (18,30-35). Previous study demonstrated germ cell death in the testis of rosuvastatin-treated rats at puberty (18) and now we showed the persistence of spermatogonias and spermatocytes death in the adult testis.

In addition, the animals treated with the dose of 3 mg/Kg of rosuvastatin at prepuberty exhibited during adult life lower height of seminiferous epithelium, reaffirming the toxic potential of rosuvastatin in spermatogenesis, when administered at the period in which spermatogenesis is not yet completely established.

Under androgenic stimulation and androgen receptor expression the Sertoli cells secrete factors that enables meiotic cells survive in the seminiferous epithelium (36,37). In this study, there was decreased expression of AR in the Sertoli cells on stages I-VI and IX-XIII in the rosuvastatin-exposed animals with spermatocytes death on stages IX-XIV.

In the rosuvastatin-exposed groups, the epididymis showed several histopathologic alterations, such as clear cells hypertrophy in the cauda epididymis and higher frequency of leukocyte infiltrate, which may be associated with delayed epididymal development, as previously reported (18), and with an increase in inflammatory response.

Hormonal measurements showed diminished serum FSH, LH and testosterone concentrations at sexual maturity, presumably due to a dysregulation of the hypothalamic-pituitary-gonadal (HPG) axis after exposure to rosuvastatin at prepuberty (18).

Testosterone is an steroid hormone and acts as a facilitator to the beginning of the male sexual behavior, because acts on central nervous system increasing nitric oxide production, modifying neuronal excitability and neurotransmitters releasing, such as dopamine in several brain areas, leading as response, to the male sexual interest (38,39). In this work, there was delay in the latency for the first intromission in the treated groups in comparison to the control group, probably due to androgen depletion, which may have retarded the beginning of intromission during sexual behavior.

Previous studies report that statins may contribute to reduce body weight (18,40,41). In this work, the exposure to rosuvastatin during prepuberty provoked discrete diminution on final body weight of adult animals, indicating that rosuvastatin administration may diminish body weight gain from puberty until adult life. The reproductive and vital organ weights at adulthood were not affected by rosuvastatin exposure during prepuberty, suggesting that this medication does not produce systemic toxicity since prepuberty.

In conclusion, this study shows, for the first time, that prepubertal exposure to rosuvastatin provoked long term adverse reproductive effects in male rats, with decreased sperm production and motility, increased incidence of sperm abnormalities and deleterious alterations in the testis and epididymis. These alterations are probably associated with a dysregulation of the HPG axis and diminished testosterone at sexual maturity.

Acknowledgments

The authors are grateful to São Paulo Research Foundation (FAPESP) for its financial support as part of Masters Scholarship (Grant #011/15065-5), to José Eduardo Bozano, from the

Department of Morphology, Institute of Biosciences, UNESP, Botucatu/SP – Brazil, for excellent technical assistance. We are also grateful to Dr. Janete Aparecida Anselmo Franci and Dr. Ruither de Oliveira Gomes Carolino for the hormonal dosages.

Disclosure summary

The authors have nothing to disclose.

References

1. Jiménez EG, Ferre JA. Estatinas: Características y efectos sobre el control lipídico en el niño y adolescente obeso. *Rev Clin Med Fam* 2011;4(1):69-75.

2. Kwiterovich PO, Jr. Recognition and management of dyslipidemia in children and adolescents. *The Journal of clinical endocrinology and metabolism.* 2008;93(11):4200-4209.

3. Cook S, Kavey RE. Dyslipidemia and pediatric obesity. *Pediatric clinics of North America.* 2011;58(6):1363-1373, ix.

4. Izar MCO, Fonseca MIH, Fonseca FAH. Dislipidemias. *Rev Bras Med.* 2011;68(12):59-73.

5. Istvan ES, Deisenhofer J. Structural mechanism for statin inhibition of HMG-CoA reductase. *Science.* 2001;292(5519):1160-1164.

6. Endres M. Statins: potential new indications in inflammatory conditions. *Atherosclerosis Supplements.* 2006;7(1):31-35.

7. Tandon V, Bano G, Khajuria V, Parihar A, Gupta S. Pleiotropic effects of statins. *Int J Pharmacol.* 2005;37(2):77-85.

8. McTaggart F. Comparative pharmacology of rosuvastatin. *Atherosclerosis Supplements.* 2003;4(1):9-14.

9. Holdgate GA, Ward WH, McTaggart F. Molecular mechanism for inhibition of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase by rosuvastatin. *Biochemical Society transactions.* 2003;31(Pt 3):528-531.

10. Fonseca FAH. Farmacocinética das estatinas. *Arquivos Brasileiros de Cardiologia.* 2005;85(5):9-14.

11. Vaughan CJ, Gotto AM, Jr. Update on statins: 2003. *Circulation.* 2004;110(7):886-892.

12. Penning-van Beest FJ, Termorshuizen F, Goettsch WG, Klungel OH, Kastelein JJ, Herings RM. Adherence to evidence-based statin guidelines reduces the risk of hospitalizations for acute myocardial infarction by 40%: a cohort study. *European heart journal.* 2007;28(2):154-159.

13. Zawatski W, Lee MM. Male pubertal development: are endocrine-disrupting compounds shifting the norms? *Journal of Endocrinology.* 2013;218(2):R1-R12.

14. Stoker TE, Laws SC, Guidici DL, Cooper RL. The effect of atrazine on puberty in male Wistar rats: An evaluation in the protocol for the assessment of pubertal development and thyroid function. *Toxicol Sci.* 2000;58(1):50-59.

15. Mantovani A, Fucic A. Puberty dysregulation and increased risk of disease in adult life: Possible modes of action. 2013.

16. Perobelli JE, Alves TR, de Toledo FC, Fernandez CD, Anselmo-Franci JA, Klinefelter GR, Kempinas Wde G. Impairment on sperm quality and fertility of adult rats after antiandrogen exposure during prepuberty. *Reproductive toxicology.* 2012;33(3):308-315.

17. Perobelli JE, Patrao MT, Fernandez CD, Sanabria M, Klinefelter GR, Avellar MC, Kempinas WD. Androgen deprivation from pre-puberty to peripuberty interferes in proteins expression in pubertal and adult rat epididymis. *Reproductive toxicology.* 2013;38:65-71.

18. Leite GAA, Rosa JL, Sanabria M, Cavariani MM, Anselmo-Franci JA, Pinheiro PFF, Kempinas WG. Delayed reproductive development in pubertal male rats exposed to the hypolipemiant agent rosuvastatin since prepuberty. 2014.

19. Park A. Statins have few side effects, but should more people be taking them? *Health and family* 2013. Accessed December 24, 2013.

20. Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2008;22(3):659-661.

21. Ahlenius S, Larsson K. Apomorphine and Haloperidol-Induced Effects on Male-Rat Sexual-Behavior - No Evidence for Actions Due to Stimulation of Central Dopamine Autoreceptors. *Pharmacol Biochem Be.* 1984;21(3):463-466.

22. Seed J, Chapin RE, Clegg ED, Dostal LA, Foote RH, Hurtt ME, Klinefelter GR, Makris SL, Perreault SD, Schrader S, Seyler D, Sprando R, Treinen KA, Veeramachaneni DNR, Wise LD. Methods for assessing sperm motility, morphology, and counts in the rat, rabbit, and dog: A consensus report. *Reproductive toxicology.* 1996;10(3):237-244.

23. Filler R. Methods for evaluation of rats epididymal sperm morphology. In: Chapin RE, Heindel JH, eds. *Male reproductive toxicology*. San Diego, California: Academic Press; 1993:334-343.

24. Robb GW, Amann RP, Killian GJ. Daily sperm production and epididymal sperm reserves of pubertal and adult rats. *Journal of reproduction and fertility.* 1978;54(1):103-107.

25. Leblond CP, Clemont Y. Spermatogenesis of rat, mouse, hamster and guinea pig as revealed by the periodic acid-fuchsin sulfureous acid technique. *American Journal of Anatomy.* 1952;90:167-215.

26. Perreault SD, Cancel AM. Significance of incorporating measures of sperm production and function into rat toxicology studies. *Reproduction.* 2001;121(2):207-216.

27. França LR, Avelar GF, Almeida FF. Spermatogenesis and sperm transit through the epididymis in mammals with emphasis on pigs. *Theriogenology.* 2005;63(2):300-318.

28. Klinefelter GR, Hess RA. Toxicology of the male excurrent ducts and accessory sex glands. In: Dekker M, ed. *Reproductive and Developmental Toxicology*. New York: Ed. KS Korach; 1998:553-591.

29. Perreault SD. Gamete toxicology: the impact of new technologies. In: Dekker M, ed. *Reproductive and Developmental Toxicology*. New York: Ed. KS. Korach; 1998:635-654.

30. Qi XF, Zheng L, Lee KJ, Kim DH, Kim CS, Cai DQ, Wu Z, Qin JW, Yu YH, Kim SK. HMG-CoA reductase inhibitors induce apoptosis of lymphoma cells by promoting ROS generation and regulating Akt, Erk and p38 signals via suppression of mevalonate pathway. *Cell Death Dis.* 2013;4.

31. Tanaka T, Tatsuno I, Uchida D, Moroo I, Morio H, Nakamura S, Noguchi Y, Yasuda T, Kitagawa M, Saito Y, Hirai A. Geranylgeranyl-pyrophosphate, an isoprenoid of mevalonate cascade, is a critical compound for rat primary cultured cortical neurons to protect the cell death induced by 3-hydroxy-3-methylglutaryl-CoA reductase inhibition. *Journal of Neuroscience.* 2000;20(8):2852-2859.

32. Araki M, Maeda M, Motojima K. Hydrophobic statins induce autophagy and cell death in human rhabdomyosarcoma cells by depleting geranylgeranyl diphosphate. *Eur J Pharmacol.* 2012;674(2-3):95-103.

33. Marcuzzi A, Zanin V, Piscianz E, Tricarico PM, Vuch J, Girardelli M, Monasta L, Bianco AM, Crovella S. Lovastatin-induced apoptosis is modulated by geranylgeraniol in a neuroblastoma cell line. *International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience.* 2012;30(6):451-456.

34. Lee SK, Kim YS. Phosphorylation of eIF2 alpha attenuates statin-induced apoptosis by inhibiting the stabilization and translocation of p53 to the mitochondria. *International journal of oncology.* 2013;42(3):810-816.

35. Gopalan A, Yu WP, Sanders BG, Kline K. Simvastatin inhibition of mevalonate pathway induces apoptosis in human breast cancer cells via activation of JNK/CHOP/DR5 signaling pathway. *Cancer Lett.* 2013;329(1):9-16.

36. Stanton PG, Sluka P, Foo CF, Stephens AN, Smith AI, McLachlan RI, O'Donnell L. Proteomic changes in rat spermatogenesis in response to in vivo androgen manipulation; impact on meiotic cells. *PloS one.* 2012;7(7):e41718.

37. De Gendt K, Swinnen JV, Saunders PT, Schoonjans L, Dewerchin M, Devos A, Tan K, Atanassova N, Claessens F, Lecureuil C, Heyns W, Carmeliet P, Guillou F, Sharpe RM, Verhoeven G. A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. *Proceedings of the National Academy of Sciences of the United States of America.* 2004;101(5):1327-1332.

38. Hull EM, Lorrain DS, Du J, Matuszewich L, Lumley LA, Putnam SK, Moses J. Hormone-neurotransmitter interactions in the control of sexual behavior. *Behavioural brain research.* 1999;105(1):105-116.

39. Gerardin DC, Pereira OC, Kempinas WG, Florio JC, Moreira EG, Bernardi MM. Sexual behavior, neuroendocrine, and neurochemical aspects in male rats exposed prenatally to stress. *Physiology & behavior.* 2005;84(1):97-104.

40. Salem NA, Assaf N, Ahmed HH. Pleiotropic effects of rimonabant and simvastatin on obesity associated multiple metabolic risk factors in rats. *European review for medical and pharmacological sciences.* 2012;16(6):797-807.

41. Famer D, Crisby M. Rosuvastatin reduces gliosis and the accelerated weight gain observed in WT and ApoE-/- mice exposed to a high cholesterol diet. *Neuroscience letters.* 2007;419(1):68-73.

Legends of figures

Fig. 1. Sperm motility of rats from control (n=10) and rosuvastatin-treated groups (n=10 per group). Values expressed as median. Kruskal-Wallis followed by Dunns test. $p < 0.05$.

Fig. 2. . Histopathological analysis of the seminiferous epithelium and the epididymis of rats on PND110. Photomicrograph of testicular and epididymal sections from control group (1A and 2A-4A) and treated with 3mg (1B and 2B-4B) or 10mg of rosuvastatin (1C and 2C-4C). Observe the presence of acidophilic cells (arrows) that include spermatogonias (1C) and spermatocytes (1B) in the seminiferous epithelium. On epididymis note the presence of clear cell hyperplasia (arrows) (2B and 2C) and leukocyte infiltrates (asterisks) (3B, 3C, 4B and 4C). Hematoxiline and Eosin (HE). Scale bar = 50 µm.

Fig. 3. Immunostaining of AR in adult rat testis from groups exposed to saline solution (control group, *n* = 5 animals), or to rosuvastatin at doses of 3 mg/kg (*n* = 5 animals) or 10 mg/kg (*n* =5 animals). Note the differential nuclear staining at AR along the stages. Observe a decrease of staining at Sertoli cells on stages IX-XIV. Scale bar = 20µm.

Fig. 4. Serum FSH, LH and testosterone levels (ng/ml) in adult male rats at 110 days of age. Values expressed as mean ± SEM. ANOVA followed by Tukey's test. $p < 0.05$.

Table 1.

Sperm morphology, testicular histology and spermatogenesis kinetics from control and rosuvastatin-treated groups.

Sperm morphology (%) and testicular evaluations	Experimental Groups		
	Control (n=10)	**3mg/Kg (n=10)**	**10mg/Kg (n=9)**
Normal shaped sperm	97.50 (97.00 - 98.00) a	93.25 (92.50 - 95.13) b	93.00 (90.13 - 94.00) b
Abnormalities of the sperm head	0.75 (0.38 - 0.75) a	2.25 (1.00 - 3.13) b	2.00 (1.38 - 2.75) ab
Abnormalities of the sperm flagellum	1.50 (0.50 - 2.63) a	4.00 (4.00 - 4.38) b	5.00 (4.38 - 7.13) b
Isolated sperm head	0.75 (0.38 - 1.13) a	2.25 (1.00 - 3.00) b	1.75 (1.38 - 2.13) ab
Isolated sperm flagellum	0.25 (0 - 0.50) a	3.00 (2.38 - 3.50) b	3.75 (2.50 - 4.63)b
Sperm with cytoplasmic droplet	12.25 (8.88 - 15.50) a	15.50 (10.25 - 17.63) a	8.75 (5.75 - 13.63) a
Normal seminiferous tubules	95.75 (94.50 - 97.00) a	88.00(86.50 - 89.50) b	88.50 (86.50 - 89.00) b
Seminiferous tubules with acidophilic cells	4.25 (2.50 - 4.63) a	10.75 (9.75 - 11.25) b	11.00 (10.50 - 12.13) b
Stages I - VI	31.00 (28.50 - 31.75) a	37.00 (35.50 - 42.50) b	42.00 (39.75 - 46.75) b
Stages VII - VIII	35.50 (32.50 - 38.50) a	29.00 (26.25 - 32.50) b	24.00 (21.50 - 25.75) b
Stages IX - XIII	29.00 (25.00 - 32.00) a	26.50 (23.75 - 34.50) a	29.50 (24.50 - 33.50) a
Stage XIV	6.00 (4.75 - 7.50) a	5.00 (3.75 - 7.25) ab	2.50 (2.00 - 5.00) b

Values are expressed as median (Q1 - Q3). Different letters indicate statistically significant differences among the groups ($p<0.05$). Kruskal Wallis followed by Dunns test.

Table 2.

Sperm counts, daily sperm production and testicular morphometry from control and rosuvastatin-treated groups.

Sperm counts and testicular morphometry	Experimental Groups		
	Control (n=10)	3mg/Kg (n=10)	10mg/Kg (n=9)
Mature spermatid number (10^6/testis)	242.40 ± 6.26 a	214.20 ± 9.57 b	269.80 ± 5.60 a
Mature spermatid number (10^6/g testis)	149.50 ± 2.98 a	126.80 ± 5.23 b	156.30 ± 4.11 a
Daily sperm production (10^6/testis/day)	39.74 ± 1.03 a	35.11 ± 1.57 b	44.22 ± 0.92 a
Relative sperm production (10^6/g testis/day)	24.52 ± 0.49 a	20.78 ± 0.86 b	25.62 ± 0.67 a
Tubular diameter (µm)	274.70 ± 3.27	272.60 ± 4.96	286.40 ± 6.46
Height of the seminiferous epithelium (µm)	80.49 ± 0.65 a	77.41 ± 0.80 b	81.20 ± 0.85 a
Area of the seminiferous tubules ($µm^2$)	58,150 ± 1,476	58,530 ± 2131	64,660 ± 2931
Luminal area of the seminiferous tubules ($µm^2$)	10,200 ± 514	11,120 ± 1,066	12,250 ± 1,141

Values are expressed as mean ± SEM. Different letters indicate statistically significant differences among the groups ($p<0.05$). ANOVA followed by Tukey test.

Table 3.

Sexual behavior from control and rosuvastatin-treated groups.

Sexual Behavior	Experimental Groups		
	Control (n=8)	**3mg/Kg (n=8)**	**10mg/Kg (n=8)**
Latency to the first mount (s)	70.00 ± 14.54	147.90 ± 42.31	168.40 ± 54.42
Latency to the first intromission (s)	70.38 ± 13.89	197.50 ± 42.58	198.00 ± 58.47
Number of intromissions until the first ejaculation	18.13 ± 2.77	18.63 ± 3.08	14.00 ± 1.60
Latency to the first ejaculation (s)	785.50 ± 103.00	918.90 ± 112.90	816.00 ± 136.70
Latency to the first post-ejaculatory mount (s)	317.00 ± 30.21	313.10 ± 34.24	327.60 ± 26.25
Latency to the first post-ejaculatory intromission (s)	318.50 ± 30.85	319.10 ± 35.12	327.60 ± 26.25
Number of post-ejaculatory intromissions	8.75 ± 0.82	10.00 ± 1.96	9.375 ± 2.19
Number of ejaculations	2.25 ± 0.25	1.88 ± 0.23	2.50 ± 0.27

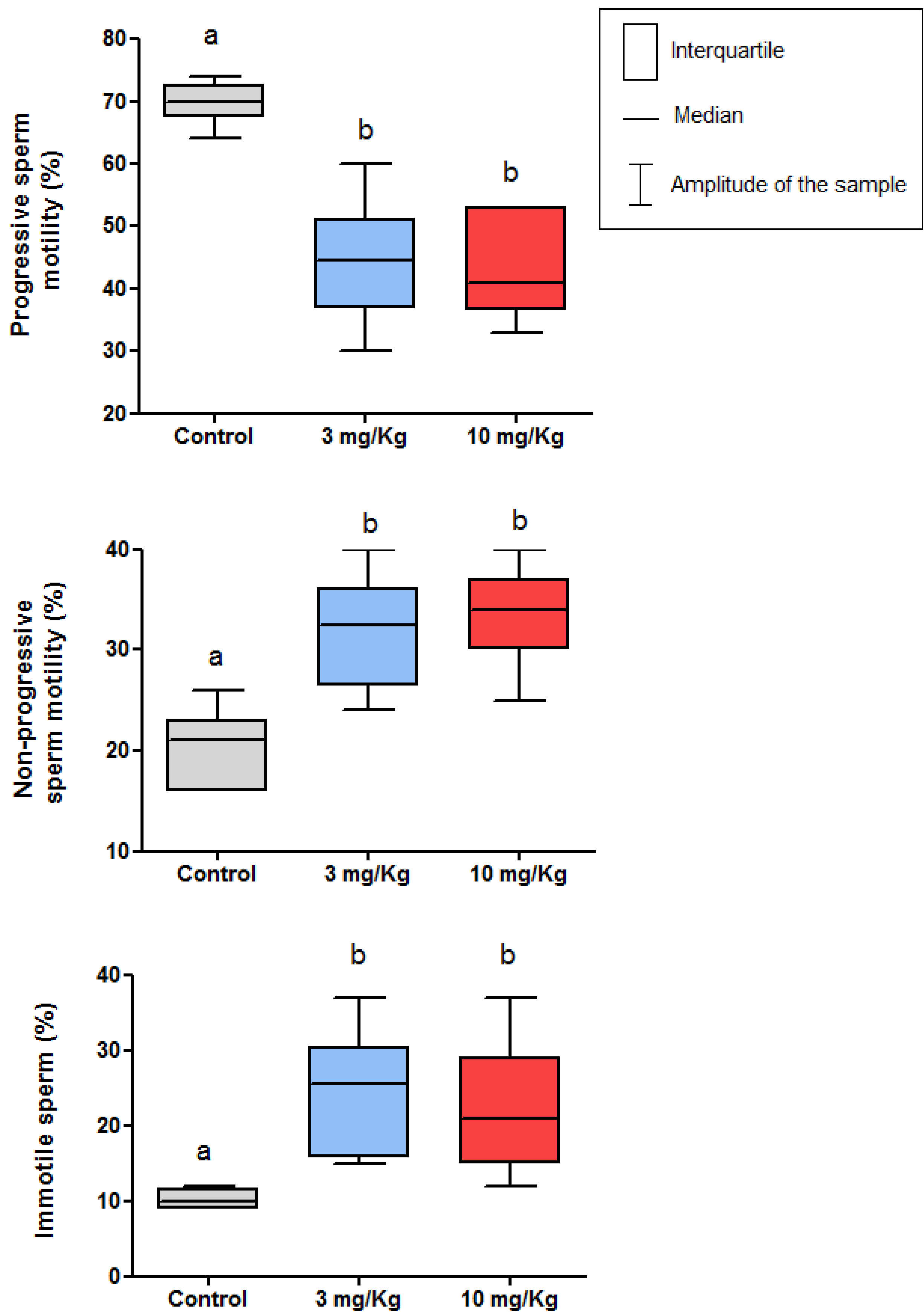
Values are expressed as mean ± SEM, $p>0.05$. ANOVA followed by Tukey test.

Table 4.

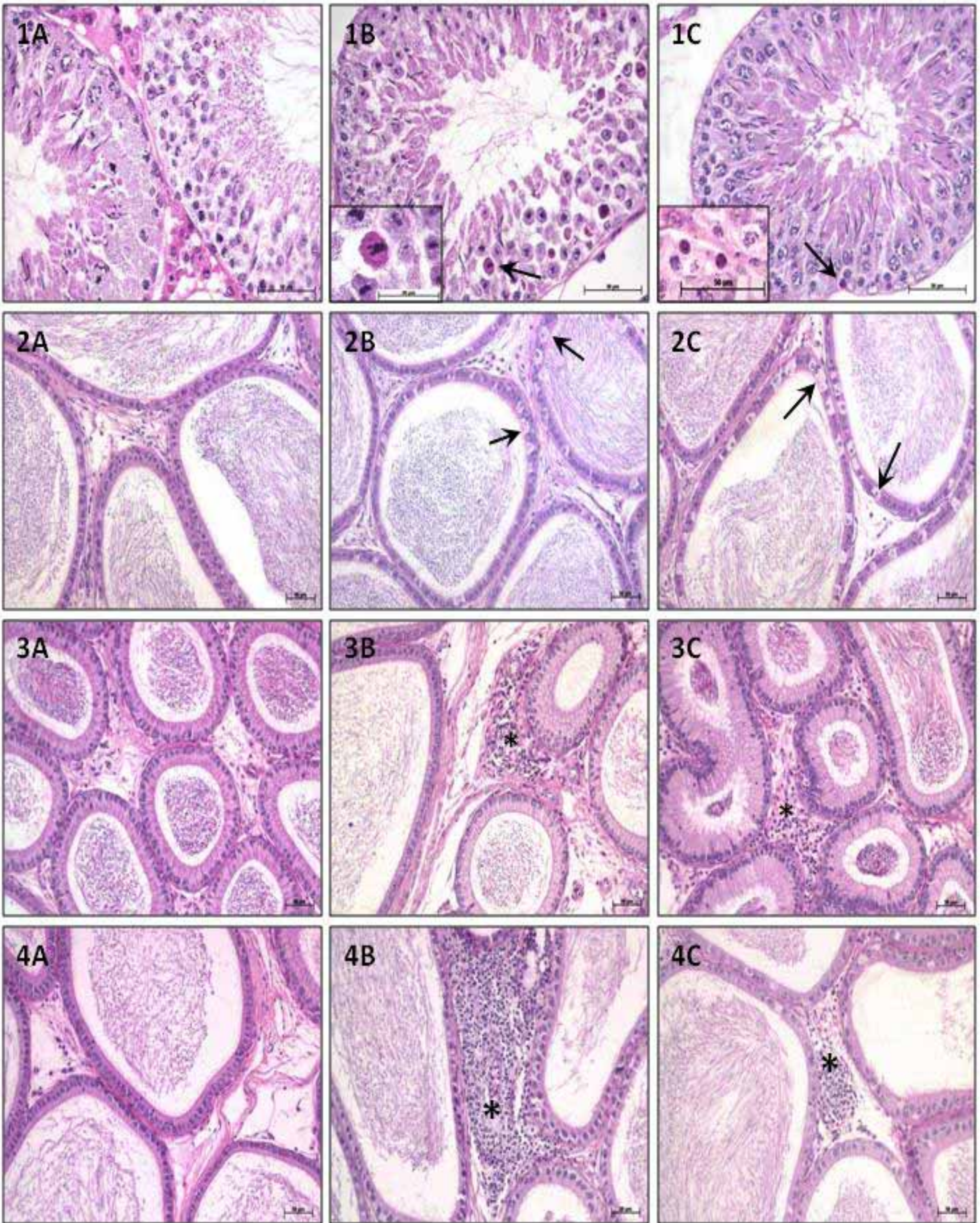
Final body weight and reproductive and vital organ weights from control and rosuvastatin-treated groups.

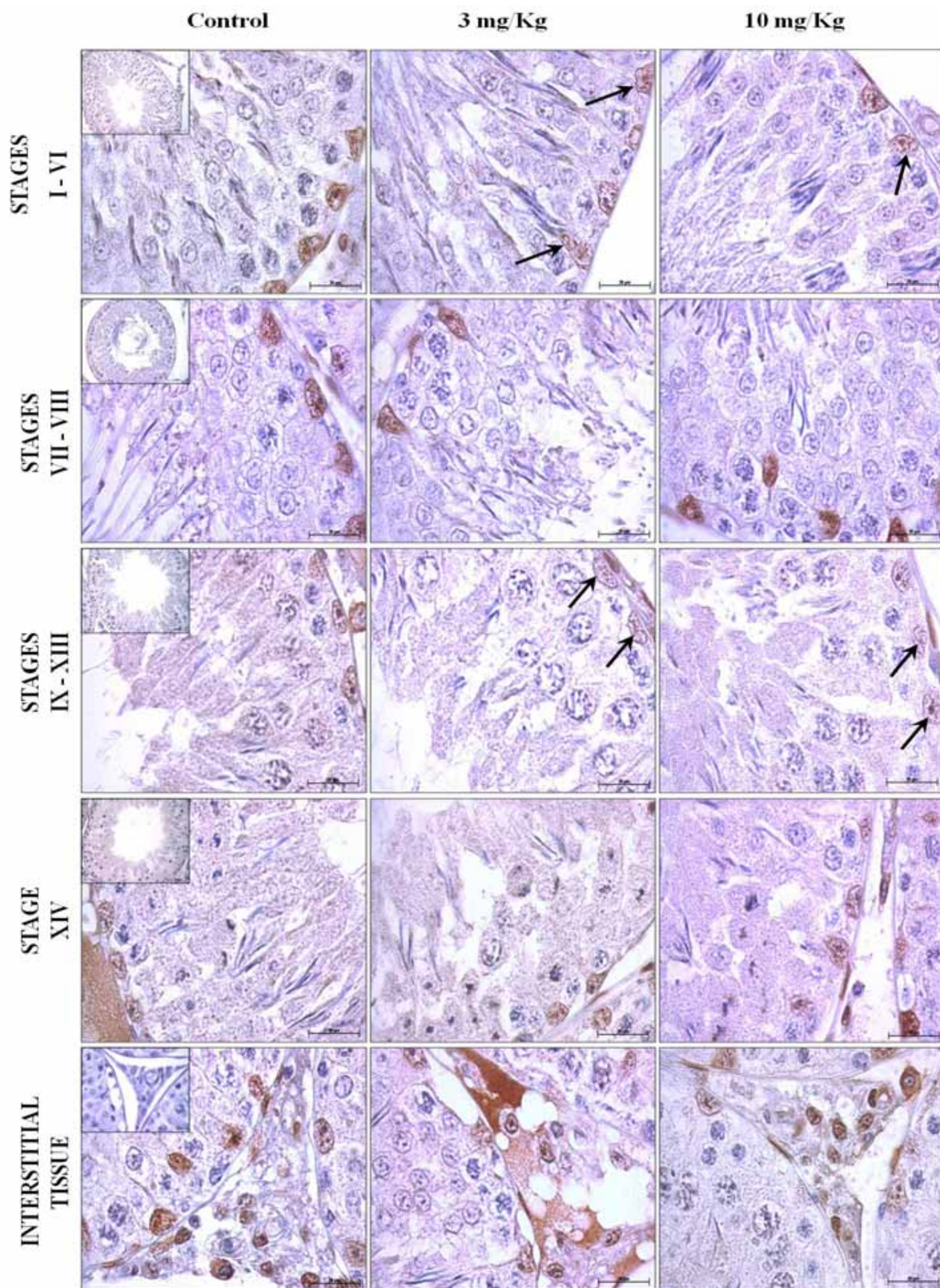
Parameters	Experimental Groups		
	Control (n=10)	**3mg/Kg (n=10)**	**10mg/Kg (n=9)**
Body Weight (g)	483.30 ± 11.56	445.40 ± 12.38	458.60 ± 13.95
Testis (g)	1.70 ± 0.05	1.77 ±0.03	1.81 ± 0.06
Epididymis(mg)	630.50 ± 20.41	634.50 ± 16.68	629.50 ± 20.25
Vas deferens (mg)	99.19 ± 11.32	91.03 ± 6.31	85.72 ± 2.50
Prostate (mg)	481.50 ± 39.88	447.60 ± 32.11	459.70 ± 38.00
Full seminal gland (g)	1.45 ± 0.05	1.37 ± 0.06	1.34 ± 0.09
Empty seminal gland (mg)	499.20 ± 25.29	507.90 ± 31.92	553.7 ± 57.26
Pituitary (mg)	10.69 ± 0.48	9.77 ± 0.32	9.52 ± 0.53
Adrenal (mg)	31.48 ± 1.99	30.73 ± 1.92	30.11 ± 2.56
Thyroid (mg)	23.43 ± 0.99	19.29 ± 1.05	22.71 ± 1.60
Liver (g)	18.59 ± 0.57	16.83 ± 0.43	17.92 ± 0.63
Kidney (g)	1.65 ± 0.04	1.56 ± 0.06	1.62 ± 0.07

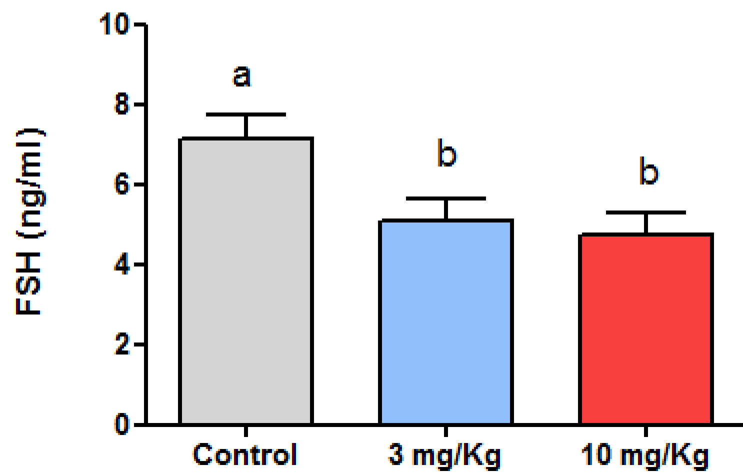
Values are expressed as mean ± SEM. Different letters indicate statistically significant differences among the groups ($p<0.05$) and the absence of letters indicate $p>0.05$. ANOVA followed by Tukey test.



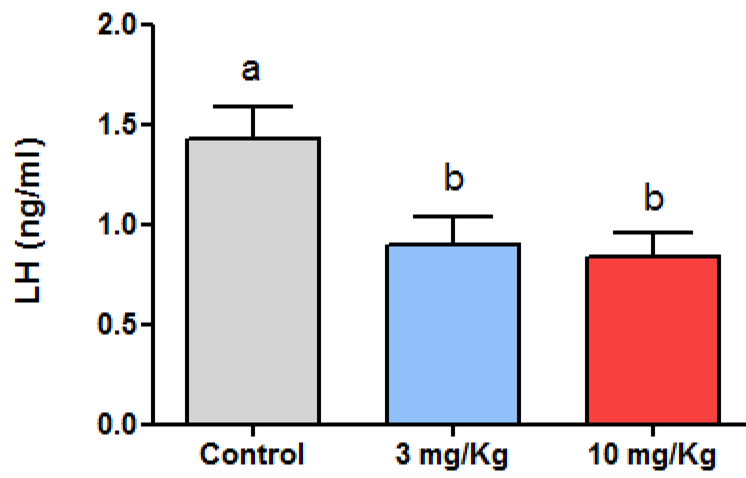
a
b
b
Progressive sperm motility (%)
80
70
60
50
40
30
20
Control
3 mg/Kg
10 mg/Kg
Interquartile
Median
Amplitude of the sample
b
b
a
Non-progressive sperm motility (%)
40
30
20
10
Control
3 mg/Kg
10 mg/Kg
b
b
a
Immotile sperm (%)
40
30
20
10
0
Control
3 mg/Kg
10 mg/Kg


1A
1B
1C
2A
2B
2C
3A
3B
3C
4A
4B
4C

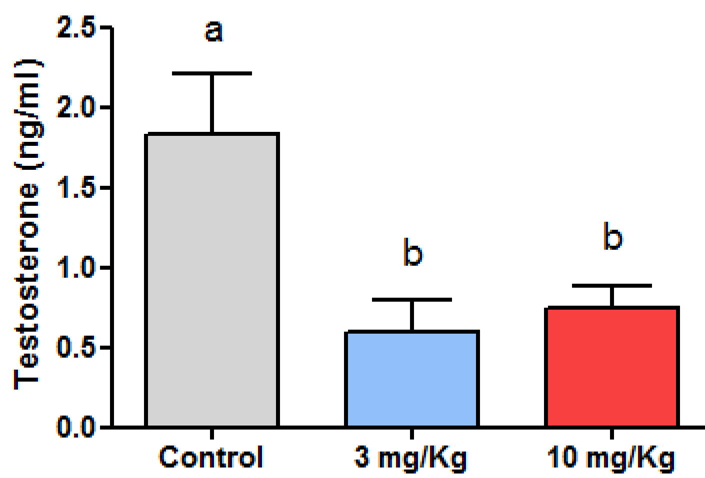

Control
3 mg/Kg
10 mg/Kg
STAGES I - VI
STAGES VII - VIII
STAGES IX - XIII
STAGE XIV
INTERSTITIAL TISSUE



FSH (ng/ml)
10
8
6
4
2
0
a
b
b
Control
3 mg/Kg
10 mg/Kg



LH (ng/ml)
2.0
1.5
1.0
0.5
0.0
a
b
b
Control
3 mg/Kg
10 mg/Kg



Testosterone (ng/ml)
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
Control
3 mg/Kg
10 mg/Kg

Conclusão

Conclui-se que a administração de rosuvastatina à ratos juvenis atrasou a instalação da puberdade e o desenvolvimento epididimário, e provocou efeitos deletérios tardios sobre a produção e qualidade espermática, o comportamento sexual e a morfologia testicular e epididimária na idade adulta. Essas alterações estiveram associadas a uma possível desregulação do eixo hipotalâmico-hipofisário-gonadal e à diminuição das concentrações séricas de testosterona. Assim sendo, os resultados desse trabalho, realizado com ratos, sinalizam para um potencial risco reprodutivo na espécie humana, decorrente da exposição de crianças à rosuvastatina.

Referências

Adam O. & Laufs U. (2008) Antioxidative effects of statins. *Arch Toxicol* **82**, 885-92.

Araki M., Maeda M. & Motojima K. (2012) Hydrophobic statins induce autophagy and cell death in human rhabdomyosarcoma cells by depleting geranylgeranyl diphosphate. *European Journal of Pharmacology* **674**, 95-103.

Azzarito C., Boiardi L., Vergoni W., Zini M. & Portioli I. (1996) Testicular function in hypercholesterolemic male patients during prolonged simvastatin treatment. *Horm Metab Res* **28**, 193-8.

Ballantyne C.M., Miller E. & Chitra R. (2004) Efficacy and safety of rosuvastatin alone and in combination with cholestyramine in patients with severe hypercholesterolemia: a randomized, open-label, multicenter trial. *Clin Ther* **26**, 1855-64.

Bellosta S., Paoletti R. & Corsini A. (2004) Safety of statins - Focus on clinical pharmacokinetics and drug interactions. *Circulation* **109**, 50-7.

Bianco S.D. (2012) A potential mechanism for the sexual dimorphism in the onset of puberty and incidence of idiopathic central precocious puberty in children: sex-specific kisspeptin as an integrator of puberty signals. *Front Endocrinol (Lausanne)* **3**, 149.

Blystone C.R., Furr J., Lambright C.S., Howdeshell K.L., Ryan B.C., Wilson V.S., Leblanc G.A. & Gray L.E., Jr. (2007) Prochloraz inhibits testosterone production at dosages below those that affect androgen-dependent organ weights or the onset of puberty in the male Sprague Dawley rat. *Toxicological Sciences* **97**, 65-74.

Bohm M., Herrmann W., Wassmann S., Laufs U. & Nickenig G. (2004) Does statin therapy influence steroid hormone synthesis? *Z Kardiol* **93**, 43-8.

Campo V.L. & Carvalho I. (2007) Estatinas hipolipêmicas e novas tendências terapêuticas. *Quim Nova* **30**, 425-30.

Caslake M.J., Stewart G., Day S.P., Daly E., McTaggart F., Chapman M.J., Durrington P., Laggner P., Mackness M., Pears J. & Packard C.J. (2003) Phenotype-dependent and -independent actions of rosuvastatin on atherogenic lipoprotein subfractions in hyperlipidaemia. *Atherosclerosis* **171**, 245-53.

Ceballos L.T., Angelín B.P. & Ruiz-García C. (2008) Uso de estatinas durante la infancia. *An Pediatr (Barc)* **68**, 385-92.

Chan D.C., Watts G.F., Barrett P.H., Beilin L.J., Redgrave T.G. & Mori T.A. (2002) Regulatory effects of HMG CoA reductase inhibitor and fish oils on apolipoprotein B-100 kinetics in insulin-resistant obese male subjects with dyslipidemia. *Diabetes* **51**, 2377-86.

Cook S. & Kavey R.E. (2011) Dyslipidemia and pediatric obesity. *Pediatr Clin North Am* **58**, 1363-73, ix.

Corder E.H., Saunders A.M., Strittmatter W.J., Schmechel D.E., Gaskell P.C., Small G.W., Roses A.D., Haines J.L. & Pericak-Vance M.A. (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* **261**, 921-3.

Cyrus-David M.S., Weinberg A., Thompson T. & Kadmon D. (2005) The effect of statins on serum prostate specific antigen levels in a cohort of airline pilots: A preliminary report. *Journal of Urology* **173**, 1923-5.

Davis H.R., Jr., Zhu L.J., Hoos L.M., Tetzloff G., Maguire M., Liu J., Yao X., Iyer S.P., Lam M.H., Lund E.G., Detmers P.A., Graziano M.P. & Altmann S.W. (2004) Niemann-Pick C1 Like 1 (NPC1L1) is the intestinal phytosterol and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis. *J Biol Chem* **279**, 33586-92.

De Angelis G. (2004) The influence of statin characteristics on their safety and tolerability. *Int J Clin Pract* **58**, 945-55.

Dogru M.T., Basar M.M., Simsek A., Yuvanc E., Guneri M., Ebinc H. & Batislam E. (2008) Effects of statin treatment on serum sex steroids levels and autonomic and erectile function. *Urology* **71**, 703-7.

Dostal L.A., Juneau P. & Rothwell C.E. (2001) Repeated analysis of semen parameters in beagle dogs during a 2-year study with the HMG-CoA reductase inhibitor, atorvastatin. *Toxicological Sciences* **61**, 128-34.

Dostal L.A., Whitfield L.R. & Anderson J.A. (1996) Fertility and general reproduction studies in rats with the HMG-CoA reductase inhibitor, atorvastatin. *Fundamental and Applied Toxicology* **32**, 285-92.

Edison R.J. & Muenke M. (2004) Mechanistic and epidemiologic considerations in the evaluation of adverse birth outcomes following gestational exposure to statins. *American Journal of Medical Genetics Part A* **131A**, 287-98.

Endres M. (2006) Statins: potential new indications in inflammatory conditions. *Atheroscler Suppl* **7**, 31-5.

Favareto A.P., Fernandez C.D., da Silva D.A., Anselmo-Franci J.A. & Kempinas Wde G. (2011) Persistent impairment of testicular histology and sperm motility in adult rats treated with Cisplatin at peri-puberty. *Basic Clin Pharmacol Toxicol* **109**, 85-96.

Feleszko W., Zagozdzon R., Golab J. & Jakobisiak M. (1998) Potentiated antitumour effects of cisplatin and lovastatin against MmB16 melanoma in mice. *Eur J Cancer* **34**, 406-11.

Fonseca F.A.H. (2005) Farmacocinética das estatinas. *Arquivos Brasileiros de Cardiologia* **85**, 9-14.

Galluzzi L., Maiuri M.C., Vitale I., Zischka H., Castedo M., Zitvogel L. & Kroemer G. (2007) Cell death modalities: classification and pathophysiological implications. *Cell Death and Differentiation* **14**, 1237-43.

Garcia-Rodriguez L.A., Masso-Gonzalez E.L., Wallander M.A. & Johansson S. (2008) The safety of rosuvastatin in comparison with other statins in over 100,000 statin users in UK primary care. *Pharmacoepidemiol Drug Saf* **17**, 943-52.

Giuliano I.C.B. & Caramelli B. (2008) Dislipidemias na infância e na adolescência. *Pediatria (São Paulo)* **29**, 275-85.

Goettsch W.G., Heintjes E.M., Kastelein J.J., Rabelink T.J., Johansson S. & Herings R.M. (2006) Results from a rosuvastatin historical cohort study in more than 45,000 Dutch statin users, a PHARMO study. *Pharmacoepidemiol Drug Saf* **15**, 435-43.

Gopalan A., Yu W.P., Sanders B.G. & Kline K. (2013) Simvastatin inhibition of mevalonate pathway induces apoptosis in human breast cancer cells via activation of JNK/CHOP/DR5 signaling pathway. *Cancer Letters* **329**, 9-16.

Gotto A.M., Jr. (2006) Statins, cardiovascular disease, and drug safety. *Am J Cardiol* **97**, 3C-5C.

Holdgate G.A., Ward W.H. & McTaggart F. (2003) Molecular mechanism for inhibition of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase by rosuvastatin. *Biochem Soc Trans* **31**, 528-31.

Hosokawa A., Bar-Oz B. & Ito S. (2003) Use of lipid-lowering agents (statins) during pregnancy. *Canadian Family Physician* **49**, 747-9.

Hunninghake D.B., Stein E.A., Bays H.E., Rader D.J., Chitra R.R., Simonson S.G. & Schneck D.W. (2004) Rosuvastatin improves the atherogenic and atheroprotective lipid profiles in patients with hypertriglyceridemia. *Coronary Artery Disease* **15**, 115-23.

Istvan E. (2003) Statin inhibition of HMG-CoA reductase: a 3-dimensional view. *Atheroscler Suppl* **4**, 3-8.

Istvan E.S. & Deisenhofer J. (2001) Structural mechanism for statin inhibition of HMG-CoA reductase. *Science* **292**, 1160-4.

Izar M.C.O., Fonseca M.I.H. & Fonseca F.A.H. (2011) Dislipidemias. *Rev Bras Med* **68**, 59-73.

Jiménez E.G. & Ferre J.A. (2011) Estatinas: Características y efectos sobre el control lipídico en el niño y adolescente obeso. *Rev Clin Med Fam.* **4**, 69-75.

Johnson L., Welsh T.H.J. & Wilker C.E. (1997) Anatomy and physiology of the male reproductive system and potential targets of toxicants. In: *Comprehensive Toxicology* (eds. by Boekleheide K, Chapin RE, Hoyer PB & Harris C), pp. 5-61. Pergamon, New York.

Kang H.S., Gutin B., Barbeau P., Owens S., Lemmon C.R., Allison J., Litaker M.S. & Le N.A. (2002) Physical training improves insulin resistance syndrome markers in obese adolescents. *Med Sci Sports Exerc* **34**, 1920-7.

Klop B., Elte J.W. & Cabezas M.C. (2013) Dyslipidemia in obesity: mechanisms and potential targets. *Nutrients* **5**, 1218-40.

Kwiterovich P.O., Jr. (2008) Recognition and management of dyslipidemia in children and adolescents. *J Clin Endocrinol Metab* **93**, 4200-9.

Lagadic-Gossmann D., Huc L. & Lecureur V. (2004) Alterations of intracellular pH homeostasis in apoptosis: origins and roles. *Cell Death and Differentiation* **11**, 953-61.

Lee S.K. & Kim Y.S. (2013) Phosphorylation of eIF2 alpha attenuates statin-induced apoptosis by inhibiting the stabilization and translocation of p53 to the mitochondria. *Int J Oncol* **42**, 810-6.

Liao J.K. & Laufs U. (2005) Pleiotropic effects of statins. *Annu Rev Pharmacol Toxicol* **45**, 89-118.

Ludman A., Venugopal V., Yellon D.M. & Hausenloy D.J. (2009) Statins and cardioprotection--more than just lipid lowering? *Pharmacol Ther* **122**, 30-43.

Mantovani A. & Fucic A. (2013) Puberty dysregulation and increased risk of disease in adult life: Possible modes of action. Reproductive Toxicology.

Martin P.D., Mitchell P.D. & Schneck D.W. (2002) Pharmacodynamic effects and pharmacokinetics of a new HMG-CoA reductase inhibitor, rosuvastatin, after morning or evening administration in healthy volunteers. *Br J Clin Pharmacol* **54**, 472-7.

McGill H.C., Jr., McMahan C.A., Herderick E.E., Zieske A.W., Malcom G.T., Tracy R.E., Strong J.P. & Pathobiological Determinants of Atherosclerosis in Youth Research G. (2002) Obesity accelerates the progression of coronary atherosclerosis in young men. *Circulation* **105**, 2712-8.

McTaggart F. (2003) Comparative pharmacology of rosuvastatin. *Atheroscler Suppl* **4**, 9-14.

Ostlund R.E., Jr. (2002) Phytosterols in human nutrition. *Annu Rev Nutr* **22**, 533-49.

Ouyang L., Shi Z., Zhao S., Wang F.T., Zhou T.T., Liu B. & Bao J.K. (2012) Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. *Cell Proliferation* **45**, 487-98.

Penning-van Beest F.J., Termorshuizen F., Goettsch W.G., Klungel O.H., Kastelein J.J. & Herings R.M. (2007) Adherence to evidence-based statin guidelines reduces the risk of hospitalizations for acute myocardial infarction by 40%: a cohort study. *Eur Heart J* **28**, 154-9.

Perobelli J.E., Alves T.R., de Toledo F.C., Fernandez C.D., Anselmo-Franci J.A., Klinefelter G.R. & Kempinas Wde G. (2012) Impairment on sperm quality and fertility of adult rats after antiandrogen exposure during prepuberty. *Reprod Toxicol* **33**, 308-15.

Perobelli J.E., Patrao M.T., Fernandez C.D., Sanabria M., Klinefelter G.R., Avellar M.C. & Kempinas W.D. (2013) Androgen deprivation from pre-puberty to peripuberty interferes in proteins expression in pubertal and adult rat epididymis. *Reprod Toxicol* **38**, 65-71.

Pollack P.S., Shields K.E., Burnett D.M., Osborne M.J., Cunningham M.L. & Stepanavage M.E. (2005) Pregnancy outcomes after maternal exposure to simvastatin and lovastatin. *Birth Defects Research Part a-Clinical and Molecular Teratology* **73**, 888-96.

Qi X.F., Zheng L., Lee K.J., Kim D.H., Kim C.S., Cai D.Q., Wu Z., Qin J.W., Yu Y.H. & Kim S.K. (2013) HMG-CoA reductase inhibitors induce apoptosis of lymphoma cells by promoting ROS generation and regulating Akt, Erk and p38 signals via suppression of mevalonate pathway. *Cell Death & Disease* **4**.

Rosenson R.S. (2004) Current overview of statin-induced myopathy. *Am J Med* **116**, 408-16.

Rubenfire M., Brook R.D. & Rosenson R.S. (2010) Treating mixed hyperlipidemia and the atherogenic lipid phenotype for prevention of cardiovascular events. *Am J Med* **123**, 892-8.

SBC (2007) IV Diretriz Brasileira Sobre Dislipidemias e Prevenção da Aterosclerose. pp. 1-19. Arquivos Brasileiros de Cardiologia.

Schachter M. (2004) Chemical, pharmacokinetic and pharmacodynamic properties of statins: an update. *Fundamental & Clinical Pharmacology* **19**, 117-25.

Seth A. & Sharma R. (2013) Childhood obesity. *Indian J Pediatr* **80**, 309-17.

Sondike S.B., Copperman N. & Jacobson M.S. (2003) Effects of a low-carbohydrate diet on weight loss and cardiovascular risk factor in overweight adolescents. *J Pediatr* **142**, 253-8.

Stoker T.E., Laws S.C., Guidici D.L. & Cooper R.L. (2000) The effect of atrazine on puberty in male Wistar rats: An evaluation in the protocol for the assessment of pubertal development and thyroid function. *Toxicological Sciences* **58**, 50-9.

Tanaka T., Tatsuno I., Uchida D., Moroo I., Morio H., Nakamura S., Noguchi Y., Yasuda T., Kitagawa M., Saito Y. & Hirai A. (2000) Geranylgeranyl-pyrophosphate, an isoprenoid of mevalonate cascade, is a critical compound for rat primary cultured cortical neurons to protect the cell death induced by 3-hydroxy-3-methylglutaryl-CoA reductase inhibition. *Journal of Neuroscience* **20**, 2852-9.

Tandon V., Bano G., Khajuria V., Parihar A. & Gupta S. (2005) Pleiotropic effects of statins. *Int J Pharmacol* **37**, 77-85.

Tiwari A. (2006) An overview of statin-associated proteinuria. *Drug Discov Today* **11**, 458-64.

Vaughan C.J. & Gotto A.M., Jr. (2004) Update on statins: 2003. *Circulation* **110**, 886-92.

Vicencio J.M., Galluzzi L., Tajeddine N., Ortiz C., Criollo A., Tasdemir E., Morselli E., Ben Younes A., Maiuri M.C., Lavandero S. & Kroemer G. (2008) Senescence, apoptosis or autophagy? *Gerontology* **54**, 92-9.

Woo K.S., Chook P., Yu C.W., Sung R.Y., Qiao M., Leung S.S., Lam C.W., Metreweli C. & Celermajer D.S. (2004) Effects of diet and exercise on obesity-related vascular dysfunction in children. *Circulation* **109**, 1981-6.

Zhang J., Yang Z.Z., Xie L., Xu L., Xu D. & Liu X.F. (2013) Statins, autophagy and cancer metastasis. *International Journal of Biochemistry & Cell Biology* **45**, 745-52.