CAROLINE MARIA CHRISTANTE

"INFLUÊNCIA DA OBESIDADE MATERNA NA DIFERENCIAÇÃO NEONATAL DAS CÉLULAS GERMINATIVAS MASCULINAS"

Campinas, 2013

UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

CAROLINE MARIA CHRISTANTE

"INFLUÊNCIA DA OBESIDADE MATERNA NA DIFERENCIAÇÃO NEONATAL DAS CÉLULAS GERMINATIVAS MASCULINAS"

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) *Caroline Maria Christante* e aprovada pela Comissão Julgadora.

Dissertação apresentada ao Instituto de Biologia da UNICAMP para obtenção do Título de Mestra em Biologia Celular e Estrutural, na área de Biologia Celular.

Orientadora: Profa. Dra. Rejane Maira Góes
Co-Orientadora: Profa. Dra. Maria Etelvina Pinto

Campinas, 2013

FICHA CATALOGRÁFICA ELABORADA POR
MARA JANAINA DE OLIVEIRA – CRB8/6972
BIBLIOTECA DO INSTITUTO DE BIOLOGIA - UNICAMP

C461i

Christante, Caroline Maria, 1987-
Influência da obesidade materna na diferenciação neonatal das células germinativas masculinas / Caroline Maria Christante. – Campinas, SP: [s.n.], 2013.

Orientador: Rejane Maira Góes.
Coorientador: Maria Etelvina Pinto Fochi.
Dissertação (mestrado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Testículos. 2. Células germinativas. 3. Obesidade. 4. Ratos neonatos. I. Góes, Rejane Maira. II. Pinto, Maria Etelvina. III. Universidade Estadual de Campinas. Instituto de Biologia. IV. Título.

Informações para Biblioteca Digital

Título em Inglês: Influence of maternal obesity on neonatal germ cell differentiation
Palavras-chave em Inglês:
Testis
Germ cells
Obesity
Neonate rats
Área de concentração: Biologia Celular
Titulação: Mestra em Biologia Celular e Estrutural
Banca examinadora:
Rejane Maira Góes [Orientador]
Estela Sasso Cerri
Arielle Cristina Arena
Data da defesa: 22-02-2013
Programa de Pós Graduação: Biologia Celular e Estrutural

Campinas, 22 de fevereiro de 2013

BANCA EXAMINADORA

Profa. Dra. Rejane Maira Góes (Orientadora) _____
Assinatura

Profa. Dra. Arielle Cristina Arena _____
Assinatura

Profa. Dra. Estela Sasso Cerri _____
Assinatura

Profa. Dra. Mary Anne Heidi Dolder _____
Assinatura

Profa. Dra. Sandra Maria Miraglia _____
Assinatura

AGRADECIMENTOS

À minha orientadora Profa. Dra. Rejane Maira Góes por todos os ensinamentos recebidos, pela dedicação e pela paciência durante essa jornada. Obrigada pela oportunidade e pela confiança depositada em mim, Rejane.

À minha co-orientadora Maê por toda ajuda, desde o início até a finalização desse trabalho. Sua calma e sua confiança me encorajaram a acreditar e a seguir em frente.

Ao Prof. Dr. Sebastião Roberto Taboga, meu primeiro orientador, pela oportunidade de estágio um dia oferecida no Laboratório de Microscopia e Microanálises (LMM), que despertou em mim o gosto e o amor pela pesquisa.

Ao técnico Luiz Roberto Falleiros Júnior pelo apoio e pelo carinho. A sua alegria contagiante e o seu bom humor tornaram, sem dúvidas, os meus momentos no LMM bem mais felizes. Ter conhecido você e a sua família linda foi um grande presente para mim.

À secretária Liliam pela dedicação, pela competência e pelo cuidado especial para com todos os alunos do Programa de Pós-Graduação em Biologia Celular e Estrutural.

Às amigas Ana Paula, Bianca, Carol Frandsen, Carol Negrin, Cintia, Elô, Júlia, Mariana Pulegio, Mariana Marcielo, Marianna Zanatelli, Marina, Pati, Simone e Vanessa pelos momentos especiais que passamos juntas nas festas, confraternizações, almoços, intervalos dos protocolos, etc. Porque nada teria tido o mesmo saber nem o mesmo valor sem a presença de vocês! Muito obrigada, principalmente, pela disposição não apenas para ajudar com os assuntos relacionados ao projeto, mas também pela disposição em me ouvir e me entender.

Aos amigos Diego, Manoel e Ricardo que sempre usaram de toda a gentileza para esclarecer as minhas dúvidas na bancada. Obrigada pelo convívio tão agradável e por serem três carinhas tão do bem.

Ao amigo Thiago pelo suporte técnico e por toda ajuda recebida tanto no laboratório quanto no biotério. Sem a sua ajuda teria sido bem mais difícil, meu amigo.

Às amigas Andressa, Elisa, Emerielle (Leli) e Leila pela hospitalidade, aos amigos que eu fiz durante as disciplinas do mestrado e à minha prima Liz por todo o apoio recebido no período em que eu estive em Campinas. Vocês contribuíram para que o ano de 2011 fosse um dos anos mais especiais da minha vida!

À CAPES pela bolsa recebida para a realização das minhas atividades.

Ao meu pai Luis Antonio, minha eterna referência de ser humano. Seu caráter, sua garra e sua alegria de viver sempre serviram de exemplo para mim. Obrigada por nunca ter medido esforços para que eu pudesse chegar até aqui.

À minha mãe Fannya pelo respeito às minhas decisões e por me amar e me aceitar como eu sou. Muito obrigada pelo carinho e pela admoestação sempre oferecidos nos momentos certos. Obrigada por nem sempre ter passada a mão na minha cabeça e por ter sido, durante toda a minha vida, a minha maior incentivadora.

À minha irmã Natacha pelo companheirismo, pela cumplicidade, pelo carinho que nos une e por sempre ter sido a minha mais fiel ouvinte.

Ao meu Deus e Senhor por me capacitar e me fortalecer dia a dia, e por me oferecer o amor mais puro, mais verdadeiro e mais desinteressado que existe.

SUMÁRIO

I. RESUMO

Os gonócitos são os precursores das células germinativas masculinas, encontrados durante o período fetal e neonatal. Sua diferenciação neonatal é fundamental para a função reprodutora e envolve retomada da atividade proliferativa, movimentação para a base do epitélio seminífero e diferenciação em espermatogônia. Sabe-se que, no período fetal, a atividade proliferativa dessas células é andrógeno dependente, e que a obesidade altera o cenário dos hormônios sexuais, levando a um aumento dos níveis de andrógenos em mulheres. Contudo, pouco é conhecido sobre os efeitos da obesidade materna sobre o desenvolvimento do aparelho genital masculino, em particular no período neonatal, e as implicações com o cenário hormonal. Sendo assim, o objetivo desse estudo foi avaliar se a obesidade materna interfere no desenvolvimento neonatal do testículo de ratos Wistar, tanto no que se refere às suas características morfológicas gerais, quanto à diferenciação das células germinativas. A obesidade materna foi induzida pelo tratamento, por 15 semanas, com dieta contendo 20% de lipídeos saturados. Foram utilizados filhotes machos de mães normais e obesas nas idades de 0,5, 4,5, 7,5 e 14,5 dias pós-parto (dpp). Os testículos foram processados para microscopias de luz e eletrônica de transmissão. Os cortes histológicos submetidos à reação imunocitoquímica para hormônio anti-Mülleriano (AMH) foram utilizados para a determinação da densidade numérica (Nv) de gonócitos e estimativa da densidade de gonócitos reposicionados para a periferia do túbulo. Também foi avaliado os níveis de apoptose, após imunocitoquímicas para Caspase 3 ativada, e a localização dos receptores de andrógeno (AR) e de estrógeno (ERα e ERβ). Nossos resultados revelaram uma diminuição no peso dos filhotes de mães obesas, ao nascimento, mas nenhuma variação foi observada para a distância anogenital. O peso das gônadas foi significantemente menor para os animais obesos de 4,5 dpp, resultando em uma variação do índice gonadossomático, nessa idade. A Nv de gonócitos e os níveis de apoptose não variaram nos neonatos de mães obesas em comparação com o grupo controle, em nenhuma das idades consideradas. No entanto, o número de gonócitos reposicionados para a base do epitélio seminífero, aos 4,5 dpp, foi aproximadamente o dobro nos testículos de animais controle em comparação com os submetidos à obesidade materna. Também foi observado que esse tipo celular não possui imunoreatividade para AR. Entretanto, as células de origem mesenquimal e o citoplasma das células de Leydig fetais, de maneira geral, são

imunomarcados. Com relação aos níveis de andrógenos, verificou-se que as médias obtidas para os animais sujeitos à obesidade materna, de 4,5 até 14,5 dpp, são muito semelhantes às encontradas para as idades anteriores no grupo controle, sendo possível inferir que a obesidade materna afete a esteredoigênese e promova alterações na concentração de testosterona, no período peri-natal. De maneira geral, nossos dados indicam que a obesidade materna afeta os níveis de esteroides sexuais e, consequentemente, o padrão de diferenciação dos gonócitos. Contudo, tais alterações parecem ocorrer nos primeiros dias de vida, principalmente na idade de 4,5 dpp, com uma recuperação do desenvolvimento testicular ainda no período pré-púbere.

II. ABSTRACT

The gonocytes are the precursors of male germ cells, found during fetal and neonatal period. Their differentiation is critical for neonatal reproductive function and involves resumption of proliferative activity, drive to the base of the seminiferous epithelium and differentiation into spermatogonia. It is also known that in the neonatal period the proliferative activity of these cells is androgen-dependent, and that the obesity changes the sex hormones scenario, leading to increased androgens levels in women. However, little is known about the effects of maternal obesity on the male genital tract development, particularly in the neonatal period, and the implications with hormonal scenario. Therefore, the objective of this study was to evaluate whether maternal obesity interferes with rat testis development, both with regard to their overall morphology, as the differentiation of germ cells. Obesity was induced by treating, for 15 weeks, with a diet containing 20% of saturated lipids. We used male offspring from normal and obese mothers at 0.5, 4.5, 7.5 and 14.5 days postpartum (dpp). The testes were processed for light microscopy and transmission electron microscopy. The sections subjected to immunocytochemistry for anti-Müllerian Hormone (AMH) were used to determine the numerical density (Nv) of gonocytes and density estimation of repositioned gonocytes to the periphery of the tubule. We also assessed the apoptosis levels after immunocytochemistry for activated Caspase 3, and location of androgen receptors (AR) and estrogen (ERα and ERβ). Our results showed a decrease in the weight of pups born from obese mothers, at birth, but no change was observed for the anogenital distance. The gonad weight was significantly lower in obese animals at 4.5 dpp, resulting in a variation of the gonadosomatic index in this age. The Nv of gonocytes and apoptosis levels did not differ in newborns subjected to maternal obesity compared with the control group, for any age considered. However, the number of repositioned gonocytes to the base of the seminiferous epithelium at 4.5 dpp was approximately twice in control group compared with the maternal obesity group. It was also observed that this cell type has no AR immunoreactivity. However, mesenchymal cells and the cytoplasm of the fetal Leydig cells generally are immunostained. With respect to androgens levels, it was found that the mean for the animals subjected to maternal obesity from 4.5 to 14.5 dpp are very similar to those found in earlier ages in the control group, it is possible to infer that maternal obesity affects steroidogenesis and promotes changes in

testosterone concentration, in the perinatal period. Overall, our data indicate that maternal obesity affects the sex steroids levels and, consequently, the pattern of differentiation of gonocytes. However, these changes seem to occur in the first days of life, especially at the age of 4.5 dpp, with a recovery of testicular development in the pre-pubertal age.

III. INTRODUÇÃO

A diferenciação das gônadas, durante o desenvolvimento embrionário, envolve a migração das células germinativas primordiais (PGC) extra-embrionárias, situadas no endoderma do saco vitelínico (Magre e Jost, 1980). A invasão do mesoderma intermediário, situado ventro-lateralmente ao mesonefron, por essa população de células migratórias, leva à formação do primórdio gonadal, também conhecido como crista genital, com capacidade de originar o testículo ou o ovário. Após alcançarem as cristas genitais, as PGC passam a ser designadas gonócitos (Clermont e Perey, 1957; Magre e Jost, 1980). Nas fêmeas, tanto as células somáticas quanto as células germinativas permanecem "desorganizadas", o oposto do que ocorre nos testículos, nos quais os precursores das células de Sertoli se organizam ao redor dos gonócitos para a formação dos cordões seminíferos (Magre e Jost, 1991). No rato, este evento ocorre 13,5-14,5 dias pós-concepção (dpc) (Magre e Jost, 1980). Assim, o termo gonócito foi originalmente proposto por Clermont e Perey (1957) para designar as células germinativas originadas no período fetal, a partir do momento em que estas ocupam o interior dos cordões sexuais primitivos, até sua diferenciação neonatal em espermatogônias (Fig. 1). Existe muita controvérsia na literatura com relação a essa nomenclatura e, além de gonócito, os termos pré ou pró-espermatogônia também foram utilizados (Culty, 2009). Alguns autores consideram o termo pró-espermatogônia mais apropriado para descrever a fase de transição entre os gonócitos e as espermatogônias (Ohbo et al., 2003; Pinto et al., 2010) e outros acreditam não ser necessário considerar a existência de uma fase especial de espermatogônia entre os gonócitos e as espermatogônias diferenciadas (Yoshida et al., 2006).

Os gonócitos, assim como as PGC, são células muito volumosas, com um núcleo proeminente contendo um ou dois nucléolos, cercado por um citoplasma em forma de anel (Clermont e Perey, 1957; Baillie, 1964; Culty, 2009) (Fig. 2A-D). Apesar das semelhanças morfológicas, estudos *in vitro* têm demonstrado diferenças funcionais entre esses dois tipos celulares. Os gonócitos, por exemplo, não podem sobreviver sem a presença das células de Sertoli, enquanto as PGC podem ser co-cultivadas com outros tipos de células somáticas (Resnick et al., 1992; van Dissel-Emiliani et al., 1993). A diferenciação das PGC em gonócitos marca a transição de uma célula com potencial múltiplo para células

germinativas embrionárias, de potencial mais restrito e relacionado ao desenvolvimento das células da linhagem germinativa masculina (Matsui, Zsebo e Hogan, 1992; de Rooij, 1988). Embora os gonócitos tenham sido caracterizados há muito tempo, a biologia dessas células e os mecanismos que regulam sua diferenciação ainda são pouco compreendidos, em comparação com outros tipos celulares do testículo (Culty 2009).

Os eventos envolvidos com a diferenciação dos gonócitos são bastante estudados em roedores de laboratório, como o camundongo e o rato. Esses estudos indicam que eles apresentam duas fases de proliferação: uma ainda no período fetal e outra no neonatal, intercaladas por um período de quiescência (Culty, 2009) (Fig. 1). No rato, a primeira fase proliferativa ocorre entre 13,5 e 17,5 dpc, enquanto o período de quiescência se estende do 17,5 dpc até o nascimento (Magre e Jost, 1980; Orth, 1982; McGuiness e Orth, 1992a, b) (Fig. 1). A atividade mitótica dos gonócitos de ratos é retomada entre os dias 1-4 pós-parto (dpp), quando também ocorre a sua diferenciação em espermatogônias A (Clermont e Perey, 1957; Orth, 1982; McGuiness e Orth, 1992a, b; Prépin et al., 1994; De Miguel et al., 1997; Boulogne et al., 1999). Outros autores sugerem que tais eventos ocorram após o dia 5 pós-parto (Zogbi et al., 2012). Segundo Clermont e Perey (1957), os gonócitos desaparecem totalmente a partir de 10 dpp. Em outros roedores, como o hamster e o gerbilo, o padrão de diferenciação é similar, embora nesse último eles sejam observados até o fim da segunda semana de vida (Miething, 1992; Pinto et al., 2010).

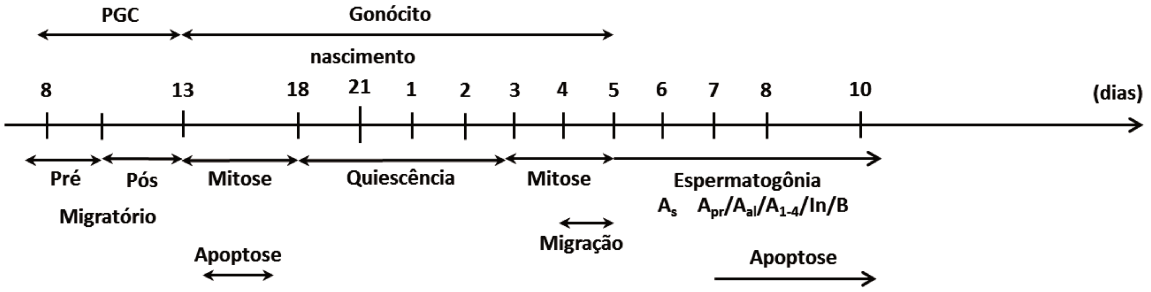


Figura 1. Esquema dos eventos envolvidos com o desenvolvimento das células da linhagem germinativa masculina do rato, durante o período embrionário e perinatal (Modificado de Culty, 2009).

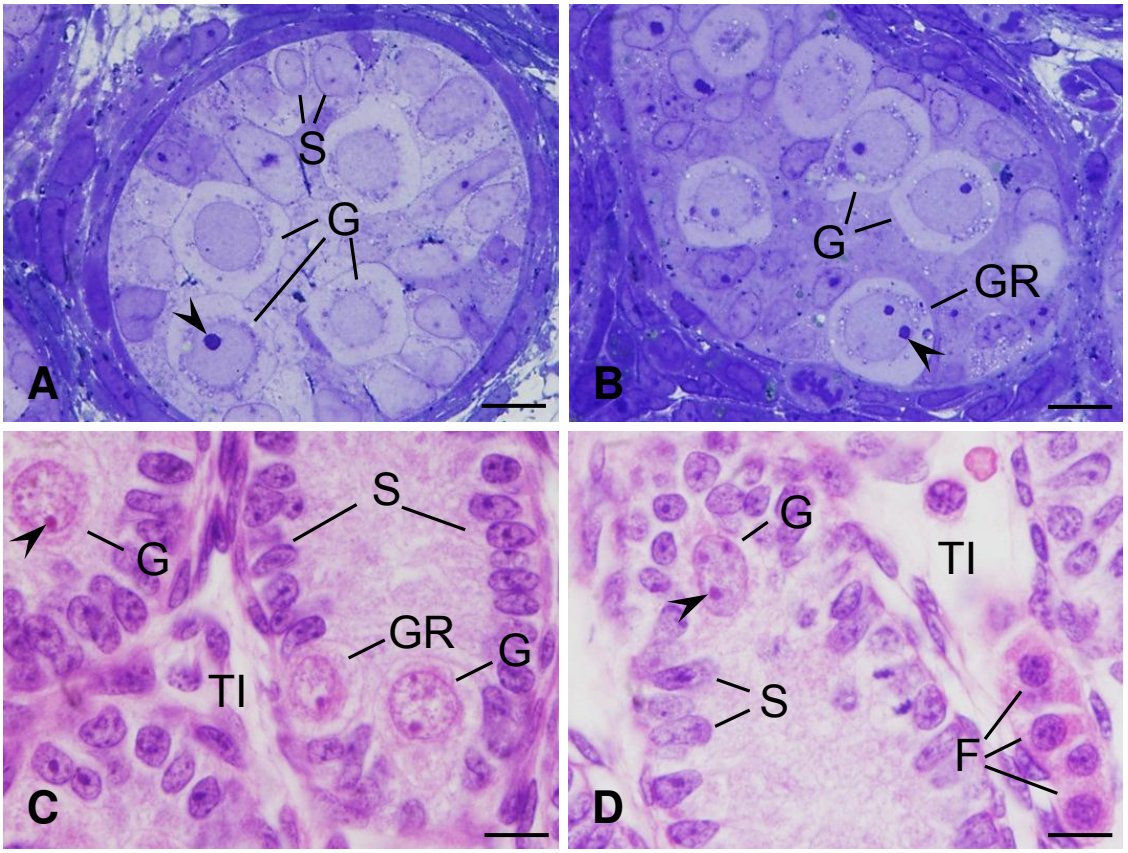


Figura 2. Aspéctos da morfologia e da diferenciação dos gonócitos. (A, B) Cortes semifinos de testículo de ratos neonatos de 0,5 dpp corados com Azul de Toluidina. (C, D) Cortes histológicos de testículo de ratos de 4,5 dpp corados com HE. Legenda: F = células de Leydig fetais; G = gonócitos; GR = gonócitos relocados; S = células de Sertoli; cabeças de seta = nucéolos. Barra = 100µm.

Existe muita controversa com relação aos processos relacionados à diferenciação dos gonócitos, durante os primeiros dias de vida neonatal. Os processos de proliferação, diferenciação e morte são fundamentais para o correto estabelecimento da espermatogênese, sendo que alterações em algum dos processos anteriormente mencionados podem levar ao surgimento de tumores de células germinativas, bem como a espermatogênese deficiente (Culty, 2009).

Um evento indispensável para a diferenciação dos gonócitos em espermatogônias, durante o período neonatal, é seu reposicionamento (“relocation”), ou seja, a migração do centro para a base dos cordões seminíferos em desenvolvimento e adesão à membrana basal

(Roosen-Runge e Leik, 1968; Clark e Eddy, 1975; McGuiness e Orth, 1992a). Em ratos, o reposicionamento se inicia um dia após o início da proliferação neonatal, o que sugere que esses eventos são independentes e, provavelmente, regulados por mecanismos distintos (McGuinness and Orth, 1992a). Os gonócitos relocam devido a sua migração ativa (McGuinness and Orth, 1992b), e seu comportamento migratório é mediado por fatores intratesticulares (McGuinness and Orth, 1992a) (Fig. 3). Na maioria dos roedores estudados, como o rato, o hamster e o gerbilo, o período proliferativo neonatal coincide com um pico de morte dos gonócitos por apoptose (2-7 dpp, no caso do rato) (Roosen-Runge e Leik, 1968; Miething, 1992; Boulogne et al., 1999). Em estudo recente sobre o desenvolvimento dos gonócitos em ratos, Zogbi et al. (2012) observaram que o número de gonócitos, por corte histológico do testículo, decresce entre 19 dpc a 5 dpp, em paralelo com o aumento do comprimento dos túbulos seminíferos, enquanto seu número total no órgão não varia. Esses dados, associados ao pequeno número de gonócitos apoptóticos, na primeira semana de vida, indicam que a redução do número de gonócitos, por corte testicular, é devido à sua redistribuição ao longo dos cordões seminíferos e não à sua morte.

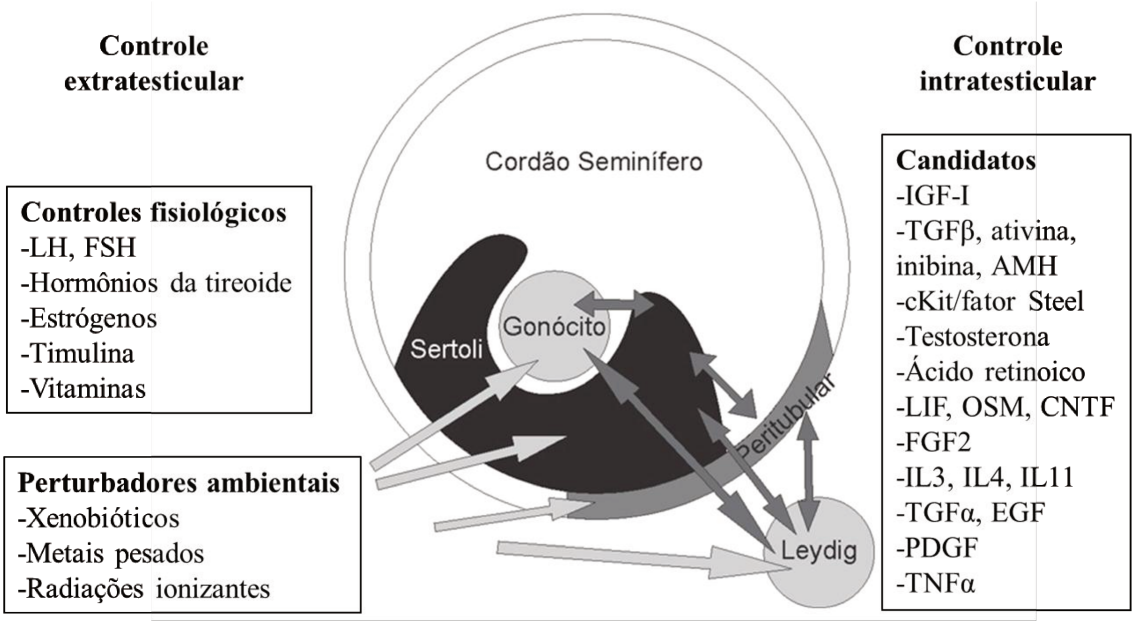


Figura 3. Fatores intra e extratesticulares envolvidos no controle do desenvolvimento testicular, durante o desenvolvemento fetal e neonatal. Adaptado de Rouiller-Fabre et al. (2003).

A diferenciação de gonócitos em espermatogônias e a origem das espermatogônias-tronco também são pontos bastante controversos. Vários estudos sugeriram que os gonócitos neonatais correspondem a uma população heterogênea de células, das quais apenas uma parte conserva as propriedades de células tronco, enquanto as demais estariam destinadas a tornarem-se células diferenciadas (Roosen-Runge e Leik, 1968; Kluin e de Rooij, 1981; de Rooij 1998, Orwig et al., 2002). Orwig et al. (2002), a partir de análises de suspensões celulares de testículo de ratos com 0 – 4 dpp, propuseram a existência de duas subpopulações de gonócitos, designados com pseudópodos e redondos. Os gonócitos com pseudópodos e redondos foram encontrados em quantidade semelhante, durante os quatro primeiros dias de vida pós-natal (45% e 42,6% do total de gonócitos, respectivamente), sendo que os primeiros foram capazes de produzir e manter colônias de espermatogênese após serem transplantados em testículos inférteis e os últimos, em sua maioria, sofreram apoptose. Essas populações possivelmente são análogas aos gonócitos em migração e em degeneração, propostos por outros autores (McGuinness e Orth, 1992a,b; Roosen-Runge e Leik, 1968; van Dissel-Emiliani et al., 1993).

A descrição dos eventos envolvidos na transição de gonócitos para espermatogônias indiferenciadas (A single, A paired e A aligned) e diferenciadas (A1 – A4, Intermediárias e B) tem sido muito dificultada pela falta de marcadores específicos para diferenciação dessas três populações celulares. Atualmente, tem sido descritos vários marcadores para essas populações celulares em alguns roedores e em humanos (Culty, 2009). Uma dessas moléculas é o receptor de tirosina quinase c-Kit, que é expresso pelas PGC, suprimido em gonócitos neonatais e espermatogônias indiferenciadas e novamente expresso pelas espermatogônias diferenciadas (Ohbo et al., 2003; Ohmura et al., 2004). Em um elegante estudo realizado por Yoshida et al. (2006), a expressão de c-kit foi analisada durante o desenvolvimento neonatal do testículo de camundongos, juntamente com a expressão do fator de transcrição neurogenina 3 (Ngn3), um marcador específico para espermatogônias indiferenciadas, e ausente nos gonócitos. A identificação da linhagem de gonócitos e de espermatogonias indiferenciadas e diferenciadas permitiu concluir que parte dos gonócitos dá origem direta às espermatogônias diferenciadas, levando à primeira onda de espermatogênese, e parte origina espermatogônias indiferenciadas, que irá fornecer espermatogônias para a espermatogênese, estando relacionada ao estabelecimento do ciclo

do epitélio seminífero (Fig. 3). Permanece por ser demonstrado se isso também se aplica a outras espécies de roedores e mamíferos.

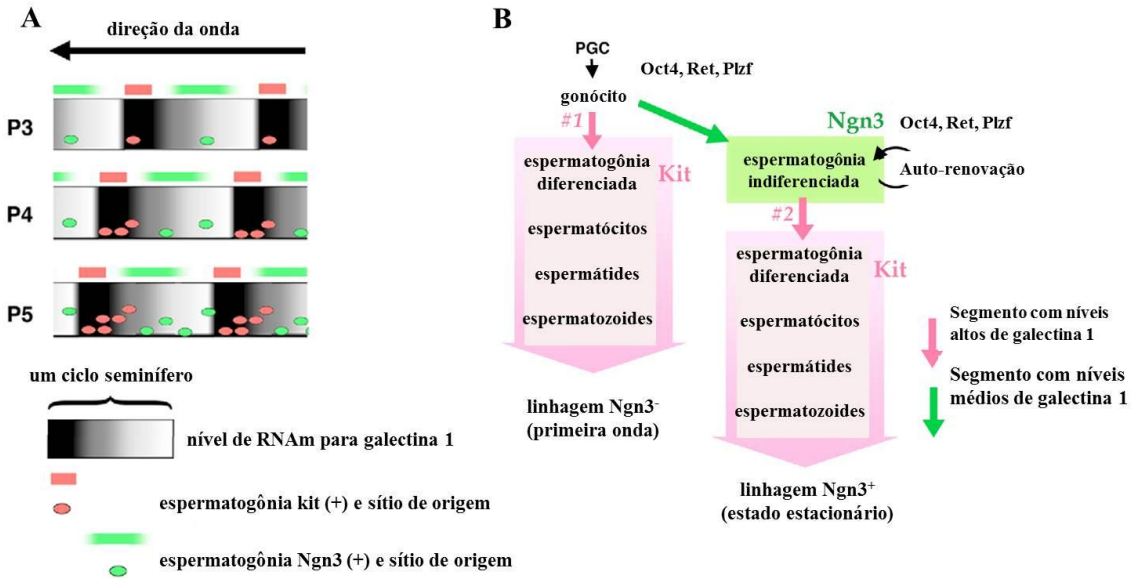


Figura 3. As duas linhagens de espermatogônias e o ciclo do Epitélio seminífero. A: as espermatogônias c-kit$^+$ (ovais vermelhos) surgem nos estágios com um alto nível de expressão de galectina1, enquanto as espermatogônias Ngn3$^+$ (ovais verdes) preferem segmentos com nível médio de galectina1. O nível de expressão de galectina1 é mostrado pelo gradiente: preto = maior; branco = menor. B: na primeira semana pós-natal, os gonócitos se diferenciam em espermatogônias diferenciadas c-Kit$^+$ (seta rosa 1) ou indiferenciadas Ngn3$^+$. A transição de espermatogônias indiferenciada para espermatogônias diferenciadas está intimamente relacionada ao ciclo do epitélio seminífero, e as células c-Kit$^+$ são estabelecidas em estágios com alta expressão de galectina1 (seta rosa 2) (Modificado de Yoshida et al., 2006).

Nas últimas duas décadas inúmeros estudos tem fornecido informações importantes sobre os mecanismos que regulam os processos envolvidos com a diferenciação dos gonócitos. Os hormônios esteroides e desreguladores endócrinos tem um papel preponderante nesses processos. Desreguladores endócrinos são agentes exógenos que interferem no funcionamento do sistema endócrino, por provocarem a diminuição, o aumento ou a substituição de hormônios naturais, ou ainda por bloquearem a ação destes (Santamarta, 2001). Constatou-se que alguns casos de insensibilidade a andrógeno e de baixos níveis desse hormônio estão associados a um alto risco de câncer testicular, devido

ao desenvolvimento alterado dos gonócitos (Skakkeback et al., 2001; Fisher, 2004). Também se constatou que a exposição de ratas prenhes a anti-andrógenos durante o período de 8 a 15 dpc afetou a espermatogênese dos adultos (Anway et al., 2005). No entanto, o mesmo tratamento entre 15 e 20 dpc não apresentou nenhum efeito (Cupp et al., 2003; Uzumco et al., 2004). Recentemente, Merlet et al. (2007) observaram um aumento no número de gonócitos, devido a maior atividade proliferativa, em camundongos mutantes com a síndrome da feminilização testicular (Tfm), os quais não apresentam receptor de andrógenos (AR). Além disso, o cultivo de gonócitos fetais na presença de dihidrotestosterona (DHT) resultou em diminuição de sua atividade proliferativa (Merlet et al., 2007). Esses autores também demonstraram a presença de mRNA para AR, bem como de AR funcional nessas células. Portanto, os dados acima indicam que os gonócitos têm um período crítico de sensibilidade a andrógenos, durante sua fase de proliferação, no período fetal, e são alvo direto de andrógenos endógenos e de desreguladores endócrinos que agem como antiandrógenos (Merlet et al., 2007).

Além da ação androgênica, estudos têm demonstrado a importância dos estrógenos e de agentes ambientais com ação estrogênica sobre o desenvolvimento dos testículos na vida fetal e neonatal (Sharpe e Skakkebaek, 1993; Delbès et al., 2004, 2007). Enquanto a expressão de receptor de estrógeno do tipo α (ERα) parece se restringir as células de Leydig (Fisher et al., 1997), nos testículos de ratos, tanto de fetos (20 dpc) quanto de adultos, o receptor de estrógeno do tipo β (ERβ) é expresso por múltiplos tipos celulares, incluindo o núcleo das células de Sertoli, células peritubulares, células de Leydig fetais e gonócitos. Tal resultado demostra que os estrógenos exercem um papel significante sobre as funções testiculares e, provavelmente, têm efeitos diretos sobre a função e a maturação das células germinativas (Saunders et al., 1998).

De acordo com Sharpe e Skakkebaek (1993), moléculas semelhantes a estrógeno podem prejudicar a fertilidade de machos adultos por agirem durante o desenvolvimento das gônadas. A exposição a esse hormônio durante a vida fetal e neonatal também pode afetar as funções reprodutivas do adulto. Tais evidências levaram a proposta da ‘‘hipótese do estrógeno’’ (Sharpe e Skakkebaek, 1993). Delbès et al. (2004) mostrou, com o uso de camundongos nocaute para receptor de estrógeno, que estrógenos endógenos inibem o desenvolvimento dos testículos fetais e neonatais. A inativação do ERβ levou a um

aumento de 50% no número de gonócitos em neonatos de dois dias e não modificou o número de células de Sertoli e de células de Leydig, nem os níveis de testosterona testicular. Por outro lado, a inativação do ERα não alterou o número de gonócitos, mas a secreção de testosterona foi maior nos animais de 13,5 dpc e 2 dpp (Delbès et al., 2004).

Considerando-se a ação dos hormônios esteroides sobre as gônadas em desenvolvimento, e sabendo-se que a obesidade está associada a múltiplas alterações do sistema endócrino, incluindo concentrações anormais de hormônios na circulação sanguínea, devido a alterações no padrão de secreção e/ou metabolismo e de transporte hormonal alterado (Kirschner, 1982), é possível inferir que essa desordem metabólica afete o processo de diferenciação das células germinativas.

A obesidade é um preocupante problema de saúde pública, pois além do sistema endócrino, compromete vários outros sistemas orgânicos, a exemplo do sistema cardiovascular, além de prejudicar a função reprodutora em ambos os sexos (Hajer et al., 2008; Hammoud et al., 2006; Hartz et al., 1979; Lake et al., 1997; Pasquali, 2006; Pasquali et al., 2007). Também é alarmante a crescente incidência de obesidade e sobrepeso em mulheres em idade reprodutiva (Khashan e Kenny, 2009). No que se refere ao aparelho genital, estudos epidemiológicos têm demonstrado que a obesidade feminina está associada ao início precoce da puberdade, distúrbios menstruais, anovulação intermitente ou crônica, excesso de andrógeno e maior susceptibilidade ao desenvolvimento de certos tipos de câncer de mama e endométrio (Pasquali, 2006; Pasquali et al., 2007). Acredita-se que com o aumento da prevalência de estilos de vida sedentários e de mudanças na dieta, a obesidade esteja emergindo como uma importante causa de efeitos adversos à saúde, incluindo a infertilidade (Hammoud et al., 2006).

Sabe-se que as desordens do metabolismo materno podem influenciar o ambiente intra-uterino e induzir disfunções metabólicas na prole, mediadas por mecanismos fisiológicos e/ou epigenéticos (Simonds e Budge, 2009; Catalano et al., 2009). Nas últimas décadas, sólidas evidências de pesquisas experimentais e clínicas resultaram na elaboração do conceito de programação fetal, segundo o qual um estímulo agudo ou crônico, durante a gestação, pode estabelecer uma resposta permanente no feto e prejudicar o funcionamento de certos sistemas no início da vida pós-natal ou na vida adulta (Simons e Budge, 2009). Assim, estudos em longo prazo têm mostrado que a obesidade materna, durante a gestação,

está associada a um maior risco de obesidade e de diabetes tipo 2 para a prole (Dabelea et al., 2000). Dados da literatura também mostram que bebês nascidos de mulheres com sobrepeso ou obesas tiveram um aumento da massa gorda, em comparação com bebês nascidos de mães não obesas, além do risco aumentado de se tornarem adolescentes obesos e de apresentarem desregulação metabólica (Boney et al., 2005, Sewell et al., 2006). Também se constatou que fetos de mães obesas desenvolvem a resistência à insulina, uma possível causa da síndrome metabólica em adultos (Catalano et al., 2009). Além disso, um estudo com roedores comparando o efeito de dietas com baixo teor de gordura e ricas em gordura, com ou sem correção da ingestão calórica, revelou que a obesidade e as alterações hormonais observadas na prole são decorrentes da obesidade e não do excesso de gordura (White et al., 2009).

A exposição intra-uterina ao excesso de andrógenos em primatas e ovelhas induz alterações na secreção de LH, na sensibilidade aos pulsos de LH, na concentração de progesterona e no metabolismo de insulina (Forsdike et al., 2007; Abbott et al., 2009; Steckler et al., 2009). O LH é o principal estímulo fisiológico para a produção de andrógeno pelas células da teca ovariana. A hiperinsulinemia, associada à obesidade, contribui para a hiperandrogenemia, uma vez que a insulina age como uma co-gonadotrofina, juntamente com o LH, nas células da teca, aumentando a produção de andrógenos (Cara e Rosenfield, 1988; Nestler et al., 1998). No entanto, os efeitos intra-uterinos das alterações endócrinas causados pela obesidade materna sobre a histofisiologia do aparelho genital masculino são pouco estudados. Também não existem estudos que investiguem os efeitos da obesidade, durante a gestação, sobre a diferenciação neonatal do testículo, em particular dos gonócitos.

Apesar da diferenciação dos gonócitos ser fundamental para o sucesso da espermatogênese e fertilidade do adulto, os mecanismos envolvidos ainda são pouco conhecidos. A análise da diferenciação dos gonócitos também é a base para a compreensão da etiologia de importantes patologias testiculares, como o carcinoma *in situ* (Almstrup et al., 2007). Apesar disso, não existem investigações que avaliem as interferências do estado nutricional da mãe sobre o desenvolvimento dessas células, seja no período fetal, seja no neonatal. Tais investigações se tornam muito necessárias no caso da obesidade, tendo em vista a crescente incidência dessa desordem na maioria dos países, em especial em crianças e em mulheres em idade reprodutiva (James et al, 2004; Khashan e Kenny, 2009). Uma vez que a

obesidade altera o eixo hipotálamo-hipófise-gonadal (Pasquali, 2006; Pasquali et al., 2007) e o cenário hormonal intra-uterino (Simonds e Budge, 2009; Catalano et al., 2009), é razoável supor que ela possa levar a perturbações nos processos envolvidos na diferenciação dos gonócitos, sabidamente influenciados pelos hormônios sexuais (Boulogne et al., 1999).

IV. OBJETIVOS

O objetivo do presente trabalho foi avaliar se a obesidade materna afeta a diferenciação dos gonócitos e o desenvolvimento testicular dos filhotes, durante o período neonatal. Essa questão foi investigada pela análise da densidade numérica de gonócitos, ao nascimento, e dos processos envolvidos com a sua diferenciação, como o reposicionamento para a periferia do túbulo, proliferação, apoptose e transição para espermatogônias.

V. ARTIGO

Maternal obesity disturbs the postnatal development of gonocytes in the rat without impairment of testis structure at pre-pubertal age

Caroline Maria Christante[1, 2], Sebastião Roberto Taboga[1], Maria Etelvina Pinto-Fochi[1], Rejane Maira Góes[1*]

[1] Department of Biology, Institute of Biosciences, Letters and Exact Sciences – São Paulo State University – IBILCE/UNESP, 15054-000, São José do Rio Preto, SP, Brazil.

[2] Department of Anatomy, Cell Biology, Physiology and Biophysics, Institute of Biology, State University of Campinas - UNICAMP, 13083-970, Campinas, SP, Brazil.

Corresponding author: Prof. Dr. Rejane Maira Góes. Rua Cristóvão Colombo, 2265, CEP 15054–000, São José do Rio Preto, SP, Brazil. Telephone +55 17 32212391, Fax +55 17 32212390. E-mail remagoes@ibilce.unesp.br

Short title: Gonocyte neonatal development following maternal obesity

Abstract

In this study, we evaluated whether maternal obesity affects testis development and gonocyte differentiation in the rat from 0.5 to 14.5 postnatal days. Male Wistar rats were used at 0.5, 4.5, 7.5 and 14.5 days postpartum. These rats were born from obese mothers, previously fed with a high-fat diet (20% saturated fat), for 15 weeks, or normal mothers that had received a balanced murine diet (4% lipids). Maternal obesity (MO) did not affect testis weight or histology at birth, but changed the migratory behavior of gonocytes. The density of relocated cells was higher in MO pups at 0.5 dpp and decreased at 4.5 dpp and differed from those of control pups, where density increased exponentially from 0.5 to 7.5 dpp. The numerical density of gonocytes within seminiferous cords did not vary in MO, in relation to control neonates, for any age considered, but the testis weight was 50% lower at 4.5 dpp. A wide variation in plasmatic testosterone and estrogen levels was observed among the groups, during the first week of age, and MO pups exhibited higher steroid concentrations at 4.5 dpp in comparison to controls. At this age, steroid levels were negatively correlated with cell proliferation and density of relocated gonocytes at seminiferous epithelium for control and MO pups. At 7.5 dpp, the testicular size and other parameters of gonocyte development are retrieved. In conclusion, maternal obesity and saturated lipid diets disturb gonocyte development and sexual steroid levels, during the first days of life, with recovery at pre-pubertal age.

Introduction

Due to the increasing incidence and the numerous organic complications associated with obesity, this disorder has been considered the fifth leading cause of death and a serious global public health problem (WHO, 2012). A review concerning the genetics of obesity, with an emphasis on established obesity susceptibility loci identified through candidate gene and genome-wide studies, indicated that the contribution of genetic loci to body weight increase is less than 2% (Loos, 2009). Therefore, environmental factors, such as high saturated fat in diets, are thought to have pivotal roles in overweight and obesity (Cascio et al., 2012).

Epidemiological data show that over 64% of women of child-bearing age were overweight or obese in the United States, in 2008, and about one third of pregnant women were obese (King, 2006; Sullivan et al., 2011). As both maternal nutrition and obesity influence the development of mammals, during the intrauterine or breast-feeding phases (Sullivan et al., 2010), scientific interest was directed to elucidate the influence of maternal obesity on the physiology of organs and health, during childhood and adult life. Epidemiological and animal model studies indicated that maternal obesity at conception alters gestational metabolic adjustments and affects placental, embryonic, and fetal growth and also post-natal development (Catalano and Ehrenberg, 2006; Catalano et al., 2009; Simonds and Budge, 2009; Sullivan et al., 2011). Several lines of evidence show that acute or chronic stimuli, during gestation, can induce a permanent response in fetus and impair the physiology of several organic systems and this phenomena was designed fetal programming (Boney et al., 2005; Catalano et al., 2009; Dabelea et al., 2000; Sewell et al., 2006; Simons and Budge, 2009). The effects of maternal obesity on fetal programming of

energy balance and adiposity (Sullivan et al., 2010), cardiovascular and renal systems (Nistala et al., 2011) had been described. However, the knowledge about consequences of maternal obesity on fetal programming and histophysiology of genital system is incipient, and there is no information about the potential interference of maternal obesity on testis development at neonatal life.

The perinatal development of testis involves the proliferation and differentiation of three cell populations of adult organ – Sertoli cells, adult Leydig cells and germ cells (Orth, 1982, Boulogne et al., 1999, Mendis-Handagama and Ariyaratne, 2001). The germ cells in fetal and neonatal testis, named gonocytes (Clermont and Perey, 1957), are derived from the migratory primordial germ cells, which colonize the genital ridge between 13,5-14,5 days post coitum (dpc), in the rat (Magre and Jost, 1980). The gonocytes have been characterized a long time ago, but the regulatory mechanism which control their differentiation are still little understood, compared with other cell types of testis (Culty, 2009). The differentiation of gonocytes in rodents involves a proliferative period in fetal development (from 13,5 to 17,5 dpc in the rat), a quiescent period during perinatal life, followed by resumption of mitosis in the first days of age (from 1 to 4 dpp for the rat) (Magre and Jost, 1980; Orth, 1982; McGuiness and Orth, 1992a, b; Prépin et al., 1994; De Miguel et al., 1997; Boulogne et al., 1999). Indeed, the relocation of gonocytes to the periphery of seminiferous cords is crucial for their posterior differentiation into spermatogonia (Roosen-Runge and Leik, 1968; Clark and Eddy, 1975; McGuiness and Orth, 1992a), and the non-relocated gonocytes die by apoptosis in the majority of rodents (Miething, 1992; Boulogne et al., 1999; Pinto et al., 2010). There are solid evidences about the inhibitory action of androgens on the proliferation of fetal gonocytes (Merlet et al., 2007), whereas estrogen, via ERB, inhibits apoptosis and enhances gonocyte development

(Delbès et al., 2004, 2007; Vigueras-Villaseñor et al., 2006). Considering that female obesity alters the scenario of sexual steroids (Kirschner, 1982) is reasonable to assume that maternal obesity may interfere in the gonocyte differentiation.

In the present study, we evaluated if a high-fat diet and maternal obesity affect the neonatal testicular development of Wistar rats, with emphasis on processes involved with gonocyte differentiation.

Results

Body weight, testicular weight and GSI

Maternal obesity decreased the mean body weight of pups at birth in 11% ($p = 0.018$, Table 1), but did not alter the anogenital distance (C = 2.05 ± 0.21 mm and MO = 2.05 ± 0.14 mm). The testicular weight (Table 1) did not change at birth, but was approximately 20% lower for pups from obese mother at 4.5 dpp. The GSI of these pups was also lower at this age (Table 1). The body weight, testicular weight and GSI of male offspring subjected to maternal obesity were recovered from 7.5 dpp onwards (Table 1).

Morphological analysis and repositioning of gonocytes

The analysis of paraffin (not shown) and semi-thin (Fig. 1A, B) sections showed no marked changes in testis histology or gonocyte morphology, for the pups subjected to maternal obesity. At birth, the majority of the gonocytes were located in the center of the seminiferous cords (Fig. 1A-D). The density of relocated gonocytes increased exponentially in control rats from 0.5 to 7.5 dpp (Fig. 1C-F, H). In contrast, the pups subjected to maternal obesity had a higher density of relocated gonocytes at 0.5 dpp, compared with the

control group, with a reduction at 4.5 dpp and a posterior recovery at 7.5 dpp (Fig. 1H). The stereological analysis of testes indicated an increase in volume density of seminiferous cords and a decrease in volume density of the interstitial tissues, during post-natal testis development. Neither of which was affected by maternal obesity (Table 2).

Numerical density (Nv) of gonocytes

As expected, the numerical density of gonocytes, per volume of seminiferous cord, dramatically decreased during the first days of age, being very small in animals at 7.5 dpp (Fig. 1I). These results reflect the differentiation of gonocytes into spermatogonia. No difference in Nv of gonocytes was observed for the pups subjected to maternal obesity, compared with the controls, for any of the ages studied (Fig. 1I). As previously mentioned, the volume density of seminiferous cords did not vary between the groups submitted to maternal obesity and their respective controls (Table 2); however, the testicular volume was significantly lower ($p = 0.03$) in control animals at 4.5 dpp, compared with pups from obese mothers (Fig. 1J). Therefore, the total number of gonocytes, per testis, decreased in the maternal obesity group at this age, compared with controls.

Cell proliferation and apoptosis

There were no statistical differences in mitotic density figures in the seminiferous epithelium, among controls and pups subjected to maternal obesity, at 7.5 dpp (Fig. 2A). However, the numerical values show a peak of cell proliferation at 4.5 dpp in controls, whereas the cell proliferation is higher at birth and decays at 4.5 and 7.5 dpp, in the group subjected to maternal obesity (Fig. 2A).

The immunocytochemistry showed that the non-relocated gonocytes were PCNA-negative, but became PCNA-positive after relocation (Fig. 2B-F). The Sertoli cells were PCNA-positive at 4.5 and 7.5 dpp (Fig. 2B-F).

All neonatal gonocytes at 0.5 dpp showed cytoplasmic staining for activated caspase 3 (Fig. 3B, C). These gonocytes exhibited normal morphology and were not considered in apoptosis. Also, for animals at 0.5 dpp, apoptotic cells were not detected in either the control group or those subjected to maternal obesity (Fig. 3B, C). Apoptotic cells/bodies, which were intensely labeled by activated caspase 3, corresponding to advanced apoptosis stages, were detected at 4.5 dpp (Fig. 3A). The density of apoptotic cells/bodies increased slightly from 4.5 to 7.5 dpp and dramatically from 7.5 to 14.5 dpp (Fig. 3A). Maternal obesity did not alter the pattern of apoptosis in seminiferous cords (Figs. 3A-E).

Immunocytochemical analyses

The immunocytochemical analysis of neonatal rat testes indicated that postnatal gonocytes did not show immunoreactivity for androgen receptor (Fig. 4A-C). No remarkable changes could be observed, between the controls and those pups born from obese mothers, with respect to the location of AR in gonocytes, at any age considered, using immunocytochemistry. Immunocytochemistry for estrogen receptors revealed that gonocytes of rats at 4.5 dpp exhibited cytoplasmic localization for ERA (Fig. 4H, I), and ERB with nuclear localization (Fig. 4E-G). Apparently, there was an intense staining increase for ERB in gonocytes of animals subjected to maternal obesity.

Testicular maturation in 14.5 dpp rats

The evaluation of testis maturation in 14.5 dpp control rats indicated that the majority of seminiferous cords, at this age, contained spermatocytes in primary leptotene, a lower percentage contained B spermatogonia and pre-leptotene and only a small percentage of tubules contained spermatocytes in zygotene (Fig. 5A-F). An increase in the percentage of tubules containing A spermatogonia ($p = 0.99$) and a reduction of those containing leptotene and zygotene primary spermatocytes ($p = 0.053$), in the testis of rats subject to maternal obesity (Fig. 5G) was observed, but these alterations were not statistically significant.

Testosterone and estrogen levels

Testosterone and estrogen plasma levels were very variable among animals, with no statistical differences between the control and the maternal obesity groups (Fig. 6). Pups subjected to maternal obesity exhibited lower plasmatic testosterone levels, at birth, in comparison with controls (Fig. 6A). The opposite was observed for estrogen levels (Fig. 6B). At 4.5 dpp, animals exposed to maternal obesity had higher testosterone and estrogen levels, compared with the controls (Fig. 6). At 14.5 dpp, the testosterone levels were very similar and the estrogen levels were almost identical, in the two groups, from 7.5 dpp onwards (Fig. 6).

Discussion

In the present study, we used a model of obesity based on a high-fat diet to evaluate the consequences of maternal obesity on neonatal testis development and on male germ

cells differentiation in Wistar rats. Maternal obesity did not affect anogenital distance, testis weight or histology at birth. A marked increase in the number of relocated gonocytes was observed, to control group, from 0.5 to 4.5 dpp and a light increase was seen from 4.5 to 7.5 dpp, the age at which most of the gonocytes have been repositioned. Such migratory behavior is in agreement with the literature (Roosen-Runge and Leik, 1968; Clark and Eddy, 1975; McGuiness and Orth, 1992a). It should be mentioned that despite the presence of gonocytes in the periphery of seminiferous cords at 0.5 dpp, it can be inferred that these cells are still separated from the basal lamina by thin extensions of Sertoli cells, as shown by McGuiness and Orth (1992a), which are not identified by immunocytochemistry to AMH at light microscopy. The offspring of obese mothers do not follow the same pattern of migration, because we did not observe an increase in relocated gonocytes at 4.5 dpp, but instead saw a decline, compared to 0.5 dpp. Therefore, it can be assumed that maternal obesity affects the migratory behavior and relocation of gonocytes, in the first days of life. There was no significant difference in the density of relocated gonocytes at 7.5 dpp, between control and maternal obesity pups, indicating that disturbances in the migratory process, in the early days of age, are adjusted.

The migration from the center to the periphery of the seminiferous cords during neonatal development is a crucial event for the cell cycle and mitosis resumption and differentiation of gonocytes into spermatogonial lineage (Roosen-Runge and Leik, 1968; McGuinness and Orth, 1992a; Tres and Kierszenbaum, 2005). In the present study, we observed an increase in density of mitotic figures in the seminiferous epithelium of control rats from 0.5 to 4.5 dpp, while the pups subjected to maternal obesity showed a decrease from 0.5 to 7.5 dpp. Interestingly, for both the controls and pups born from obese mothers there was a relationship between relocated gonocyte density and proliferation in the

seminiferous epithelium at 0.5 dpp and 4.5 dpp. Additionally, only relocated gonocytes were PCNA-positive. These findings are in accordance with previous observations (McGuinness and Orth, 1992a) and reinforce the suggestion that migration to the periphery of the seminiferous cord is related to the resumption of proliferative activity of rat gonocytes, in neonatal life.

The immunocytochemistry for activated caspase 3 indicated a diffuse cytoplasmic labeling in gonocytes at 0.5 dpp, as observed by Zogbi et al. (2012). The cytoplasm of all gonocytes, at this age, was labeled and a slight cytoplasmic staining could be seen until 7.5 dpp. This diffuse cytoplasmic staining for activated caspase 3 was observed in normal gonocytes and was not considered an apoptosis indicator, unlike that for apoptotic cells/bodies detected from 4.5 to 14.5 dpp, which represented advanced apoptosis stages (Fig. 3B-E). The presence of activated caspase 3 in these cells can be explained by the fact it is involved not only in the cell death system, but also in the self-renewal and differentiation processes of germ cells (Fujita et al., 2008). According to Dejosez et al. (2008), caspase 3 also recognizes critical pluripotency factors for embryonic stem cell function.

We observed increasing levels of apoptosis in control rats, during the first week of age, which probably refers to gonocyte degeneration, since previous studies have shown that about 25-30% of such cells die at 7 dpp in this rodent (Boulogne et al., 1999; Lehraiki et al., 2011). These authors also demonstrated that there is no apoptosis of Sertoli cells in the perinatal period (Boulogne et al., 1999). Already high apoptosis levels observed at 14.5 dpp relate to the cells in the first wave of spermatogenesis. No significant difference was observed in the apoptotic cell frequency, among neonates born from obese and control

mothers, for any age evaluated, demonstrating that the factors that regulate the apoptosis of gonocytes, in the neonatal period, were not affected by maternal obesity.

The numerical density of gonocytes decreases exponentially, during the first week of life, due to their transition into spermatogonia. Maternal obesity did not affect the numerical density of gonocytes at birth or during the first week of age. However, despite testicular volume remaining practically unchanged in the maternal obesity group at 0.5 dpp, it was approximately 50% lower at 4.5 dpp, compared with the control group. This indicates a reduction in the total number of gonocytes at 4.5 dpp. At 7.5 dpp, gonocyte density and testicular volume of rats submitted to maternal obesity had recovered.

The hormonal dosage using high sensitivity ELISA and five animals per group indicated wide variations in plasma testosterone levels, especially at ages below 14.5 dpp, with no statistical differences observed, for any age. However, the analysis of the numerical values obtained for each animal and the means for each group showed a different pattern variation for serum testosterone levels, during the first week, with a decrease in the control pups and an increase in the maternal obesity group, at 4.5 dpp. Pups born from obese mothers showed higher estrogen levels, in the first week of age, with a peak at 4.5 dpp. A comparison between testosterone levels and cell proliferation also showed an inverse relationship of these parameters, for both studied groups. Merlet et al. (2007) have shown that androgens exert an inhibitory role on fetal gonocyte proliferation in mice with testicular feminization (Tfm). Our data suggest that the inhibitory action also extends to the neonatal period. However, this action is direct during fetal life (Merlet et al., 2007), whereas in postnatal life it is probably due to indirect mechanisms acting via paracrine factors produced by peritubular cells, because androgen receptors have not been detected in fetal or neonatal rat gonocytes (Williams et al., 2001). Still, in relation to androgen levels it

has been found that the obtained means for animals subject to maternal obesity from 4.5 to 14.5 dpp are very similar to those found in earlier ages of the control group (Fig. 6A). Therefore, it is possible that maternal obesity affects steroidogenesis and anticipates testosterone concentration changes in the perinatal life, with consequences for gonocyte differentiation. There are epidemiological studies showing that maternal obesity anticipates female puberty (Connor et al., 2012); however, to our knowledge, this is the first study that examines the effects of maternal obesity on sex hormones and testicular development, during neonatal period, in a rodent model.

Besides the density of gonocytes, comparisons of other parameters determined here, between maternal obesity and control groups, such as gonocyte migration, proliferation and apoptosis and plasma levels of estrogen and testosterone, as well as analysis of the degree of maturation and early testicular spermatogenesis, indicate the changes that occur in the first days of life, especially at 4.5 dpp, are normalized at 7.5 dpp. The degree of maturation and testicular volume of this organ at 7.5 and 14.5 dpp in pups subject to maternal obesity were similar to controls, suggesting an apparent recovery of germ cell development and steroidogenic capacity. It was confirmed, in another study in our laboratory, in which we found that rats subjected to the same model of maternal obesity, followed by breastfeeding in normal mothers, showed a decline of ~10% in sperm production and 5% in testosterone levels in adulthood. However, due to the wide inter-individual variations, no statistical difference was found with the control group, in that study (Reame V, Pinto ME, Ribeiro DL, Taboga SR, Góes RM 2012, unpublished observations). The trends in sperm count reductions and hyperandrogenemia, together with the current data about alterations in testicular development, in the first few days of life, suggest that maternal obesity affects testis development, during the fetal period, leading to developmental reprogramming of

organs and consequences for adult life. This is very worrying for male reproductive function when considering the influence of numerous other environmental factors that individuals are exposed throughout life, as these could be exacerbated by discrete impairment induced by maternal obesity. The main difficulty in the interpretation of the obesity model used here is in discriminating the effects of maternal obesity from those resulting from high saturated lipid consumption. Our data show that imbalances in steroids levels are an important component of this situation. It is well known that maternal obesity and high saturated lipids consumption causes reprogramming at hypothalamic level, influencing satiety and adiposity mechanism control in postnatal life (Ikenasio-Thorpe et al., 2007; Benkalfat, 2011). In this obesity model, mothers are insulin-resistant and exhibit hyperleptinemia. Insulin is not transported across the placenta, but leptin is (Smith and Waddell, 2003). Whereas leptin has an anti-steroidogenic action, it is possible that many of the observed changes are consequences of the increase in this hormone, in fetal life, since this has been previously observed for this model of obesity (Ribeiro et al., 2012). The influence of leptin in testis alterations reported here is currently being examined by our group.

Taken together, our data indicate that maternal obesity affects the pattern of gonocyte differentiation, in the first days of life, probably as a result of alterations in sex steroid levels. However, testicular development recovery occurs at the end of the pre-pubertal period. These results show that the first postnatal days are crucial to the repair of germ cell development and steredoigenic activity in testis of rats affected by maternal obesity.

Material and Methods

Animals and experimental design

Male and female Wistar rats (5 weeks old) were obtained from Anilab Animais de Laboratório e Comércio Ltda (Paulínia, SP, Brazil). The animals were kept in the Animal Breeding Center of São Paulo State University - UNESP, Institute of Biosciences, Humanities and Exact Sciences - IBILCE, at controlled temperature (23-25°C), humidity (40-60%), under 12 h light/dark cycle and treated with filtered water "ad libitum". The experimentation procedures were performed in accordance with the rules of the Ethics Committee on Animal Experimentation (CEUA / UNESP, protocol 22/2009).

Maternal obesity was induced by treatment for 15 weeks with a high fat diet (20% fat; 4.8 Kcal/g), acquired from Agroceres (Campinas, SP, Brazil). This model of obesity and diet was previously standardized by researchers at the Laboratory of Experimental Clinical Medicine, Faculty of Medicine of Botucatu, UNESP (Nascimento et al., 2008). Females of the control group and the males used for breeding received the standard normal calorie (normo-calorica) diet (4% fat; 3.2 Kcal/g) produced by the same manufacturer. Obese females (20 weeks old) were mated with thin adult males (12 weeks old) and normal females of the same age were mated with adult rats. The pregnancy was confirmed by the presence of vaginal plug. The birth date of pups was defined as day 0.5 postpartum (dpp) and the identification of gender was based on anogenital distance, which was measured with a digital caliper (King Tools, digital caliper, 0-150 mm). A total of 48 male offspring of normal and obese mothers aged 0.5, 4.5, 7.5 and 14.5 dpp (n = 6), each one born from different families, were used. The pups were killed by CO_2 inhalation and their weight was determined before death. Then, the pups were decapitated for blood collection, and testes

were dissected and weighed. The gonadosomatic index (GSI) was obtained from the formula GSI = (Testicular Weight / Body Weight) x 100.

Light microscopy

The right testes were fixed by immersion for 6 h in Bouin fluid, washed in 70% alcohol for the removal of picric acid and processed for inclusion in Paraplast (Histosec, Merck, Darmstadt, Germany). Serial sections of whole testes (5 μm thick) were made, part of which was used for routine histological analysis and part was subjected to immunocytochemistry.

The left testes were immersed in 2.5% glutaraldehyde, 1% tannic acid, 3.5% sucrose and 5 mM calcium chloride in 0.1 M cacodylate buffer (pH 7.4) at 4°C. Small incisions were made in the tunica albuginea of the gonads of animals at 4.5 and 7.5 dpp to improve fixative penetration. After 1 h in this solution, testes were cut into smaller cubes (1-2 mm thickness) and fixed for 4 h in the same solution, following washing in buffer, and post-fixation in osmium tetroxide 1%. Specimens were dehydrated in acetone and embedded in Araldite 502 (Electron Microscopy Sciences, Hatfield, PA, USA). One-μm thick sections were stained with a 1% solution of toluidine blue and 1% borax in water for light microscopy analysis.

Immunocytochemistry

Immunocytochemical reactions were performed for anti-Müllerian hormone (AMH), androgen receptor (AR), estrogen receptors (ERA and ERB) and activated Caspase 3. Briefly, paraffin sections were immersed in citrate buffer (pH 6), at 92°C, for 45 min, for antigen retrieval. The blocking of endogenous peroxidase was achieved by incubation with

3% H_2O_2 in methanol, for 20 min. Then, the tissue sections were incubated with 5% non-fat milk in PBS, for 30 min, to block non-specific protein-linkage. Incubations with primary antibodies were performed overnight at 4°C in 1% BSA using the following antibodies and dilutions: 1:75 rabbit IgG anti-human AR (Santa Cruz, sc-816), 1:100 goat IgG anti-human AMH (Santa Cruz, sc-6886), 1:50 rabbit IgG anti-human ERα (Santa Cruz, sc-8974), 1:50 rabbit IgG anti-human ERβ (Santa Cruz, sc-542), and 1:200 rabbit IgG anti-human activated Caspase 3 (Abcam, ab-13847).

The above mentioned reactions were then incubated with the biotinylated secondary antibody, followed by ABC avidin-biotin complex Kit (Santa Cruz Biotechnology), for 45 min, at 37°C. Sections were revealed with diaminobenzidine (DAB) for approximately one minute and counterstained with hematoxylin.

Also, double immunocytochemical reactions for proliferating cell nuclear antigen protein (PCNA) and VASA were performed. In this case, the blockages were used as described above, but the sections were incubated overnight at 4°C with 1:50 primary antibody rabbit IgG anti-human VASA (Santa Cruz , sc-67185), followed by incubation with 1:50 primary antibody mouse IgG anti-human PCNA (Santa Cruz, SC-56), for 1 h at 37°C. The incubation with Rat and Mouse Double Stain kit (Biocare) proceeded for 1 h. PCNA was revealed with DAB as described above and VASA with Vulcan Fast Red Chromogen (PAP). The negative control was obtained by omission of the primary antibody.

Numerical density of gonocytes

The numerical density (Nv) of gonocytes, or the number of these cells in a given tissue volume, was estimated for the seminiferous cords. This parameter was estimated in order to examine whether the number of gonocytes, in the same volume of seminiferous

cord, varied among the different experimental groups. These analyses were conducted based on the procedures of Rosen-Runge and Leik (1968) and Zogbi et al. (2012).

For this purpose, we primarily determined testicular volume (mm^3). Testicular volume for the pups at 0.5 and 4.5 dpp was determined by the Cavalieri method (Gundersen et al., 1988). One of the 20 histological sections had its area determined using the Image Pro-Plus software (Media Cybernetics version 4.5, MD, USA), and testicular volume was calculated by multiplying the sum of the areas by 100, a value that is related to the distance between two successive sections (20 x 5 μm). The testicular volume of 7.5 and 14.5 dpp rats was equal to fresh testis weight (g), without correction for density. Subsequently, the seminiferous cord volume density, in the testis, was determined, based on Weibel (1963). For each animal, we used two different histological sections and 10 random fields, per section, examined with 20x objective. After application of the M132 reticle, the volume density was determined by the percentage of dots which covered the seminiferous cords. Based on seminiferous cord volume density, the absolute volume of seminiferous cord was calculated, for each organ. Then, the crude counting (CC) of gonocytes was estimated using five histological sections, from approximately equidistant regions of the testis, for each animal. The tissue sections were previously submitted to immunocytochemistry for AMH and gonocytes appeared unmarked against AMH-positive cytoplasm of Sertoli cells. For estimation of CC, only the gonocytes with evident nuclei were counted. The Nv was calculated as the ratio between CC and seminiferous cord volume.

Density of relocated gonocytes, cell proliferation and apoptosis

The density of relocated gonocytes and mitotic cells, in seminiferous epithelium, was determined for 0.5, 4.5 and 7.5 dpp pups. We used three different histological sections, per testis, for each animal, subjected to immunocytochemistry for AMH, which were analyzed at 40x objective, in all of its extension. For each tissue section, the gonocytes at the base of seminiferous cord (relocated gonocytes) were counted, using the Image Pro-Plus software (Media Cybernetics version 4.5, MD, USA). We considered the cells with typical morphology, situated at the periphery of the seminiferous cords, without AMH-positive cytoplasmic processes of the Sertoli cells involving their basis, as relocated gonocytes. The other gonocytes were considered non-relocated.

The cell proliferation in seminiferous epithelium was estimated following the same tissue samples and procedures described above, in which the mitotic figures were counted. The values obtained were expressed as number of mitotic figures/mm^3 of seminiferous cords, previously determined as described for gonocyte count.

The estimation of apoptosis in seminiferous epithelium was made for four animals, in groups from 4.5 to 14.5 dpp, since only after 4.5 days postpartum apoptotic cells were observed. We used three histological sections for each animal subjected to immunocytochemistry for activated caspase 3. The total area of the histological section was estimated from images digitized at 40x magnification, using the above program, excluding the region corresponding to the tunica albuginea. The total number of apoptotic cells, per histologic section, was divided by the corresponding area.

Grade of testicular maturation

The grade of testicular maturation and onset of spermatogenesis were evaluated for animals at 14.5 dpp. One testis from each animal (n = 6, per group) was scanned under a microscope and the seminiferous cords staged according to the most advanced cell type of spermatogenic lineage, as follows: A spermatogonia – both undifferentiated (A_{single}, A_{paired} and $A_{aligned}$) and differentiated (A1-4) were considered; B spermatogonia; pre-leptotene or "resting" - the final period of DNA replication; leptotene primary spermatocytes and zygotene primary spermatocytes. These cells were identified as described by Clermont and Perey (1957), with 20x objective, in histological sections stained with HE. At least 150 tubules were evaluated, for each animal, and the data were expressed as percentages of tubules according to the most advanced cell type present.

Hormone dosage

The blood samples were collected immediately after decapitation, centrifuged at 3000 rpm, for plasma separation, and frozen at -20°C, for the subsequent analysis of testosterone and estrogen levels. The measurements were performed by capture/sandwich ELISA (antibody-antigen-antibody), using specific commercial kits (Enzo Life Sciences International Inc., PA, USA), with high sensitivity (5.67 pg/ml and 14.0 pg/ml, respectively) and coefficients of variation for inter-assay of 11.3 pg/mL (for testosterone) and 8.3 pg/mL (for estrogen). The readings were taken in reader Epoch™ Multi-Volume Spectrophotometer System (BioTek Instruments, VT, USA).

Statistical analyses

Statistical analysis of the collected data was performed in Statistica 7.0 software (Copyright © StatSoft, Inc. 1984-2004) and T-test, $p \leq 0.05$ was considered statistically significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

Funding was provided by National Research Council – CNPq (fellowship to R M Góes - Grant number 306258/2011), Coordinanting Body for Training University – level personnel - CAPES (Master fellowship to C M Christante) and São Paulo State Research Foundation - FAPESP (Grant to R M Góes - Grant number 2011/01612-4, Post-doctoral fellowship to M E Pinto-Fochi - Grant number 2009/16071-9 and Technical Training fellowship to Thiago Feres Pissolato - Grant number 2011/10739-8).

Acknowledgements

The authors are gratefully to technical assistance of Luiz Roberto Faleiros Jr. and Thiago Feres Pissolato.

References

Benkalfat NB, Merzouk H, Bouanane S, Merzouk SA, Bellenger J, Gresti J, Tessier C & Narce M 2011 Altered adipose tissue metabolism in offspring of dietary obese rat dams. Clinical Sciences **121** 19-28.

Boney CM, Verma A, Tucker R & Vohr BR 2005 Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. Pediatrics **115** 290-296.

Boulogne B, Olaso R, Levacher C, Durand P & Habert R 1999 Apoptosis and mitosis in gonocytes of the rat testis during foetal and neonatal development. International Journal of Andrology **22** 356-365.

Cascio G, Schiera G & Di Liegro I 2012 Dietary fatty acids in metabolic syndrome, diabetes and cardiovascular diseases. Current Diabetes Reviews **8** 2-17.

Catalano PM & Ehrenberg HM 2006 The short- and long-term implications of maternal obesity on the mother and her offspring. British Journal of Obstetrics and Gynaecology **113** 1126-1133.

Catalano PM, Presley L, Minium J & Hauguel-de Mouzon S 2009 Fetuses of obese mothers develop insulin resistance in utero. Diabetes Care **32** 1076-1080.

Clark JM & Eddy EM 1975 Fine structural observations on the origin and associations of primordial germ cells of the mouse. Current Opinion in Endocrinology, Diabetes and Obesity **47** 136-155.

Clermont Y & Perey B 1957 Quantitative study of the cell population of the seminiferous tubules in immature rats. American Journal of Anatomy **100** 241-267.

Connor KL, Vickers MH, Beltrand J, Meaney MJ & Sloboda DM 2012 Nature, nurture or nutrition? Impact of maternal nutrition on maternal care, offspring development and reproductive function. The Journal of Physiology **590** 2167-2180.

Culty M 2009 Gonocytes, the forgotten cells of the germ cell lineage. Birth Defects Research **87** 1-26.

Dabelea D, Hanson RL, Lindsay RS, Pettitt DJ, Imperatore G, Gabir MM, Roumain J, Bennett PH & Knowler WC 2000 Intrauterine exposure to diabetes conveys risks for type 2 diabetes and obesity: a study of discordant sibships. Diabetes **49** 2208-2211.

De Miguel MP, De Boer-Brouwer M, Paniagua R, van den Hurk R, De Rooij DG & Van Dissel-Emiliani FM 1996 Leukemia inhibitory factor and ciliary neurotropic factor promote the survival of Sertoli cells and gonocytes in coculture system. Endocrinology **137** 1885-1893.

Dejosez M, Krumenacker JS, Zitur LJ, Passeri M, Chu LF, Songyang Z, Thomson JA & Zwaka TP 2008 Ronin is essential for embryogenesis and the pluripotency of mouse embryonic stem cells. Cell **133** 1162-1174.

Delbès G, Levacher C, Pairault C, Racine C, Duquenne C, Krust A & Habert R 2004 Estrogen receptor beta-mediated inhibition of male germ cell line development in mice by endogenous estrogens during perinatal life. Endocrinology **145** 3395-3403.

Delbès G, Duquenne C, Szenker J, Taccoen J, Habert R & Levacher C 2007 Developmental changes in testicular sensitivity to estrogens throughout fetal and neonatal life. Toxicological Sciences **99** 234-243.

Fujita J, Crane AM, Souza MK, Dejosez M, Kyba M, Flavell RA, Thomson JA & Zwaka TP 2008 Caspase activity mediates the differentiation of embryonic stem cells. Cell Stem Cell **2** 595-601.

Gundersen HJ, Bendtsen TF, Korbo L, Marcussen N, Møller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sørensen FB & Vesterby A 1988 Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. Acta Pathologica, Microbiologica et Immunologica Scandinavica **96** 379-394.

Ikenasio-Thorpe BA, Breier BH, Vickers MH & Fraser M 2007 Prenatal influences on susceptibility to diet-induced obesity are mediated by altered neuroendocrine gene expression. Journal of Endocrinology **193** 31-37.

King JC 2006 Maternal obesity, metabolism, and pregnancy outcomes. Annual Review of Nutrition **26** 271-291.

Kirschner MA Schneider G, Ertel NH, Worton E 1982 Obesity, androgens, estrogens, and cancer risk. Cancer Research **42** 3281-3285.

Lehraiki A, Messiaen S, Berges R, Canivenc-Lavier MC, Auger J, Habert R & Levacher C 2011 Antagonistic effects of gestational dietary exposure to low-dose vinclozolin and genistein on rat fetal germ cell development. Reproductive Toxicology **31** 424-430.

Loos RJ 2009 Recent progress in the genetics of common obesity. British Journal of Clinical Pharmacology **68** 811-829.

Magre S & Jost A 1980 The initial phases of testicular organogenesis in the rat. An electron microscopy study. Archives D'anatomie Microscopique et de Morphologie Expérimentale **69** 297-318.

McGuinness MP & Orth JM 1992a Gonocytes of male rats resume migratory activity postnatally. European Journal of Cell Biology **59** 196-210.

McGuinness MP & Orth JM 1992b Reinitiation of gonocyte mitosis and movement of gonocytes to the basement membrane in testes of newborn rats in vivo and in vitro. The Anatomical Record **233** 527-537.

Mendi-Handagama SMLC & Ariyaratne HBS 2001 Differentiation of the adult Leydig cell population in the postnatal testis. Biology of Reproduction **65** 660-671.

Merlet J, Racine C, Moreau E, Moreno SG & Habert R 2007 Male fetal germ cells are targets for androgens that physiologically inhibit their proliferation. Proceedings of the National Academy of Sciences of the United States of America **104** 3615-3620.

Miething A 1992 Germ-cell death during prespermatogenesis in the testis of the golden hamster. Cell and Tissue Research **267** 583-590.

Nistala R, Hayden MR, Demarco VG, Henriksen EJ, Lackland DT & Sowers JR 2011 Prenatal Programming and Epigenetics in the Genesis of the Cardiorenal Syndrome. Cardiorenal Medicine **1** 243-254.

Orth JM 1982 Proliferation of Sertoli cells in fetal and postnatal rats: a quantitative autoradiographic study. The Anatomical Record **203** 485-492.

Pinto ME, Botta LS, Taboga SR & Góes RM 2010 Neonatal gonocyte differentiation in Mongolian gerbil Meriones unguiculatus involves asynchronous maturation of seminiferous cords and rapid formation of transitional cell stage. The Anatomical Record **293** 310-419.

Prépin J, Le Vigouroux P & Dadoune JP 1994 Effects of thymulin on in vitro incorporation of 3H-thymidine into gonocytes of newborn rat testes. Reproduction Nutrition Development **34** 289-294.

Ribeiro DL, Pinto ME, Rafacho A, Bosqueiro JR, Maeda SY, Anselmo-Franci JA, Taboga SR & Góes RM 2012 High-fat diet obesity associated with insulin resistance increases cell proliferation, estrogen receptor, and PI3K proteins in rat ventral prostate. Journal of Andrology **33** 854-865.

Roosen-Runge EC & Leik J 1968 Gonocyte degeneration in the postnatal male rat. American Journal of Anatomy **122** 275-299.

Sewell MF, Huston-Presley L, Super DM & Catalano P 2006 Increased neonatal fat mass, not lean body mass, is associated with maternal obesity. American Journal of Obstetrics and Gynecology **195** 1100-1103.

Sullivan EL, Grayson B, Takahashi D, Robertson N, Maier A, Bethea CL, Smith MS, Coleman K & Grove KL 2010 Chronic consumption of a high-fat diet during pregnancy causes perturbations in the serotonergic system and increased anxiety-like behavior in nonhuman primate offspring. The Journal of Neuroscience **30** 3826-3830.

Sullivan EL, Smith MS & Grove KL 2011 Perinatal exposure to high-fat diet programs energy balance, metabolism and behavior in adulthood. Neuroendocrinology **93** 1-8.

Symonds ME & Budge H 2009 Nutritional models of the developmental programming of adult health and disease. Proceedings of Nutrition Society **68** 173-178.

Tres LL & Kierszenbaum AL 2005 The ADAM-integrin-tetraspanin complex in fetal and postnatal testicular cords. Birth Defects Research Part C: Embryo Today: Reviews **75** 130-141.

Vigueras-Villaseñor RM, Moreno-Mendoza NA, Reyes-Torres G, Molina-Ortiz D, León MC & Rojas-Castañeda JC 2006 The effect of estrogen on testicular gonocyte maturation. Reproductive Toxicology **22** 513-520.

Weibel ER 1963 Principles and methods for the morphometric study of the lung and other organs. Laboratory Investigation **12** 131-155.

Williams K, McKinnell C, Saunders PT, Walker M, Fisher JS, Turner KJ, Atanassova N & Sharpe M 2001 Neonatal exposure to potent and environmental oestrogens and abnormalities of the male reproductive system in the rat: evidence for importance of the androgen-oestrogen balance and assessment of the relevance to man. Human Reproduction Update **7** 236-247.

Zogbi C, Tesser RB, Encinas G, Miraglia SM & Stumpp T 2012 Gonocyte development in rats: proliferation, distribution and death revisited. Histochemistry Cell Biology **138** 305-322.

Table 1. Body weight, testis weight and gonadosomatic index (GSI) of control (C) or maternal obesity rats (MO) at 0.5, 4.5, 7.5 and 14.5 days postpartum (dpp). Values are mean and standard error. Statistical differences between groups C and MO are indicated by: *$p \leq 0.05$.

Age (dpp)	Groups (C e MO)	Body weight (g)	Testis wieght (mg)	GSI
0.5	C	7.33 ± 0.12	2.62 ± 0.27	0.29 ± 0.03
	MO	6.51 ± 0.28 *	2.41 ± 0.28	0.26 ± 0.04
4.5	C	12.7 ± 0.75	6.85 ± 0.41	0.77 ± 0.06
	MO	11.7 ± 0.55	5.6 ± 0.36 *	0.6 ± 0.04 *
7.5	C	16.44 ± 1.02	12.06 ± 0.50	1.26 ± 0.07
	MO	16.92 ± 0.66	12.68 ± 0.46	1.33 ± 0.06
14.5	C	26.63 ± 0.70	39.57 ± 1.76	4.13 ± 0.29
	MO	26.93 ± 0.88	37.28 ± 1.59	3.92 ± 0.22

Table 2. Relative proportion of seminiferous tubules (ST) and interstitial tissue (IT) in testis of control (C) or maternal obesity rats (GO) at 0.5, 4.5 and 7.5 days postpartum (dpp). Values are mean and standard deviation.

Age (dpp)	Groups (C e GO)	ST (%)	IT (%)
0.5	C	44.34 ± 1.7	55,65 ± 1.7
	MO	43.31 ± 2.17	56,69 ± 2.17
4.5	C	51.81 ± 9.02	48,19 ± 9.02
	MO	54.34 ± 2.44	45,65 ± 2.44
7.5	C	62.83 ± 2.55	37,17 ± 2.55
	MO	60.17 ± 2.19	39,82 ± 2.19

Figure legends

Figure 1. (A, B) Semi-thin sections, stained with toluidine blue, of testes from control (Control) or maternal (MO) obesity rats. (C-F) Histological sections of testes of rats born from control (Control) or obese mothers (MO) subjected to immunocytochemistry for anti-Müllerian hormone (AMH). The sections were counterstained with hematoxylin. The age of the animal is shown on the left. The negative control of reaction is shown in (G). Legend: G - gonocytes; arrowheads - nuclei of Sertoli cells; arrows - repositioned gonocytes. Bars = 10 μm (A, B) and 20 μm (C-G). (H) Repositioned gonocyte density in testes of control (Control) or maternal obesity (MO) rats. (I) Density number of gonocytes (Nv) in Control and MO groups. We observed a linear decrease in the numerical density of gonocytes from 0.5 to 7.5 dpp. (J) Testicular volume of neonatal rats subjected to maternal obesity (MO), compared with controls. The values are expressed per mm^3 of seminiferous tubules. * $p \leq 0.05$.

Figure 2. (A) Density of mitotic figures in testes of control (Control) and maternal obesity (MO) rats at 0.5 dpp, 4.5 and 7.5 dpp. There were no statistically significant differences. (B-F) Histological sections of testes of Control or MO groups subjected to double immunocytochemical reaction for proliferating cell nuclear antigen (PCNA) and VASA protein. The age of the animal is shown on the left. The sections were counterstained with hematoxylin. Legend: G - gonocytes; S - labeling nuclei of Sertoli cells; arrowhead - unlabeled nuclei of Sertoli cell; arrows – labeling nuclei of gonocytes. Bars = 10 μm.

Figure 3. (A) Density of apoptotic cells/bodies in the seminiferous epithelium expressed by the total area of the testis. We observed a linear increase of apoptotic cells from 4.5 to 14.5 dpp. There were no significant differences between the control group (Control) and those subjected to maternal obesity (MO). (B-E) Histological sections of testes of Control or MO groups subjected to immunocytochemistry for activated caspase 3. The age of the animal is shown on the left. The sections were counterstained with hematoxylin. Note the diffuse labeling for activated caspase 3 in the cytoplasm of the gonocytes in 0.5 dpp rats (B, C). (F) Negative control. Legend: G - gonocytes; arrowheads - cells or apoptotic bodies. Bars = 20 μm.

Figure 4. Histological sections of rat testes at 4.5 dpp of control (Control) or maternal obesity (MO) groups subjected to immunocytochemistry for androgen receptor (AR) (A-C), estrogen receptor type B (ERB) (E-G) and estrogen receptor type A (ERA) (H, I). The sections were counterstained with hematoxylin. (D, J) Negative controls. Legend: F - unlabeled nuclei of fetal Leydig cells; G - gonocytes; R - relocated gonocyte; arrowheads - labeling nuclei of Sertoli cells; arrows - aggregates of fetal Leydig cells. Bars = 10 μm (B-D, G-J) and 20 μm (A, E, F, K).

Figure 5. Testicular maturation degree analysis of control (Control) or maternal obesity (MO) groups at 14.5 dpp. (A-F) Seminiferous cords in different stages of maturation. (G) Percentage of seminiferous cords in each phase of the cycle, from A spermatogonia to spermatocyte in zygotene, at 14.5 dpp. There were no statistical differences between the control group (Control) and the maternal obesity group (MO). Legend: A = A spermatogonia; B = B spermatogonia; L = leptotene primary spermatocytes; pL = pre-

leptotene primary spermatocytes; arrowheads = zygotene primary spermatocytes. Bars = 20 μm.

Figure 6. Testosterone (A) and estrogen (B) plasmatic levels of control (Control) or maternal obesity (MO) rats, from 0.5 to 14.5 dpp. There were no statistically significant differences.

Figure 1

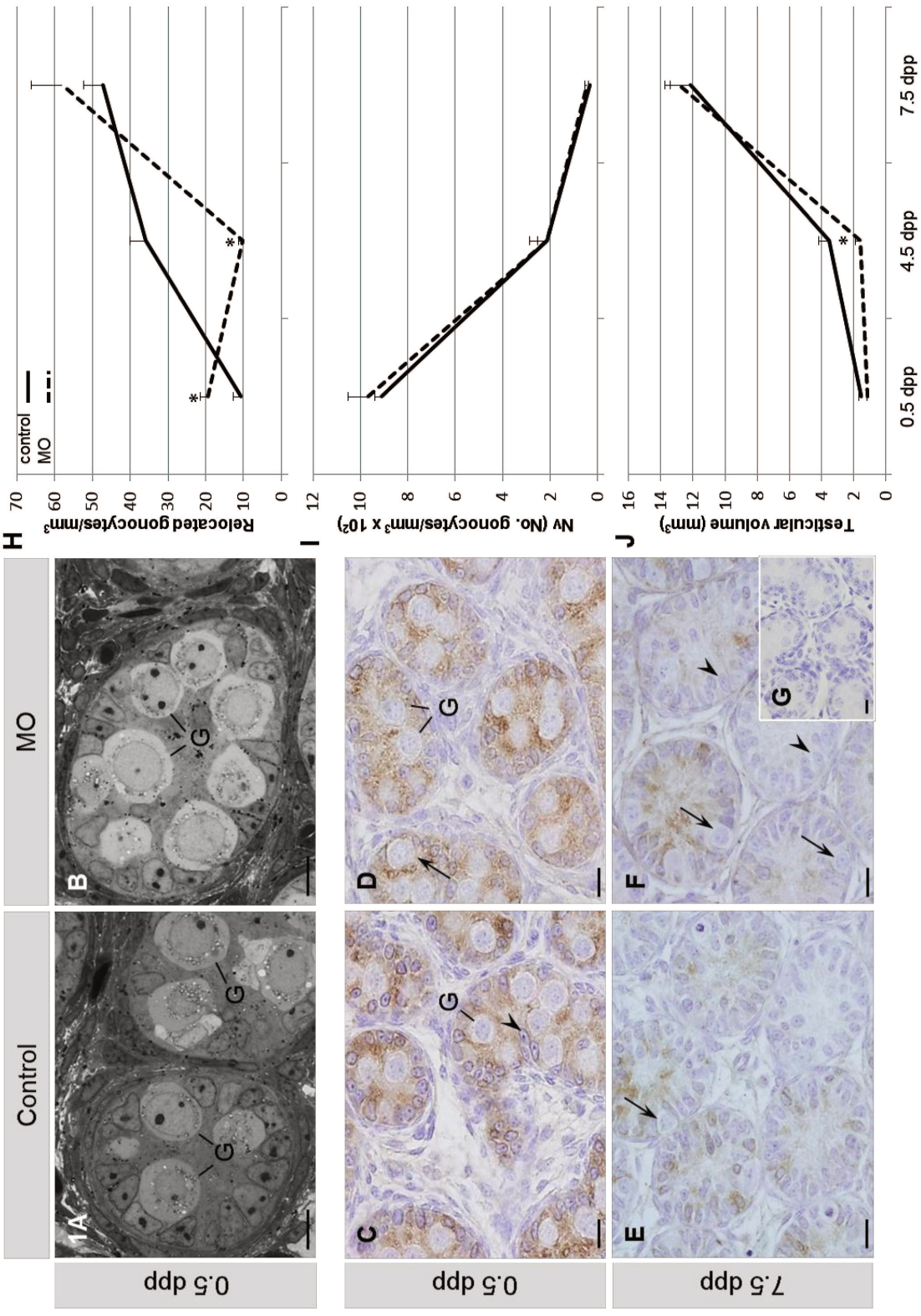

Control
MO
0.5 dpp
0.5 dpp
7.5 dpp
A
B
C
D
E
F
G
H
I
J
control
MO
Relocated gonocytes/mm3
Nv (No. gonocytes/mm3 x 10²)
Testicular volume (mm3)
0.5 dpp
4.5 dpp
7.5 dpp

Figure 2

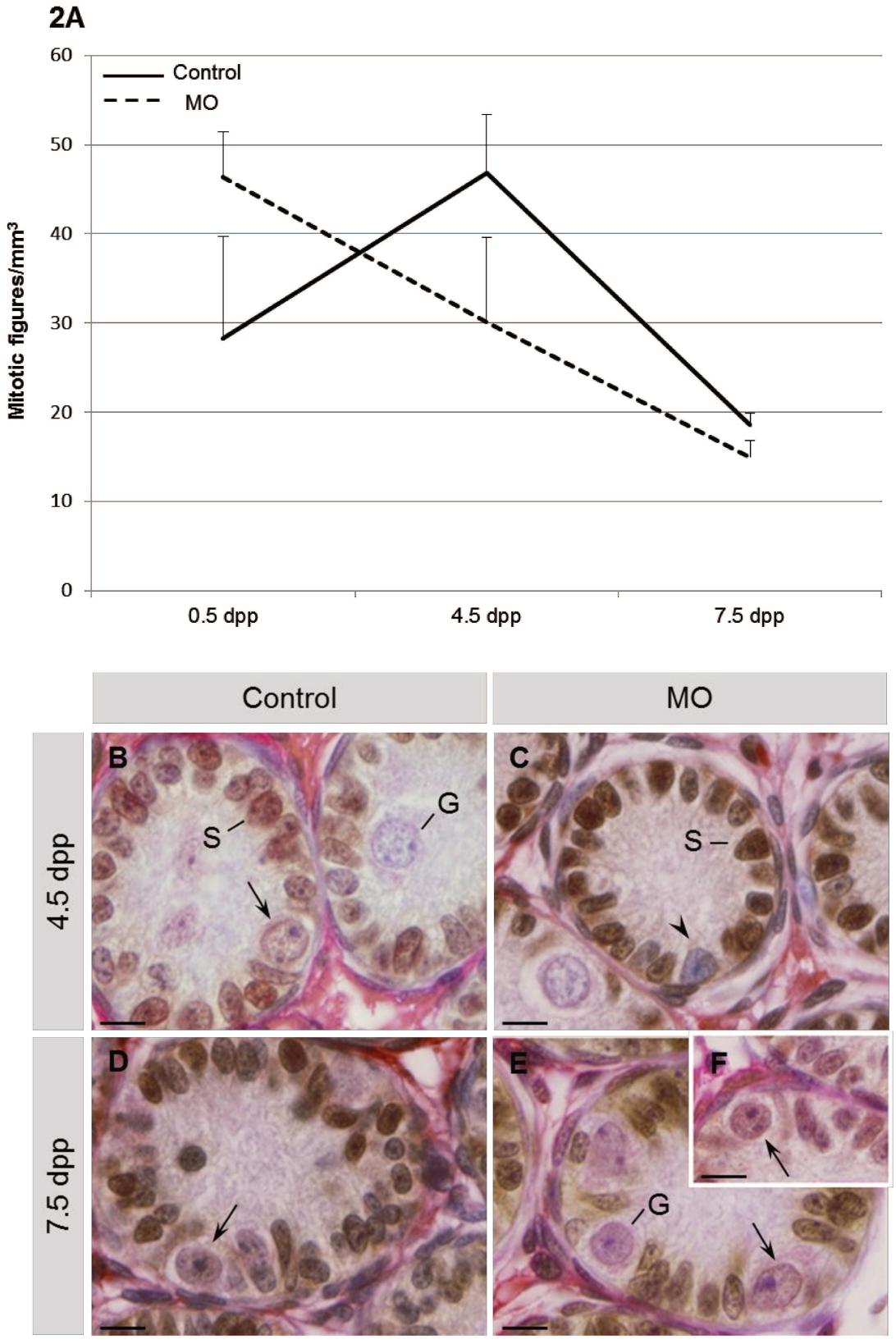

2A
Control
MO
Mitotic figures/mm³
60
50
40
30
20
10
0
0.5 dpp
4.5 dpp
7.5 dpp
Control
MO
4.5 dpp
7.5 dpp
B
C
D
E
F
S
G

Figure 3

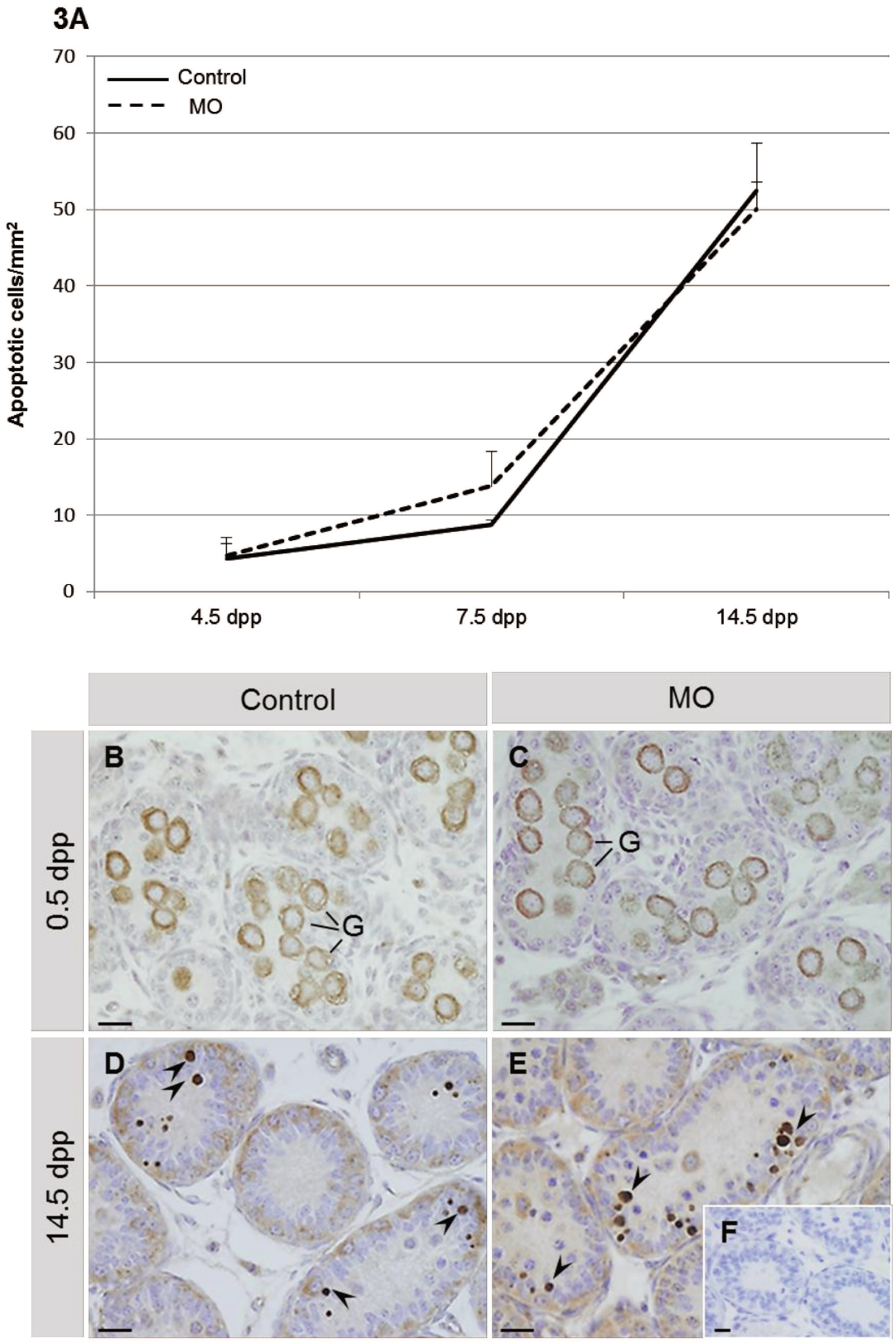

3A
70
60
50
40
30
20
10
0
Control
MO
Apoptotic cells/mm²
4.5 dpp
7.5 dpp
14.5 dpp
Control
MO
0.5 dpp
14.5 dpp
B
C
D
E
F
G
G

Figure 4

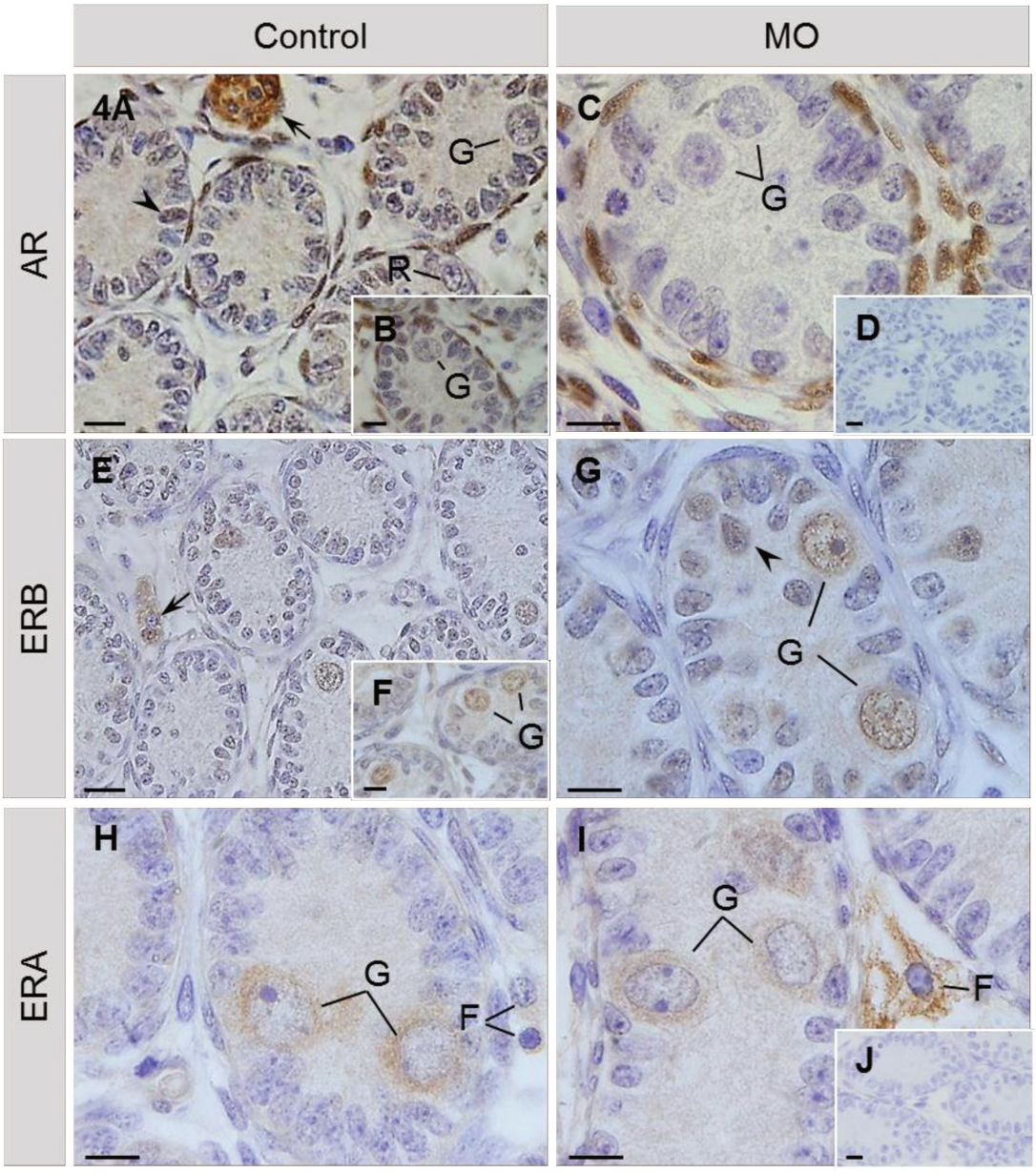


Figure 5

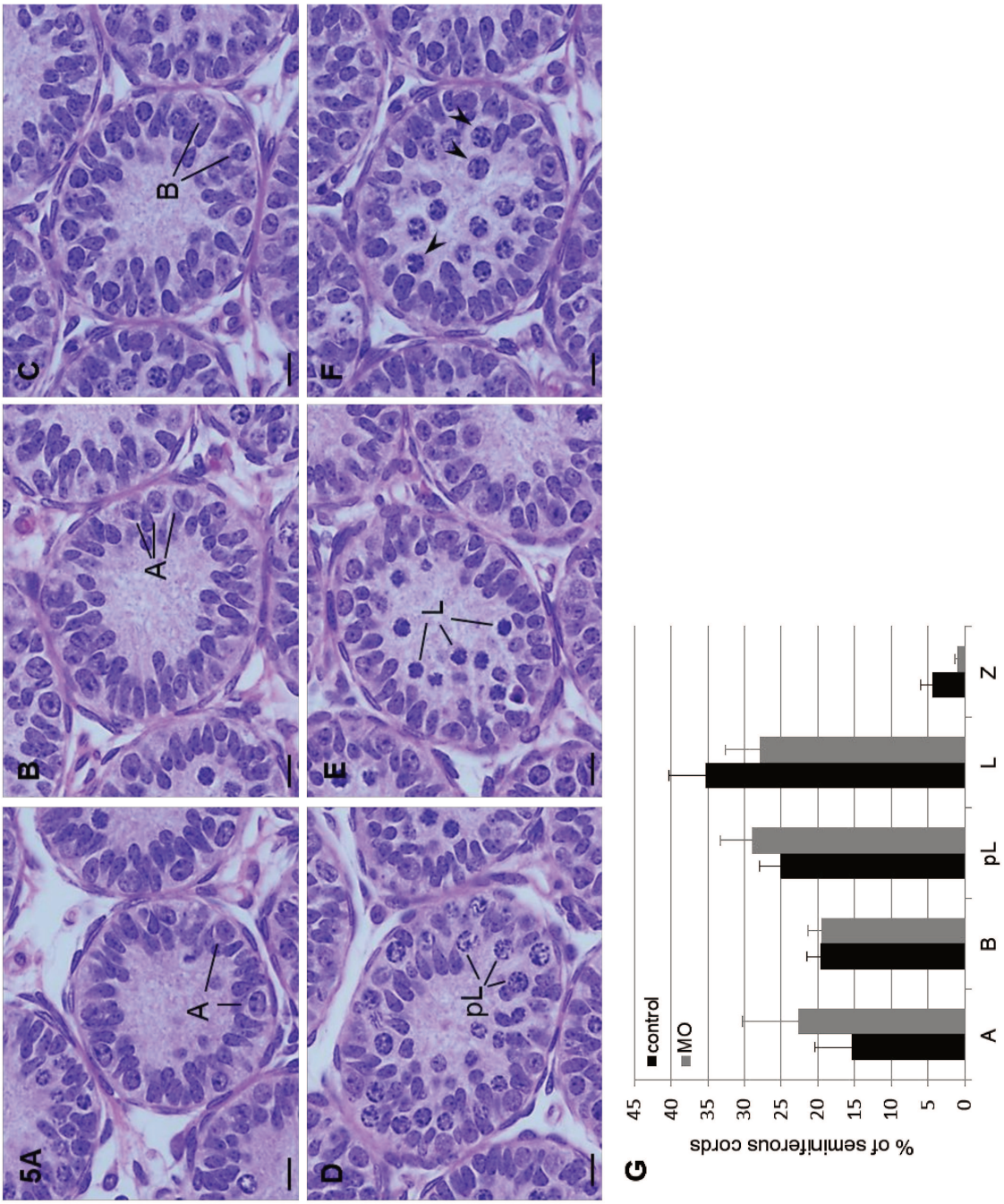
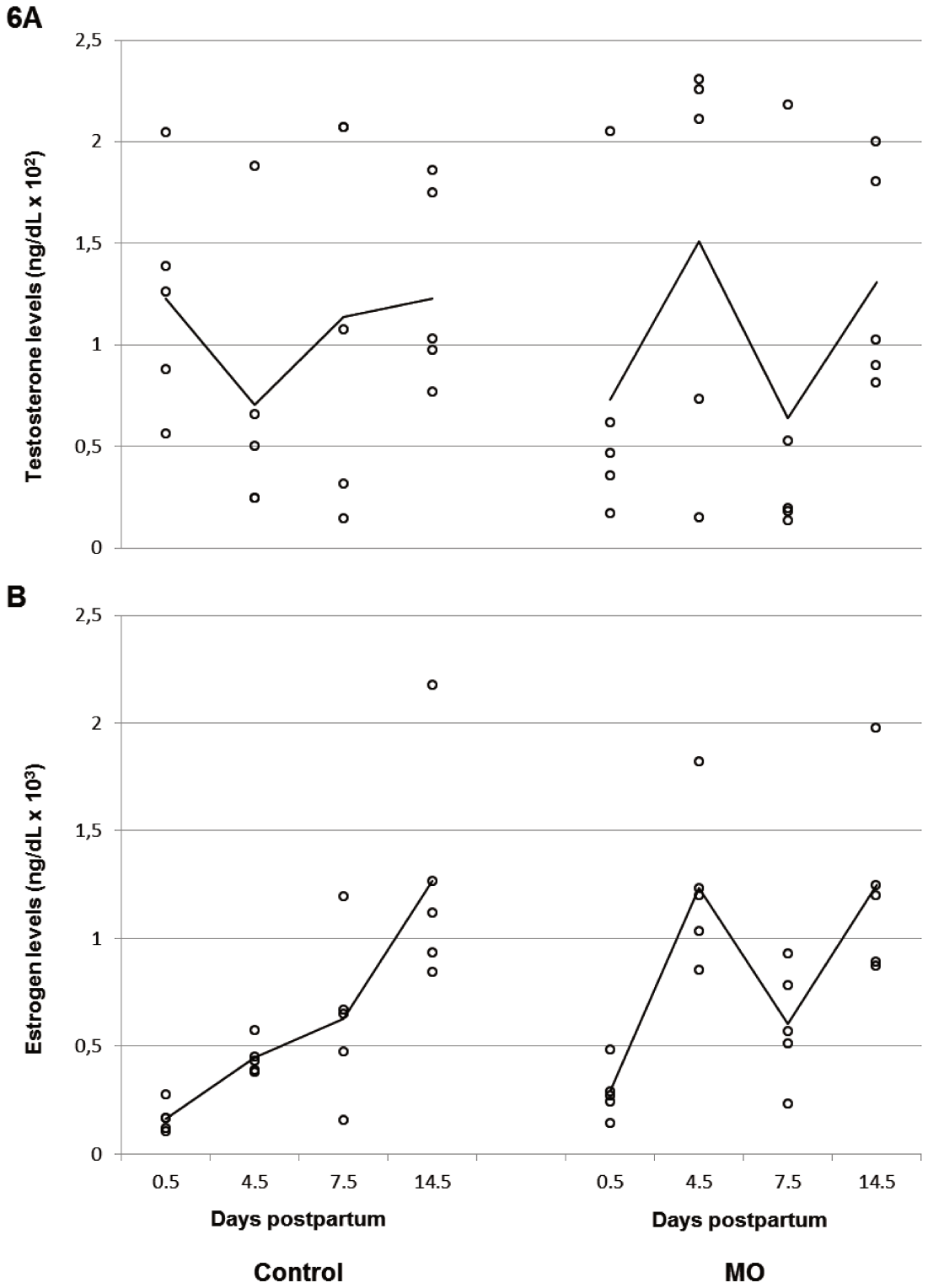

5A
A
B
A
C
B
D
pL
E
L
F
G
control
MO
% of seminiferous cords
0
5
10
15
20
25
30
35
40
45
A
B
pL
L
Z

Figure 6


6A
2,5
2
1,5
1
0,5
0
Testosterone levels (ng/dL x 10²)
B
2,5
2
1,5
1
0,5
0
Estrogen levels (ng/dL x 10³)
0.5
4.5
7.5
14.5
Days postpartum
Control
0.5
4.5
7.5
14.5
Days postpartum
MO

VI. CONSIDERAÇÕES E CONCLUSÕES GERAIS

- A retomada da atividade proliferativa dos gonócitos, em testículos de ratos neonatos, está relacionada à migração dessas células do centro para a base dos túbulos seminíferos, como descrito na literatura.

- Os achados referentes às células/corpos apoptóticos revelaram que os fatores que regulam o processo de morte das células germinativas, nas duas primeiras semanas de vida neonatal, não são afetados pela obesidade materna.

- A obesidade materna, associada ao consumo de lipídeos saturados, interfere no comportamento migratório e nos processos de relocação dos gonócitos, nos primeiros dias de vida neonatal, provavelmente devido aos desequilíbrios nos níveis de esteróides.

- As alterações que ocorrem na primeira semana de vida (principalmente na idade de 4,5 dpp) são normalizadas a partir de 7,5 dpp, não resultando em prejuízos para as células germinativas e para a função testicular, ao final da primeira semana de vida pós-natal.

VII. REFERÊNCIAS BIBLIOGÁFICAS

ABBOTT, D. H.; TARANTAL, A. F.; DUMESIC, D. A. Fetal, infant, adolescent and adult phenotypes of polycystic ovary syndrome in prenatally androgenized female rhesus monkeys. **American Journal of Primatology**, v. 71, p. 776-784, 2009.

ALMSTRUP, K. et al. Improved gene expression signature of testicular carcinoma *in situ*. **International Journal of Andrology**, v. 30(4), p. 292-303, 2007.

ANWAY, M. D. et al. Epigenetic transgenerational actions of endocrine disruptors and male fertility. **Science**, v. 308, p. 1466-1469, 2005.

BAILLIE, A. H. The histochemistry and ultrastructure of the gonocyte. **Journal of Anatomy**, v. 98, p. 641-645, 1964.

BENKALFAT, N. B. et al. Altered adipose tissue metabolism in offspring of dietary obese rat dams. **Clinical Sciences**, v. 121(1), p. 19-28, 2011.

BONEY, C. M. et al. Metabolic syndrome in childhood: association with birth weight, maternal obesity and gestational diabetes mellitus. **Pediatrics**, v. 115, p. 290-296, 2005.

BOULOGNE, B. et al. Apoptosis and mitosis in gonocytes of the rat testis during foetal and neonatal development. **International Journal of Andrology**, v. 22, p. 356-365, 1999.

CARA, J. F.; ROSENFIELD, R. L. Insulin-like growth factor I and insulin potentiate luteinizing hormone-induced androgen synthesis by rat ovarian thecal-interstitial cells. **Endocrinology**, v. 123, p. 733-739, 1988.

CASCIO, G.; SCHIERA, G.; LIEGRO, I. Dietary fatty acids in metabolic syndrome, diabetes and cardiovascular diseases. **Current Diabetes Reviews**, v. 8, p. 2-17, 2012.

CATALANO, P. M.; EHRENBERG, H. M. The short- and long-term implications of maternal obesity on the mother and her offspring, **British Journal of Obstetrics and Gynaecology**, v. 113(10), p. 1126-1133, 2006.

CATALANO, P. M. et al. Fetuses of obese mothers develop insulin resistance in utero. **Diabets Care**, p. 1076-1080, v. 32(6), p. 1076-1080, 2009.

CLARK, J. M.; EDDY, E. M. Fine structural observations on the origin and associations of primordial germ cells of the mouse. **Current Opinion in Endocrinology, Diabetes and Obesity**, v. 47, p. 136-155, 1975.

CLERMONT, Y.; PEREY, B. Quantitative study of the cell population of the seminiferous tubules in immature rats. **American Journal of Anatomy**, v. 100, p. 241-260, 1957.

CONNOR, K. L. et al. Nature, nurture or nutrition? Impact of maternal nutrition on maternal care, offspring development and reproductive function. **The Journal of Physiology**, v. 590, p. 2167-2180, 2012.

CUPP, A. S. et al. Effect of transient embryonic in vivo exposure to the endocrine disruptor methoxychlor on embryonic and postnatal testis development. **Journal of Andrology**, v. 24, p. 736-745, 2003.

CULTY, M. Gonocytes, the forgotten cell of the germ cell lineage. **Birth Defects Research**, v. 87, p. 1-26, 2009.

DABELEA, D. et al. Intrauterine exposure to obesity conveys risks for type 2 diabetes and obesity: a study of discordant sib ships. **Diabetes**, v. 49, p. 2208-2211, 2000.

de MIGUEL, M. P. et al. Leukemia inhibitory factor and ciliary neutropic factor promote the survival of Sertoli cells and gonocytes in a coculture system. **Endocrinoly**, v. 137, p. 1885-1893, 1997.

de ROOIJ, D. G. Regulation of proliferation of spermatogonial stem cells. **Journal of Cell Science**, v. 10, p. 181-194, 1988.

de ROOIJ, D. G. Stem cells in the testis. **International Journal of Experimental Pathology**, v. 79, p. 67-80, 1998.

DEJOSEZ, M. et al. Ronin is essential for embryogenesis and the pluripotency of mouse embryonic stem cells. **Cell**, v. 133, p. 1162-1174, 2008.

DELBÈS, G. et al. Estrogen receptor β-mediated inhibition of male germ cell line development in mice by endogenous estrogens during perinatal life. **Endocrinilogy**, v. 145, p. 3395-3403, 2004.

DELBÈS, G. et al. Developmental Changes in Testicular Sensitivity to Estrogens throughout Fetal and Neonatal Life. **Toxicological Sciences**, v. 99, p. 234-243, 2007.

FISHER, J; et al. Immunolocalisation of oestrogen receptor-alpha (ER-α) within the testis and excurrent ducts of the rat and marmoset monkey from perinatal life to adulthood. **Journal of Endocrinology**, v. 153, p. 485-495, 1997.

FISHER, J. S. Environmental anti-androgens and male reproductive health: focus on phthalates and testicular dysgenesis syndrome. **Reproduction**, v. 127, p. 305-315, 2004.

FORSDIKE, R. A. et al. Disordered follicle development in ovaries of prenatally androgenized ewes. **Journal of Endocrinology**, v. 192, p. 421-428, 2007.

FUJITA, J. et al. Caspase activity mediates the differentiation of embryonic stem cells. **Cell Stem Cell**, v. 2, p. 595-601, 2008.

GUNDERSEN, H. J. G. et al. Some news, simple and efficient stereological methods and their use in pathological research and diagnosis. **Acta Pathologica, Microbiologica et Immunologica Scandinavica**, v. 96, p. 379-394, 1988.

HAMMOUD, A. O. et al. Obesity and male reproductive potential. **Journal of Andrology**, v. 27(5), p. 619-626, 2006.

HAJER, G. R.; van HAEFTEN, T. W.; VISSEREN, F. L. Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. **European Heart Journal**, v. 29, p. 2959-2971, 2008.

HARTZ, A. J. et al. The association of obesity with infertility and related menstrual abnormalities in women. **International Journal of Obesity and Related Metabolic Disorders**, v. 3, p. 57-77, 1979.

IKENASIO-THORPE, B. A. et al. Prenatal influences on susceptibility to diet-induced obesity are mediated by altered neuroendocrine gene expression. **Journal of Endocrinology**, v. 193(1), p. 31-37, 2007.

JAMES, W. P. T.; RIGBY, N.; LEACH, R. The obesity epidemic, metabolic syndrome and future preventive strategies. **European Journal of Cardiovascular Prevention and Rehabilitation**, v. 11, p. 3-8, 2004.

KHASHAN, A. S.; KENNY, L. C. The effects of maternal body mass index on pregnancy outcome. **European Journal of Epidemiology**, v. 24, p. 697-705, 2009.

KING, J. C. Maternal obesity, metabolism and pregnancy outcomes. **Annual Review of Nutrition**, v. 26, p. 271-291, 2006.

KIRSCHNER, M. A. et al. Obesity, androgens, estrogens, and cancer risk. **Cancer Research**, v. 42, p. 3281-3285, 1982.

KLUIN, P. M.; de ROOIJ, D. G. A comparison between the morphology and cell kinetics of gonocytes and adult type undifferentiated spermatogonia in the mouse. **International Journal of Andrology**, v. 4, p. 475-493, 1981.

LEHRAIKI, A. et al. Antagonistic effects of gestational dietary exposure to low-dose vinclozolin and genistein on rat fetal germ cell development. **Reproductive Toxicology**, v. 31(4), p. 424-430, 2011.

LAKE, J. K.; POWER, C.; COLE, T. J. Women's reproductive health - the role of body mass index in early and adult life. **International Journal of Obesity and Related Metabolic Disorders**, v. 21, p. 432-438, 1997.

LOOS, R. J. Recent progress in the genetics of common obesity. **British Journal of Clinical Pharmacology**, v. 68, p. 811-829, 2009.

MAGRE, S.; JOST, A. The initial phases of testicular organogenesis in the rat. An electron microscopy study. **Archives D'anatomie Microscopique et de Morphologie Expérimentale**, v. 69, p. 297-318, 1980.

MAGRE, S.; JOST, A. Sertoli cells and testicular differentiation in the rat fetus. **Journal of Electron Microscopy Technique**, v. 19, p. 172-188, 1991.

MATSUI, Y.; ZSEBO, K.; HOGAN, B.L.M. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. **Cell**, v. 70, p. 841-847, 1992.

McGUINNESS, P.; ORTH, J. M. Gonocytes of male rats resume migratory activity postnatally. **European Journal of Cell Biology**, v. 59, p. 196-210, 1992a.

McGUINNESS, P.; ORTH, J. M. Reiniciation of gonocyte mitosis and movement of gonocytes to the basement membrane in testes of newborn rats in vivo and in vitro. **The Anatomical Record**, v. 233, p. 527-537, 1992b.

MERLET, J. et al. Male fetal germ cells are targets for androgens that physiologically inhibit their proliferation. **Proceedings of the National Academy of Sciences of the United States of America**, v. 104, p. 3615-3620, 2007.

MIETHING, A. Germ cell death during prespermatogenesis in the testis of the goldem hamster. **Cell and Tissue Research**, v. 267, p. 583-590, 1992.

NESTLER, J. E. et al. Insulin stimulates testosterone biosynthesis by human thecal cells from women with polycystic ovary syndrome by activating its own receptor and using inositolglycan mediators as the signal transduction system. **Journal of Clinical Endocrinology and Metabolism**, v. 83, p. 2001-2005, 1998.

NISTALA, R. et al. Prenatal Programming and epigenetics in the genesis of the cardiorenal syndrome. **Cardiorenal Medicine**, v. 1(4), p. 243-254, 2011.

OHBO, K. et al. Identification and characterization of stem cells in prepubertal spermatogenesis in mice. **Developmental Biology**, v. 258, p. 209-225, 2003.

OHMURA, M. et al. Spatial analysis of germ stem cell development in Oct-4/EGFP transgenic mice. **Archives of Histology and Cytology**, v. 67, p. 285-296, 2004.

ORTH, J. M. Proliferation of Sertoli cells in fetal and postnatal rats: a quantitative autoradiographic study. **The Anatomical Record**, v. 203, p. 485-492, 1982.

ORWIG, K. E. et al. Male germ-line stem cell potential is predicted by morphology of cells in neonatal rat testis. **Proceedings of the National Academy of Sciences of the United States**, v. 99(18), p. 11706-11711, 2002.

PASQUALI, R. Obesity, fat distribution and infertility. **Maturitas**, v. 54, p. 363-371, 2006.

PASQUALI, R.; PATTON, L.; GAMBINERI, A. Obesity and infertility. **Current Opinion in Endocrinology, Diabetes and Obesity**, v. 14, p. 482-487, 2007.

PINTO, M. E. et al. Neonatal gonocyte differentiation in mongolian gerbil *Meriones unguiculatus* involves asynchronous maturation of seminiferous cords and rapid formation of transitional cell stage. **The Anatomical Record**, v. 293, p. 310-319, 2010.

PRÉPIN, J.; Le VIGOUROUX, P.; DADOUNE, J. P. Effects of thymulin on in vivo incorporation of 3H-thymidine into gonocytes of newborn rat testes. **Reproduction Nutrition Development**, v. 34, p. 289-294, 1994.

RESNICK, J. L. et al. Long-term proliferation of mouse primordial germ cell in culture. **Nature**, v. 359, p. 550-551, 1992.

RIBEIRO, D. L. et al. High- fat diet-obesity associated with insulin resistance increases cell proliferation, estrogen receptor and PI3K proteins in rat ventral prostate. **Journal of Andrology**, v. 33(5), p. 854-865, 2012.

ROOSEN-RUNGE, E. C.; LEIK, J. Gonocyte degeneration in postnatal male rat. **American Journal of Anatomy**, v. 122, p. 275-300, 1968.

ROUILLER-FABRE, V., et al. Development of the foetal and neonatal testis. **Andrologia**, v. 35, p. 79-83, 2003.

SANTAMARTA, J. A ameaça dos disruptores endócrinos. **Agroecologia e Desenvolvimento Rural Sustentável,** v. 2(3), 2001.

SAUNDERS, P. T. K., et al. Expression of oestrogen receptor beta (ERβ) occurs in multiple cell types, including some germ cells, in the rat testis. **Journal of Endocrinology,** v. 156, p. 13-17, 1998.

SEWELL, M. F. et al. Increased neonatal fat mass and not lean body mass is associated with maternal obesity. **American Journal of Obstetrics and Gynecology**, v. 195, p. 1100-1103, 2006.

SHARPE, R. M. e SKAKKEBAEK, N. E. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? **Lancet**, v. 341, p. 1392-1395, 1993.

SKAKKEBAEK, N. E.; RAJPERT-De MEYTS, E.; MAIN, K. M. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects: Opinion. **Human Reproduction**, v. 16, p. 972-978, 2001.

SULLIVAN, E. L. et al. Chronic consumption of a high-fat diet during pregnancy causes perturbations in the serotonergic system and increased anxiety-like behavior in nonhuman primate offspring. **The Journal of Neuroscience**, v. 30(10), p. 3826-3830, 2010.

STECKLER, T. L. et al. Developmental programming: excess weight gain amplifies the effects of prenatal testosterone excess on reproductive cyclicity-implication for polycystic ovary syndrome. **Endocrinology**, v. 150, p. 1456-1465, 2009.

SULLIVAN, E. L.; SMITH, M. S.; GROVE, K. L. Perinatal expose to high-fat diet programs energy balance, metabolism and behavior in adulthood. **Neuroendocrinology**, v. 93, p. 1-8, 2011.

SYMONDS, M. E.; BUDGE, H. Nutritional models of the developmental programming of adult health and disease. **Proceedings of Nutrition Society**, v. 68, p. 173-178, 2009.

TRES, L. L.; KIERSZENBAUM, A. L. The ADAM-integrin-tetraspanin complex in fetal and postnatal testicular cords. **Birth Defects Research Part C: Embryo Today: Reviews**, v. 75(2), p. 130-141, 2005.

UZUMCO, M.; SUZUKI, H.; SKINNER M. K. Effect of the anti-androgenic endocrine disruptor vinclozolin on embryonic testis cord formation and postnatal testis development and function. **Reproductine Toxicology***,* v. 18, p. 765-774, 2004.

van DISSEL-EMILIANI, F. M. F. et al. Survival and proliferation of rat gonocytes in vitro. **Cell and Tissue Research**, v. 273, p. 141-147, 1993.

VIGUERAS-VILLASEÑOR, R. M. et al. The effect of estrogen on testicular gonocyte maturation. **Reproductive Toxicology**. V. 22(3), p. 513-520, 2006.

WEIBEL, E. R. Principles and methods for the morphometric study of the lung and other organs. **Laboratory Investigation**, v. 12, p. 131-155, 1963.

WHITE, C. L.; PURPERA, M. N.; MORRISON, C. D. Maternal obesity is necessary for programming effect of high-fat diet on offspring. **American Journal of Physiology Regulatory, Integrative and Comparative Physiology**, v. 296, p. 1464-1472, 2009.

WILLIAMS, K. et al. Neonatal exposure to potent and environmental oestrogens and abnormalities of the male reproductive system in the rat: evidence for importance of the androgen-oestrogen balance and assessment of the relevance to man. **Human Reproduction Update**, v. 7(3), p. 236-247, 2001.

YOSHIDA, S. et al. The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage. **Development**, v. 133, p. 1495-1505, 2006.

ZOGBI, C. et al. Gonocyte development in rats: proliferation, distribution and death revisited. **Histochemistry Cell Biology**, v. 138, p. 305-322, 2012.

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha Dissertação de Mestrado intitulada "INFLUÊNCIA DA OBESIDADE MATERNA NA DIFERENCIAÇÃO NEONATAL DAS CÉLULAS GERMINATIVAS MASCULINAS":

( ) não se enquadra no § 3º do Artigo 1º da Informação CCPG 01/08, referente a bioética e biossegurança.

Tem autorização da(s) seguinte(s) Comissão(ões):

( ) CIBio - Comissão Interna de Biossegurança , projeto nº _____, Instituição: _____.

(x) CEUA - Comissão de Ética no Uso de Animais , projeto nº 022/09 , Instituição: Instituto de Biociências. Letras e Ciências Exatas de São José do Rio Preto.

( ) CEP - Comissão de Ética em Pesquisa, protocolo nº _____, Instituição: _____.

** Caso a Comissão seja externa ao IB/UNICAMP, anexar o comprovante de autorização dada ao trabalho. Se a autorização não tiver sido dada diretamente ao trabalho de tese ou dissertação, deverá ser anexado também um comprovante do vínculo do trabalho do aluno com o que constar no documento de autorização apresentado.*

Aluno(a): (nome completo)

Orientador(a): (nome completo)

Para uso da Comissão ou Comitê pertinente:
(X) Deferido ( ) Indeferido

Carimbo e assinatura

Profa. Dra. ANA MARIA APARECIDA GUARALDO
Presidente da CEUA/UNICAMP

Para uso da Comissão ou Comitê pertinente:
( ) Deferido ( ) Indeferido

Carimbo e assinatura

unesp UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de São José do Rio Preto

COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL IBILCE

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado ***"Efeitos da Obesidae materna e supernutrição pós-natal sobre o aparelho genital masculino de ratos: possíveis mecanismos envolvidos nas alterações testiculares e prostáticas"*** e subprojetos relacionados (protocolo nº. 022/09 CEEA), sob responsabilidade da Profa. Dra. Rejane Maira Góes, estão de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética em Experimentação Animal, em reunião de 10/12/2009.

CERTIFICATE

UNESP / IBILCE Ethical Committee for Animal Research (CEEA) hereby certify that the scientific investigation entitled ***"Effects of Obesidae maternal and postnatal overnutrition on the male genital tract of rats: possible mechanisms involved in prostate and testicular changes"*** and related projects (protocol nº. 022/09 CEEA), on Rejane Maira Góes responsibility, is in accordance with Ethical Principles in Animal Research adopted by Brazilian College of Animal Experimentation (COBEA) and it was approved by the Committee of this Institute, on december 10th, 2009.

São José do Rio Preto, 10 de dezembro de 2009.

Profa. Dra. Rejane Maira Góes
Presidente da CEEA

Prof. Dr. Luiz Florindo
Vice-presidente da CEUA

Instituto de Biociências, Letras e Ciências Exatas – SAEPE
Rua Cristóvão Colombo, 2265 CEP 15054-000 São José do Rio Preto – SP - Brasil
Tel 17 221 2456 fax 17 221 2390
E-mail: bitonti@ibilce.unesp.br e ceea@ibilce.unesp.br