ANA CAROLINA NEGRIN

"EFEITOS COMBINADOS DA EXPOSIÇÃO AO DI-N-BUTIL FTALATO E À DIETA HIPERLIPÍDICA SOBRE A ESTRUTURA E FUNÇÃO TESTICULAR DE GERBILOS ADULTOS"

"COMBINED EFFECTS OF EXPOSURE TO DI-N-BUTYL PHTHALATE AND TO HIGH-FAT DIET ON TESTICULAR STRUCTURE AND FUNCTION OF ADULT GERBILS"

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Orientadora: Dra. Rejane Maira Góes

"COMBINED EFFECTS OF EXPOSURE TO DI-N-BUTYL PHTHALATE AND TO HIG-FAT DIET ON TESTICULAR STRUCTURE AND FUNCTION OF ADULT GERBILS"

Dissertação apresentada ao Programa de Pós-Graduação em Biologia Celular e Estrutural do Instituto de Biologia da Universidade Estadual de Campinas para obtenção do Título de Mestre(a) em Biologia Celular e Estrutural, na área de Biologia Celular.

Dissertation presented to the State University of Campinas in fulfillment of the requirements for the degree of Doctor on Cellular and Structural Biology Postgraduate Programme, in the Biology Institute, in the area of Cellular Biology.

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Endocrine disrupting chemicals (EDC), as di-n-butyl phthalate (DBP), can alter the scenary of steroid hormones or their action, impairing the testicular development and reproductive capacity in adulthood. Toxicological studies show that adult rats, when exposed to high doses of phthalates during sexual differentiation, exhibit several reproductive anomalies, such as agenesis of the epididymis and reduction in daily sperm production. Data show that exposures to 2mg/kg/day of DBP during gestation and lactation periods are enough to impair the development of the male germ cells. Furthermore, there are reports that EDC contribute to the increase of the adipogenesis by altering cell signaling of adipocytes and lipid metabolism. It is known that the male obesity can affect semen quality and increases infertility rates. Whereas that phthalates can accumulate in adipose tissue, we were interested in evaluating the effects of prolonged exposure to low doses of DBP and possible interferences of excess dietary fat for testicular function and spermatic parameters of adult gerbils. Adult female gerbils, fed high-fat (20% fat) or balanced diet (4% fat) for eight weeks, were mated with normal males. The male offspring was divided into control (C), di-n-butyl phthalate (Ph), high-fat diet (HF) and high-fat diet plus di-n-butyl phthalate (HFPh) groups. DBP (5 mg/kg/day) was administered in drinking water to pregnant and breastfeeding mothers and to offspring from weaning up to adulthood (14-week-old). Testis response was evaluated by means of microscopic and stereological analyses, sensitivity of its major cell populations to androgens and estrogens, steroidogenic capacity and spermatic efficiency. We also examined the effects on sperm reserves, sperm transit time through the epididymis and sperm motility. Alone, low doses of DBP resulted in obesity and dislipidemy at adulthood. No histological change was observed in testicular structure of these animals, but there was reduction in intratesticular estrogen synthtesis, resulting in a tendency to decrease in sperm production. The decrease in intratesticular estrogen after lifetime DBP exposure was accompanied by a ~70% increment in ERα content in the testis, that might be an adaptative response to low estrogen levels. Chronic intake of high-fat diet did not induce obesity in aldult gerbils, but led to a slight decrease in spermatic efficiency. This decrease was not associated with changes in testicular structure or steroidogenic

capacity, because serum or intratesticular testosterone and estrogen concentrations were not altered, but it can be linked to an unbalanced in androgen receptor signaling, since it was observed a decrease in AR content. The combined exposure to high-fat diet and to low doses of DBP acted synergical and negatively on intratesticular testosterone synthesis, impairing the spermatic efficiency and increasing the sperm transit time. The sperm motility was not changed in isolated or combined exposures. This study demonstrated that fat nutritional environment may adversely affect the response of testes to phthalates, and provide new informations for understanding the consequences of exposures to ECD in decreasing human sperm counts and fertility.

Key words: Di-n-butyl phthalate, high-fat diet, testes, sperm parameters, Mongolian gerbil.

Desreguladores endócrinos (DE), como o di-n-butil ftalato (DBP), podem alterar o panorama dos hormônios esteroides ou sua ação, comprometendo o desenvolvimento testicular e a capacidade reprodutiva na vida adulta. Estudos toxicológicos mostram que ratos adultos, quando expostos a altas doses de ftalatos durante a fase de diferenciação sexual, exibem diversas anomalias reprodutivas, como agenesia do epidídimo e redução na produção diária de espermatozoides. Dados mostram que exposições a 2mg/kg/dia de DBP durante a gestação e a lactação são suficientes para prejudicar o desenvolvimento das células germinativas. Além disso, há relatos de que DE contrinuem para o aumento da adipogênese por causar alterações na sinalização celular de adipócitos. É conhecido que a obesidade masculina pode afetar a qualidade do sêmen e aumenta as taxas de infertilidade. Considerando que os ftalatos podem se acumular no tecido adiposo, ficamos interessados em avaliar os efeitos da exposição prolongada a baixas doses de DBP a as possíveis interferências do excesso de lipídeos na dieta para a função testicular e os parâmetros espermáticos de gerbilos adultos. Gerbilos fêmeas adultas, alimentadas com dieta balanceada (4% de lipídeos) ou hiperlipídica (20% de lipídeos) por oito semanas, foram acasaladas com machos normais. A prole masculina foi dividida em grupos controle (C), di-n-butil ftalato (Ph), dieta hiperlipídica (HF) e hieta hiperlipídica mais di-n-butil ftalato (HFPh). DBP (5 mg/kg/dia) foi administrado na água de beber às mães durante a gravidez e lactação e aos filhotes do desmame até a idade adulta (14 semanas). A resposta do testículo foi avaliada por meio de análises microscópicas e esterológicas, da sensibilidade de suas principais populações celulares a andrógenos e estrógenos, e da produção espermática. Também foram examinados os efeitos sobre a reserva espermática, o tempo de trânsito dos espermatozoides pelo epidídimo e a motilidade espermática. Isoladamente, baixas doses de DBP resultaram em obesidade e dislipidemia nos animais adultos. Nenhuma alteração foi observada na estrutura testicular, mas a síntese de estrógeno pelo testículo foi reduzida, resultando em tendência a diminuição na eficiência espermática. A redução no estrógeno intratesticular, após exposição ao DBP, foi acompanhada de um aumento de ~70% na expressão de ERα nos testículos, que pode ser uma resposta

adaptativa aos baixos níveis deste hormônio. A ingestão crônica de dieta hiperlipídica não induziu os gerbilos adultos à obesidade, mas causou uma leve queda na eficiência espermática. Esta redução não está relacionada a alterações na estrutura testicular ou na sua capacidade esteroidogênica, mas podem estar ligadas ao comprometimento da sinalização testicular, já que foi verificada uma redução no conteúdo de AR. A exposição combinada à dieta hiperlipídica e a baixas doses de DBP atuaram sinérgica e negativamente na síntese de testosterona intratesticular, prejudicando a eficiência espermática e aumentando o tempo de trânsito dos espermatozoides pelo epidídimo. A motilidade espermática não sofreu alteração frente as exposições isoladas ou combinadas. Este estudo demonstrou que o ambiente nutricional pode interferir na resposta dos testículos frente aos ftalatos e proporciona novas informações para o entendimento das consequências da exposição aos DE para a redução na reverva espermática e fertlidade humana.

Palavras-chave: Di-n-butil ftalato, dieta hiperlipídica, testículo, parâmetros espermáticos, gerbilo da Mongólia.

" Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas Graças a Deus, não sou o que era antes"

Martin Luther King

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17β-HSD – Do inglês *17β-hydroxysteroid dehydrogenase*

AGD – Do inglês *Anogenital distance*

AR – Do inglês *Androgen receptor*

BBP – Do inglês *Butyl benzyl phthalate*

C – *Control group*

DBP – Do inglês *Di-n-butyl phthalate*

DE – Desreguladores endócrinos

DEHP – Do inglês *Di-(2-ethylhexyl) phthalate*

DEP- Do inglês *Di-ethyl phthalate*

DMP – Do inglês *Di-metyl phthalate*

DSP – Do inglês *Daily sperm production*

E_2 – Estradiol ou do inglês *Estradiol*

EDC – Do inglês *Endocrine disrupting chemicals*

ERα – Do inglês *Estrogen receptor α*

GD – Do inglês *Gestation days*

GSI – Do inglês *Gonadosomatic index*

HDL – Do inglês *High-density lipoprotein*

HF – *High-fat diet group*

HFPh – *High-fat diet plus di-n-butyl phthalate group*

IL-1β – Do inglês *Interleukin-1β*

LDL – Do inglês *Low-density lipoprotein*

LH – Do inglês *Luteinizing hormone*

LXR α – Do inglês *Liver X Receptor type α*

MBP – Do inglês *Monobutyl phthalate*

MEHP – Do inglês *Mono(ethylhexyl) phthalate*

NR – Do inglês *Nuclear receptors*

PDN – Do inglês *Postnatal days*

Ph – *Di-n-butyl phthalate group*

PPARγ – Do inglês *Peroxisome-Proliferator Activaded Receptor γ*

PVC – Do inglês *Polyvinyl chloride*

T – Testosterona ou do inglês *Testosterone*

TNF-α – Do inglês *Tumor necrosis factor-α*

Desreguladores endócrinos e di-n-butil ftalato (DBP)

A reprodução masculina envolve processos complexos e depende do desenvolvimento normal dos órgãos do aparelho genital durante a gestação, bem como de sua adequada maturação pós-natal (Jensen et al., 2000). O desenvolvimento testicular requer a proliferação e a diferenciação de três populações celulares principais: as células de Leydig, as células de Sertoli e as células germinativas (Orth, 1982; Boulogne et al., 1999; Mendi-Handagama and Ariyaratne, 2001), sendo este processo altamente influenciado pelos esteroides sexuais. A testosterona, por exemplo, rege uma série de transformações na gônada em desenvolvimento, como a proliferação das células de Sertoli, durante os períodos fetal e neonatal (Sharpe et al., 2003). Este hormônio também interfere na proliferação das células germinativas fetais ou gonócitos (Merlet et al., 2007). Os estrógenos, por sua vez, influenciam o desenvolvimento dos gonócitos e das células de Leydig (Delbès et al., 2004, 2007; Vigueras-Villasenõr et al., 2006). É amplamente conhecido que a exposição a substâncias que levam à desregulação nos hormônios sexuais, como os desreguladores endócrinos (DE) ou o estilo de vida, podem afetar a diferenciação dessas populações celulares e o desenvolvimento dos órgãos andrógeno-dependentes, comprometendo a capacidade reprodutiva (Wilson, 1978; Andrade et al., 2006; Christiansen et al., 2010; Sharpe, 2010). Como pode ser visto na Figura 1, após a diferenciação, as fases fetal/neonatal e puberal correspondem a etapas críticas do desenvolvimento testicular e distúrbios, causados por químicos ambientais ou pelo estilo de vida, em qualquer uma destas etapas podem alterar permanentemente os mecanismos homeostáticos do sistema endócrino e comprometer a espermatogênese e contagem espermática na vida adulta (IPCS, 2002; Scott et al., 2008).

De acordo com a *Environmental Protection Agency* (EPA, 1997), um desregulador endócrino é definido como "um agente exógeno que interfere na síntese, secreção, transporte, ligação, ação ou eliminação de hormônio natural do organismo, o qual é responsável pela manutenção, reprodução, desenvolvimento e/ou comportamento".

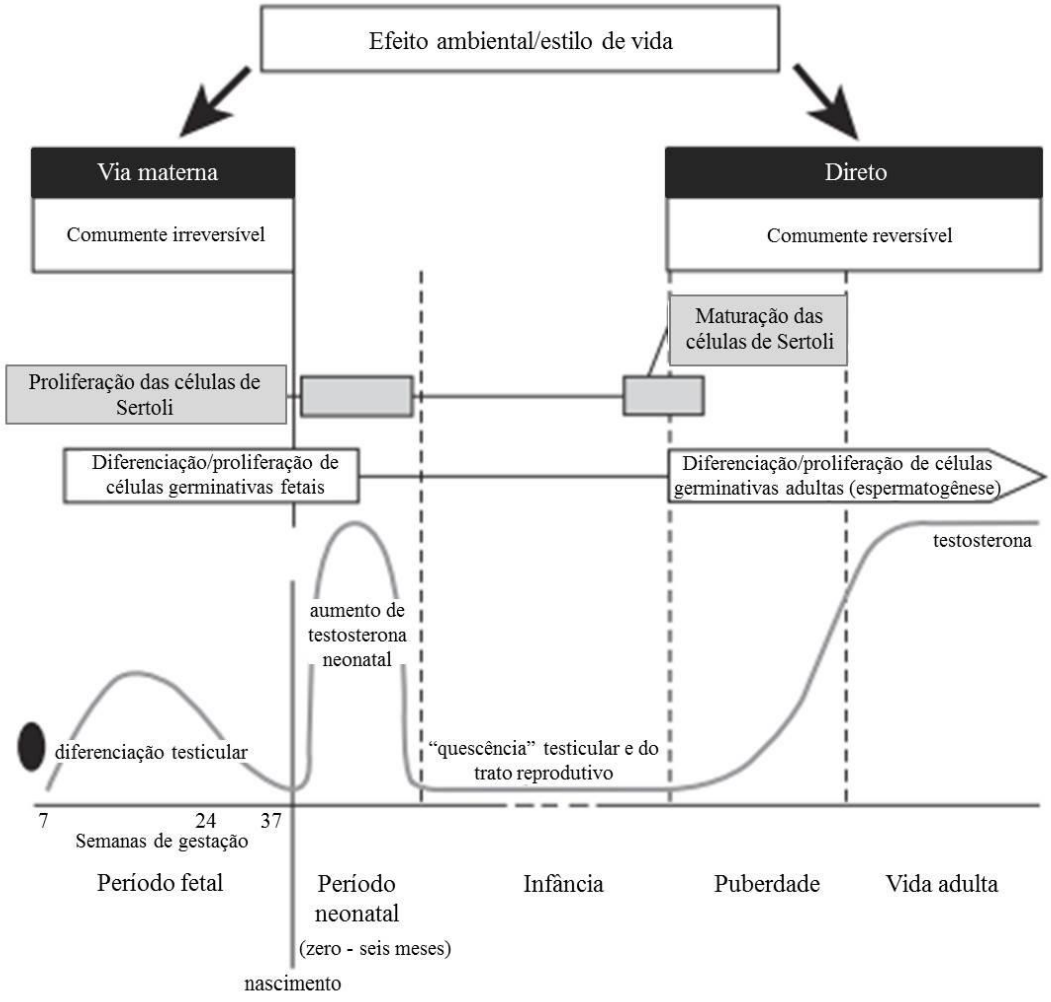

Figura 1. Tempos conhecidos em que fatores ambientais/estilo de vida podem ter impacto negativo sobre o desenvolvimento e função testicular e afetar a espermatogênese e a contagem de espermatozoides em humanos (retirado e modificado de Sharpe, 2010).

Os DE abrangem uma ampla gama de substâncias naturais ou sintéticas, geralmente encontradas no ambiente em baixas concentrações (de μg/L a ηg/L; Bila e Dezotti, 2007). Entre os compostos sintéticos encontramos os alquilfenóis, os pesticidas, os policlorados de bifenilas (PCB), os ftalatos, e o bisfenol A; entre os naturais, os estrogênios naturais e os fitoestrogênios (Loureiro, 2002; Bila e Dezotti, 2007).

Uma das categorias de DE com interesse crescente é composta pelos ésteres de ftalato (EPA, 2006), mais comumente denominados ftalatos. Os ftalatos são substâncias orgânicas derivadas do composto ácido 1,2-benzeno dicarboxílico e, geralmente, apresentam baixa toxicidade (Loureiro, 2002; Foster, 2006). Alguns exemplos dessa categoria de DE são o di-metil ftalato (DMP), di-n-butil ftalato (DBP), di-(2-etil-exil) ftalato (DEHP), butil benzil ftalato (BBP) e di-etil ftalato (DEP) (Fig. 2). Estruturalmente, os ftalatos apresentam dois grupos éster ligados a um anel benzeno (Fig. 2). A configuração *orto* é conhecida genericamente como éster de ftalato, ou apenas ftalato. Segundo Howdeshell e cols. (2008), ésteres de ftalato com 4-6 carbonos ligados na posição *orto* são considerados ativos. As configurações *meta* e *para*, por outro lado, são conhecidas, respectivamente, como isoftalatos e teraftalatos (Kluwe,1982).

Os ftalatos são produtos químicos, líquidos, incolores e inodoros, usados como plastificantes desde a década de 1940 em diversos tipos de plásticos, principalmente o PVC (cloreto de polivinila) (Loureiro, 2002). Eles constituem de 10-60% dos plásticos e conferem flexibilidade e transparência ao produto (Thomas e Thomas, 1984). Também estão presentes em uma variedade de cosméticos, como hidratantes, esmaltes, shampoos e protetores solares; brinquedos infantis; produtos alimentícios e materiais hospitalares (Mylchreest et al., 2000; Loureiro, 2002; Lottrup et al., 2006). Devido aos ftalatos não se ligarem covalentemente aos polímeros com os quais são misturados, podem ser liberados na comida, bebida e outros produtos que estiverem em contato (Thomas e Thomas, 1984; Huber et al., 1996). Sua produção aumenta proporcionalmente à indústria de plásticos e estão disseminados em todos os ecossistemas do mundo, sendo lançados no ambiente a partir de despejos domésticos e industriais, efluentes aquosos e emissões atmosféricas (Loureiro, 2002). As principais fontes de exposição humana e animal aos ftalatos são a ingestão de alimentos e líquidos que tenham tido contado com plásticos, tintas e outros constituintes das suas embalagens (Blount et al., 2000; Mylchreest et al., 2000; Loureiro, 2002), bem como a inalação e o contato dérmico (Kavloc et al., 2002).

A determinação de um panorama geral para a ingestão diária média de ftalatos é bastante difícil, já que essa estimativa depende de vários aspectos, como idade, sexo e níveis de exposição ambiental a que a população está sujeita. Na Alemanha, por exemplo,

estudo realizado com 85 adultos mostrou que a ingestão média diária dos ftalatos DEP, DBP e DEHP é 2,32 μg/kg/dia, 5,22 μg/kg/dia e 10,3 μg/kg/dia, respectivamente (Koch et al., 2003). Recentemente, Frederiksen e cols. (2011) estimaram que a ingestão diária média de DEP, DBP e DEHP por crianças e adolescentes dinamarqueses foi de 1,09 μg/kg/dia para o DEP, 4,29 μg/kg/dia para o DBP e 4,04 μg/kg/dia para o DEHP. Inúmeros estudos mostram que além de serem encontrados em amostras de urina humana (Blount et al., 2000; Koch et al., 2003, 2007; Kondo et al., 2010; Frederiksen et al., 2011), metabólitos de ftalatos também estão presentes no leite materno (Mortensen et al., 2005; Main et al., 2006) e no fluido amniótico (Silva et al., 2004; Calafat et al., 2006).

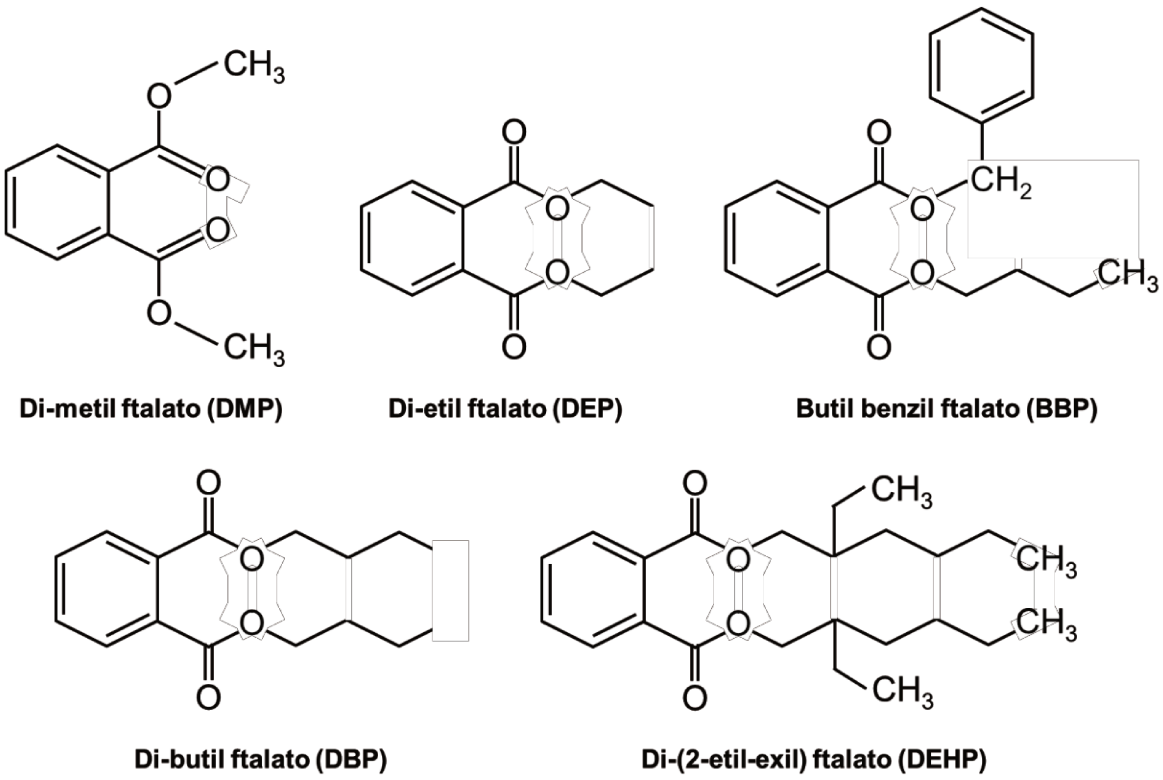

Figura 2. Estrutura química se alguns ftalatos (retirado e modificado de Olujimi et al., 2010).

Os principais plastificantes ftálicos produzidos no Brasil são o DEHP, o BBP e o DBP, sendo o DEHP e DBP os mais largamente utilizados pela indústria. O DBP, juntamente com o DEHP, é o principal ftalato encontrado nos alimentos embalados em

plástico. Este composto, extremamente resistente à lavagem, também tem sido amplamente utilizado na preparação de repelentes de insetos e esmaltes (Loureiro, 2002).

Assume-se que a toxicidade do DBP esteja associada à atividade biológica de seu principal metabólito, o monoéster monobutil ftalato (MBP) (Fig. 3; Latini, 2005). Após a absorção, a metabolização e a excreção do DBP, e de outros ftalatos, na urina e nas fezes são consideradas rápidas (Latini, 2005). Estudo de Tanaka e cols. (1978) revelou que mais de 90% do DBP administrado intravenosa ou oralmente é excretado na urina e nas fezes de ratos Wistar machos dentro de 48h após sua administração. Em humanos, a taxa de excreção renal observada foi de aproximadamente 70% do MBP (Tanaka et al., 1978; Anderson et al., 2001).

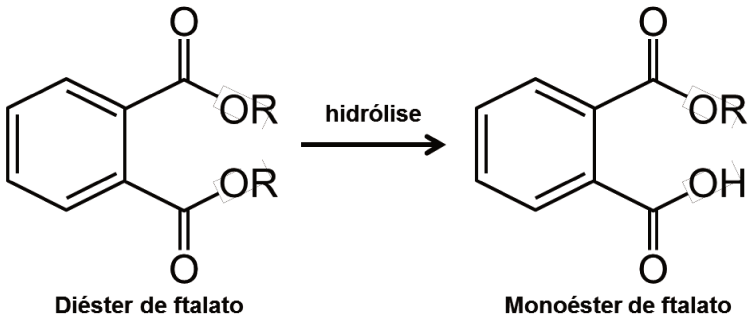

Figura 3. Estrutura química genérica de ésteres de ftalato e seu metabílito (retirado e modificado de Hauser e Calafat, 2005).

Certos ftalatos têm sido apontados como desreguladores da organogênese do trato genital masculino dos filhotes quando administrados em ratas durante a prenhez (Fisher, 2004). Estudos revelam que a exposição a altas doses de ftalatos durante os períodos críticos do desenvolvimento está associada ao aumento de hipospadias e criptorquidia, além de redução da distância anogenital e dos pesos dos epidídimos e das glândulas acessórias sexuais (Moore et al., 2001; Fisher, 2004; Kim et al., 2010). Também há dados indicando ser o testículo um dos principais órgãos afetados por estes desreguladores, exibindo hipotrofia (Moore et al., 2001; Carruthers e Foster, 2005; Heng et al., 2012), atrofia dos túbulos seminíferos (Gray e Gangolli, 1986), além de redução na produção diária de

espermatozoides e na qualidade do sêmen (Murature et al., 1987; Sharpe, 2013). Na Tabela 1 são relacionados esses e outros estudos de exposição ao DBP e DEHP, e os efeitos para o testículo e o epidídimo.

Estudo recente de Scarano e cols. (2010) demonstrou que a exposição ao DBP durante a gestação e lactação pode não causar danos para função testicular na vida adulta. Neste estudo, ratos Wistar foram expostos *in utero* a 100 mg/kg/dia desse ftalato. Os testículos fetais apresentaram agregados maiores de células de Leydig, células germinativas multinucleadas e aumento dos componentes intersticiais. No entanto, nos animais adultos não foram observadas alterações nos parâmetros espermáticos, nos níveis de testosterona ou na histologia epididimal. Mylchreest e cols. (2000) utilizaram esta e outras doses de DBP (0,5; 5; 50; 100 e 500 mg/kg/dia) durante a gestação tardia de ratos Sprague-Dawley e verificaram que apenas a mais alta dose causou perturbações no trato reprodutor masculino. Dentre elas estão malformações, como ausência ou malformação dos vasos deferentes, hipospadia, perda da cabeça e corpo do epidídimo, e lesões testiculares, como degeneração dos túbulos seminíferos e hiperplasia intersticial.

Apesar de escassos, também encontramos dados de efeitos causados pela exposição a baixas doses de DBP. Bao e cols. (2011) expuseram ratos Sprague-Dawley púberes a doses variadas deste ftalato (0,1; 1; 10; 100 e 500 mg/kg/dia) por um período de 30 dias. Foram verificadas severa atrofia dos túbulos seminíferos e perda da espermatogênese em altas doses. Em baixas doses (0,1; 1 e 10 mg/kg/dia), a exposição levou a alteração na expressão de proteínas importantes para a espermatogênese, como vimentina e heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1), além de estimulação na produção de estradiol. Estes e outros dados de estudos experimentais com roedores demonstram que exposições variadas aos ftalatos em períodos críticos de desenvolvimento fetal e neonatal causam efeitos prejudiciais, permanentes ou não, na prole masculina (Mylchreest et al., 1998, 2000; Carruthers e Foster, 2005; Foster, 2006; Scarano et al., 2010). Para alguns autores, os efeitos adversos causados pelos ftalatos ativos seriam devido à sua ação anti-androgênica nos órgãos andrógeno-dependentes (Mylchreest et al., 1998; 2000; Foster, 2006; Rider et al., 2009). Para outros, os efeitos seriam causados pela ação estrogênica, através da ligação aos receptores de estrógeno (Jobling et al., 1995; Takeuchi

et al., 2005; Filipiak et al, 2011), no entanto, os mecanimos de ação dos ftalatos ainda são pouco elucidados.

Tabela 1. Avaliação dos efeitos da exposição ao DBP e ao DEHP sobre o sistema reprodutor masculino, especialmente sobre o testículo e o epidídimo.

Modelo animal	Ftalato utilizado	Dose utilizada	Período de exposição	Efeitos causados	Referência
Ratos Sprague-Dawley	DEHP	375, 750, 1.500 e 3.000 mg/kg/dia	Gestação e lactação (GD3 a PDN 21)	Hispopadias, criptorquidismo, redução no peso do epidídimo	Moore et al., 2001
Ratos Sprague-Dawley	DBP	500 mg/kg/dia	Gestação (GD 14 e 15, GD 15 e 16, GD 16 e 17, GD 17 e 18, GD 18 e 19 e GD 19 e 20)	Redução no peso epididimal (GD 17 e 18), edema testicular e malformação epididmal (GD 17 e 17)	Carruthers e Foster, 2005
Ratos Sprague-Dawley	DBP	250, 500, 750 e 1000 mg/kg/dia	Gestação (GD 14 a 18)	Criptorquidismo (250, 500 e 750 mg/kg/dia), hipospadias (500 e 750 mg/kg/dia) e redução nos níveis de T (todas as doses)	Jiang et al., 2007
Ratos Wistar	DEHP	250, 500 e 750 mg/kg/dia	Pré-púberes (PND 21 a 51)	Redução no peso testicular (750 mg/kg/dia)	Botelho et al., 2009
Ratos Wistar	DEHP	10, 30, 100, 300, 600 e 900 mg/kg/dia	Pós-natal (PND 1 a 16)	Redução na AGD (todas as doses), redução no número de células germinativas e hiperplasia das células de Leydig (300, 600 e 900 mg-kg/dia)	Christiansen et al., 2010
Ratos Sprague-Dawley	DBP	250, 500 e 700 mg/kg	Gestação (GD 10 a 19)	Redução no peso testicular (todas as doses), nos níveis de T e na AGD (700 mg/kg/dia)	Kim et al., 2010

GD: dias de gestação; PND: dias pós-natal; T: testosterona; AGD: distância ano-genital; E_2: estradiol.

Dieta hiperlipídica

A obesidade é um grave problema de saúde pública, com incidência crescente na maioria dos países (WHO, 2011). Esse distúrbio metabólico pode ter causas multifatoriais (Symonds et al., 2013) e está associado a múltiplas alterações do sistema endócrino, incluindo concentrações anormais de hormônios na circulação sanguínea, devido a alterações no padrão de secreção, metabolismo ou transporte (Kirschner, 1982).

No que se refere ao aparelho genital, estudos epidemiológicos têm demonstrado que a obesidade feminina está associada ao início precoce da puberdade, a distúrbios menstruais, à anovulação intermitente ou crônica, ao excesso de andrógenos e maior susceptibilidade ao desenvolvimento de certos tipos de câncer de mama e endométrio (Pasquali, 2006; Pasquali et al., 2007). Os efeitos deletérios da obesidade sobre os órgãos reprodutores e fertilidade no sexo masculino são comparativamente menos investigados. No entanto, com o aumento do número de indivíduos obesos, maior ênfase tem sido dada aos efeitos negativos desse distúrbio sobre a função reprodutiva neste sexo (Pasquali, 2006; Pasquali et al., 2007; Hammoud et al., 2008; Hajer et al., 2008; Kasturi et al., 2008).

Há relatos mostrando que o acúmulo de tecido adiposo em homens está associado à diminuição dos níveis séricos de testosterona total e livre (Giagulli et al., 1994, Mah e Wittert, 2010; Landry et al., 2013). É conhecido também que a obesidade masculina leva a disfunção erétil (Chung et al., 1999; Esposito et al., 2004), redução da qualidade do sêmen (número, motilidade e morfologia dos espermatozóides) e aumento nas taxas de infertilidade (Guzick et al., 2001; Hammoud et al., 2006; Kort et al., 2006).

A obesidade é frequentemente atribuída ao estilo de vida ocidental, que combina dietas ricas em lipídeos e calorias e atividade física reduzida (Desai et al., 2013). Está bem estabelecido que o consumo crônico de dieta hiperlipídica pode causar aumento do peso corporal, dos índices de adiposidade, além de diminuir a sensibilidade à insulina (Jen, 1980; Hill et al., 1992; Guo e Jen, 1995). Ademais, estudo publicado recentemente mostrou que a obesidade, induzida a ratos adultos pelo consumo da dieta hiperlipídica, não alterou a quantidade de espermatozoides do testículo e epidídimo, mas afetou a motilidade dos espermatozoides e reduziu o potencial de fertilidade dos machos (Fernandez et al., 2011).

Nas últimas décadas, sólidas evidências de pesquisas experimentais e clínicas resultaram na elaboração do conceito de programação fetal, segundo o qual muitas doenças encontradas na idade adulta são devidas a influências ambientais durante o desenvolvimento embrionário. Assim, estímulos agudos ou crônicos durante a gestação podem estabelecer uma resposta permanente no feto e prejudicar o funcionamento de certos sistemas no início da vida pós-natal ou na vida adulta (Oken e Gillman, 2003; Symonds e Budge, 2009). Isso se aplica, em particular, às desordens do metabolismo materno, as quais influenciam o ambiente intra-uterino e podem induzir disfunções metabólicas na prole, mediadas por mecanismos fisiológicos e/ou epigenéticos (Symonds e Budge, 2009; Catalano et al., 2009). Uma variedade de estudos em diversos modelos experimentais, incluindo roedores e primatas não humanos, indica que a exposição materna ao consumo de dieta hiperlipídica influencia na programação metabólica da prole e pode resultar em obesidade no indivíduo adulto (Sullivan et al., 2011), especialmente por alterar a sensibilidade à leptina (Walker et al., 2008).

Desreguladores endócrinos e adipogênese

Embora mudanças na dieta e atividade física sejam, sem dúvida, fatores chaves para o aumento da incidência de obesidade, há um crescente interesse na possibilidade da exposição aos DE contribuírem para esse aumento (Hatch et al., 2010; Sargis et al., 2010; Lubrano et al., 2013; Manikkam et al., 2013).

Estudos apontam que a exposição ambiental a certos produtos químicos durante o desenvolvimento pode ter algum efeito na indução da obesidade na idade adulta (Newbold, 2010; Tang-Péronard et al., 2011). Estes químicos têm sido denominados obesogênicos ou desreguladores metabólicos, baseado na hipótese de Bruce Blumberg (2006) de que uma variedade de DE regula inapropriadamente o metabolismo de lipídeos, promovendo a adipogênese (Grün e Blumberg, 2006; Lubrano, 2008).

Os mecanismos moleculares pelos quais este fenômeno acontece ainda não são totalmente compreendidos. No entanto, conforme destacado em uma recente revisão, desreguladores endócrinos, como o bisfenol A, os pesticidas organoclorados e os ftalatos, podem afetar a adiposidade e o peso corporal por alterar a sinalização de células envolvidas

na regulação da diferenciação e maturação dos adipócitos (Hatch et al., 2010). Uma das hipóteses é que essa regulação aconteça via ativação de *peroxisome-profilerator activated receptor γ* (PPAR γ), um membro da superfamília de receptores nucleares (NR), os quais ativam a principal via de diferenciação dos adipócitos e de estocagem de energia (Evans, 2004). Outro NR fortemente regulado pela ação dos ftalatos é o *Liver X Receptor type α* (LXR α), o qual é, principalmente, um regulador da homeostase de lipídeos e colesterol (Muczynski et al., 2012). Estudo *in vitro* de exposição de testículos e ovários fetais humanos ao mono(etilhexil) ftalato (MEHP) demonstrou que LXR α tem sua atividade aumentada nas células somáticas, culminando em maior síntese de colesterol e lipídeos (Muczynski et al., 2012).

É sabido que os ftalatos são compostos lipofílicos e podem se acumular no tecido adiposo (Williams e Blanchfield, 1974; Jobling et al., 1995). Entretanto, pouco é conhecido sobre os efeitos do consumo de dieta rica em lipídeos para a ação dos compostos ftálicos.

Gerbilo da Mongólia

O gerbilo da Mongólia (*Meriones unguiculatus*, Fig. 4), também conhecido como esquilo da Mongólia, é um pequeno roedor da subfamília Gerbillinae, proveniente das regiões áridas da China e da Mongólia (Schwentker, 1963). Segundo Razolli e cols. (2003), as colônias de laboratório disponíveis para a investigação científica em todo o mundo foram desenvolvidas a partir de 20 pares importados do Japão em 1954, descendentes de gerbilos originalmente capturados no leste da Mongólia na década de 1930. Segundo esses autores, a endogamia resultou em perda significativa de diversidade genética desses roedores de laboratório. Introduzidos nas Américas como nova proposta experimental nos anos cinquenta por Victor Schwentker, os gerbilos, durante muito tempo, ficaram limitados aos Estados Unidos como animais de excelência para a pesquisa biomédica (Robinson, 1974), em especial nas áreas de neurofisiologia, parasitologia e patofisiologia do sistema áudio-vestibular (Muller e Nielsen, 1979; Nawaa et al., 1994).

A grande vantagem desses animais para estudos experimentais reside no fato deles serem consideravelmente menores que os ratos (machos em torno de 100 gramas e fêmeas,

85 gramas – Kramer, 1964), mas essencialmente maiores que os camundongos e hamsters (Willians, 1974).

Figura 4. Gerbilo da Mongólia (*Meriones unguiculatus*).

Nas últimas décadas, o gerbilo tem sido utilizado em inúmeros estudos desenvolvidos no Brasil (Pinheiro et al., 2003; Segatelli et al., 2004; Pinto et al., 2010a, b; Taboga et al., 2009) ou em outros países (Saltzman et al., 2008; Beu et al., 2009; Juana et al., 2010), focalizando diferentes aspectos do aparelho genital masculino. Em nosso grupo de pesquisa, esses estudos foram iniciados pelo Prof. Dr. Sebastião Roberto Taboga e têm avaliado o complexo prostático do macho (Pinheiro et al., 2003; Campos et al., 2006; Góes et al., 2007; Rochel et al., 2007) e a próstata feminina (Fochi et al., 2008; Oliveira et al., 2011; Perez et al., 2011; Rochel-Maia et al., 2011) durante o desenvolvimento ou em diferentes situações de manipulação hormonal. Recentemente, uma nova frente de pesquisa sobre a ação de DE tem focalizado as próstatas masculina e feminina do gerbilo. Esses estudos têm demonstrado que trata-se de um roedor bastante aplicável aos estudos de toxicologia reprodutiva, devido à alta susceptibilidade a lesões nessa glândula (Perez et al., 2011, 2012).

Vários aspectos da biologia reprodutiva do gerbilos são conhecidos. Sabe-se que sua gestação dura de 24 a 26 dias (Norris e Adams, 1981) e que a implantação ocorre no 8º dia de gestação, por exemplo. Aspectos da biologia testicular deste roedor também estão descritos. Segatelli e colaboradores descreveram as características estruturais do testículo

adulto, a duração da espermatogênese, composta por 12 estágios, e os aspectos da diferenciação das espermátides relacionados à formação do acrossomo (Segatelli et al., 2000, 2002, 2004). Recentemente, foram obtidos em nosso laboratório dados sobre os mecanismos envolvidos com a diferenciação neonatal dos gonócitos nessa espécie (Pinto et al., 2010b), as populações de células de Leydig (Pinto et al., 2010a) e sua a cinética de diferenciação desde o nascimento até a senescência. Também examinamos as fases do desenvolvimento testicular e descrevemos os principais parâmetros espermáticos dessa espécie, como a produção espermática diária, reserva espermática e tempo de trânsito no epidídimo (dados não publicados). Em conjunto, essas informações fornecem uma base sólida para os estudos experimentais sobre a função testicular nesse roedor frente à exposição a desreguladores endócrinos ou condições obesogênicas. Tendo em vista que o testículo é altamente susceptível aos DE e que danos funcionais nesse órgão, resultantes dessa exposição, podem comprometer a produção espermática e afetar diretamente a reprodução, ficamos interessados em analisar os efeitos de um desses desreguladores, o di-n-butil ftalato, nesse modelo animal.

A maior parte dos estudos sobre os efeitos dos ftalatos no trato genital masculino utiliza ratos ou camundongos como modelo experimental (Mylchreest et al., 2000; Moore et al., 2001; Lee et al., 2004; Foster, 2005; Andrade et al., 2006; Gaido et al., 2007; Jiang et al., 2007; Botelho et al., 2009; Kim et al., 2010; Bao et al., 2011). Esses estudos focam, principalmente, a exposição nos períodos pré-natal e/ou lactacional (Mylchreest et al., 2000; Moore et al., 2001; Lee et al., 2004; Kim et al., 2004; Foster, 2005; Carruthers e Foster, 2005; Andrade et al., 2006; Jiang et al., 2007; Kim et al., 2010) e o principal ftalato administrado é o DEHP (Agarwal et al., 1986; Gray e Gangolli, 1986; Moore et al., 2001; Andrade et al., 2006; Botelho et al., 2009; Vo et al., 2009, Christiansen et al., 2010). Além disso, os efeitos são analisados logo após o nascimento ou desmame (Foster, 2005; Christiansen et al., 2010), sendo desconhecidos os efeitos dos compostos ftálicos administrado do período pré-natal até a vida adulta. Em adição, a maioria dos experimentos utiliza-se de doses altas de ftalatos (100 a 3.000 mg/kg/dia; Moore et al., 2001; Carruthers and Foster, Kim et al., 2010; Scarano et al., 2010). Pernanecem por serem melhor esclarecidos os efeitos da exposição a baixas doses de ftalatos, como o DBP.

Embora o papel deletério da exposição aos ésteres de ftalato sobre o desenvolvimento do sistema genital seja bem conhecido, no presente estudo buscou-se examinadar as consequências de períodos de exposição mais prolongados, através de toda a vida, a doses mais baixas de DBP sobre a estrutura e função testicular do gerbilo, de maneira a simular uma exposição mais próxima à que ocorre no ambiente.

Recentemente, têm sido desenvolvidas em nosso laboratório investigações sobre as consequências da exposição ao ambiente obesogênico, através do consumo de dieta hiperlipídica, durante a gestação e outras fases do desenvolvimento, sobre a capacidade esteroidogênica das células de Leydig de ratos Wistar (Proc. FAPESP 2011/01612-4), a produção testicular de espermatozóides (Proc. FAPESP 2011/14815-8) e a histofisiologia prostática (Proc. FAPESP 2011/03596-6). Tendo em vista que a associação entre os efeitos da obesidade sobre a função testicular do gerbilo com a exposição a DE ainda é pouco investigada em estudos experimentais, consideramos que o presente estudo poderá trazer

novos subsídios para a compreensão de como essas situações isoladas afetam a estrutura e a função testicular e os efeitos da suas interações para a função reprodutiva desse roedor. Os resultados obtidos com esse estudo experimental também poderão trazer novas informações sobre os mecanismos celulares envolvidos na resposta testicular frente a obesidade e aos DE, as quais podem auxiliar no esclarecimento dos prejuízos reprodutivos descritos para o homem.

O objetivo deste estudo foi avaliar os efeitos da exposição prolongada, da gestação até a maturidade sexual, a baixas doses de DBP e as possíveis interferências do excesso de lipídeos na dieta sobre os parâmetros espermáticos e a capacidade esteroidogênica de gerbilos adultos. A resposta do testículo frente a essas condições foi avaliada com base (i) em análises microscópicas e esterológicas, (ii) nas dosagens dos esteroides sexuais, (iii) na sensibilidade aos andrógenos e estrógenos das principais populações celulares do testículo e (iv) na produção espermática. Também foram examinados os efeitos sobre (v) a reserva espermática, (vi) o tempo de trânsito dos espermatozoides através do epidídimo e (vii) a motilidade dos espermatozoides.

Os resultados obtidos nesse estudo serão apresentados na forma de manuscrito, o qual foi submetido e se encontra em fase de análise.

Additive effects of dietary fat and low doses of di-n-butyl phthalate on sperm parameters in Mongolian gerbils

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Abbreviations: DBP, di-n-butyl phthalate; EDC, endocrine disrupting chemicals; DEHP, di(2-ethylhexyl) phthalate; GSI, gonadosomatic index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; DSP, daily sperm roduction; PPARγ, peroxisome proliferator activaded receptor γ; MEHP, mono(ethylhexyl) phthalate; LXR-α, liver X receptor type-α.

Abstract

Toxicological studies indicate that di-n-butyl phthalate (DBP) and other endocrine disrupting chemicals (EDC) alter sexual steroid hormone scenary and induce reproductive anomalies, when administered during sexual differentiation. However, the consequences of low doses of DBP throughout a life on reproductive male function remain to be elucidated. EDC can accumulate in adipose tissue and interfere in adipogenesis and lipid metabolism. Indeed, male obesity affects negatively semen quality and fertility rates. Because the consequences of combined effects of phthalates and excess dietary fat on reproductive parameters are still unknown, we evaluated the effects of prolonged exposure to low DBP dose and the possible interferences of excess dietary fat for testicular function of adult gerbils. Adult female gerbils, fed high-fat or balanced diet for eight weeks, were mated with normal males. The offspring was divided in control (C), di-n-butyl phthalate (Ph), high-fat diet (HF) and high-fat diet plus di-n-butyl phthalate (HFPh) groups. 5 mg/kg body weight/day of DBP was administered in drinking water to pregnant and breastfeeding mothers and to offspring from weaning up to adulthood. High-fat diet contained 20% of fat against 4% of balanced diet. Lifelong low DBP doses resulted in obesity and dislipidemy at adulthood. No changes were verified in testes structure, however, it was reduced the testicular estrogen synthesis and was observed a tendency to reduction by 10% in the spermatic efficiency. Chronic exposure to high-fat diet did not induce obesity in animals, but led to a decrease in AR expression and a trend to decrease in the spermatic efficiency (~4%). The combined exposure reduced by 83% the intratesticular testosterone concentration and decreased by 15% the spermatic efficiency. This study shows that the excess of dietary fat and low doses of DBP throughout a life acted synergistically compromising the spermatogenic and steroidogenic function of testes in the gerbil. These results demonstrate for the first time that fat nutritional environment may worsen the response of testes to phthalates and vice versa.

Key words: di-n-butyl phthalate, high-fat diet, testicular function, sperm parameters, Mongolian gerbil

1. Introduction

Di-n-butyl phthalate (DBP) is a plasticizer widely used in industrial products such as children's toys, food packaging, medical devices and cosmetic formulations (Mylchreest et al., 2000; Lottrup et al., 2006; Filipiak et al., 2011). This and others phthalates, including di(2-ethylhexyl) phtalate (DEHP), denominated endocrine disrupting chemicals (EDC), are known to alter sexual steroid hormone profiles and prejudice testicular development and other androgen-dependent organs, impairing reproductive capacity in adulthood (Wilson, 1978; Andrade et al., 2006; Christiansen et al., 2010). Potential primary routes of exposure to phthalates are inhalation, ingestion and dermal contact (Kavlock et al., 2002).

Toxicological studies in male rat offspring exposed to some phthalate esters, such as DBP, during sexual differentiation indicate the occurrence of several reproductive anomalies, such as agenesis of the epididymis (Foster, 2006), testicular atrophy (Moore et al., 2001; Carruthers and Foster, 2005; Heng et al., 2012), seminiferous tubule atrophy (Gray and Gangolli, 1986) and reduction in daily sperm production and semen quality (Murature et al., 1987; Sharpe, 2013) during adulthood. Harmful effects of phthalates are, in part, associated with suppression of the androgen signaling pathway and a decrease in fetal testosterone levels, demonstrating the anti-androgenic potential of phthalic compounds (Mylchreest et al., 1999; Parks et al., 2000; Gray et al., 2006). There are also data showing that these compounds, at low doses, have low estrogenic activity *in vitro* (Jobling et al., 1995; Zacharewski et al., 1998); however, this is a controversial issue, because estrogenic activity has not been observed in some studies *in vivo* (Gray et al., 1998; Grande et al., 2007; Lehraiki et al., 2009).

Data about human exposure to phthalates are quite variable. According to Blount et al. (2000), human exposure to DBP is estimated between 0.84 and 113 µg/kg/day. Most toxicological studies were based on high dose exposures to phthalates (100 to 3,000 mg/kg/day; Moore et al., 2001; Carruthers and Foster, 2005; Kim et al., 2010; Scarano et al., 2010) and a few studies assessed the low dose exposure (Andrade et al., 2006; Christiansen et al., 2010; Bao et al., 2011). These studies focused on the effects of exposure to phthalates during critical periods of testicular development, such as fetal, neonatal and

pubertal. Lee et al. (2004) showed that exposure to 2 mg/kg/day of phthalates during gestation and lactation was sufficient to impair germ cell development. A recent study (Bao et al., 2011) using administration of different doses of DBP for 30 days during pubertal development of rats indicated the occurrence of abnormalities in testes and epididymis's development, severe seminiferous tubule atrophy and loss of spermatogenesis under high doses (100 and 500 mg/kg/day). At low doses (0.1, 1 and 10 mg/kg/day), no morphological changes were observed, however, there was an increase in the expression of important proteins required for spermatogenesis, including heterogeneous nuclear ribonucleoprotein A2/B1 and vimentin (HNRNPA2B1; Bao et al., 2011). The effects of low doses throughout a life remain to be determined, including the various stages of testicular development.

Laboratory and epidemiological studies have demonstrated that exposure to EDC contributes to adipogenesis and an increase in obesity (Hatch et al., 2010; Lubrano et al., 2013; Manikkam et al., 2013). Studies indicate that some EDC categories, such as bisphenol A and phthalates, can lead to obesity by altering cell signaling involved in the regulation of adipocyte differentiation and maturation (Hatch et al., 2010). Although phthalates are lipophilic compounds, with a tendency to accumulate in adipose tissue (Jobling et al., 1995; Hatch et al., 2010), little is known about the effect of an excess of dietary fat on phthalate metabolism and action, particularly during development.

It is known that obesity in males affects testosterone production (Landry et al., 2013), semen quality (number, motility and morphology of sperm) and increases the infertility rates (Guzick et al., 2001; Hammoud et al., 2006; Kort et al., 2006; MacDonald et al., 2010; Sharpe, 2013). Experimental study with rodents revealed that obesity induced by a high-fat diet did not alter the amount of sperm in the testis and epididymis, but affected sperm motility and reduced the fertility potential of male rats (Fernandez et al., 2011).

Most studies of phthalate effects on the male genital tract use rats or mice as an experimental models (Mylchreest et al., 2000; Moore et al., 2001; Andrade et al., 2006; Foster, 2006; Gaido et al., 2007; Christiansen et al., 2010; Kim et al., 2010; Bao et al., 2011). There are some differences among rodents in the response of genital organs to phthalates and even among rat strains (Howdeshell et al., 2008; Muczynski et al., 2012). The Mongolian gerbil (*Meriones unguiculatus*) is a laboratory rodent largely used in studies

concerning the biology of male genital organs (Pinheiro et al., 2003; Segatelli et al., 2002; Segatelli et al., 2004; Góes et al., 2007; Beu et al., 2009; Taboga et al., 2009; Juana et al., 2010; Pinto et al., 2010a, b). Recent reports from our laboratory have indicated that this rodent is an excellent model to assess the consequences of EDC in prostatic lesions (Perez et al., 2011, 2012); however, the interference of these disrupters on testicular function is unknown.

Thus, the objective of this study was to evaluate the effects of prolonged exposure, from gestation until sexual maturity, to low doses of DBP and the possible interferences of excess dietary fat on sperm parameters and steroidogenic capacity of adult gerbils.

2. Material and Methods

2.1. Animals and experimental design

Mongolian gerbils (*Meriones unguiculatus)* were maintained in polyethylene cages under controlled light (12-hour light/dark cycle) and temperature (22±2°C) conditions in the Animal Research Group in Reproductive Biology of Institute of Biosciences, Humanities and Exact Sciences (IBILCE/UNESP), São José do Rio Preto, Brazil. Gerbils had free access to food and water, which was provided to the animals in glass bottles. The experimental procedures were approved by the Institutional Committee for Ethics in Animal Experimentation (060/2012 CEUA / UNESP).

Sixty newly weaned female gerbils (4 weeks old) were randomly divided into two groups, which were fed a balanced or high-fat diet (Table 1) for a period of 8 weeks. There was no difference in body weight gain of females after this period (33.8 ± 5.5g and 30.8 ± 5.2g, $p = 0.1186$, respectively). After 8 weeks, females were mated with normal males (12 to 14 weeks old). After confirmation of mating by vaginal swabs (day 0 of pregnancy), half of the females from each group were exposed to di-n-butyl phthalate (DBP; Sigma Chemical Co., St. Louis, MO, USA). DBP was dissolved in ethanol 95% and stored in aliquots at -70° C. Aqueous solutions of DBP in 0.01% of ethanol were prepared three times a week and provided to the animals in glass bottles protected from the light with adhesive Silver Tape. The dilution of DBP was conducted to result in a dose of

5mg/kg/day. This dosage is considered low for this (Bao et al., 2011) and other phthalates (Andrade et al., 2006). The average consumption of water with DBP in the females fed a balanced or high-fat diet was 14.1±4.2 mL/day and 12.1±2.3 mL/day, respectively. A correction in DBP was made to compensate for this difference.

Male offspring (n = 15 animals/group, 1 animal/family) of four weeks of age were weighed and randomly selected to compose the following groups: 1) Control group (C): control male offspring; 2) Di-n-butyl phthalate group (Ph): male offspring exposed to low doses of DBP during gestation, lactation until sexual maturity; 3) High-fat diet group (HF): male offspring exposed to high-fat diet during gestation, lactation until sexual maturity; 4) High-fat diet plus di-n-butyl phthalate group: male offspring exposed to high-fat diet and low doses of DBP during gestation, lactation until sexual maturity, concomitantly.

The high-fat diet was standardized by the Experimental Laboratory of Internal Medicine, Medical School of Botucatu - UNESP and produced by Agroceres (Rio Claro, SP, Brazil). This diet contains 20% lipids relative to the total amount of fat versus 4% in the balanced diet (Table 1), and its composition has been described by Leopoldo et al. (2011).

Pups were exposed to the same doses of DBP described above from their mothers. At the end of the experiment, 14-week-old male gerbils were euthanized by CO_2 inhalation and decapitated.

2.2. Weight of reproductive organs

The wet weights of the right testes and epididymes were measured, and the gonadosomatic index (GSI) was determined based on the formula: [(testicular weight/body weight) x 100].

2.3. Characterization of metabolic state

The body weight, abdominal circumference and adiposity index were determined. The adiposity index was estimated by the formula [(sum of epididymal, retroperitoneal and visceral fat/body weight) x 100], according to Taylor and Phillips (1996). Furthermore, the lipid profile was evaluated in the serum of animals by enzymatic tests to assess total

cholesterol, HDL, LDL and triglycerides levels (In vitro Diagnóstica Ltda, Itabira, MG, Brazil). Eight animals per experimental group were analyzed, and each sample was evaluated in duplicate in a Spectro Thermo Scientific Evolution 300.

2.4. Hormone levels

2.4.1. Serum testosterone and estradiol levels

After decapitation, blood was collected (between 7:30 and 11:00 a.m.) from the ruptured cervical vessels for the determination of the serum concentrations of testosterone and estradiol. Serum was obtained after centrifugation (3,000 rpm, 20 min) and was frozen at -80◦C until analyses of the hormones were carried out. The hormone concentration were measured by ELISA capture/sandwich (antibody-antigen-antibody) using specific commercial kits (testosterone and estradiol-17β, Cayman Chemical Company, MI, USA), according to the manufacturer's instructions. The detection limits for testosterone and estradiol were 6 and 19pg/mL, respectively. The readings were performed in a reader SpectraMax Plus 384 (Molecular Devices, CA, EUA) at 405 ηm. Nine animals were analyzed per experimental group and samples were analyzed in duplicate.

2.4.2. Intratesticular testosterone and estradiol levels

The left testis of five animals was removed and decapsulated, and the parenchyma was sliced into ~100mg pieces and processed for the extraction of steroids. In this processing, the fragments were homogenized twice in PBS and incubated in a tube with diethyl ether for 10 minutes, the first time at room temperature and the second on dry ice. The supernatants were removed and transferred to another tube and stored until complete evaporation of the diethyl ether. The material was then resuspended in PBS and stored at -80°C until the determination of intratesticular testosterone and estradiol concentrations (Cayman Chemical Company, MI, USA).

2.5. Histological and stereological analysis

The testes of five gerbils were removed and fixed in Bouin's fluid for 12h. Fragments were washed several times in 70% alcohol, processed for inclusion in Paraplast

(Merck, Darmstadt, Germany) and used for general histological, immunohistochemical and stereological analyses.

The relative volume of each testicular tissue component was calculated using a Weibel multipurpose graticulate with 130 points (Weibel, 1966). The following components were estimated: seminiferous epithelium, lumen of seminiferous tubules, nuclei of Sertoli cells, peritubular tissue, Leydig cells, others cells of interstitial tissue, blood vessels and lymphatic spaces. The microscopic fields of each animal, submitted to AR immunohistochemistry and counterstained with Harris's hematoxylin, were analyzed using the software Image-Pro Plus (Media Cybernetics version 4.5 for Windows, MD, USA) and chosen at random.

Stereological analysis was performed in twenty microscopic fields at 200x magnification in a total of 100 microscopic fields per group (five animals). In summary, the relative values were determined by counting the coincident points of the test grid and dividing them by the total number of points.

2.6. Immunohistochemistry

AR, ERα and 17β-HSD (sc816, sc-542 and sc-32872, respectively; Santa Cruz Biotechnology, CA, USA) primary antibodies were used for immunohistochemistry analyses. Immunohistochemistry reactions were performed using the avidin–biotin complex (ABC) kit (Santa Cruz Biotechnology, CA). Antigenic retrieval in tissue sections was performed in citrate buffer at high temperature (92°C) for 45 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 20 min, followed by three washes in PBS and then incubation in 5% nonfat dry milk in PBS for 45 min to block nonspecific binding. Sections were incubated with the primary antibody at 4°C overnight. The slides were then incubated with biotinylated secondary antibody at 37°C followed by peroxidase-conjugated avidin–biotin complexes and diaminobenzidine (DAB). The sections were counterstained with Harris's hematoxylin. Negative controls for all immunocytochemical reactions were obtained by omission of the primary antibody.

2.7. Western blotting analysis

AR, ERα and 17β-HSD levels were quantified in testes samples by Western blotting. For each analysis total extracts of the left testis of four animals per experimental group were used. Total extracts were obtained from 100 mg of tissue fragments, homogenized in RIPA buffer (Sigma, St. Louis, MO, USA) and Triton X100. Following centrifugation of the homogenate, the protein present in the supernatant were quantified by the Bradford method (Bradford, 1976), and 100 μg protein was subjected to SDS-PAGE. After electrophoresis, proteins were transblotted onto a nitrocellulose membrane (GE Healthcare). The blot was blocked with 5% nonfat dry milk in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% Tween-20) for 30 min, incubated overnight at 4°C with 2% BSA in TBST containing a 1:300-500 dilution of the primary antibodies (AR - sc-816, ERα - sc-542 and 17β-HSD - sc-32872; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-β-actin (Santa Cruz Biotechnology), washed (3 × 10 min) in TBST, and incubated for 1h at 4°C with secondary antibody, followed by three washes (10 min) in TBST. Antibody detection was revealed using the ELC chemiluminescent detection kit (Healthcare). The protein expression was normalized to the β-actin values (Santa Cruz Biotechonology , CA, USA - sc-47778). The density of immunolabeled bands was analyzed using the Image J 1.34 (Wayne Rasband, Research Services Banch, National Institute of Mental Health, Bethesda, Maryland) densitometry program.

2.8. Cytokine analysis

The serum and intratesticular concentrations of proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were quantified by ELISA capture/sandwich (antibody-antigen-antibody) using specific commercial kits (R&D System, Minneapolis, MN, USA).

2.9. Sperm count, daily sperm production and sperm transit time in the epididymis

The spermatids resistant to homogenization (stages 13, 14 and 15 of spermiogenesis) and the spermatozoa present in the caput/corpus and cauda of the epididymis were estimated as described by Robb et al. (1978), with modifications by Fernandes et al. (2007). To calculate the daily sperm production (DSP), the number of

spermatids per testis was divided by 5.81, which is the number of days in which mature and resistant to homogenization spermatids are present in the seminiferous epithelium (unpublished data). Then, the DSP per gram was calculated in order to determine the efficiency of the process (Berndtson, 1977; Johnson et al., 2000). The sperm transit time through the epididymal caput/corpus and cauda was obtained by dividing the number of spermatozoa present in each of these regions by the DSP.

2.10. Sperm motility

Immediately after euthanasia, the left cauda of the epididymis was collected. Sperm were obtained with the aid of a needle by means of rinsing with 1.0 mL of modified HTF medium (Human Tubal Fluid, IrvineScientific) at 34°C. A Makler counting chamber (Sefi-Medical, Haifa, Israel) warmed to 34°C was loaded with a small aliquot of sperm solution (about 10 μL). Sperm motility evaluation was assessed by visual estimation (100 spermatozoa per animal, in duplicate) under a phase-contrast microscope (Olympus BX60) at 200x magnification. Spermatozoa were classified as: immotile, motile without progression and motile with progressive movement.

2.11. Statistical analysis

Statistical analyses were evaluated by GraphPad InStat (GraphPad Software Inc. 1992-1998) software. The quantitative results were evaluated by parametric statistical tests (ANOVA followed by Tukey's tests) and nonparametric statistical tests (Kruskal-Wallis tests followed by Dunn's tests). Samples obtained by ELISA were analyzed by Grubbs's tests (GraphPad Software) to verify the outliers' presence. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Body weight and adiposity index

A high-fat diet did not affect body weight at weaning (four weeks), however, when accompanied by DBP exposure, body weights increases by approximately 28% compared

to control animals (Table 2). There was an increasing trend in body weight for all experimental groups at 14 weeks of age, however, there was only a statistical difference in the Ph group, in which the weight gain was ~10% (Table 2). Similarly, the adiposity index was higher (~41%) only in this group compared to control group (Table 2). There were no changes in abdominal circumference in the animals at four or 14-week-old (data not shown).

3.2. Reproductive organ weights

Neither high-fat diet nor exposure to low doses of DBP, or the combination of both treatments, resulted in changes in testicular weight and in GSI (Fig. 1A). The weight of the epididymis was 13 and 17% higher in the Ph and HFPh groups, respectively, compared to the C group (Fig. 1B). Analysis of epididymal caput/corpus and cauda separately indicated that the change in epididymal weight was due to the increase in weight of the cauda (Fig. 1B). However, this change was significant (~ 19%) only in the HFPh group with respect to the C group (Fig. 1B).

3.3. Lipid profile

The lipid profiles of the animals are shown in Figure 2. Total cholesterol levels were higher in the Ph group with respect to the HFPh group. Similarly, the Ph group showed increases serum triglycerides levels. Ph and HF groups showed the lowest LDL levels. Combined exposure to DBP and to high-fat diet normalized triglycerides, LDL and total cholesterol levels. HDL levels did not differ between the experimental groups.

3.4. Serum and intratesticular hormone levels

Serum testosterone (T) and estradiol (E_2) levels had no significant variations among experimental groups, as shown in Figures 3A and B, respectively. However, intratesticular T concentration was lower in the HFPh group (Fig. 3C), and intratesticular E_2 concentration diminished in the Ph group (Fig. 3D).

3.5. Histology and stereology

No drastic changes or tissue damage were observed in the testes of animals exposed to low doses of DBP, to high-fat diet, or to both treatments (not shown).

In general, stereological analyses showed no significant changes in testicular components of the treated groups (Ph, HF and HFPh groups) (Table 3). Only the HFPh group exhibited a higher relative frequency of Sertoli cell nuclei and lower relative frequency of lymphatic space than the C group, and the HF group exhibited a higher relative frequency of lumen.

3.6. AR, ERα and 17β-HSD expression in testes

AR expression, assessed by Western blotting, was lower in the HF group than in the other groups. In this group, a decrease of ~35% in average AR content was observed (Figs. 4A and B). The Ph and HFPh groups showed lower mean values of AR content than the C group, but the decrease was less pronounced (~17 and ~21%, respectively) (Figs. 4A and B). Such variations in AR expression could not be detected by immunohistochemistry (Figs. 4C-F).

ERα expression did not change significantly in HF and HFPh groups but higher expression was observed in the Ph group in comparison to the C and HF groups (increase of 70 and 76%, respectively) (Figs. 5A and B). This increased expression was also observed by immunocytochemistry (Figs. 5C-F).

There was no change in expression of 17β-HSD among the four experimental groups (Fig. 6).

3.7. Cytokine levels

Concentrations of TNF-α did not differ in the serum or testis in experimental groups (Figs. 7A and C). IL-1β levels increased mildly in the serum of the HF and HFPh groups (Fig. 7B) and increased mildly in the testis of the Ph group (Fig. 7D), but without statistical significance.

3.8. Sperm parameters and motility

Isolated exposure to low doses of DBP or high-fat diet did not affect sperm parameters, except for increasing the sperm counts in the caput/corpus of the epididymis in the HF group (Table 4). However, in both isolated treatments, there was a tendency for a reduction in spermatic efficiency (DSP/g testis). The combined exposure to DBP and to high-fat diet decreased the spermatid number per gram of testis by ~15% in comparison to the control group, as well as the spermatic efficiency (Table 4). Moreover, the HFPh group exhibited higher transit times in the caput/corpus (~ 39%) and cauda (34%) of the epididymis than the C group and a tendency of increase in sperm concentration in the epididymis (Table 4).

High-fat diet and DBP, individually or in association, did not alter the sperm motility (Fig. 8).

4. Discussion

DBP, as well as other phthalic esters and their active metabolites, are important environmental endocrine disrupting chemicals and resulting in adverse effects on the male reproductive system when administered at high doses during critical periods of gonadal development (Gangolli, 1982; Moore et al., 2001; Carruthers and Foster, 2005; Jiang et al., 2007). However, the consequences of prolonged exposure to lower doses of DBP on sperm production and steroidogenesis remain to be elucidated. To assess this question, in this study we used a novel experimental design of exposing to Mongolian gerbils to 5 mg/kg/day doses of di-n-butyl phthalate, from gestation to adulthood, in order to simulate a continuous exposure similar to what potentially occurs in humans and other mammals.

The exposure of gerbils to this DBP dose during intrauterine and post-natal life resulted in a trend toward a reduction in spermatic efficiency by about 10%, and resulted in a slight increase in sperm transit time in the epididymis. Such exposures did not disrupt testosterone synthesis or affect testicular AR and 17β-HSD expression, but reduced intratesticular E_2 synthesis and increased ERα expression in this organ. Persistent exposure to low doses of DBP also resulted in obesity and dislipidemy in adulthood. Our metabolic data support the "obesogen hypothesis" of Grün and Blumberg (2006), which postulates

that a variety of EDC regulate inappropriately the lipid metabolism, promoting adipogenesis and obesity. Phthalates are known to affect weight homeostasis by disrupting steroid hormones, and estrogen exposure during development is associated with an increase of adipocyte numbers in adulthood (Grün and Blumberg, 2006; Heindel and vom Saal, 2009). Some phthalates also act as agonists of peroxisome-proliferator activated receptors γ (PPARγ), which play critical roles in adipogenesis and lipid metabolism (Hurst and Waxman, 2003; Grün and Blumberg, 2006; Lubrano et al., 2013).

The mechanism of action of phthalates in testis development has been investigated by *in vitro* studies (Jobling et al., 1995; Takeuchi et al., 2005; Chauvigné et al., 2009; Lambrot et al., 2009; Lehaiki et al., 2009; Muczynski et al., 2012). Evidence from *in vivo* studies indicates that exposure to high doses of phthalates (above 100mg/kg/day) during critical periods of testis development induces antiandrogenic effects and impairs testosterone production and testicular descent in different rodents (Parks et al., 2000; Fisher et al., 2003; Howdeshell et al., 2008). However, alterations in interactions of Sertoli cells and gonocytes induced by some phthalates occur independently from testosterone synthesis inhibition (Gaido et al., 2007; Scott et al., 2007). Most of the information concerning the *in vivo* effects of phthalates on the male genital system and fertility are based on high doses and administration in a restrict stage of development (Moore et al., 2001; Christiansen et al., 2010; Bao et al., 2011), leaving the effects of persistent exposure to phthalates relatively unknown. Our data demonstrate that the lifelong intake of DBP has an inhibitory effect on intratesticular E_2 concentration without influencing serum E_2 or androgen levels. When low (0.1 mg/kg/day) or high (500 mg/kg/day) doses of DBP were administered to male Wistar rats for 30 days in the puberal period, an increase in serum E_2 concentration was observed in adulthood (Bao et al., 2011). The intratesticular steroid concentrations were not examined in that study. The discrepant effects on serum steroid concentrations observed by Bao et al. (2011) and in this present study may be due to differences in animal models, DBP dosage and also in the exposure phase, once the steroidogenic activity is differentially regulated in immature and mature testis. In the mature testis, E_2 is produced by conversion of T by the aromatase enzyme of Leydig cells, a process that is under LH regulation (Valladares and Payne, 1979; Akingbemi, 2005; Ishikawa et al., 2006). The

general histological and stereological analyses did not show relevant alterations in Leydig cells, indicating that structural alterations probably did not occurred. It is possible that the continuous intake of DBP since gestation altered the hypothalamic-pituitary axis and LH secretion, which were not examined here. An increase in serum LH levels was observed after puberal exposure of rats to 0.1 or 10 mg/kg/day of DBP (Bao et al., 2011). However, a study based on the culture of rat fetal testis demonstrated that mono(ethylhexyl) phthalate (MEHP), the metabolite of DEHP, inhibits testosterone production independently from the receptivity of Leydig cells to LH (Chauvigné et al., 2009). Thefore, it is possible that DBP exposure in fetal and perinatal periods may have affected the responsiveness of Leydig cells to LH or resulted in other functional reprogramming. Thus, the decrease in intratesticular estrogen may be due to alterations in the expression or activity of aromatase, for example. Changes in brain aromatase have been observed in rats exposed to low DEHP doses *in utero* or during lactational periods (Andrade et al., 2006). Kim et al. (2003) observed that high DEHP doses (100 mg/kg/day) reduced aromatase expression in the testis of prepubertal rats. These data show that changes in aromatase expression are associated with exposure to this compounds. Interestingly, the decrease in intratesticular E_2 after lifetime DBP exposure was accompanied by a ~70% increment in ERα content in the testis. The increased ERα expression in the testis might be an adaptative response to low E_2 levels.

Although our data clearly show that prolonged DBP exposure results in a trend of low sperm production, it is not possible to discriminate if deleterious effects of DBP occurred during the fetal, neonatal or puberal/adult periods. The decrease in spermatic efficiency was not accompanied by relevant alterations on testis morphology, on the frequency of Leydig and Sertoli at adulthood or on spermatogenesis, which apparently occurred normally. Indeed, reductions on sperm counts were not associated with alterations in serum testosterone concentration and intratesticular AR expression, but involved estradiol concentration and signaling changes in the testis in adulthood. Based on these findings, we considered that chronic exposure to DBP did not significantly affect spermatogenesis at adulthood, but disturbed steroid signaling in the adult testis. The

interference in Sertoli cell-germ cell interactions or other functional impairments in Sertoli cells at adulthood requires further investigation.

One rational explanation for the lower sperm counts observed here after DBP exposure may be an interference in the development of germ cell populations during the fetal or neonatal phases. There is solid evidence that phthalate exposure impairs the development of gonocytes. Lambrot et al. (2009), using organotypic cultures of human fetal testis, showed that 10^{-4} M of MEPH reduced the germ cell numbers by 40% by increasing their apoptosis. Phthalates are also reported to have a weak estrogenic action, and *in vitro* studies have demonstrated that 10^{-5} M of DBP and other phthalates, such as butyl-benzyl phthalate, inhibit the binding of estradiol to fish estrogen receptors, acting as agonists and stimulating the transcriptional activity of estrogen-dependent genes (Jobling et al., 1995). Takeuchi et al. (2005) also showed, using Chinese Hamster Ovary (CHO) cells, that phthalate diesters, such as DBP, have estrogenic activity mediated by ERα, anti-estrogenic activity mediated by ERβ and AR-mediated antiandrogenic activity. Although *in vitro* studies support the estrogenic and anti-androgenic action of phthalates, data from Lehraiki et al. (2009) using the organotypic culture of testis from estrogen-deficient mice (ERαKO or ERβKO) or androgen (Tfm) receptors indicates that the effects of MEHP on the reduction of germ cells did not involve these receptors. Recently, results from the same group indicated that MEHP up-regulated the expression of the nuclear receptor Liver X Receptor type α (LXR α), which stimulates the mRNA expression of SREBP1c and SREBP2. In turn, SREBP1c and SREBP2 upregulate the transcriptional levels of triglycerides and cholesterol synthesis enzymes (Muczynski et al, 2012). The up regulation is observed in Sertoli cells and not in germ cells. Because the Ph group exhibited elevated triglycerides in adulthood, it is possible that DBP interfered in LXRα and SREBP1c expressions and affected the number of germ cells if it also occurred during fetal or neonatal development. Additional analyses focusing on the gestational and lactational effects of DBP are necessary to clarify the influence of this dose of DBP on fetal/neonatal testis development, gonocyte differentiation and the relation to steroids receptors or LXRα in this rodent. Such analyses are underway in our laboratory. It is noteworthy that although the decline in spermatic efficiency may have been mild, it is biologically significant given

that it was caused by a single compound, while human populations are exposed to complex mixtures of environmental chemicals whose dose and effects could be cumulative (Howdeshell et al., 2008).

In contrast to the effects of DBP exposure, the excess fat during gestation/lactation followed by persistent ingestion of 20% of lipids did not induce obesity in gerbils at adulthood. Previous studies indicate that at least 15 weeks of high-fat diet are necessary to induce weight gain in the Wistar rat (Nascimento et al., 2008; Fernandez et al., 2011; Ribeiro et al., 2012), and the present data show that gestational and lactational exposure probably did not contribute to reduce the duration of this time exposure.

With regard to sperm parameters, similar to those observed after DBP exposure, the excess of dietary fat also led to a tendency of reduction by ~4% in spermatic efficiency, and a slight increase in sperm transit time in the epididymis. However, high-fat diet alone did not alter serum steroid levels and steroid biosynthesis in the testis, but decreased AR expression in this organ. Several clinical and experimental studies have shown a negative influence of adiposity on androgen serum levels, sperm production and fertility (Giagulli et al., 1994; Hammoud et al., 2008; MacDonald et al., 2010). Fernandez et al. (2011) observed that after 15 weeks of high-fat diet consumption the sperm counts in testes were not affected, but sperm motility was reduced and E_2 serum levels increased in Wistar rats. Other recent results from our laboratory indicated a reduction of ~17% in sperm counts of Wistar rats from obese mothers exposed to a high-fat diet (unpublished data). A decrease in AR expression was also observed in the testes of these rats, which was more pronounced if followed by high-fat diet consumption during post natal development (unpublished data). Therefore, the excess of fats in the diet or via maternal nutrition seems to interfere in expression of the AR expression in androgen-dependent organs in adulthood, independently of the obesity status. Thus, the trend of sperm production reduction observed here probably reflects the impairment in AR signaling in testes.

When DBP exposure was combined with excess of dietary fat, the impairment in spermatic efficiency was higher and a reduction of about 15% was observed, indicating the additive effects of exposure. Similar to that was observed in individual exposures, no perturbations on testis weight or histology were detected after concomitant exposure to

DBP and to high-fat diet. Furthermore, these gerbils also exhibited lipid profiles and adiposity indexes similar to those of control gerbils at adulthood. However, in spite of the lack of changes observed in serum hormone levels, intratesticular testosterone synthesis was impaired. A reduction of 83% in intratesticular testosterone was observed in treated animals after combined exposures. As previously mentioned, several studies indicate that phthalates, such as DBP, have antiandrogenic activity (Fisher et al., 2003; Barlow et al., 2003; Howdeshell et al., 2008). In fetal testes, the mechanism by which DBP causes a reduction in intratesticular T levels is through decreasing androgen production by Leydig cells (Lambright et al., 2003). Lehmann et al. (2004) revealed that the exposure to low (0.1 and 1.0 mg/kg/day) and to high (50, 100 and 500 mg/kg/day) DBP doses decreases the expression of genes involved in cholesterol transport and in steroidogenesis, and consequently, decreases testosterone synthesis in fetal testes. In adult testes, Show et al. (2003) revealed that low intratesticular testosterone concentrations impair the integrity of vimentin filaments present in Sertoli cells, and spermatogenesis in rats. The authors suggest that changes in vimentin filaments may represent a general mechanism used by Sertoli cell to release adherent germ cells or to communicate a death signals to germ cells after reduction of intratesticular T, causing damage to sperm production.

Alterations in the vimentin cytoskeleton also occured after exposure to phthalic esters. Kleymenova et al. (2005) exposed Sprague-Dawley rats to 500mg/kg/day of DBP *in utero* and observed alterations in the vimetin cytoskeleton of fetal Sertoli cells and a loss in Sertoli cell-gonocyte contact. In another study, MEHP altered Sertoli cell vimentin filaments and germ cell apoptosis in young rat testes (Richburg and Boekelheide, 1996). In this present study, testes were analyzed in adulthood and no drastic change was verified in testes or serum hormones of animals exposed to DBP, only small changes in sperm production. In addiction, there were few changes in spermatic efficiency in animals exposed to a high-fat diet. Therefore, it is possible to conclude that the combinaed exposures acted synergistically and impaired intratesticular testosterone production, reducing the spermatic efficiency and the DSP. Proteins directly responsible for T synthesis in rat testes include StAR, P450scc, 3β-HSD, 17α-hidroxylase/C17,20-lyase and 17β-HSD (Payne and Youngblood, 1995; Miller and Strauss, 1999). Here, 17β-HSD expression did

not show any variation between groups, as well as the number of Leydig cells, but the expression of the other proteins was not investigated.

Despite normalization in lipid metabolism in adulthood, the group exposed to DBP and to a high-fat diet exhibited an ~28% increment in body weight at weaning, unlikely that observed in the groups exposed only to these treatments. Therefore, it is possible that increased body weight could be accomplished by an unbalance in sexual steroid and leptin concentrations of mothers and offspring, as has been previously reported by White et al. (2009) and by Landry et al. (2013). If so, the inbalance in sex steroid milieu during gestation and neonatal life might be interfered with in the Leydig cell differentiation, or in other paracrine regulatory factors, thus affecting their future secretory capacity. In addiction, it is also possible that low sperm counts may be due to the influence of high-fat maternal intake and increased body weight of offspring on gonocyte differentiation. In fact, we recently observed that male Wistar rats born from obese mothers induced by the same high-fat diet showed alterations in serum steroids and gonocyte differentiation (Christante et al, 2013) and a reduction in spermatic efficiency of 30% (unpublished data).

Some cytokines, such as IL-1α, IL-1β and TNF-α, are known for their importance in spermatogenesis, since they are involved in maintenance of the immunosuppressive microenvironment of the seminiferous tubules (Hedger and Meinhardt, 2003). These cytokines are usually produced in testes, yet in low concentrations, being produced at higher concentrations in inflammation situations (Matsuzawa, 2006) or during adipogenesis (Desai and Ross, 2011). As seen in our results, the serum IL-1β and TNF-α concentrations did not vary significantly among the experimental groups. Similarly, the intratesticular levels changed slightly, showing that exposure to DBP or to a high-fat diet, or both treatments, did not affect the production of inflammatory cytokines. Therefore, the drop in sperm production here observed was not related to alterations in these cytokines.

High-fat diet and DBP exposure also had an effect on sperm transit time through the epididymis and epididymal sperm concentration. The sperm transit time was ~39% and 34% higher in the caput/corpus and cauda of the epididymis, respectively, and the sperm concentration was slightly higher, despite the lower daily sperm production verified in this group. These data associated with the increase in the epididymis cauda weight demonstrate

abnormal sperm storage in the epididymis. The epididymis is an important organ in which spermatozoa undergo maturation (Orgebin-Crist, 1969) and sperm transit through this organ is modulated by androgens and the contractile activity of smooth muscle (Garcia et al., 2012; Borges et al., 2013). Moreover, epididymal secretory activity is androgen-dependent (Dhar and Setty, 1976). As previously stated, intratesticular testosterone synthesis was impaired in animals subjected to concomitant exposures. Thus, we can conclude that the increase in sperm transit time and in sperm storage was due to a reduction in intratesticular androgen levels. Some studies indicate that many environmental chemicals are associated with decreased numbers of sperm stored in the cauda epididymis, with little or no reduction in sperm production (Klinefelter and Suarez, 1997; Goyal et al., 2001). This suggests that the epididymis is the target of these toxic substances, and that the transit of sperm through the epididymis is accelerated by these exposures (Klinefelter and Suarez, 1997). In this present study, DBP associated with high-fat diet impaired sperm production but increased the sperm reserves and sperm transit time in the epididymis. Indeed, no change was observed in sperm motility, leading us to believe that there was no drastic problem with sperm maturation. We can not infer that no losses to fertility occurred in this rodent, because analyses for this purpose were not performed.

This study shows that lifetime low DBP doses exposure results in obesity and dislipidemy at adulthood. Even though such exposure did not induce evident alterations in testis structure, this exposure impaired testicular estrogen synthesis and resulted in a tendency to decrease sperm production. The chronic intake of high-fat diet also was associated with a trend to reduction sperm production. In this case, the reduction was not related to obesity or sexual steroid alterations, but was probably due to an impairment in AR content and signaling. In combination, both exposures act synergically in damaging intratesticular testosterone synthesis and sperm production. This is the first report to demonstrate that the fat nutritional environment may adversely affect the response of testes to phthalates, and provides new information for understanding the consequences of exposure to ECD in decreasing human sperm counts and fertility, as previously indicated (Sharpe and Skakkebaek, 2008; Swan, 2008; Merzenich et al., 2010).

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6. References

Akingbemi BT. 2005. Estrogen regulation of testicular function. *Reprod Biol Endocrinol* 3:51. doi:10.1186/1477-7827-3-51

Andrade AJM, Grande SW, Talsness CE, Gerickea C, Grote K, Golombiewski A, Sterner-kock A, and Chahoud I. 2006. A dose response study following *in utero* and lactational exposure to di-(2-ethylhexyl) phthalate (DEHP): reproductive effects on adult male offspring rats. *Toxicology* 228, 85-97. doi: 10.1016/j.tox.2006.10.005

Bao AM, Man XM, Guo XJ, Dong HB, Wang FQ, Sun H, Wang YB, Zhou ZM, and Sha JH. 2011. Effects of di-n-butyl phthalate on male rat reproduction following pubertal exposure. *Asian J. Androl* 13(5), 702-9. doi:10.1038/aja.2011.76

Barlow NJ, Phillips SL, Wallace DG, Sar M, Gaido KW, and Foster PMD. 2003. Quantitive changes in gene expression in fetal rat testes following exposure to di(n-butyl) phthalate. *Toxicol Sci* 73, 431-451. doi: 10.1093/toxsci/kfg087

Berndtson WE. 1977. Methods for quantifying mammalian spermatogenesis: a review. *J Anim Sci* 44, 818-33.

Beu CC, Orsi AM, and Domeniconi RF. 2009. Structure of the lining epithelium of the cauda epididymis of the golden hamster. *Anat Histol Embryol* 38(1), 49-57. doi: 10.1111/j.1439-0264.2008.00891.x

Blount BC, Silva MJ, Caudill SP, Needham LL, Pirkle JL, Sampson EJ, Licier GW, Jackson RJ, and Brock JW. 2000. Levels of seven urinary phthalate metabolites in a human reference population. *Environ Health Perspect* 108, 979-982.

Borges CS, Missassi G, Pacini ESA, Kigutu LRA, Sanabria M, Silva RF, Banzato TP, Perobelli JE, Pupo AS, and Kempinas WDG. 2013. Slimmer or fertile? Pharmacological mechanismis involved in reduce sperm quality and fertility in rats exposed to the anorexigen sibutramine**.** *Plos One* 8(6). doi: 10.1371/journal.pone.0066091

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248–254.

Carruthers CM, and Foster PMD. 2005. Critical window of male reproductive tract development in rats following gestational exposure to di-n-butyl phthalate. *Birth Defects Res* B 74, 277–285. doi: 10.1002/bdrb.20050

Chauvigné F, Menuet A, Lesné L, Chagnon MC, Chevrier C, Regnier JF, Angerer J, and Jégou B. 2009. Time- and dose-related effects of di-(2-ethylhexyl) phthalate and its main metabolites on the function of the rat fetal testis *in vitro*. *Environ Health Perspect* 117(4), 515-521. doi: 10.1289/ehp.11870

Christante CM, Taboga SR, Pinto-Fochi ME, and Góes RM. 2013. Maternal obesity disturbs the postnatal development of gonocytes in the rat without impairment of testis structure at prepubertal age. *Reproduction* 146, 549-558. doi: 10.1530/REP-13-0037

Christiansen S, Boerg J, Axelstad M, Dalgaard M, Vinggaard AM, Metzdorff SB, and Hass U. 2010. Low-dose perinatal exposure to di(2-ethylhexyl) phthalate induces anti-androgenic effects in male rats. *Reprod Toxicol* 30, 313-321. doi:10.1016/j.reprotox.2010.04.005

Desai M, and Ross MG. 2011. Fetal programming of adipose tissue: effects of intrauterine growth restriction and maternal obesity/high-fat diet. *Semin Reprod Med* 293, 237-45. doi: 10.1055/s-0031-1275517.

Dhar JD, and Setty BS. 1976. Studies on the physiology and biochemistry of mammalian epididymis: effect of flutamide, a nonsteroidal antiandrogen, on the epididymis of the rat. *Fertil Steril* 27(5), 566-576.

Fernandes GS, Arena AC, Fernandez CDB, Mercadante A, Barbisan LF, and Kempinas WG. 2007. Reproductive effects in male rats exposed to diuron. *Reprod Toxicol* 23(1), 106-112. doi:1 0.1016/j.reprotox.2006.09.002

Fernandez CBD, Bellentani FF, Fernandes GSA, Perobelli JE, Favareto APA, Nascimento AF, Cicogna AC, and Kempinas WDG. 2011. Diet-induced obesity in rats leads to a decrease in sperm motility. *Reprod Biol Endocrinol* 9:32.

Fisher JS, Macpherson S, Marchetti N, and Sharpe RM. 2003. Human 'testicular dysgenesis syndrome': a possible model using *in utero* exposure of the rat to dibutyl phthalate. *Hum Reprod* 18, 1383-1394. doi: 10.1093/humrep/deg273

Filipiak E, Walczak-Jedrzejowska R, Krupinski M, Oszukowsha E, Marchlewska K, Dlugonski J, Kula K. and Slowikowka-Hilczer J. 2011. Di(*n*-butyl) phthalate has no effect on the rat prepubertal testis despite its estrogenic activity *in vitro*. *Folia Histochem Cytobiol* 49(4), 685-689. doi: 10.5603/FHC.2011.0092

Foster PMD. 2006. Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. *Int J Androl* 29, 140-147. doi:10.1111/j.1365-2605.2005.00563.x

Gaido KW, Hensley JB, Liu D, Wallace DG, Borghoff S, Johnson KJ, Hall SJ, and Boekelheide K. 2007. Fetal mouse phthalate exposure shows that gonocyte multinucleation is not associated with decrease testicular testosterone. *Toxicol Sci* 97(2), 491-503. doi: 10.1093/toxsci/kfm049

Gangolli SD. 1982. Testicular effects of phthalate esters. *Environ Health Perspect* 45, 77-84.

Garcia PV, Barbieri MF, Perobelli JE, Consonni SR, Mesquita SFP, Kempinas WDG, and Pereira LAV. 2012. Morphometric-stereological and functional epididymal alterations and a decrease in fertility in rats trated with finasteride and after a 30-day post-treatment recovery period. *Fertil Steril* 97, 1444-1451. doi:10.1016/j.fertnstert.2012.03.025

Giaugulli VA, Kaufman JM, and Vermeulen A. 1994. Pathogenesis of the decrease androgen levels in obese men. *J Clin Endocrinol Metab* 79, 997-1000.

Grande SW, Andrade AJM, Talsness CE, Grote K, Golombiewski A, Sterner-Kock A, and Chahoud I. 2007. A dose-reponse study following *in utero* and lactational exposure to di-(2-ethylhexyl) phthalate (DEHP): reproductive effects on adult female offspring rats. *Toxicology* 229(1-2), 114-122. doi: 10.1016/j.tox.2006.10.005

Gray LEJr. 1998. Xenoendocrine disrupters: laboratory studies on male reproductive effects. *Toxicol Lett* 102-103, 331-335. doi: 10.1016/S0378-4274(98)00327-0

Gray LEJr, Wilson VS, Stoker T, Lambright C, Furr J, Noriega N, Howdeshell K, Ankley GT, and Guillette L. 2006. Adverse effects of environmental antiandrogens and androgens on reproductive development in mammals. *Int J Androl* 29, 96-104. doi: 10.1111/j.1365-2605.2005.00636.x

Gray TJB, and Gangolli SD. 1986. Aspects of the Testicular Toxicity of Phthalate Esters. *Environ Health Perspect* 65, 229-235.

Góes RM, Zanetoni C, Tomiosso TC, Ribeiro DL, and Taboga SR. 2007. Surgical and chemical castration induce differential histological response in prostate lobes of Mongolian gerbil. *Micron* 38(3), 231-236. doi: 10.1016/j.micron.2006.06.016

Goyal HO, Graden TD, Mansour M, Williams CS, Kamaleldin A, and Srivastava KK. 2001. Diethylstilbestrol-treated adult rats with altered epididymal sperm numbers and sperm motility parameters, but withought alterations in sperm production and sperm morphology. *Biol Reprod* 64(3), 927-934. doi: 10.1095/biolreprod64.3.927

Guzick DS, Overstreet JW, Factor-Litvak P, Brazil CK, Nakajima ST, Coutifaris C, Carson SA, Cisneros P, Steinkampf MP, Hill JA, Xu D, and Vogel DL. 2001. Sperm morphology, motility, and concentration in fertile and infertile men. *N Engl J Med* 345, 1388-1393. doi: 10.1056/NEJMoa003005

Grün F, and Blumberg B. 2006. Environmental obesogens: organotins and endocrine disruption via nuclear receptor signaling. *Endocrinology* 147, 50-55. doi: 10.1210/en.2005-1129

Hammoud AO, Gibson M, Peterson CM, Hamilton BD, and Carrel DT. 2006. Obesity and male reproductive potential**.** *J Androl* 27(5), 619-626. doi: 10.2164/jandrol.106.000125

Hammoud AO, Gibson M, Peterson CM, Meikle AW, and Carrel DT. 2008. Impact of male obesity on infertility: a critical review of the current literature. *Fertil Steril* 90, 897-904. doi: 10.1016/j.fertnstert.2008.08.026

Hatch EE, Nelson JW, Stahlhut RW, and Webster TF. 2010. Association of Endocrine Disruptors and Obesity: Perspectives from Epidemiologic Studies. *Int J Androl* 33(2), 324-332. doi:10.1111/j.1365-2605.2009.01035.x.

Hedger MP, and Meinhardt A. 2003. Cytokines and the immune-testicular axis. *J Reprod Immunol* 58, 1-26. doi: 10.1016/S0165-0378(02)00060-8

Heindel JJ, and vom Saal FS. 2009. Role of nutrition and environmental endocrine disrupting chemicals during the perinatal period on the aetiology of obesity. Mol Cell Endocrinol 304(1-2), 90-96. doi: 10.1016/j.mce.2009.02.025

Heng K, Anand-Ivell R, Teerds K, and Ivell R. 2012. The endocrine disruptors dibutyl phthalate (DBP) and diethylstilbestrol (DES) influence Leydig cell regeneration following ethane dimethane sulphonate treatment of adult male rats. *Int J Androl* 35, 353-363. doi: 10.1111/j.1365-2605.2011.01231.x

Howdeshell KL, Wilson VS, Furr J, Lambright CR, Rider CV, Blystone CR, Horchkiss AK, and Gray LEJr. 2008. A mixture of five phthalate esters inhibits fetal testicular testosterone production in the Sprague-Dawley rat in a cumulative, dose-additive manner. *Toxicol Sci* 105(1), 153-165. doi: 10.1093/toxsci/kfn077

Hurst CH, and Waxman DJ. 2003. Activation of PPARα and PPARγ by environmental phthalate monoesters. *Toxicol Sci* 74(2), 297-308. doi: 10.1093/toxsci/kfg145

Ishikawa T, Glidewell-Kenney C, and Jameson JL. 2006. Aromatase-independent testosterone conversion into estrogenic steroids inhibited by 5α-reductase inhibitor. *J Steroid Biochem Mol Biol* 98, 133–138. doi: 10.1016/j.jsbmb.2005.09.004

Jiang J, Ma L, Yuan L, Wang X, and Zhang W. 2007. Study on developmental abnormalities in hypospadiac male rats induced by maternal exposure to di-n-butyl phthalate (DBP). *Toxicology* 232, 286-293. doi: 10.1016/j.tox.2007.01.018

Jobling S, Reynolds T, White R, Parker MG, and Sumpter JP. 1995. A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environ Health Perspect* 103(6), 582-587.

Johnson L, Varner DD, Roberts ME, Smith TL, Keillor GE, and Scrutchfield WL. 2000. Efficiency of spermatogenesis: a comparative approach. *Anim Reprod Sci* 60-61, 471–80. doi: 10.1016/S0378-4320(00)00108-1

Juana L, Bárbara VG, Martín MT, Agustín C, Guillermo RB, and Guadalipe O. 2010. Neither testosterone levels nor aggression decrease when the male Mongolian gerbil (*Meriones unguiculatus*) displays paternal behavior. *Horm Behav* 57(3), 271-275. doi: 10.1016/j.yhbeh.2009.12.007

Kavlock R, Boekelheide K, Chapin R, Cunningham M, Faustman E, Foster PMD, Golub M, Henderson R, Hinberg I, Little R, Seed J, Shea K, Tabacova S, Tyl R, Williams P, and Zacharewski T. 2002. Center for the evaluation of risks to human reproduction:

phthalates expert panel report on the reproductive and developmental toxicity of di(2-ethylhexyl) phthalate. *Reprod Toxicol* 16, 529-653.

Kim H, Saito K, Ishizuka M, Kazusaka A, and Fujita S. 2003. Short period exposure to si-(2-ethylhexyl) phthalate regulates testosterone metabolism in testis of pubertal rats. *Arch Toxicol* 77, 446-451. doi: 10.1007/s00204-003-0466-7

Kim TS, Jung KK, Kim SS, Kang IH, Baek JH, Nam H, Hong S, Lee BM, Hong JT, Oh KW, Kim HS, Han SY, and Kang TS. 2010. Effects of in utero exposure to di(n-butyl) phthalate on development of male reproductive tracts in sprague-dawley rats. *J Toxicol Environ Health A* 73, 1544–1559. doi: 10.1080/15287394.2010.511579

Kleymenova E, Swanson C, Boekelheide K, and Gaido KW. 2005. Exposure in utero to di(n-butyl) phthalate alters the vimentin cytoskeleton of fetal rat Sertoli cells and disrupts Sertoli cell-gonocyte contact. *Biol Reprod* 73, 482-490. doi: 10.1095/biolreprod.104.037184

Klinefelter GR, and Suarez JD. 1997. Toxicant-induced acceleration of epididymal sperm transit: androgen-dependent proteins may be involved. *Reprod Toxicol* 11(4), 511-519. doi: 10.1016/S0890-6238(97)00018-X

Kort HI, Massey JB, Elsner CW, Mitchell-Leef D, Shapiro DB, Witt MA, and Roudebush WE. 2006. Impact of body mass index values on sperm quantity and quality. *J Androl* 27, 450-452. doi: 10.2164/jandrol.05124

Lambright CS, Wilson VS, Furr JR, Wolf CJ, Noriega N, and Gray LEJr. 2003. Effects of endocrine disrupting chemicals on fetal testes hormone production. *Toxicologist* 72:272.

Lambrot R, Muczynski V, Lécureuil C, Angenard G, Coffigny H, Pairaut C, Moison D, Frydman R, Habert R, and Rouiller-Fabre V. 2009. Phthalates impair germ cell development in the human fetal testis *in vitro* without chance in testosterone production. *Environ Health Perspect* 117(1), 32-37. doi:10.1289/ehp.11146

Landry D, Cloutier F, and Marton LJ. 2013. Implications of leptin in neuroendocrine regulation of male reproduction. *Reprod Biol* I3, I-14. doi: 10.1016/j.repbio.2012.12.001

Lee KY, Shibutani M, Takagi H, Kato N, Takigami S, Uneyama C, Hirose M. 2004. Diverse developmental toxicity of di-n-butyl phthalate in both sexes of rat offspring after maternal exposure during the period from late gestation through lactation. *Toxicology* 203, 221-238. doi: 10.1016/j.tox.2004.06.013

Lehraiki A, Racine C, Krust A, habert R, and Levacher C. 2009. Phthalates impair germ cell number in the mouse fetal testis by an androgen- and estrogen-independent mechanism. *Toxicol Sci* 111(2), 372-382. doi:10.1093/toxsci/kfp153

Leopoldo AS, Lima-Leopoldo AP, Sugizaki MM, do Nascimento AF, de Campos DHS, Luvizotto RAM, Castardeli E, Alvez CAB, Brum PC, and Cicogna AC. 2011. Involvement of L-type calcium channel and SERCA2a in myocardial dysfunction induced by obesity. *J Cell Physiol* 226, 2934-2942. doi: 10.1002/jcp.22643

Lottrup G, Andersson AM, Leffers H, Mortensen GK, Toppari J, Skakkebaek NE, and Main KM. 2006. Possible impact of phthalates on infant reproductive health. *Int Jo Androl* 29, 172–180. doi: 10.1111/j.1365-2605.2005.00642.x

Lubrano C, Genovesi G, Specchia P, Costantini D, Mariani S, Petrangeli E, Lenzi A, and Gnessi L. 2013. Obesity and metabolic comorbidities: environmental diseases? *Oxid Med Cell* 2013. doi: 10.1155/2013/640673.

MacDonald AA, Herbison GP, Showell M, and Farquhar CM. 2010. The impact of body mass index on semen parameters and reproductive hormones in human males: a systematic review with meta-analysis. *Hum Reprod Update* 16(3), 293-311. doi: 10.1093/humupd/dmp047

Manikkan M, Tracey R, Guerrero-Bosagna C, and Skinner MK. 2013. Plastics derived endocrine disruptors (BPA, DEHP and DBP) induce epigenetic transgenerational inheritance of obesity, reproductive disease and sperm epimutations. *Plos One* 8(1). doi: 10.1371/journal.pone.0055387

Matsuzawa Y. 2006. Therapy insight: adipocytokines in metabolic syndrome and related cardiovascular disease. *Nat Clin Pract Card* 31, 35-42. doi:10.1038/ncpcardio0380

Merzenich H, Zeeb H, and Blettner M. 2010. Decreasing sperm quality: a global problem? *BMC Public Health* 10(24). doi:10.1186/1471-2458-10-24

Miller WL, and Strauss JF. 1999. Molecular pathology and mechanism of action of the steroidogenic acute regulatory protein, StARProceedings of Xth International Congresso n Hormonal Steroids, Quebec, Canada, 17-21 June 1998. *J Steroid Biochem Mol Biol* 69(1-6), 131-141. doi: 10.1016/S0960-0760(98)00153-8

Moore RW, Rudy TA, Lin T, Ko K, and Peterson RE. 2001. Abnormalities of sexual development in male rats with *in utero* and lactational exposure to the antiandrogenic plasticizer di(2-ethylhexyl) phthalate. *Environ Health Perspect* 109(3), 229-237.

Muczynski V, Lecureuil C, Messiaen S, Guerquin MJ, N´Tumba-Byn T, Moison D, Hodroj W, Benjelloun H, Beijer J, Livera G, Frydman R, Benachi A, Habert R, and Rouiller-Fabre V. 2012. Cellular and molecular effects of MEHP involving LXRα in human fetal testis and ovary. *Plos One* 7(10). doi:10.1371/journal.pone.0048266

Murature DA, Tang SY, Steinhardt G, and Dougherry RC. 1987. Phthalate esters and semen quality parameters. *Biomed Environ Mass Spectrom* 13, 473-477. doi: 10.1002/bms.1200140815

Mylchreest E, Sar M, Cattley RC, and Foster PMD. 1999. Disruption of androgen-regulated male reproductive development by di(*n*-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol Appl Pharmacol* 156(2), 81-95. doi: 10.1006/taap.1999.8643

Mylchreest E, Wallace DG, Cattley RC, and Foster PMD. 2000. Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to di(n-butyl) phthalate during late gestation**.** *Toxicol Sci* 55, 143–151. doi: 10.1093/toxsci/55.1.143

Nascimento AF, Sugizaki MM, Leopoldo AS, Luvizotto RAM, Nogueira CR, and Cicogna AC. 2008. A hypercaloric pellet-diet cycle induces obesity and co-morbidities in Wistar rats. *Arq Bras Endocrinol Metab* 52(6), 968-974.

Orgebin-Crist MC. 1969. Studies on the function of the epididymis. *Biol Reprod* 1, 155-175. doi: 10.1095/biolreprod1.Supplement_1.155

Parks LG, Ostby JS, Lambright CR, Abbott BD, Klinefelter GR, Barlow NJ, Gray LEJr. 2000. The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicol Sci* 58, 339-349. doi: 10.1093/toxsci/58.2.339

Payne AH, Youngblood GL. 1995. Regulation of expression of steroidogenic enzymes in Leydig cells. *Biol Reprod* 52, 217-225. doi: 10.1095/biolreprod52.2.217

Perez APS, Biancardi MF, Góes RM, Santos FA, and Taboga SR. 2011. Exposure to ethinylestradiol during prenatal development and postnatal supplementation with testosterone causes morphophysiological alterations in the prostate of male and female adult gerbils. *Int J Exp Path* 92, 121-130. doi: 10.1111/j.1365-2613.2010.00756.x

Perez APS, Biancardi MF, Vilamaior PSL, Góes RM, Santos FCA, and Taboga SR. 2012. Microscopic comparative study of the exposure effects of testosterone cypionate and

ethinylestradiol during prenatal fife on the prostatic tissue of adult gerbils. *Microsc Res Tech* 00, 000-000. doi: 10.1002/jemt.22034

Pinheiro PFF, Almeida CCD, Segatelli TM, Martinez M, adovani CR, and Martonez FE. 2003. Structure of the pelvic and penile urethra – relationship with the ducts of the sex accessory glands of the Mongolian gerbil (*Meriones unguiculatus*). *J Anat* 202, 431-444. doi: 10.1046/j.1469-7580.2003.00181.x

Pinto ME, Botta LS, Taboga SR, and Góes RM. 2010a. Neonatal gonocyte differentiation in Mongolian gerbil *Meriones unguiculatus* involves asynchronous maturation of seminiferous cords and rapid formation of transitional cell stage. *Anat Rec* 293(2), 310–419. doi: 10.1002/ar.21050

Pinto ME, Egydio FM, Taboga SR, Mendis-Handagama SMLC, and Góes RM. 2010b. Differentiation of Leydig cells in the Mongolian gerbil. *Microsc Res Tech* 73(2), 119–127. doi: 10.1002/jemt.20763

Ribeiro DL, Pinto ME, Rafacho A, Bosqueiro JR, Maeda SY, Anselmo-Franci JA, Taboga SR, and Góes RM. 2012. High-fat diet obesity associated with insulin resistance increases cell proliferation, estrogen receptor, and PI3K proteins in rat ventral prostate. *J Androl* 338, 854-865. doi: 10.2164/jandrol.111.016089

Richburg JH, and Boekelheide K. 1996. Mono-(2-ethylhexyl) phthalate rapidly alters both Sertoli cell vimentin filaments and germ cell apoptosis in young rat testes. *Toxicol Appl Pharmacol* 137, 42-50. doi: 10.1006/taap.1996.0055

Robb GW, Amann RP, and Killian GJ. 1978. Daily sperm production and epididymal sperm reserves of pubertal and adult rats. *J Reprod Fertil* 54(1), 103-117. doi: 10.1530/jrf.0.0540103

Scarano WR, Toledo FC, Guerra MT, Pinheiro PFF, Domeniconi RF, Felisbino SL, Campos SGP, Taboga SR, and Kempinas WG. 2010. Functional and Morphological Reproductive Aspects in Male Rats Exposed to Di-n-Butyl Phthalate (DBP) in Utero and During Lactation. *J Toxicol Environ Health A* 73, 972–984. doi: 10.1080/15287391003751760

Scott HM, Hutchinson GR, Mahood IM, Hallmark N, Welsh M, De Gendt K, Verhoeven G, O'Shaughnessy P, and Sharpe RM. 2007. Role of androgens in fetal testis development and dysgenesis. *Endocrinology* 148, 2027–2036. doi: 10.1210/en.2006-1622

Segatelli TM, Almeida CCD, Pinheiro PFF, Martinez M, Padovani CR, and Martinez FE. 2002. Kinetics of spermatogenesis in the Mongolian gerbil (*Meriones unguiculatus*).*Tissue Cell* 34(1), 7-13. doi: 101054/tice.2001.0218

Segatelli TM, França LR, Pinheiro PFF, Almeida CCD, Martinez M, and Martinez FE. 2004. Spermatogenic Cycle Length and Spermatogenic Efficiency in the Gerbil (*Meriones unguiculatus*). *J Androl* 25(6), 872-880. doi: 10.1002/j.1939-4640.2004.tb03156.x

Sharpe RM. 2010. Environmental/lifestyle effects on spermatogenesis. *Phil. Trans. R. Soc. B* 365, 1697-1712.

Sharpe RM, and Skakkebaek NE. 2008. Testicular dysgenesis syndrome: mechanistic insights and potential downstream effects. *Fertil Steril* 89(2), e33-e38. doi: 10.1016/j.fertnstert.2007.12.026

Show MD, Anway MD, Folmer JS, and Zirkin BR. 2003. Reduced intratesticular testosterone concentrations alters the polymerization state of the Sertoli cell intermediate filament cytoskeleton by degradation of vimentin. *Endocrinology* 144(12), 5530-5536. doi: 10.1210/en.2003-0735

Swan SH. 2008. Environmental phthalate exposures in relation to reproductive outcomes and other health endpoints in humans. *Environ Res* 108(2), 177-184. doi: 10.1016/j.envres.2008.08.007

Taboga SR, Vilamaior PSL, and Góes RM. 2009. Modulação androgênica e estrogênica na próstata: uma abordagem em modelos experimentais de roedores com enfoque na biologia estrutural. *Arq Bras Endocrinol Metab* 53(8).

Takeuchi S, Iida M, Kobayashi S, Jin K, Matsuda T, Kojima H. 2005. Differential effects of phthalate esters on transcriptional activities via human estrogen receptors α and β, and androgen receptor. *Toxicology* 210, 223-233. doi: 10.1016/j.tox.2005.02.002

Taylor BA, and Phillips SJ. 1996. Detection of obesity QTLs on mouse chromosomes 1 and 7 by selective DNA pooling. *Genomics* 34(3), 389-398. doi: 10.1006/geno.1996.0302

Valladares LE, and Payne AH. 1979. Induction of testicular aromatization by luteinizing hormone in mature rats. *Endocrinology* 105(2). doi: 10.1210/endo-105-2-431

Weibel ER, Kistler GS, and Scherle WF. 1966. Practical stereological methods for morphometric cytology. *J Cell Biol* 30, 23-38. doi: 10.1083/jcb.30.1.23

White CL, Purpera MN, and Morrison CD. 2009. Maternal obesity is necessary for programming effect of high-fat diet on offspring. *Am J Physiol Regul Integr Comp Physiol* 196, 1464-1472. doi: 10.1152/ajpregu.91015.2008

Wilson JD. 1978. Sexual differentiation. *Annu Rev Physiol* 40, 279-306. doi: 10.1146/annurev.ph.40.030178.001431

Zacharewski TR, Meek MD, Clemons JH, Wu ZF, Fielden MR, and Matthews JB. 1998. Examination of the *in vitro* and *in vivo* estrogenic activities of eight commercial phthalates esters. *Toxicol Sci* 46:282-293. doi: 10.1093/toxsci/46.2.282

Table 1. Composition of diets used in this study.

Composition	**Balanced diet**	**High-fat diet[#]**
Proteins (%)	22	20
Carbohydrates (%)	48	37
Lipids (%)	4	20
Others* (%)	26	23
Calories (Kcal/g)	3.16	4.8

* Others: vitamins, minerals and water.

[#] High-fat diet was composed of hypercaloric diets nutritionally identical, except for the added flavoring addictive (cheese, bacon, chocolate or vanilla).

Table 2. Body weight (g) and adiposity index of gerbils from control (C), di-n-butyl phthalate (Ph), high-fat diet (HF) and high-fat diet plus di-n-butyl phthalate (HFPh) groups.

Biometric parameters n=15	**Experimental groups**			
	C	**Ph**	**HF**	**HFPh**
Body weight (4 weeks) (g)	19.33 ± 4.11^{a}	18.21 ± 3.52^{a}	19.7 ± 5.4^{a}	24.7 ± 5.5^{b}
Body weight (14 weeks) (g)	59.24 ± 7.38^{a}	65.11 ± 7.38^{b}	$61.5 \pm 5.35^{a,b}$	$64 \pm 5.11^{a,b}$
Adiposity index (14 weeks)	2.18 ± 0.83^{a}	3.08 ± 0.96^{b}	2.30 ± 0.83^{a}	$2.53 \pm 0.81^{a,b}$

Values expressed as means ± standard deviations.

^{a,b} indicate statistical differences between groups ($p \leq 0.05$).

Table 3. Stereological analysis of the testicular componentes of Mongolian gerbil from control (C), di-n-butyl phthalate (Ph), high-fat diet (HF) and high-fat diet plus di-n-butyl phthalate (HFPh) groups.

Stereological analysis	**Experimental groups**			
	C	**Ph**	**HF**	**HFPh**
Seminiferous tubule	92.69 ± 2.95	92.25 ± 2.96	93.12 ± 2.48	93.08 ± 3.16
Epithelium	75.33 ± 4.53	75.27 ± 5.0	74.1 ± 5.43	75.26 ± 5.43
Lumen	12.72 ± 4.97[a,b]	11.76 ± 4.98[a]	14.15 ± 6.69[b]	12.63 ± 5.98[a,b]
Nuclei of Sertoli cells	3.04 ± 1.47[a]	3.76 ± 1.79[a,b]	3.55 ± 1.61[a,b]	3.75 ± 1.65[b]
Peritubular tissue	1.6 ± 1.17	1.46 ± 1.15	1.32 ± 1.05	1.43 ± 1.08
Interstitial tissue	.31 ± 2.95	7.75 ± 2.96	6.88 ± 2.48	6.92 ± 3.16
Leydig cells	2.38 ± 1.38	2.48 ± 1.51	2.01 ± 1.32	2.49 ± 1.41
Other cells	1.41 ± 1.0	1.51 ± 1.44	1.36 ± 1.02	1.41 ± 1.35
Blood vessels	0.44 ± 1.02	0.42 ± 0.76	0.24 ± 0.56	0.44 ± 1.21
Lymphatic space	3.09 ± 1.9[a,b]	3.35 ± 1.6[a,b]	3.27 ± 1.63[a]	2.59 ± 1.63[b]

Values expressed as means ± standard deviations.

[a,b] indicate statistical differences between groups ($p \leq 0.05$).

Table 4. Sperm parameters of Mongolian gerbils from control (C), di-n-butyl phthalate (Ph), high-fat diet (HF) and high-fat diet plus di-n-butyl phthalate (HFPh) groups.

Sperm parameters	Experimental groups			
	C	**Ph**	**HF**	**HFPh**
Sperm number				
x10^6/testis	71.02 ± 9.72^{a}	67.28 ± 11.21^{a,b}	76.92 ± 6.93^{a}	63.8 ± 11.39^{b}
x10^6/g testis	149.91 ± 10.45^{a}	134.85 ± 15.53^{a,b}	143.80 ± 13.99^{a,b}	127.24 ± 15.54^{b}
Daily sperm production				
x10^6/ testis/day	12.22 ± 1.67^{a,b}	11.58 ± 1.93^{a,b}	13.24 ± 1.19^{a}	10.98 ± 1.96^{b}
x10^6/ g testis/day*	25.80 ± 3.52^{a}	23.21 ± 2.67^{a,b}	24.75 ± 2.41^{a,b}	21.90 ± 2.67^{b}
Sperm number in the caput/corpus of the epididymis (x10^6)	10.36 ± 3.57^{a}	11.56 ± 2.24^{a,b}	13.86 ± 1.73^{b}	12.58 ± 2.17^{a,b}
Sperm transit time in the caput/corpus (days)	0.84 ± 0.25^{a}	1.02 ± 0.29^{a,b}	1.05 ± 0.15^{a,b}	1.17 ± 0.25^{b}
Sperm number in the cauda of the epididymis (x10^6)	108.96 ± 24.63	125.91 ± 27.84	131.72 ± 26.43	130.68 ± 22.81
Sperm transit time in the cauda (days)	8.96 ± 1.78^{a}	10.94 ± 2.23^{a,b}	10.03 ± 2.17^{a,b}	12.01 ± 1.79^{b}
N	13	10	10	9

Values expressed as means ± standard deviations.

* Spermatic efficiency.

^{a,b} indicate statistical differences between groups ($p \leq 0.05$).

Figure legends

Figure 1. A. Testicular weight and gonadosomatic index of gerbils from control (C), di-n-butyl phthalate (Ph), high-fat diet (HF) and high-fat diet plus di-n-butyl phthalate (HFPh) groups. No change occurred in these two parameters. **B.** Total epididymal weight and caput/corpus and cauda region weights in different experimental groups, C, Ph, HF and HFPh. Note that in the Ph and HFPh groups, the epididymal weight was higher, and the caudal region of the HFPh group appeared to be more prominent. No difference was observed in the caput/corpus of the epididymis. Values are expressed as means ± standard deviations. ^{a,b} indicate statistical difference between groups ($p \leq 0.05$).

Figure 2. Lipid profile of gerbils from control (C), di-n-butyl phthalate (Ph), high-fat diet (HF) and high-fat diet plus di-n-butyl phthalate (HFPh) groups. In this analysis,total cholesterol, HDL, LDL and triglyceride values (mg/dL) were obtained. The HFPh group showed normal levels near those seen in the C group. Values are expressed as means ± standard deviations. * represent the statistical difference between groups ($p \leq 0.05$).

Figure 3. Hormonal profile of animals from control (C), di-n-butyl phthalate (Ph), high-fat diet (HF) and high-fat diet plus di-n-butyl phthalate (HFPh) groups. **A and B.** Serum testosterone (T) and estradiol (E_2) levels. **C and D.** Intratesticular T and E_2 concentrations. Note that intratesticular T levels were lower in the HFPh group and intratesticular E_2 levels were lower in the Ph group. Individual values of animals from each experimental group (circles) and the median value per group (line) are represented. * represent the statistical difference between groups ($p \leq 0.05$).

Figure 4. Androgen receptor (AR) expression by testicular cells of Mongolian gerbils from control (C), di-n-butyl phthalate (Ph), high-fat diet (HF) and high-fat diet plus di-n-butyl phthalate (HFPh) groups. **A**. AR and β-actin content wre obtained by Western blotting analysis. **B.** The rate of AR with respect to β-actin. β-actin was used as a positive control. **C - F.** AR immunohistochemistry in testicular sections of animals from C (**C**) Ph (**D**), HF (**E**) and HFPh (**F**) groups. Note that the AR expression was lower in the HF group, which is not detected by immunohistochemistry. Values are expressed as means ± standard deviations. ^{a,b} indicate statistical differences between groups ($p \leq 0.05$).

Figure 5. Estrogen receptor α (ERα) expression by testicular cells of Mongolian gerbils from control (C), di-n-butyl phthalate (Ph), high-fat diet (HF) and high-fat diet plus di-n-butyl phthalate (HFPh) groups. **A**. ERα and β-actin levels were obtained by Western blotting analysis. **B.** The rate of ERα with respect to β-actin. β-actin was used as a positive control. **C - F.** ERα immunohistochemistry in testicular sections of animals from C (**C**) Ph (**D**), HF (**E**) HFPh (**F**) groups. Note the higher ERα expression in the Ph group. Values are expressed as means ± standard errors. ^{a,b} indicate statistical differences between groups ($p \leq 0.05$). Scale bar: 50 μm.

Figure 6. Testicular 17β-HSD protein expression on control (C), di-n-butyl phthalate (Ph), high-fat diet (HF) and high-fat diet plus di-n-butyl phthalate (HFPh) groups. **A.** 17β-HSD and β-actin levels were determinated by Western blotting analysis. **B.** The rate of 17β-HSD with respect to β-actin. β-actin was used as a positive control. Values are expressed as means ± standard errors.

Figure 7. Serum (**A** and **B**) and intratesticular (**C** and **D**) TNF-α and IL-1β cytokine concentrations of animals from control (C), di-n-butyl phthalate (Ph), high-fat diet (HF) and high-fat diet plus di-n-butyl phthalate (HFPh) groups. Values are expressed as means ± standard deviations.

Figure 8. Sperm motility in control (C), di-n-butyl phthalate (Ph), high-fat diet (HF) and high-fat diet plus di-n-butyl phthalate (HFPh) groups. Sperm were analyzed according to the movement type: progressive movement, non-progressive movement and immotile. No change was observed in this analysis. Values are expressed as means ± standard deviations. n = 8 animals/group.

Figure 1

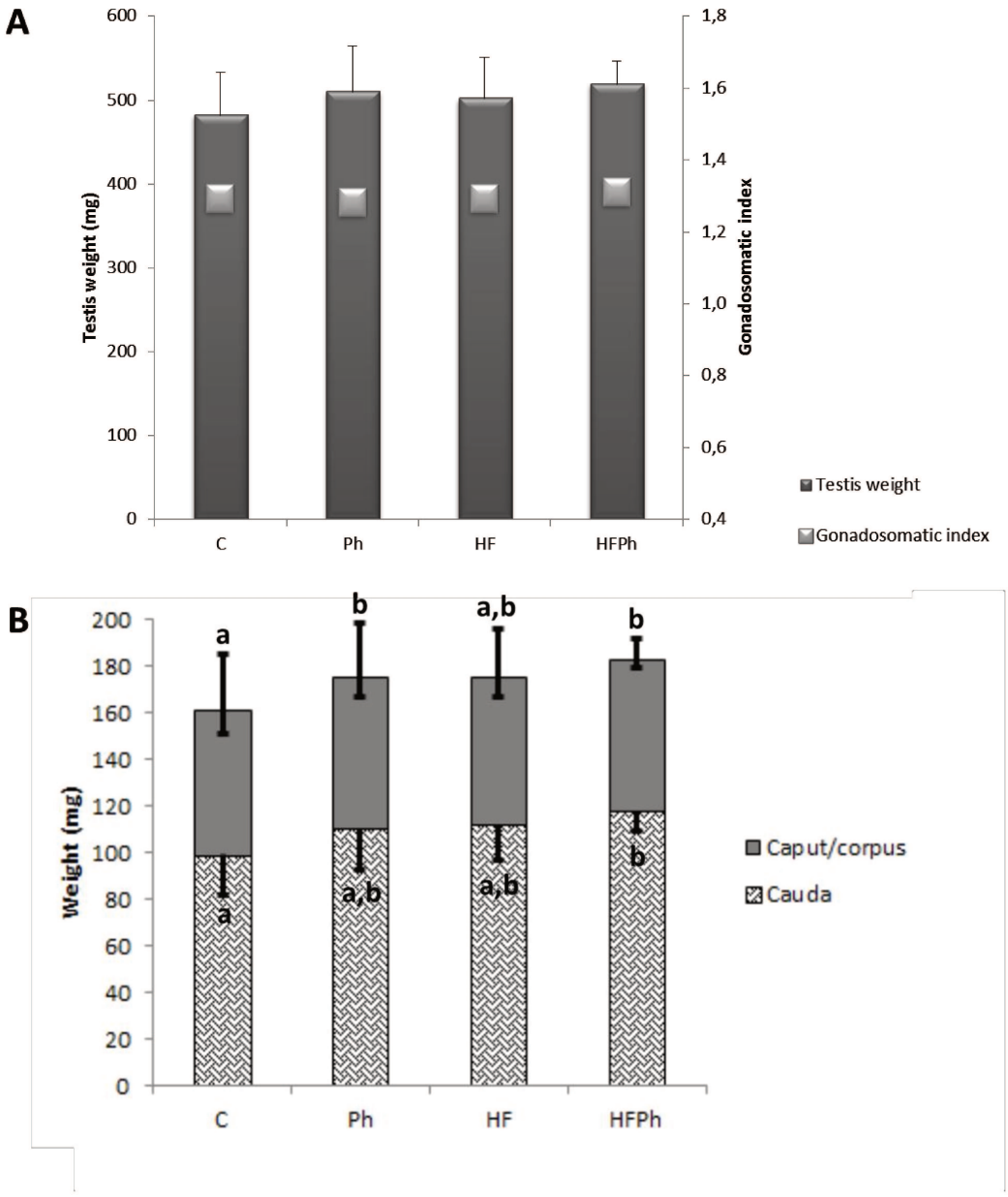

A
600
500
400
300
200
100
0
Testis weight (mg)
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
Gonadosomatic index
C
Ph
HF
HFPh
Testis weight
Gonadosomatic index
B
200
180
160
140
120
100
80
60
40
20
0
Weight (mg)
a
b
a,b
b
a
a,b
a,b
b
C
Ph
HF
HFPh
Caput/corpus
Cauda

Figure 2

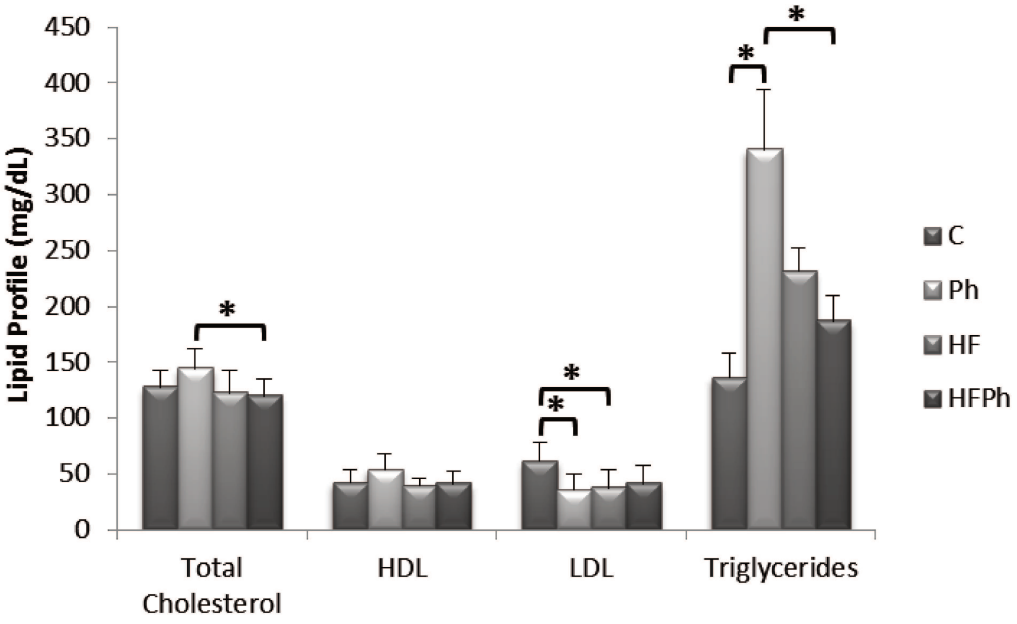

Lipid Profile (mg/dL)
450
400
350
300
250
200
150
100
50
0
Total Cholesterol
HDL
LDL
Triglycerides
C
Ph
HF
HFPh
*
*
*
*
*
*

Figure 3

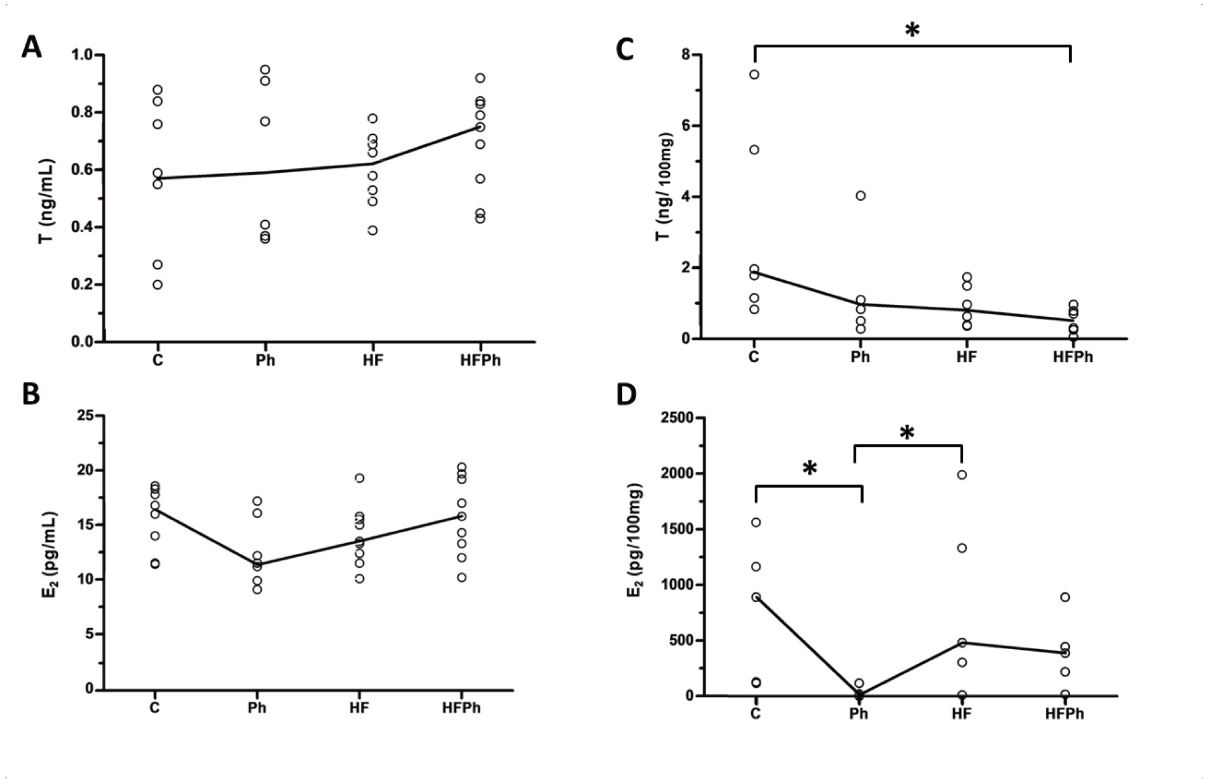

A
T (ng/mL)
1.0
0.8
0.6
0.4
0.2
0.0
C
Ph
HF
HFPh
B
E2 (pg/mL)
25
20
15
10
5
0
C
Ph
HF
HFPh
C
T (ng/ 100mg)
8
6
4
2
0
*
C
Ph
HF
HFPh
D
E2 (pg/100mg)
2500
2000
1500
1000
500
0
*
*
C
Ph
HF
HFPh

Figure 4

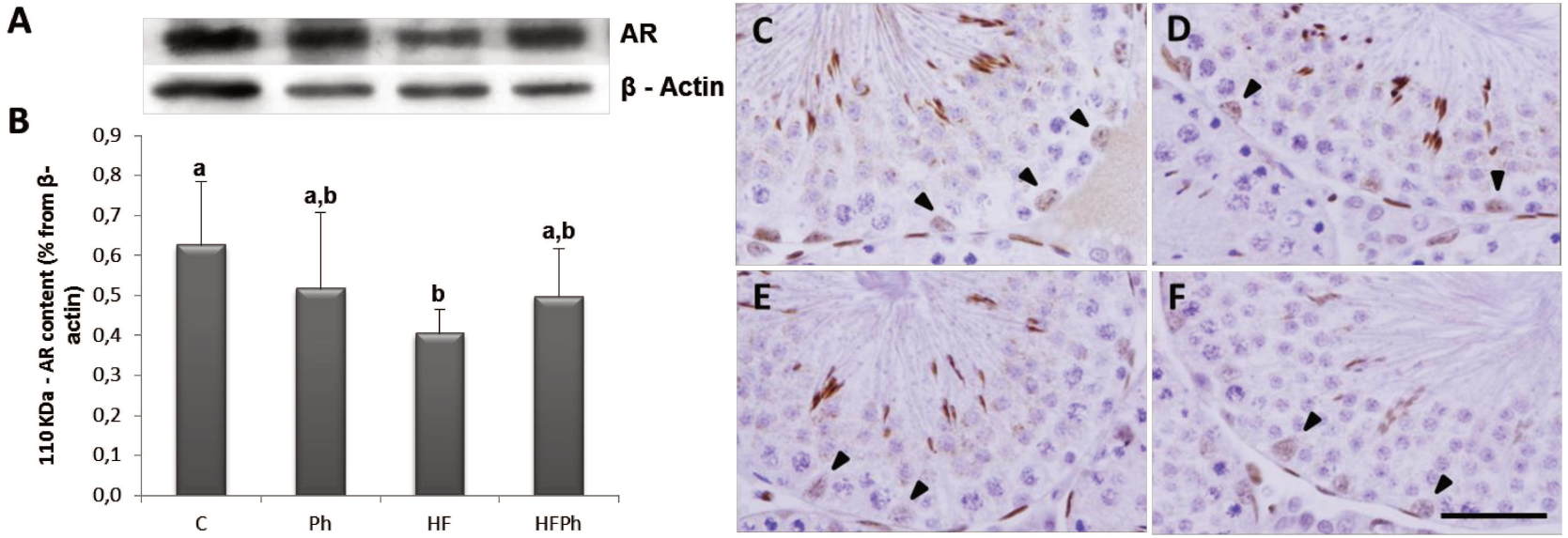

A
AR
β - Actin
B
110 KDa - AR content (% from β-actin)
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
a
a,b
b
a,b
C
Ph
HF
HFPh
C
D
E
F

Figure 5

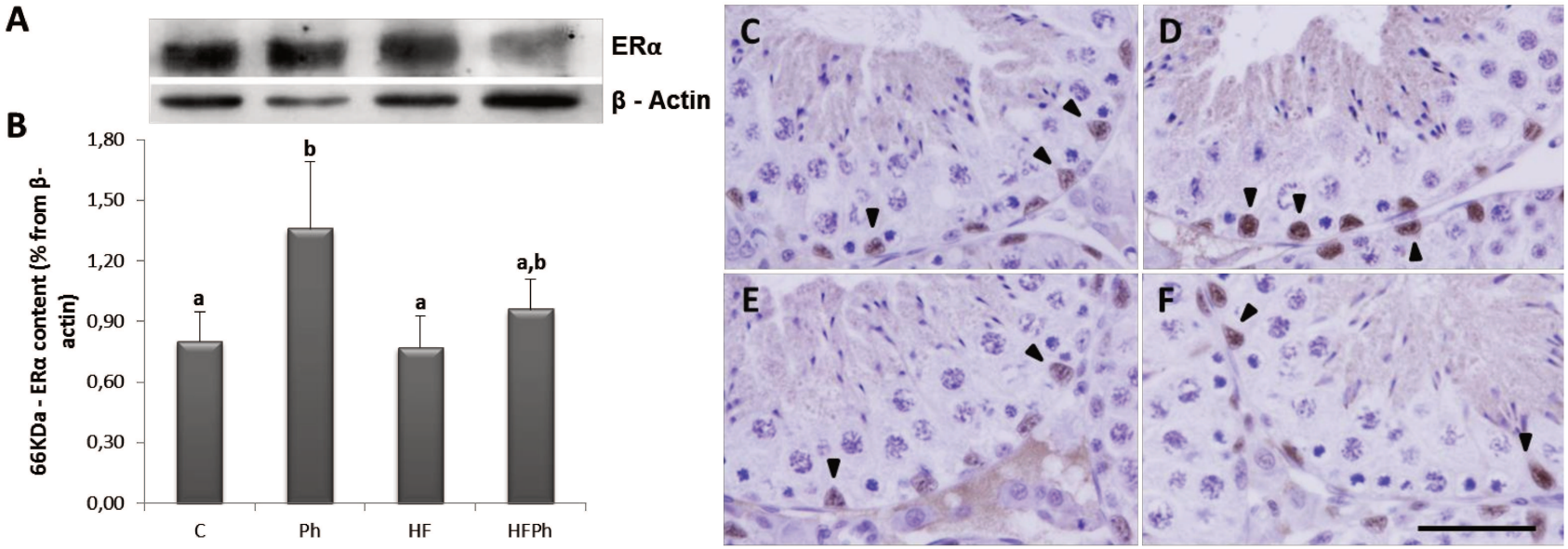

A
ERα
β - Actin
B
66KDa - ERα content (% from β-actin)
1,80
1,50
1,20
0,90
0,60
0,30
0,00
a
b
a
a,b
C
Ph
HF
HFPh
C
D
E
F

Figure 6

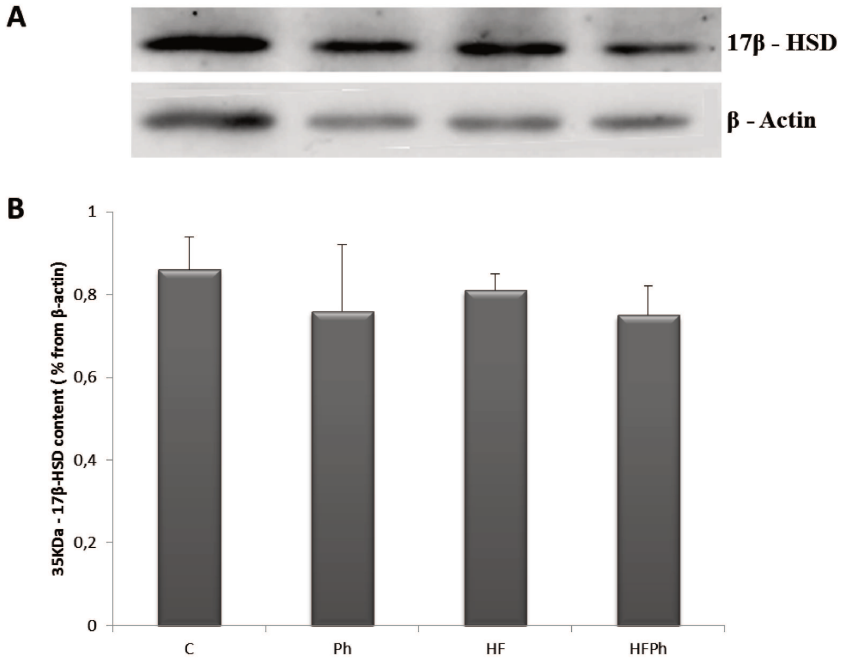

A
17β - HSD
β - Actin
B
35KDa - 17β-HSD content (% from β-actin)
1
0,8
0,6
0,4
0,2
0
C
Ph
HF
HFPh

Figure 7

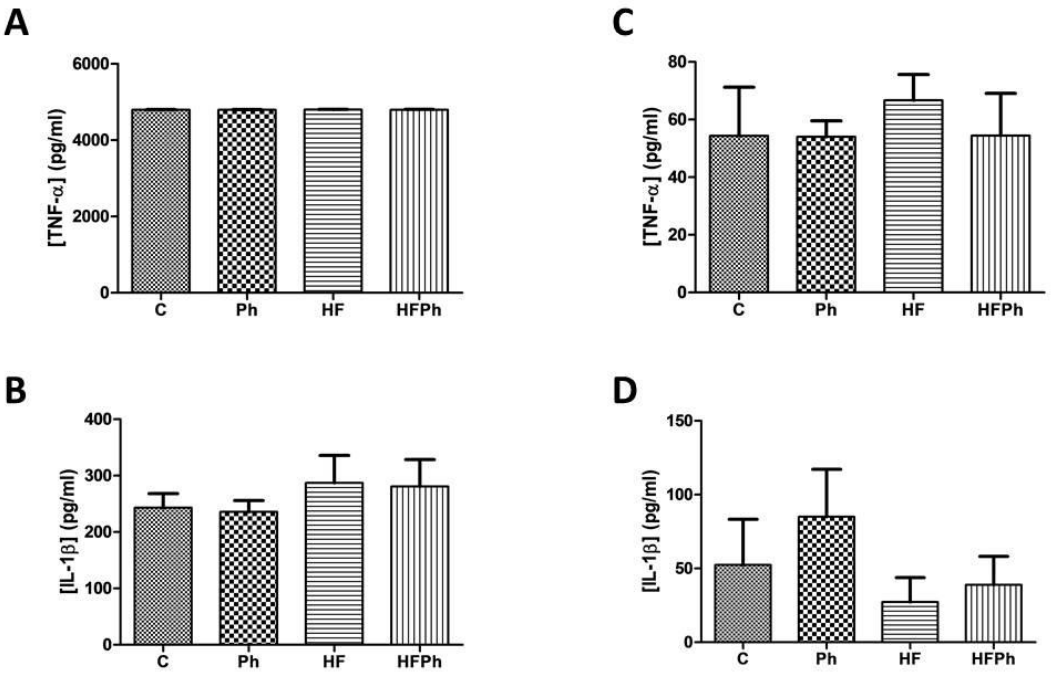
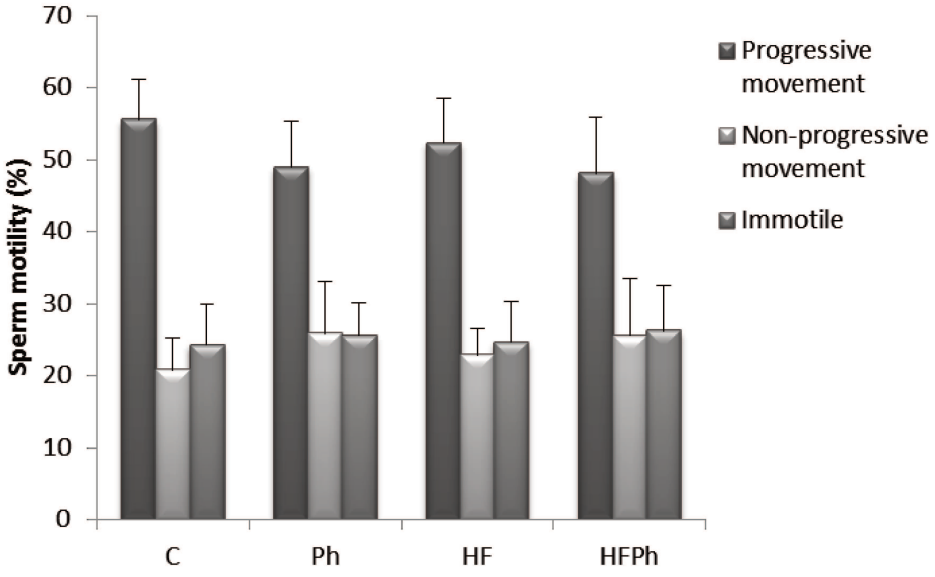

A
[TNF-α] (pg/ml)
6000
4000
2000
0
C
Ph
HF
HFPh
C
[TNF-α] (pg/ml)
80
60
40
20
0
C
Ph
HF
HFPh
B
[IL-1β] (pg/ml)
400
300
200
100
0
C
Ph
HF
HFPh
D
[IL-1β] (pg/ml)
150
100
50
0
C
Ph
HF
HFPh

Figure 8


70
60
50
40
30
20
10
0
Sperm motility (%)
C
Ph
HF
HFPh
Progressive movement
Non-progressive movement
Immotile

Supplementary material

Table I. Profile of saturated and unsaturated fatyy acids of the diets (%).

Fatty acids	Balanced diet	High-fat diet
Caproic (c6:0)	0.00	0.02
Caprylic (c8:0)	0.03	0.03
Capric (c10:0)	0.02	0.05
Lauric (c12:0)	0.33	0.25
Myristic (c14:0)	0.30	0.33
Palmitic (c16:0)	16.56	15.09
Heptadecanoic (c17:0)	0.02	0.08
Stearic (c18:0)	3.90	4.36
Palmitoleic (c16:1)	0.06	0.15
Oleic (c18:1n9c)	27.96	37.94
Linoleic (c18:2n6c)	47.10	40.83
α-linoleic acid (c18:3n3)	3.72	0.87
Saturated fatty acids	21.16	20.21
Unsaturated fatty acids	78.84	79.79

O estudo experimental de exposição contínua dos gerbilos da Mongólia, desde gestação até a idade adulta, a baixas doses do desregulador endócrino di-n-butil ftalato (DBP), à dieta hiperlipídica ou a ambos permitiu concluir que:

1) A exposição conjunta a baixas doses de DBP (5 mg/kg peso corporal/dia) e à dieta hiperlipídica durante a gestação e lactação aumentou o peso corporal da prole masculina ao desmame e resultou em tendência à obesidade na idade adulta.

2) A exposição prolongada a baixas doses de DBP teve efeito obesogênico e dislipidêmico.

3) A exposição prolongada ao DBP não afetou a síntese de testosterona nem a expressão testicular de AR e 17β-HSD, mas reduziu a síntese intratesticular de estradiol e aumentou a expressão de ERα nesse órgão.

4) O excesso de lipídeos na dieta não afetou a biossíntese testicular de hormônios esteroides, mas diminuiu a expressão de AR no testículo.

5) A exposição isolada a baixas doses de DBP ou à dieta hiperlipídica ocasionou uma tendência à redução da eficiência espermática (produção diária de espermatozoides/g de testículo) nesse roedor, provavelmente devido ao comprometimento da síntese intratesticular de estrógenos, nos animais expostos ao DBP, e da sinalização celular, vista a baixa expressão de AR, nos animais expostos ao excesso de lipídeos.

6) A exposição conjunta a baixas doses de DBP e à dieta hiperlipídica teve efeito cumulativo sobre a eficiência espermática, causando uma redução de 15% neste parâmetro. Este prejuízo pode ter sido devido à redução de 83% na produção de testosterona intratesticular, que possivelmente comprometeu a espermatogênese deste roedor.

7) A exposição conjunta a baixas doses de DBP e à dieta hiperlipídica teve efeito sinérgico sobre o tempo de trânsito dos espermatozoides através do epidídimo, aumentando-o em 35%. Esse aumento foi devido ao maior tempo de armazenamento dos espermatozoides na causa do epidídimo. Nenhuma alteração na motilidade dos espermatozoides foi constatada para esse grupo ou para os grupos de exposição isolada.

Agarwal DK, Eustis S, Lamb JCIV, Reel JR, and Kluwe WM. 1986. Effects of di(2-ethylhexyl) phthalate in the gonadal pathophysiology, sperm morphology, and reproductive performance of male rats. *Environ Health Perspect* 65, 343-350.

Anderson WA, Castle L, Scotter MJ, Massey RC, and Sringall C. 2001. A biomarker approach to measuring human dietary exposure to certain phthalate diesters. *Food Addit Contam* 18, 1068-1074.

Andrade AJM, Grande SW, Talness CE, Gerickea C, Grote K, Golombiewski A, Sterner-Kock A, and Chahoud I. 2006. A dose response study following in utero and lactational exposure to di-(2-ethylhexyl) phthalate (DEHP): Reproductive effects on adult male offspring rats. *Toxicology* 228, 85-97.

Bao AM, Man XM, Guo XJ, Dong HB, Wang FQ, Sun H, Wang YB, Zhou ZM, and Sha JH. 2001. Effects of di-n-butyl phthalate on male rat reproduction following pubertal exposure. *Asian J Androl* 13(5), 702-709.

Beu CC, Orsi AM, and Domeniconi RF. 2009. Structure of the lining epithelium of the cauda epididymis of the golden hamster. *Anat Histol Embryol* 38(1), 49-57.

Bila DM, and Dezotti M. 2007. Desreguladores endócrinos no meio ambiente: efeitos e consequências. *Quim Nova* 30(3), 651-666.

Blount BC, Silva MJ, Caudill SP, Needham LL, Pirkle JL, Sampson EJ, Licier GW, Jackson RJ, and Brock JW. 2000. Levels of seven urinary phthalate metabolites in a human reference population. *Environ Health Perspect* 108, 979-982.

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

Botelho GGK, Golin M, Bufalo AC, Morais RN, Dalsenter PR, and Andrade AJM. 2009. Reproductive effects of di(2-ethylhexyl) phthalate in immature male rats and its relation to cholesterol, testosterone, and thyroxin levels. *Arch Environ Contam Toxicol* 57, 777-784.

Bradley EL, Burden RA, Leon I, Mortimer DN, Speck DR, and Castle L. 2013. Determination of phthalate diesters in foods. *Food Addit Contam Part A* 30, 722-734.

Calafat AM, Brock JW, Silva MJ, Gray LEJr, Reidy JÁ, Barr DB, and Needham LL. 2006. Urinary and amniotic fluid levels of phthalate monoesters in rats after the oral administration of di(2-ethylhexyl) phthalate and di-n-butyl phthalate. *Toxicology* 217, 2-30.

Campos SGP, Zanetoni C, Góes RM, and Taboga SR. 2006. Biological behavior of the gerbil ventral prostate in three phases of postnatal development. *Anat Rec A* 288, 723–733.

Carruthers CM, and Foster PMD. 2005. Critical window of male reproductive tract development in rats following gestational exposure to di-n-butyl phthalate. *Birth Defects Res B* 74, 277-285.

Catalano PM, Presley L, Minium J, and Mouzon H. 2009. Fetuses of obese mothers develop insulin resistance *in utero*. *Diabets Care* 32(6), 1076-1080.

Christiansen S, Boerg J, Axelstad M, Dalgaard M, Vinggaard AM, Metzdorff SB, and Hass U. 2010. Low-dose perinatal exposure to di(2-ethylhexyl) phthalate induces anti-androgenic effects in male rats. *Reprod Toxicol* 30, 313-321.

Chung WS, Sohn JH, and Park YY. 1999. Is obesity an underlying factor in erectile dysfunction? *Eur Urol* 36, 68-70.

Delbès G, Levacher C, Pairault C, Racine C, Duquenne C, Krust A, and Habert R. 2004. Estrogen receptor β-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, and Levacher C. 2007. Developmental changes in testicular sensitivity to estrogens throughout fetal and neonatal life. *Toxicol Sci* 99, 234–243.

Desai M, Beall M, and Ross MG. 2013. Developmental origins of obesity: programmed adipogenesis. *Curr Diab Rep* 13, 27-33.

Esposito K, Giugliano F, Di Palo C, Giuliano G, Marfella R, D'Andrea F, D'Armiento M, and Giuliano D. 2004. Effect of lifestyle changes on erectile dysfunction in obese men: a randomized controlled trial. *J Am Med Assoc* 291, 2978-2984.

Fernandez CBD, Bellentani FF, Fernandes GSA, Perobelli JE, Favareto APA, Nascimento AF, Cicogna AC, and Kempinas WDG. 2011. Diet-induced obesity in rats leads to a decrease in sperm motility. *Reprod Biol Endocrinol* 9, 32.

Filipiak E, Walczak-Jedrzejowska R, Krupinski M, Oszukowsha E, Marchlewska K, Dlugonski J, Kula K. and Slowikowka-Hilczer J. 2011. Di(*n*-butyl) phthalate has no effect on the rat prepubertal testis despite its estrogenic activity *in vitro*. *Folia Histochem Cytobiol* 49(4), 685-689.

Fisher JS. 2004. Environmental anti-androgens and male reproductive health: focus on phthalates and testicular dysgenesis syndrome. *Reproduction* 127, 305-315.

Fochi RA, Perez APS, Bianchi CV, Rochel SS, Goes RM, Vilamaior PS, Taboga, SR, Santos FC. 2008. Hormonal oscillations during the estrous cycle influence the morphophysiology of the gerbil (Meriones unguiculatus) female prostate (skene paraurethral glands). *Biol Reprod* 79, 1084-1091.

Foster PMD. 2006. Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. *Int J Androl* 29, 140-147.

Frederiksen H, Aksglaede L, Sorensen K, Skakkebaek NE, Juul A, and Andersson AM. 2011. Urinary excretion of phthalate metabolites in 129 healthy Danish children and adolescents: estimation of daily phthalate intake. *Environ Res* 111, 656-663.

Gaido KW, Hensley JB, Liu D, Wallace DG, Borghoff S, Johnson KJ, Hall SJ, and Boekelheide K. 2007. Fetal mouse phthalate exposure shows that gonocyte multinucleation is not associated with decrease testicular testosterone. *Toxicol Sci* 97(2), 491-503.

Giagulli VA, Kaufman JM, and Vermeulen A. 1994. Pathogenesis of the decreased androgen levels in obese men. *J Clin Endocrinol Metab* 79, 997-1000.

Góes RM, Zanetoni C, Tomiosso TC, Ribeiro DL, and Taboga SR. 2007. Surgical and chemical castration induce differential histological response in prostate lobes of Mongolian gerbil. *Micron* 38(3), 231-236.

Gray TJB, and Gangolli SD. 1986. Aspects of the Testicular Toxicity of Phthalate Esters. *Environ Health Perspect* 65, 229-235.

Grun F, and Blumberg B. 2006. Environmental obesogens: organotins and endocrine disruption via nuclear receptor signaling. *Endocrinology* 147, 50-55.

Guo F, and Jen KLC. 1995. High-fat feeding during pregnancy and lactation affects offspring metabolism in rats. *Physiol Behav* 57(4), 681-686.

Guzick DS, Overstreet JW, Factor-Litvak P, Brazil CK, Nakajima ST, Coutifaris C, Carson SA, Cisneros P, Seinkampf MP, Hill JA, Xu D, and Vogel DL. 2001. Sperm

morphology, motility, and concentration in fertile and infertile men. *N Engl J Med* 345, 1388-1393.

Hajer GR, Van Haeften TW, and Visseren FL. 2008. Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. *Eur Heart J* 29, 2959-2971.

Hammoud AO, Gibson M, Peterson CM, Hamilton BD, and Carrel DT. 2006. Obesity and male reproductive potential**.** *J Androl* 27(5), 619-626.

Hammoud AO, Gibson M, Peterson CM, Meikle AW, and Carrel DT. 2008. Impact of male obesity on infertility: a critical review of the current literature. *Fertil Steril* 90, 897-904.

Hatch EE, Nelson JW, Stahlhut RW, and Webster TF. 2010. Association of Endocrine Disruptors and Obesity: Perspectives from Epidemiologic Studies. *Int J Androl* 33(2), 324-332.

Hauser R, and Calafat AM. 2005. Phthalates and human health. *Occup Environ Med* 62, 806–818.

Heng K, Anand-Ivell R, Teerds K, and Ivell, R. 2012. The endocrine disruptors dibutyl phthalate (DBP) and diethylstilbestrol (DES) influence Leydig cell regeneration following ethane dimethane sulphonate treatment of adult male rats. *Int J Androl* 35, 353-363.

Hill JO, Lin D, Yakubu F, and Peters JC. 1992. Development of dietary obesity in rats: Influence of amount and composition of dietary fat. *Int J Obes* 16, 321-333.

Howdeshell KL, Wilson VS, Furr J, Lambright CR, Rider CV, Blystone CR, Horchkiss AK, and Gray LEJr. 2008. A mixture of five phthalate esters inhibits fetal testicular testosterone production in the Sprague-Dawley rat in a cumulative, dose-additive manner. *Toxicol Sci* 105(1), 153-165.

Huber WW, Grasl-Kraupp B, and Schulte-Hermann R. 1996. Hepatocarcinogenic potential of di(2-ethyl-hexyl) phthalate in rodents and its implications on human risk. *Crit Rev Toxicol* 26, 365-481.

IPCS. 2002. Global Assessment of the State-of-the-Science of Endocrine Disruptors. http://ehp.niehs.nih.gov/who/preface.pdf.Eds

Jen KLC. 1980. The effect of high fat diet on the behavioral patterns of male rats. *Physiol Behav* 25, 373-381.

Jensen TK, Vierula M, Hjollund NH, Saaranen M, Scheike T, Saarikoski S, Suominen J, Keiski A, Toppari J, and Skakkebaek NE. 2000. Semen quality among Danish and Finnish men attempting to conceive. The Danish First Pregnancy Planner Study Team. *Eur J Endocrinol* 142, 47-52.

Jiang J, Ma L, Yuan L, Wang X, and Zhang W. 2007. Study on developmental abnormalities in hypospadiac male rats induced by maternal exposure to di-n-butyl phthalate (DBP). *Toxicology* 232, 286-293.

Jobling S, Reynolds T, White R, Parker MG, and Sumpter JP. 1995. A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environ Health Perspect* 103(6), 582-587.

Juana L, Bárbara VG, Martín MT, Agustín C, Guillermo RB, and Guadalipe O. 2010. Neither testosterone levels nor aggression decrease when the male Mongolian gerbil (*Meriones unguiculatus*) displays paternal behavior. *Horm Behav* 57(3), 271-275.

Kasturi SS, Tannir J, and Brannigan RE. 2008. The metabolic syndrome and male infertility**.** *J Androl* 29, 251-259.

Kavlock R, Boekelheide K, Chapin R, Cunningham M, Faustman E, Foster PMD, Golub M, Henderson R, Hinberg I, Little R, Seed J, Shea K, Tabacova S, Tyl R, Williams P, and Zacharewski T. 2002. Center for the evaluation of risks to human reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di(2-ethylhexyl) phthalate. *Reprod Toxicol* 16, 529-653.

Kim TS, Jung KK, Kim SS, Kang IH, Baek JH, Nam H, Hong S, Lee BM, Hong JT, Oh KW, Kim HS, Han SY, and Kang TS. 2010. Effects of in utero exposure to di(n-butyl) phthalate on development of male reproductive tracts in sprague-dawley rats. *J Toxicol Environ Health A* 73, 1544-1559.

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

Kluwe WM. 1982. Overview of phthalate ester pharmacokinetics in mammalian species. *Environ Health Perspect* 45, 3-10.

Koch HM, Becker K, Wittassek M, Seiwert M, Angerer J, and Kolossa-Gehring M. 2007. Di-n-butylphthalate and butylbenzylphthalate – urinary metabolite levels and estimated daily intakes: pilot study for the German environmental survey on children. *J Expo Sci Environ Epidemiol* 17, 378-387.

Koch HM, Drexler H, and Angerer J. 2003. An estimation of the daily intake of di(2-ethylhexyl)phthalate (DEHP) and other phthalates in the general population**.** *Int J Hyg Environ Health* 206, 77–83.

Kondo F, Ikai Y, Hayashi R, Okumura M, Takatori S, Nakazawa H, Izumi S, and Makino T. 2010. Determination of five phthalate monoesters in human urine using gas chromatography-mass spectrometry. *Bull Environ Contam Toxicol* 85, 92-96.

Kort HI, Massey JB, Eelsner CW, Mitchell-Leef D, Shapiro DB, Witt MA, and Roudebush WE. 2006. Impact of body mass index values on sperm quantity and quality. *J Androl* 27, 450-452.

Kramer AW Jr. 1964. Body and organ weights and linear measurements of the adult Mongolian gerbil. *Anat Rec* 150, 343-348.

Landry D, Cloutier F, and Marton LJ. 2013. Implications of leptin in neuroendocrine regulation of male reproduction. *Reprod Biol* I3, I-14.

Latini G. 2005. Monitoring phthalate exposure in humans. *Clin Chim Acta* 361(1-2), 20-29.

Lee KY, Shibutani M, Takagi H, Kato N, Takigami S, Uneyama C, Hirose M. 2004. Diverse developmental toxicity of di-n-butyl phthalate in both sexes of rat offspring after maternal exposure during the period from late gestation through lactation. *Toxicology* 203, 221-238.

Lottrup G, Andersson AM, Leffers H, Mortensen GK, Toppari J, Skakkebaek NE, and Main KM. 2006. Possible impact of phthalates on infant reproductive health. *Int J Androl* 29, 172-180.

Loureiro IR. 2002. *A importância e ocorrência de ftalatos em água potável e no ecossistema da Baía de Guanabara*. Tese (Doutorado em Química Analítica) – Pontifícia Universidade Católica do Rio de Janeiro. Rio de Janeiro.

Lubrano C. 2008. Obesità e inquinamento. *Sicurezza Sanitaria* 98:38-45.

Lubrano C, Genovesi G, Specchia P, Costantini D, Mariani S, Petrangeli E, Lenzi A, and Gnessi L. 2013. Obesity and metabolic comorbidities: environmental diseases? *Oxid Med Cell* 2013. doi: 10.1155/2013/640673.

Mah PM, and Wittert GA. 2010. Obesity and testicular function. *Mol Cell Endocrinol* 316(2), 180-6.

Main KM, Mortensen GK, Kaleva MM, Boisen KA, Damgaard IN, Chellakooty M, Schmidt IM, Suomi AM, Virtanen HE, Petersen DV, Andersson AM, Toppari J, and Skakkebaek NE. 2006. Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age. *Environ Health Perspect* 114, 270-276.

Manikkan M, Tracey R, Guerrero-Bosana C, and Skinner MK. 2013. Plastics Derived Endocrine Disruptors (BPA, DEHP and DBP) Induce Epigenetic Transgenerational Inheritance of Obesity, Reproductive Disease and Sperm Epimutations. *Plos One* 8(1).

Mendi-Handagama SM, and Ariyaratne HB. 2001. Differentiation of the adult Leydig cell population in the postnatal testis. *Biol Reprod* 65, 660–671.

Merlet J, Racine C, Moreau E, Moreno SG, and Habert R. 2007. Male fetal germ cells are targets for androgens that physiologically inhibit their proliferation. *PNAS* 104, 3615-3620.

Moore RW, Rudy TA, Lin T, Ko K, Peterson RE. 2001. Abnormalities of Sexual Development in Male Rats with in Utero and Lactational Exposure to the Antiandrogenic Plasticizer Di(2-ethylhexyl) Phthalate. *Environ Health Perspect* 109(3), 229-237.

Mortensen GK, Main KM, Andersson AM, Leffers H, and Skakkebaek NE. 2005. Determination of phthalate monoesters in human milk, consumer milk, and infant formula by tandem mass spectrometry (LC-MS-MS). *Anal Bioanal Chem* 382, 1084-1092.

Muller M, and Nielsen JT. 1979. Effect of superior cervical ganglionectomy on monoamine content in the epithalamic area of the Mongolian Gerbil – A fluorescence histochemical study. *Cell Tissue Res* 201(1), 1-9.

Murature DA, Tang SY, Steinhardt G, and Dougherry RC. 1987. Phthalate esters and semen quality parameters. *Biomed Environ Mass Spectrom* 13, 473-477.

Mylchreest E, Cattley RC, and Foster PMD. 1998. Male reproductive tract malformations in rats following gestational and lactational exposure to di(n-butyl) phthalate: an antiandrogenic mechanism? *Toxicol Sci* 43, 47-60.

Mylchreest E, Wallace DG, Cattley RC, and Foster PMD. 2000. Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to di(n-butyl) phthalate during late gestation**.** *Toxicol Sci* 55, 143-151.

Nawaa Y, Okada M, and Arizono N. 1994. Histochemical and cytochemical characterizations of mucosal and connective tissue mast cells of Mongolian gerbils. *Int Arch Allergy Imunol* 104, 249-54.

Newbold RR. 2010. Impact of environmental endocrine disrupting chemicals on the development of obesity. *Hormones* 9, 206-217.

Oken E, and Gillman MW. 2003. Fetal origins of obesity. *Obes Res* 11(4), 496-506.

Oliveira SM, Santos FC, Corradi LS, Góes RM, Vilamaior PSL, and Taboga SR. 2011. Microscopic evaluation of proliferative disorders in the gerbil female prostate: evidence of aging and the influence of multiple pregnancies. *Micron* 42(7), 712-7.

Olujini OO, Fatoki OS, Odendaal JP, and Okonkwo JO. 2010. Endocrine disrupting chemicals (phenol and phthalates) in the South African environment: a need for more monitoring. *Water SA* 36, 671-682.

Orth JM. 1982. Proliferation of Sertoli cells in fetal and postnatal rats: a quantitative autoradiographic study. *Anat Rec* 203. 485–492.

Pasquali R. 2006. Obesity, fat distribution and infertility. *Maturitas* 54, 363-371.

Pasquali R, Patton L, and Gambineri A. 2007. Obesity and infertility. *Curr Opin Endocrinol Diabetes Obes* 14, 482-487.

Perez APS, Biancardi MF, Góes RM, Santos FA, and Taboga SR. 2011. Exposure to ethinylestradiol during prenatal development and postnatal supplementation with testosterone causes morphophysiological alterations in the prostate of male and female adult gerbils. *Int J Exp Path* 92, 121-130.

Pinheiro PFF, Almeida CCD, Segatelli TM, Martinez M, Padovani CR, and Martinez FE. 2003. Structure of the pelvic and penile urethra – relationship with the ducts of the sex accessory glands of the Mongolian gerbil (*Meriones unguiculatus*). *J Anat* 202, 431-444.

Pinto ME, Botta LS, Taboga SR, and Góes RM. 2010a. Neonatal Gonocyte Differentiation in Mongolian Gerbil *Meriones unguiculatus* Involves Asynchronous Maturation of Seminiferous Cords and Rapid Formation of Transitional Cell Stage. *Anat Rec* 310-419.

Pinto ME, Egydio FM, Taboga SR, Mendis-Handagama SMLC, and Góes RM. 2010b. Differentiation of Leydig cells in the Mongolian gerbil. *Microsc Res Tech* 73(2), 119-127.

Razolli M, Papa R, Valsecchi P, and Nonnismarzano F. 2003. AFLP to assess genetic variation in laboratory gerbils (Meriones unguiculatus). *J Hered* 94, 507-511.

Rider VC, Wilson VS, Howdshell KL, Hotchkiss AK, Furr JR, Lambright CR, Gray LEJr. 2009. Cumulative effects of *in utero* administration of mistures of "antiandrogens" on male rat reproductive development. *Toxicol Pathol* 37, 100-113.

Robinson DG. 1974. The anatomy of the Mongolian gerbil. *Publishe's Foreword, USA*.

Rochel SS, Bruni-Cardoso A, Taboga SR, Vilamaior PSL, and Góes RM.2007. Lobe Identity in the Mongolian Gerbil Prostatic Complex: A New Rodent Model for Prostate Study. *Anat Rec* , 1233-1247.

Rochel-Maia SS, Santos FC, Vilamaior PSL, Jistulin Jr LA, Felisbino SL, Góes RM, and Taboga SR. 2011. MMP-2 and TIMP-2 in the prostates of male and female Mongolian gerbils: effects of hormonal manipulation. *Histol Histopathol* 26(11), 1423-1434.

Saltzman W, Thinda S, Higgins AL, Matsumoto WR, Ahmed S, Mcgeehan L, and Kolb EM. 2009. Effects of siblings on reproductive maturation and infanticidal behavior in cooperatively breeding Mongolian gerbils. *Dev Psychobiol* 51, 60-72.

Sargis RM, Johnson DN, Choudhury RA, and Brady MJ. 2010. Environmental endocrine disruptors promote adipogenesis in the 3T3-L1 cell line through glucocorticoid receptor activation. *Obesity* 18, 1283-1288.

Scarano WR, Toledo FC, Guerra MT, Pinheiro PFF, Domeniconi RF, Felisbino SL, Campos SGP, Taboga SR, and Kempinas WG. 2010. Functional and Morphological Reproductive Aspects in Male Rats Exposed to Di-n-Butyl Phthalate (DBP) in Utero and During Lactation. *J Toxicol Environ Health A* 73, 972-984.

Schwentker V. 1963. The gerbil – a new laboratory animal. *Veterinarian* 6, 5-9.

Scott HM, Hutchison GR, Jobling MS, McKinnell C, Drake AJ, and Sharpe RM. 2008. Relationship between androgen action in the male programing window, fetal Sertoli cell number and adult testis size in the rat. Endocrinology 149, 5280-5287.

Segatelli TM, Almeida CCD, Pinheiro PFF, Martinez M, Padovani CR, and Martinez FE. 2002. Kinetics of spermatogenesis in the Mongolian gerbil (*Meriones unguiculatus*).*Tissue Cell* 34(1), 7-13.

Segatelli TM, Almeida CC, Pinheiro PF, Martinez M, Padovani CR, and Martinez FE. 2000. Ultrastructural study of acrossome formation in Mongolian gerbil (*Meriones unguiculatus*). *Tissue Cell* 32, 508-507.

Segatelli TM, França LR, Pinheiro PFF, Almeida CCD, Martinez M, and Martinez FE. 2004. Spermatogenic Cycle Length and Spermatogenic Efficiency in the Gerbil (*Meriones unguiculatus*). *J Androl* 25(6), 872-880.

Sharpe RM. 2010. Environmental/lifestyle effects on spermatogenesis. *Phil. Trans. R. Soc. B* 365, 1697-1712.

Sharpe RM, McKinnell C, Kivlin C, and Fisher JS. 2003. Proliferations and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction* 125, 769-784.

Silva MJ, Reidy JÁ, Herbert AR, Preau Jr. JL, Needham LL, and Calafat AM. 2004. Detection of phthalate metabolites in human amniotic fluid. *Bull Environ Contam Toxicol* 72, 1226-1231.

Sullivan EL, Smith MS, and Grove KL. 2011. Perintal exposure to high-fat dieta programs energy balance, metabolism and behavior in adulthood. *Neuroendocrinilogy* 93, 1-8.

Symonds ME, and Budge H. 2009. Nutritional models of the developmental programming of adult health and disease. *Proc Nutr Soc* 68, 173-178.

Symonds ME, Budge H, and Frazier-Wood AC. 2013. Epigenetics and obesity: a relationship waiting to be explained. *Hum Hered* 75, 90-97.

Taboga SR, Vilamaior PSL, and Góes RM. 2009. Modulação androgênica e estrogênica na próstata: uma abordagem em modelos experimentais de roedores com enfoque na biologia estrutural. *Arq Bras Endocrinol Metab* 53/8.

Takeuchi S, Iida M, Kobayashi S, Jin Kazuo, Matsuda T, and Kojima H. 2005. Differential effects of phthalate esters on transcriptional activities via human estrogen receptors α and β, and androgen receptor. *Toxicology* 210, 223-233.

Tanaka A, Matsumoto A, and Yamaha T. 1978. Biochemical studies on phthalic esters. III. Metabolism of dibutyl phthalate (DBP) in animals. *Toxicology* 9, 109-123.

Tang-Péronard JL, Andersen HR, Jensen TK, Heitmann BL. 2011. Endocrine-disrupting chemicals and obesity development in humans: a review. *Obes Ver* 12, 622-636.

Thomas JA, and Thomas MJ. 1984. Biological effects of di-(2-ethyl-hexyl) phthalate and other phthalic acid esters. *Crit Rev Toxicol* 13, 283-317.

US, EPA. 2006. Toxicological Review of Dibutil Phthalate (di-n-Butil Phthalate). *U.S. Environmental protection Agency***,** CAS No. 84-74-2.

US, EPA. 1997. Special Report on Environmental Endocrine Disruption: An Effects Assessment and Analisys. *U.S. Environmental Protection Agency***,** Report No. EPA/630/R-96/012, Washington D. C.

Vigueras-Villasenõr RM, Moreno-Mendoza NA, Reyes-Torres G, Molina-Ortiz D, León MC, and Rojas-Castañeda JC. 2006. The effect of estrogen on testicular gonocyte maturation. *Reprod Toxicol* 22, 513–520.

Vo TTB, Jung E, Dang VH, Jung K, Baek J, Choi K, and Jeung E. 2009. Differential effects of flutamide and di-(2-ethylhexyl) phthalate on male reproductive organs in a rat model. *J Reprod Dev* 55(4).

Walker CD, Naef L, D'Asti E, Long H, Xu Z, Moreau A, Azeddine B. 2008. Perinatal maternal fat intake affects metabolism and hippocampal function in the offspring: a potential role for leptin. *Ann NY Acad Sci* 1144, 189-202.

Williams DT, Blanchfield BJ. 1974. Retention, excretion and metabolism of di-(2-ethylhexyl) phthalate administered orally to the rat. *Bull Environ Contam Toxicol* 11, 371–378.

Willians WM. 1974. The anatomy of Mongolian gerbil. Tumblebrook Fram, Inc., USA. 107p.

Wilson JD. 1978. Sexual differentiation. *Annu Rev Physiol* 40, 279-306.

WORLD HEALTH ORGANIZATION. **Obesity**. Disponível em: <http://www.who.int/topics/obesity/en/ >. Acesso em: 20 jul. 2011.

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Dissertação de Mestrado** intitulada "Efeitos combinados da exposição ao di-n-butil ftalato e à dieta hiperlipídica sobre a estrutura e função testicular de gerbilos adultos":

( ) não se enquadra no § 4º do Artigo 1º da Informação CCPG 002/13, 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 2012/060, Instituição: Universidade Paulista "Júlio de Mesquita Filho", São José do Rio Preto – IBILCE-UNESP.

( ) 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.*

Ana Carolina Negrin
Aluno(a): Ana Carolina Negrin

Rejane Maira Góes
Orientador(a): Rejane Maira Góes

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:
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Carimbo e assinatura

UNIVERSIDADE ESTADUAL PAULISTA
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Câmpus de São José do Rio Preto

COMISSÃO DE ÉTICA NO USO DE ANIMAIS
IBILCE/UNESP-CSJRP

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Efeitos combinados da exposição ao di-n-butil ftalado e ambiente obesogênico sobre histofisiologia prostática e testicular de gerbilos adultos"**, (protocolo nº. 060/2012 - CEUA), sob responsabilidade da Professora Doutora Rejane Maira Góes, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética no Uso de Animais deste Instituto, em reunião de 28 de março de 2012.

CERTIFICATE

Certify that the research project entitled "**Combined effects of exposure to di-n-butyl and ftalado obesogenic environment histophysiology on prostatic and testicular gerbils adult**" (protocol no. 060/2012 - CEUA), under the responsibility of Professor Dr. Rejane Maira Góes, is in accordance with the Ethical Principles in Animal Experimentation adopted by Brazilian College of Animal Experimentation (COBEA) and was approved by the Ethics Committee on Animal Use of this Institute, at the meeting of march 28th, 2012.

São José do Rio Preto, 08 de agosto de 2013.

Prof. Dr. Luiz Henrique Florindo
Vice-Presidente da CEUA

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