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

Instituto de Biociências

FUNÇÃO REPRODUTIVA DE RATAS EXPOSTAS AO FTALATO *IN UTERO* E LACTAÇÃO

MARINA TREVIZAN GUERRA

Dissertação apresentada ao Instituto de Biociências, Campus de Botucatu, Unesp, para obtenção do título de Mestre no Programa de PG em Biologia Geral e Aplicada.

BOTUCATU - SP

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Livros Grátis

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"A mente que se abre a uma nova idéia jamais voltará ao seu tamanho original"

(Albert Einstein)

Dedicatória

À minha família, meu bem mais precioso e tudo que mais me importa no mundo, eu dedico este trabalho.

Agradecimentos Pessoais

À Deus, pela fé.

"Toda vez que não viste as tuas pegadas na areia, foi porque te carreguei no colo."

À minha família (meus pais, Pedro e Edmar, e minhas irmãs, Bárbara e Simony), pelo maior amor do mundo.

"A fé de vocês em mim me fez ter mais fé em mim mesmo."

Ao Uilian, pela companhia, comprometimento, amor, compreensão, amizade e ajuda.

"É apenas com o coração que se pode ver direito; o essencial é invisível aos olhos."

Às meninas da Wilma: Dani, Carla, Ana Sílvia, Glaura, Aninha, Jú, Fabíola, Carlinha, Thaiane, Flaviha e Thaís,e aos meninos da Wilma: Wellerson, Luís, Nirso e Gustavo, que são pessoas lindas, que proporcionam um ótimo ambiente de trabalho, e me permitiram ganhar amizades maravilhosas.

"Bons amigos são bons para sua saúde."

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"Amigo: alguém que sabe de tudo a teu respeito e gosta de ti assim mesmo."

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À Profa. Dra. Wilma De Grava Kempinas, pelo apoio, pelos ensinamentos e pela amizade. Tive uma excelente profissional em quem me espelhar.

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"Um anão sobre os ombros de um gigante pode ver mais longe que o próprio gigante."

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À Dra. Janete Aparecida Anselmo Franci, pelas dosagens hormonais.

Aos ratos (em especial às fêmeas), animais de laboratório que cederam suas vidas em prol do desenvolvimento da ciência.

"Não há diferenças fundamentais entre o homem e os animais nas suas faculdades mentais...os animais, como os homens, demonstram sentir prazer, dor, felicidade e sofrimento."

À todas as pessoas, que de certa maneira, trabalharam para que este trabalho fosse concluído, meu muito obrigada!

Resumo

Marina Trevizan Guerra

Ftalatos são contaminantes ambientais usados como plasticidas na produção de PVC, cosméticos, praguicidas, lubrificantes e produtos de cuidado pessoal. Estudos que relatam os efeitos do ftalatos no sistema reprodutor feminino são particularmente esparsos e restritos a exposição a altas doses em ratos. No presente estudo, ratas prenhes foram tratadas com 100mg/kg/dia de di-η-butil ftalato (DBP) ou somente com o veículo (grupo controle), do DG 12 ao DPN 21. Os efeitos foram avaliados nas mães e na prole feminina. Os resultados mostraram que todos os parâmetros foram comparáveis entre os grupos, embora tenha ocorrido um aumento significativo no peso corpóreo fetal após a exposição ao DBP. Entretanto, o peso corpóreo ao nascimento foi normal. Baseado nestes dados, podemos concluir que, nestas condições experimentais, o DBP não alterou o desenvolvimento nem a função reprodutiva feminina.

Abstract

Marina Trevizan Guerra

Phthalates are environmental contaminants used as plasticizers in PVC manufacturing, in the production of cosmetics, pesticides, lubricants and personal care products. Studies that report effects of phthalates on female reproductive health are particularly sparse and restricted to high - dose exposure in rats. In the present study, pregnant rats were treated with 100mg/kg/day of Di-n-butyl-phthalate (DBP) or only the vehicle (control group), from GD 12 to PND 21. The effects were evaluated on the dams and in the female offspring. Results showed that all parameters were comparable between groups, although there was a significant increase in the fetal weight after DBP exposure. However, the body weight at birth was normal. Based on these data we can conclude that, in these experimental conditions, DBP did not disturb the reproductive development or function of female rats.

Sumário

Ftalatos e contaminação ambiental

A população mundial é regularmente exposta a uma infinidade de produtos químicos provenientes da indústria. Esta exposição ocorre através de alimentos, água, ar, ou contato direto com uma variedade de produtos, e a rota de exposição (ingestão, inalação, absorção pela pele ou administração intravenosa) pode influenciar significativamente a toxicidade do químico (Health Care Without Harm, 2002).

Uma classe de produtos químicos que vem chamando a atenção por ser amplamente encontrada como substância contaminante no meio ambiente (International Programme on Chemical Safety [IPCS], 1992, 1997) são os ésteres de ftalato, cujo uso industrial teve início em 1930. A produção anual mundial chega a milhões de toneladas e o uso abrange centenas de bens de consumo e produtos industriais. São utilizados na fabricação de cosméticos, praguicidas, lubrificantes e produtos de cuidado pessoal, e como plasticidas para conferir flexibilidade a plásticos como polivinil cloreto (PVC), nitrocelulose e éter de celulose. Devido ao grande volume de produção, uso abrangente e contaminação ambiental, humanos são expostos a estes compostos diariamente através da ingestão, inalação e exposição dermal (Blount et al., 2000).

Aproximadamente 90% da produção global de ftalatos é destinada para uso em plástico PVC para fabricação de diversos materiais (Bizzari et al., 2000), como materiais médico-hospitalares (bolsas de sangue, soro e soluções intravenosas, catéteres, máscaras para inalação de oxigênio, luvas e tubos) e embalagens plásticas maleáveis de uso comercial como mamadeiras, brinquedos e garrafas (Mylchreest et al., 2000; Lehmann et al., 2004; Lottrup et al., 2006). Os outros 10% são utilizados em produtos diversos como adesivos, detergentes, capacitores elétricos, sprays de cabelo, tintas, solventes, óleos lubrificantes, loções corporais, esmaltes, perfumes e produtos farmacêuticos (Houlihan et al., 2000; Centers of Disease Control and Prevention - CDC, 2001). A maioria destes produtos não traz a informação da presença de ftalatos em suas composições.

Estrutura dos ftalatos

As moléculas de ftalato não se ligam covalentemente ao polímero e, desta forma, desprendem-se facilmente para o ar, água, saliva, sangue e soluções intravenosas (Nass, 1977; Kemi, 2001; US Food and Drug Administration - FDA, 2001). Dependendo das circunstâncias de uso, de 2 a 50% do ftalato presente no produto pode ser desprendido (Kemi, 2001). Por serem lipossolúveis, os ftalatos difundem-se mais facilmente para soluções lipídicas.

Os ftalatos representam uma ampla família de substâncias químicas que contêm um anel de benzeno, dois grupos carbonila, e dois grupos álcool para gerar uma estrutura diéster (figura 1) (Health Care Without Harm, 2002). As formas diésteres destas substâncias químicas são rapidamente hidrolisadas por esterases no intestino, fígado e sangue, em formas de monoésteres, que são considerados metabólitos tóxicos finais (Loverkamp-Swan and Davis, 2003).

Ftalatos normalmente seguem uma rota metabólica de pelo menos dois passos: a fase I, cujo principal evento é a hidrólise da molécula, seguida pela fase II, onde ocorre a conjugação. Na fase I, os ftalatos diésteres são hidrolisados em seus metabólitos monoésteres primários, num processo catalisado por lipases e esterases no intestino (Calafat et al., 2006; Rusyn et al., 2006). A fase I normalmente é conhecida como detoxificação, mas estudos mostram que ftalatos diésteres tornam-se mais bioativos quando hidrolisados a monoésteres (Heindel et al., 1992). Na fase II, os metabólitos são conjugados através da ação de enzimas, tornando-os hidrofílicos, e deste modo, facilmente excretados na urina (figura 2) (Frederiksen et al. 2007).

(a)

Di(-2-ethilhexil) ftalato

Di-isononil ftalato

Di-n-butil ftalato

Butil-benzil ftalato   (b)
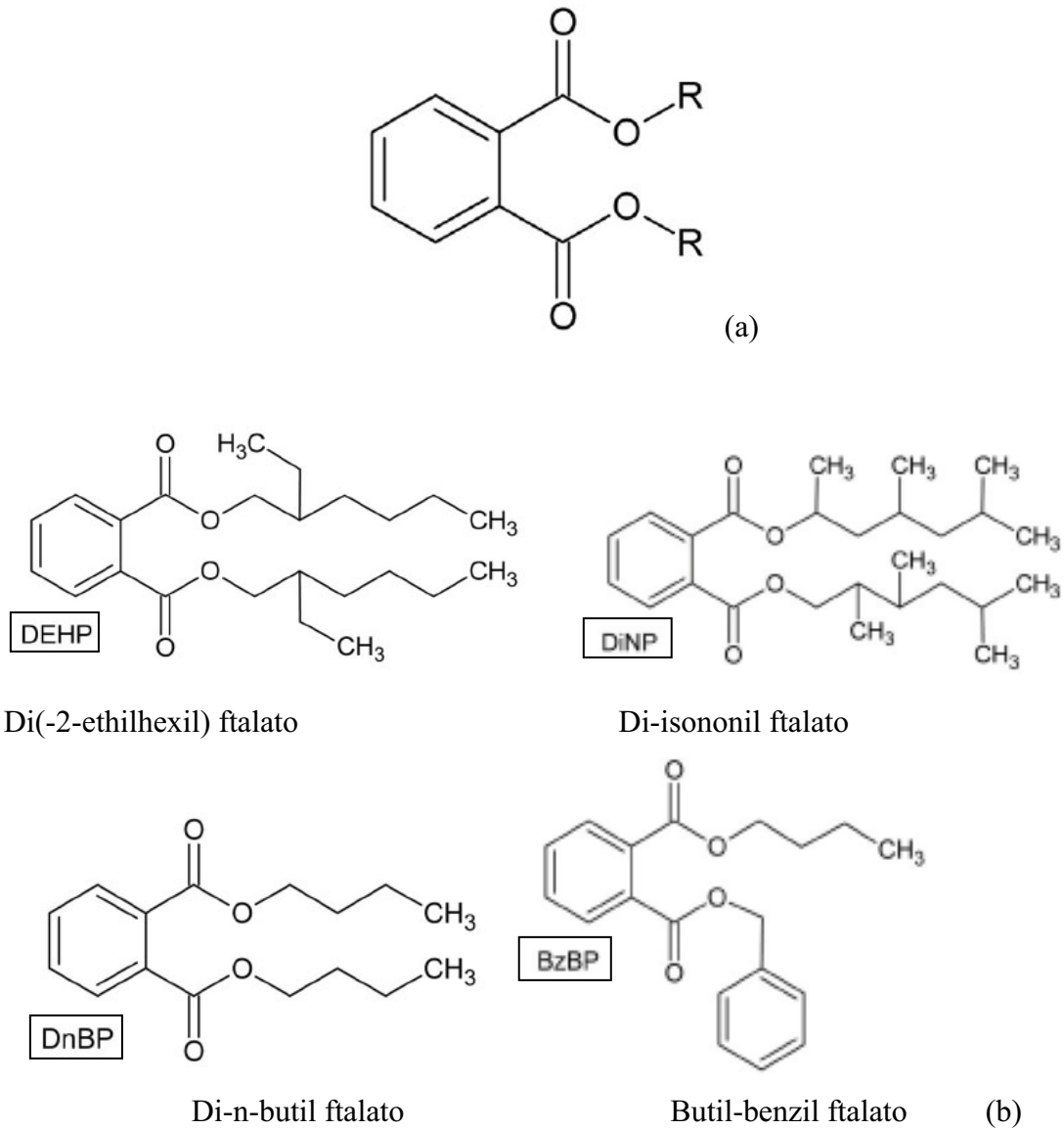
Figura 1. Estrutura básica comum a todas as moléculas de ftalato (a) e estruturas moleculares dos ftalatos comumente utilizados na indústria (b) (Frederiksen et al., 2007).
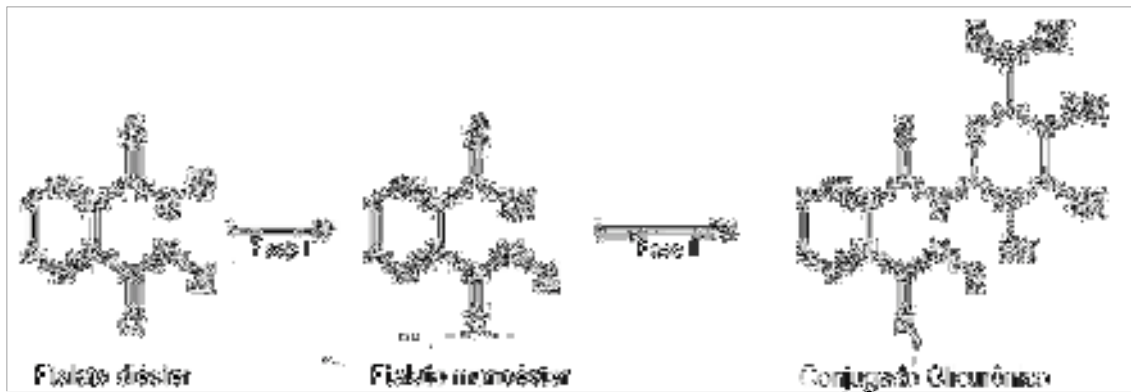
Figura 2. Rotas metabólicas dos ftalatos: fase I, que resulta em um metabólito monoéster, seguida pela fase II, gerando metabólitos hidrofílicos, e assim, facilmente excretáveis (Frederiksen et al., 2007).

Ftalatos em produtos de consumo

Produtos de cuidado pessoal, materiais de construção, roupas, brinquedos, adesivos, tintas, praguicidas, filmes e vasilhames podem conter ftalato. A concentração em alguns produtos algumas vezes ultrapassa 20% do peso total do produto (Agency for Toxic Substances and Disease Registry, 1995); outros podem conter níveis de ftalato entre 6-80% do peso total (DiGangi, 1999; NTP-CERHR, 2000; Kemi, 2001).

Di-η-butil ftalato (DBP), um dos tipos de ftalato mais utilizados na indústria, é usado na fabricação de cosméticos, brinquedos, papéis de parede, tintas e adesivos. O Di-(2-etilhexil) ftalato (DEHP) é o principal ftalato utilizado na fabricação de materiais médico-hospitalares e peças de automóveis. Di-isononil ftalato (DINP) é usado em brinquedos infantis, sapatos, automóveis e recipientes para armazenamento de alimentos (Health Care Without Harm, 2002).

No ano de 2000, pesquisadores do CDC (Centers of Disease Control and Prevention) encontraram sete tipos diferentes de ftalatos em amostras biológicas de 298 pessoas testadas, e todas elas apresentaram um tipo específico de ftalato em comum como contaminante no corpo, o DBP (Houlihan et al., 2002). Estes dados evidenciam que a exposição a ftalatos é maior e mais comum do que se suspeitava (Blount et al., 2000).

Avaliando-se o sexo e a idade mais propensos a exposição ao ftalato, descobriu-se que mulheres em idade reprodutiva podem chegar a uma exposição até 20 vezes maior que a população de um modo geral, acima dos níveis seguros estabelecidos (Environmental Protection Agency – EPA, 1990; Blount et al., 2000; Kohn et al., 2000). Altas concentrações de ftalatos nessas mulheres podem ter como fonte o uso de cosméticos e produtos de beleza, em geral, contaminados com este tipo de substância química (Houlihan et al., 2002).

Descrição do sistema reprodutor de rata

        O sistema reprodutor é responsável por produzir e transportar os gametas para a reprodução sexual e, nas fêmeas, atua também protegendo e nutrindo o embrião (Walker & Homberger, 1997). Nas ratas, o sistema reprodutor é formado basicamente por vagina, tubas uterinas, útero e ovários (Anderson et al., 2004). A seguir, uma breve descrição dos órgãos envolvidos pelo sistema reprodutor feminino, segundo Walker & Homberger (1997), Hoyer, (2005), e Junqueira e Carneiro (2008).

Vagina

        As paredes da vagina não possuem glândulas e consistem de três camadas: mucosa, muscular e adventícia. O muco presente no lúmen da vagina origina-se nas glândulas da cérvix uterina. A lâmina própria da mucosa vaginal é constituída por tecido conjuntivo frouxo rico em fibras elásticas, linfócitos e neutrófilos (que em determinadas fases do ciclo, passam para o lúmen da vagina) (Figura 3).
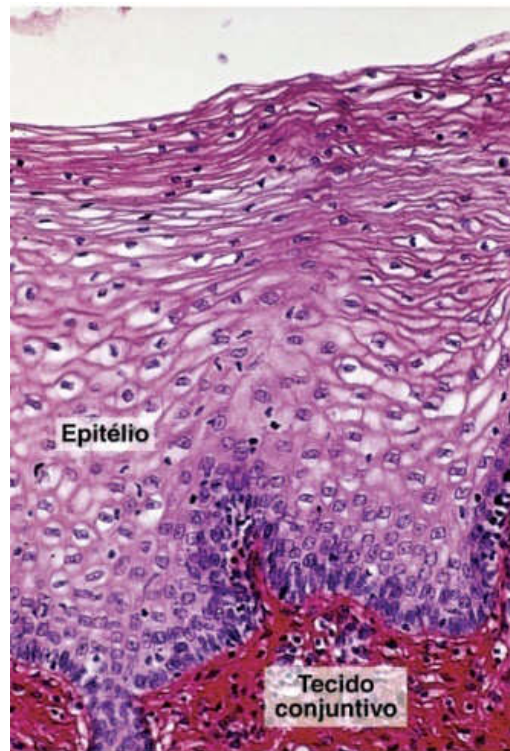
Figura 3. Epitélio pavimentoso estratificado não queratinizado da vagina, apoiado em um tecido conjuntivo denso. O citoplasma das células epiteliais é claro por causa do acúmulo de grande quantidade de glicogênio. Fotomicrografia. Médio aumento (Junqueira e Carneiro, 2008).

Tubas uterinas

As tubas uterinas são dois tubos musculares de grande mobilidade. Uma de suas extremidades abre-se na cavidade peritoneal (infundíbulo), próximo ao ovário, e possui prolongamentos em forma de franjas (fímbrias). A outra extremidade é denominada intra-mural e atravessa a parede do útero, abrindo-se no interior deste órgão. A parede da tuba uterina é composta por uma camada de mucosa, uma camada de músculo liso e uma serosa.

A mucosa da tuba apresenta dobras longitudinais, que são numerosas na região da ampola e diminuem em direção ao útero. A camada mucosa é formada por um epitélio colunar simples, lâmina própria e tecido conjuntivo frouxo. O epitélio possui células ciliadas (que se movimentam em direção ao útero) e células secretoras (que secretam muco necessário para nutrição e proteção do ovócito e promoção da capacitação espermática) (figura 4).
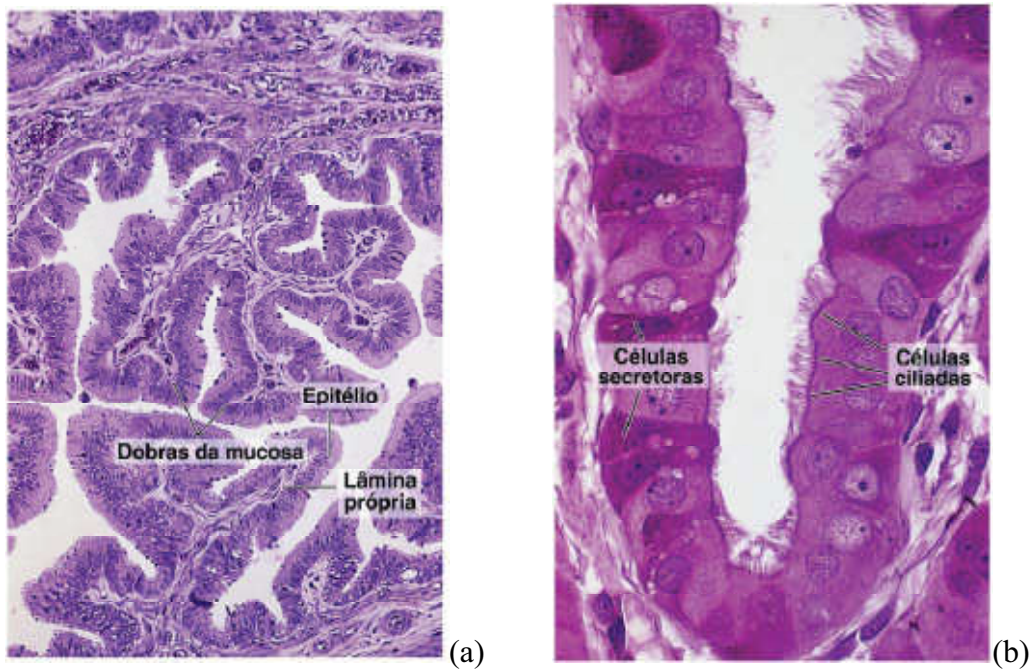
Figura 4. Parte da parede da tuba uterina. (a) A mucosa intensamente preagueada indica que esta região está próxima ao ovário. (b) O epitélio que reveste a tuba é formado por células colunares ciliadas e secretoras não ciliadas (Junqueira e Carneiro, 2008).

Útero

O útero é um órgão em forma de Y e consiste num par de cornos uterinos alongados independentes entre si. Caudalmente, esses cornos são rodeados por musculatura, formando um pequeno corpo uterino e logo abaixo deste corpo, encontra-se a cérvix uterina. A parede do útero é relativamente espessa e formada de três camadas. Externamente há uma serosa (mesotélio e tecido conjuntivo) ou adventícia (tecido conjuntivo apenas), dependendo da porção do órgão. As outras camadas são o miométrio (espessa camada de músculo liso) e o endométrio (ou mucosa uterina) (figura 5).

O miométrio é composto por fibras musculares lisas separadas por tecido conjuntivo. Os músculos se distribuem em quatro camadas: a primeira e a quarta camadas são compostas principalmente de fibras longitudinais e as camadas intermediárias contêm grandes vasos sanguíneos que irrigam o órgão. Durante a gestação, esta camada passa por um período de grande crescimento como resultado de processos de hiperplasia e hipertrofia, e algumas células musculares adquirem características ultra-estruturais de células secretoras de proteínas e sintetizam ativamente colágeno, cuja quantidade aumenta significativamente no útero.

O endométrio contém células que revestem a cavidade uterina e se organizam em um epitélio colunar simples, com células secretoras (figura 6); contém também glândulas tubulares simples que podem se ramificar nas porções mais profundas, próximas ao miométrio. Pode ser dividido em duas subcamadas: camada basal (mais profunda, contém tecido conjuntivo e a parte inicial das glândulas uterinas) e camada funcional (formada por tecido conjuntivo e porção final das glândulas uterinas e pelo epitélio superficial), que sofre as principais alterações durante o ciclo reprodutivo feminino.

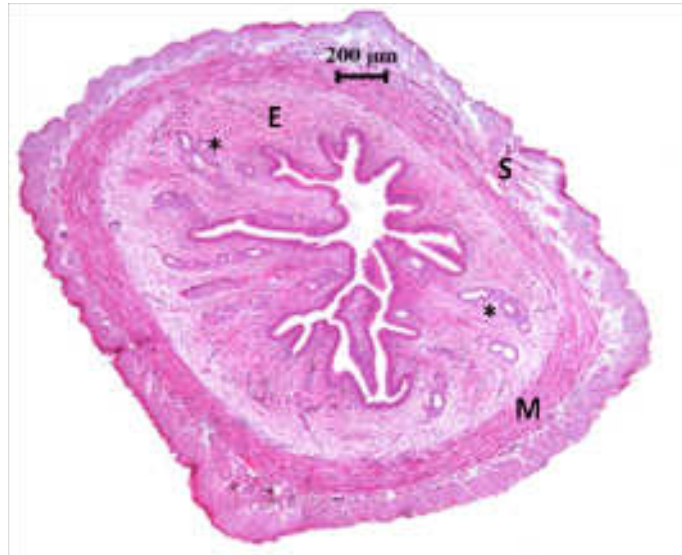**Figura 5**. Fotomicrografia ilustrativa de corte transversal de corno uterino de rata, em fase de estro. E=endométrio (mucosa), com glândulas uterinas (asteriscos); M=miométrio; S=serosa. Aumento de 50X, coloração HE. (Guerra, 2006).
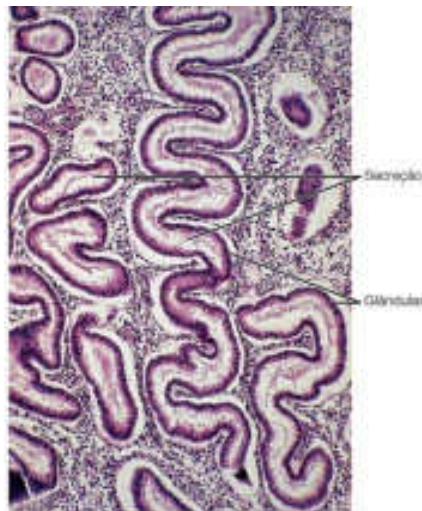

Figura 6. Durante a fase luteal as glândulas uterinas se tornam tortuosas e o seu lúmen é preenchido por secreção. Fotomicrografia. HE. Médio aumento (Junqueira e Carneiro, 2008).

Ovários

As principais funções do ovário são a produção de células germinativas femininas (os ovócitos) e hormônios sexuais femininos. Os ovários constituem-se de uma massa sólida de células e tecidos. Podem ser divididos em medula (no centro do ovário, constituído de tecido conjuntivo, vasos sanguíneos, nervos e vasos linfáticos) e córtex (constituído por tecido conjuntivo denso e folículos, que envolvem os ovócitos). O tecido conjuntivo cortical forma uma camada densa em volta do ovário, é desprovido de folículos e recebe o nome de túnica albugínea. A periferia do córtex ovariano logo abaixo da túnica albugínea é composta por várias camadas de folículos primordiais. Cada um desses folículos contém ovócito primário circundado por uma camada de células foliculares achatadas. Como o ovário funciona também como um órgão endócrino, produz hormônios responsáveis pelo crescimento e desenvolvimento dos folículos primordiais até atingirem o tamanho de folículo de Graaf (folículo antral ou pré-ovulatório), estando pronto para ovulação. Após a ovulação, há a formação do corpo lúteo a partir das células remanescentes do folículo maduro rompido durante a ovulação (figura 7).

Os folículos, juntamente com o corpo lúteo, são glândulas endócrinas. Os primeiros se desenvolvem sob estímulo do FSH (hormônio folículo estimulante) secretado pela adenohipófise, e secretam estrógeno, que é responsável pelo crescimento uterino e desenvolvimento endometrial. O corpo lúteo desenvolve-se por influência do LH (hormônio luteinizante), também secretado pela adenohipófise, e secreta principalmente progesterona. Este hormônio é necessário para o desenvolvimento final e aumento da vascularização do endométrio, preparando-o para uma possível implantação de um óvulo fertilizado. Ele também realiza a manutenção do endométrio proliferado e da placenta nos primeiros estágios de gestação.
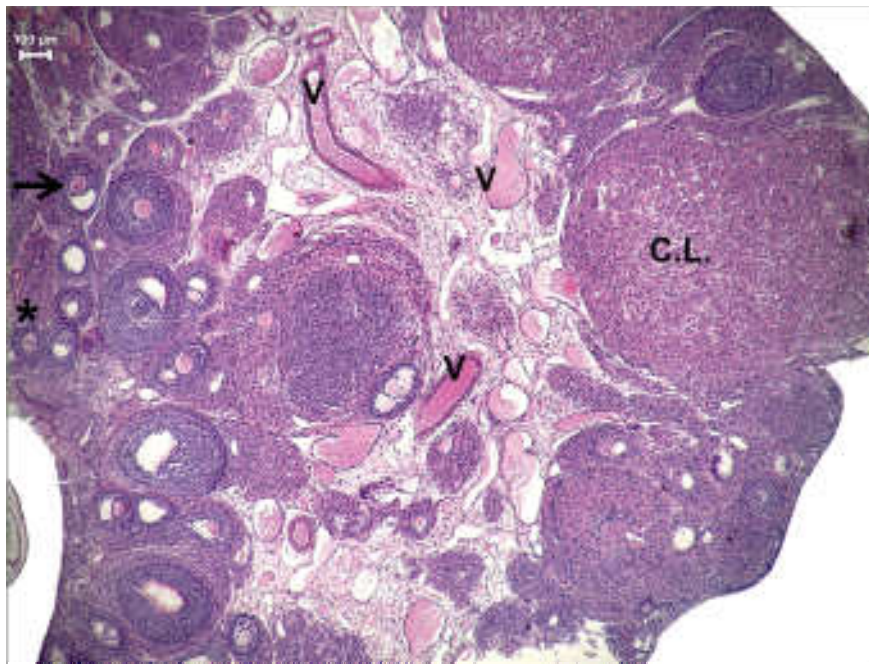
Figura 7. Fotomicrografia ilustrativa de corte longitudinal de ovário de rata. CL: corpo lúteo; seta: folículo antral; asterisco: folículo pré-antral, v: vasos sanguíneos. Aumento de 10X, coloração HE.

Puberdade

A puberdade é caracterizada por rápidas mudanças fisiológicas, tais como crescimento acentuado e maturação das gônadas e do cérebro. Este evento marca o período de transição de um indivíduo em estado não-reprodutivo para reprodutivo. O principal hormônio envolvido com a regulação da instalação da puberdade é o hormônio liberador de gonadotrofinas (GnRH) no hipotálamo. Este hormônio é responsável por estimular a liberação de FSH e LH na adenohipófise (Terasawa et al., 2001), que por sua vez, atuam nas gônadas (Walker & Homberger, 1997), levando a um aumento nos níveis de estradiol sanguíneo. A idade da abertura vaginal e ocorrência do primeiro estro são indicativos deste evento (US EPA, 1996; Guyton & Hall, 2002) (figura 8). Durante o ciclo estral, flutuações nos níveis de estrógeno e progesterona exercem profundo efeito na proliferação do epitélio e citodiferenciação dos órgãos do sistema reprodutor feminino (Boutin and Cunha, 1997) influenciando o crescimento, a função e a diferenciação estes órgãos. Substâncias químicas conhecidas como desreguladores endócrinos podem implicar em numerosos processos fisiológicos que afetam a saúde reprodutiva normal (McLachlan et al., 2001).
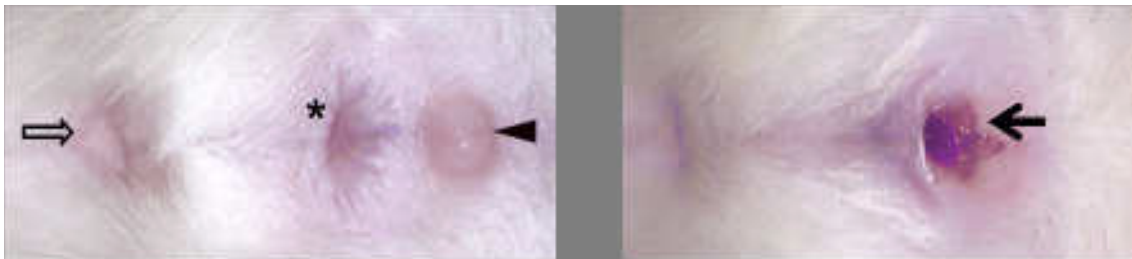

Figura 8. Aspecto macroscópico da genitália externa de rata, mostrando : cabeça de seta: uretra; asterisco: vagina fechada; seta vazada: ânus; seta: abertura vaginal (Poulet et al., 2005).

Ciclo estral

As ratas fêmeas são animais poliéstricos, o ciclo reprodutivo tem a duração de cerca de 4 a 5 dias, e em torno de 4 a 12 ovócitos são ovulados por ciclo. O ciclo estral de roedores é composto por quatro diferentes fases, que são classificadas de acordo com a predominância de tipos celulares observados em lavados vaginais em microscopia de luz (Chahoud and Kwasigroch, 1977; US EPA, 1996; Marcondes et al., 2002): células epiteliais nucleadas (proestro); células epiteliais queratinizadas (estro); células queratinizadas e nucleadas e leucócitos (metaestro); leucócitos e muco (diestro) (figura 9).

A fase de proestro corresponde à fase folicular ou proliferativa, onde os folículos estão em desenvolvimento, produzindo estrógeno, e tem duração média de 12 horas. O estro está relacionado ao período de ovulação e receptividade sexual da fêmea e dura cerca de 14 horas. Metaestro é a fase correspondente a atividade secretória do corpo lúteo e tem a duração de 21 horas. Finalmente, a fase de diestro corresponde à regressão do corpo lúteo e dura 57 horas. A constância desta fase por longos períodos pode significar imaturidade ou infecção.
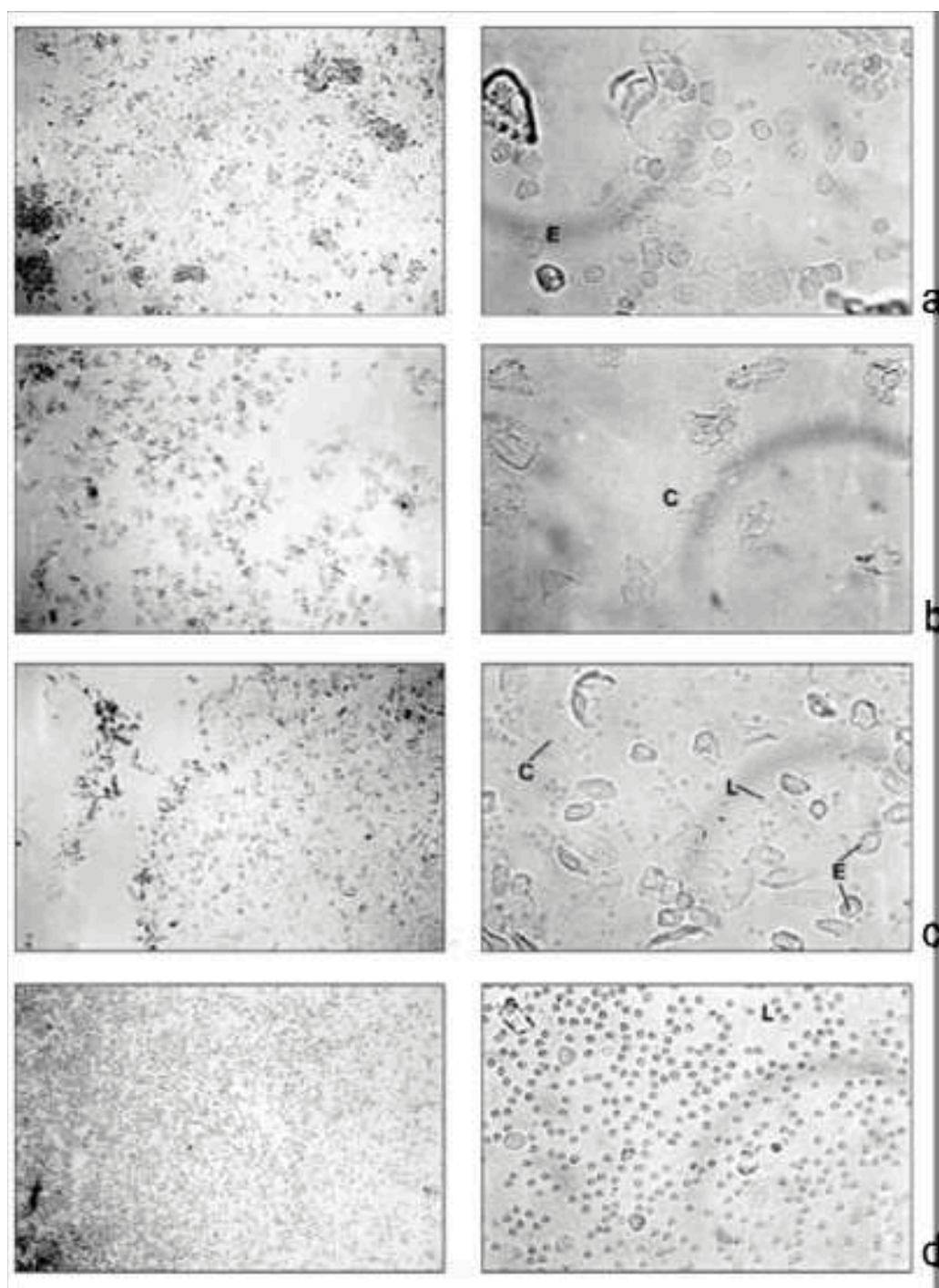
Figura 9. Fotomicrografia de lavado vaginal a fresco, de rata fêmea na fase de (a) proestro, (b) estro, (c) metaestro e (d) diestro, sob microscopia de luz. E=células epiteliais; C=células cornificadas; L=leucócitos. A prevalência desses três tipos celulares foi utilizada para determinação das fases do ciclo estral (Marcondes et al., 2002).

Ftalatos e reprodução feminina

Algumas substâncias encontradas como contaminantes ambientais são conhecidas como desreguladores endócrinos ou "xenohormônios", assim chamados porque mimetizam ou bloqueiam hormônios endógenos, interferindo na síntese, secreção, transporte, ligação, ação ou eliminação de hormônios naturais do corpo, alterando os níveis hormonais normais e a função endócrina no organismo (Kavlock et al., 1996; National Resoucers Defense Council, 1998; Wu et al., 2002). Várias destas substâncias já foram identificadas por apresentar esta atividade, incluindo hormônios naturais e sintéticos, praguicidas, plasticidas e produtos industriais (World Health Organization, 2002; Brevini et al., 2005). Os ésteres de ftalato são plasticidas suspeitos de apresentar uma atividade hormonal desregulatória (Witorsch, 2002; Grande et al., 2006). Os ftalatos são considerados agentes anti-androgênicos e também possuem alguma atividade estrogênica (Jobling et al., 1995; Harris et al., 1997; Zacharewski et al., 1998; Yu et al., 2003).

Estudos que relatam efeitos dos ftalatos sobre a saúde reprodutiva feminina são particularmente esparsos e restritos a exposição a altas doses em ratas adultas. Avaliações toxicológicas realizadas por Mylchreest et al. (2000), revelaram que a NOAEL (No Observed Adverse Effect Level) para o DBP é 50 mg/kg e a LOAEL (Lowest Observed Adverse Effect Level) é igual a 100 mg/kg, para ratos machos expostos a este composto durante a vida intra-uterina, com base em parâmetros reprodutivos (peso corpóreo, distância ano-genital, número de aréolas/mamilos, análise de órgãos reprodutivos e separação prepucial) .

Os ésteres de ftalato possuem a capacidade de atravessar a placenta e passar também pelo leite materno, tornando- se, assim, um risco significante para exposição de fetos em desenvolvimento e recém-nascidos (Dostal et al., 1987; Latini et al., 2003). Organismos imaturos são especialmente susceptíveis a desreguladores endócrinos porque o sistema reprodutor encontra-se em desenvolvimento e mudanças relativamente pequenas nos níveis hormonais endógenos podem resultar em mudanças estruturais e funcionais permanentes (World Health Organuization, 2002).

Alguns estudos sugerem uma associação entre exposição humana a ftalatos e alterações nos parâmetros reprodutivos (Tabacova, 1999; Cólon et al., 2000). Além disso, vários estudos têm demonstrado que os ftalatos podem afetar negativamente a função reprodutiva de animais de laboratório (Heindel et al., 1989; Davis et al., 1994). Estudos realizados demonstram que ftalatos são agentes tóxicos ao sistema genital em

machos (Wine et al., 1997, Mylchreest et al., 1999, 2000). Ratas expostas a altas doses (500 a 750 mg/Kg) dos dias 12 a 21 de gestação produzem fetos machos com múltiplas malformações de epidídimo, ducto deferente, vesícula seminal e próstata dorsolateral e ventral (Mylchreest et al., 1999, 2000).

Estudos revelam que os ovários são órgãos-alvo para toxicidade de ftalatos em animais adultos (Davis et al., 1994; Lovekamp-Swan et al., 2003). Ciclo estral prolongado, alterações dos níveis séricos hormonais, ausência de ovulações (Davis et al., 1994), diminuição na duração da prenhez (Latini et al., 2003), ovários císticos (Fan et al. 1998) e patogêneses de endometriose (Cobellis et al., 2003; Reddy et al., 2006) constituem alguns dos efeitos adversos encontrados em fêmeas expostas a altas doses de ftalato.

Capítulo

Este trabalho deu origem ao artigo "Reproductive development and function of female rats exposed to di-η-butyl phthalate (DBP) *in utero* and lactation" que sera submetido ao periódico "Reproductive Toxicology".

Reproductive development and function of female rats exposed to di-η-butyl phthalate (DBP) *in utero* and lactation

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Running Title: DBP and female reproduction

Reproductive development and function of female rats exposed to di-η-butyl phthalate (DBP) *in utero* and lactation

Abstract

Phthalates are environmental contaminants used as plasticizers in PVC manufacturing, in the production of cosmetics, pesticides, lubricants and personal care products. Studies that report effects of phthalates on female reproductive health are particularly sparse and restricted to high - dose exposure in rats. In the present study, pregnant rats were treated with 100mg/kg/day of Di-n-butyl-phthalate (DBP) or only the vehicle (control group), from GD 12 to PND 21. The effects were evaluated on the dams and in the female offspring. Results showed that all parameters were comparable between groups, although there was a significant increase in the fetal weight after DBP exposure. However, the body weight at birth was normal. Based on these data we can conclude that, in these experimental conditions, DBP did not disturb the reproductive development or function of female rats.

Key words: maternal exposure; Di-η-butyl phthalate; female offspring; reproduction; uterus; ovary; estrous cycle.

Marina Trevizan Guerra

Introduction

People are constantly exposed to a wide range of environmental contaminants from industrial process, through air, food, water or contact with a variety of consumer products. In recent years one class of chemicals used as plasticizers called phthalates have attracted special attention of the scientific community and the general public because their high production volume, in millions of tons annually, and variety of applications. Besides, they have been suspected by acting as endocrine-disruptors, which means that they have potential to modify the normal endocrine function [1-4]. Phthalate esters are liquid plasticizers widely used in flexible polyvinyl chloride (PVC) products, personal care products, cosmetics (perfume, lotions and nail polish), paints, coatings, some pesticide formulations, and pharmaceutical products [5,6] and thus are ubiquitous low-level environmental contaminants [7,8].

Di-η-butyl-phthalate (DBP) is a phthalic acid ester with extensive use in industry in such products as a plasticizer in many products, like medical devices [9], flexible plastics (child's toys, bottles and feed bottles) and some cosmetics formulations [10-12].

Human exposure occurs primarily through contaminated food, especially high-fat foods, which may have been in contact with plastic, adhesives, or other packing materials that contain DBP.

Most of the studies related to phthalates are restricted mainly to high dose exposure [3, 13-16], where the effects are more pronounce. The exposition to phthalates for humans and animals usually happens starting from the liberation of those esters for products and plastic utensils, and that liberation is in low concentrations and is not strong. DBP, among others phthalates, is not permanently bound to the polymer matrix. As a result, it can migrate from the plastics products and leach into the environment, under certain use or disposal conditions [12, 17-20]. Phthalate metabolites where

detected in the urine of children and adults, in serum, seminal fluid, amniotic fluid, breast milk and saliva [21-26]. Estimates for human exposure to DBP range from 0.84 to 113 µg/kg/day [5, 27].

Based on that, the aim of this present study is to evaluate the possible effects of low DBP dose on female reproductive function, in rats whose mothers were exposed to low dose of DBP during pregnancy and lactation.

Materials and Methods

Animals

Adult female (60 days of age, *n*=20) and male (90 days of age, *n*=10) Wistar rats, were supplied by Central Biotherium of State University of Sao Paulo (Unesp) and were housed in polypropylene cages (43cm x 30cm x 15cm) with laboratory-grade pine shavings as bedding. Rats were maintained under controlled temperature (23 $\pm$ 1ºC) and lighting conditions (12:12-h photoperiod). Rat chow and filtered tap water were provided *at libitum*. Two non-gravid female rats were mated with one male, during the dark cycle of light, and the day of sperm detection in the vaginal smear was considered day 0 of gestation (GD 0). The gravid female were randomly assigned among the experimental groups and housed individually in cages. The experimental protocol followed the Ethical Principles in Animal Research of the Brazil College of Animal Experimentation and was approved by the Bioscience Institute/UNESP Ethical Committee for Animal Experimentation.

Treatment

Pregnant rats from treated group (*n*=10) received di-η-butyl-phthalate (DBP - Sigma Chemical Co., St. Louis, Mo) at 100 mg/kg/day, considered the LOAEL dose

[10], by gavage (oral route), from GD 12 until the end of lactation (post-natal day 21 - PND 21). Pregnant control rats received corn oil (vehicle), following the same experimental protocol. The beginning of the mothers' treatment coincided with the critical period of reproductive system development, which continues after the birth [28, 29].

Maternal body weight

Dams were weighted in alternate days, since the GD 0 until the end of lactation (PND 21) to allow the calculation of the DBP volume to be administered and the investigation of maternal toxicity, which could interfere with the development of the offspring, object of this study. Pups were weaned in PND 21 and, in the following day, the respective mothers were submitted to the euthanasia.

Fetal gonads collection and analysis

Control (*n*=5) and treated (*n*=5) pregnant dams were killed by decapitation on GD 20. After uterus and ovaries were removed, the numbers of corpora lutea, implantation sites, resorptions and live and death fetuses were recorded. From females fetuses (one per litter) the ovaries were collected, fixed in Karnovsky (2.5% glutaraldehyde, 8% de paraformoldehyde), processed to histological analysis and stained with hematoxylin and eosin. Germ cells were counted from sections of control and treated ovaries and expressed as number of cells per unit area (nº germ cells/mm^2).

Anogenital distance and number of nipples/areola

On PND 4, the anogenital distance (AGD, the distance between the anus and the genital tubercle) was measured in female pups, using a paquimeter. On

PND 13, the number of areola was recorded. Observations were scored based on the presence or absence of a nipple bud or a discoloration of the skin surrounding the nipple [10].

External signs of puberty onset

Beginning on PND 30, all females were evaluated daily for vaginal opening (VO). The day of complete VO were recorded. From the day of VO, daily vaginal smears were collected to detect the day of first estrous, characterized by the predominance of cornfied epithelial cells.

Estrous cycle

On PND 60 the estrous cyclicity of female rats was assessed on cells from daily vaginal smears, collected over a period of 15 days, as described by [30]. Every morning 10μL of 0.9% saline was instillated into the vagina and subsequently aspirated. The material was observed under light microscopy and the estrous cycle phase was determined by cytology: predominance of nucleated epithelial cells (proestrous); predominance of cornified epithelial cells (estrous); presence of cornified and nucleated epithelial cells and leukocytes (metaestrous); predominance of leukocytes (diestrous). The total frequency of each phase for every rat observed in this period was used to calculate the total length of the proestrous, estrous, metaestrous and diestrous (in days).

Analysis of reproductive organs

At PND 60, ovaries and uteri were collected from rats in estrous phase (*n*=5 in each group), fixed in Alfac's solution, dehydrated in ethanol and embedded in paraplast.

Serial sections (5μm) were obtained, mounted on glass slides and stained with hematoxylin and eosin.

In ovary, ovarian follicles and corpora lutea were counted. Follicles were classified according to [31, 32]. Primordial and primary follicles were enumerated together; oocytes surrounded by a single layer of either squamous or cuboidal epithelial cells were included. Follicles were classified as preantral when containing 2-4 layers of granulose cells with no antral space. Antral follicles were classified when containing three or more layers of granulose cells and a clearly defined antral space. Characteristics of atretic follicles included pyknotic granulose cells, disorganized granulose cells, degenerating oocyte and detachment from the basement membrane. In the uterus the endometrial height was measure, in three sections per animal using a light microscope. In each section, five different regions were analyzed, totalizing 15 measurements.

Hormonal analysis

Female pups were sacrificed on PND 60, during the estrous phase, between 8:00 and 10:00 a.m. After decapitation, trunk blood was collected and allowed to clot on a refrigerator (4ºC) for 30 minutes. Serum was collected after centrifugation and stored at -20ºC until analysis. Serum FSH, LH and progesterone concentrations were measured using a double-antibody radioimmunoassay (RIA) kit (National Institute of Arthritis, Diabetes and Kidney Diseases – NIADDK, USA). All the samples were analyzed at the same assay to avoid inter-assay mistakes.

Sexual behavior

On the first estrous after PND 80, control (n=10) and treated (n=10) female rats were used for the mating tests. Rats were maintained under controlled temperature

conditions on an inverted 12h light–dark cycle, for at least seven days, with food and water *ad libitum*. For the evaluation of female sexual behavior, sexually experienced males were allowed ten mounts on the female and the presence of lordosis was measured. Results were expressed as the lordosis quotient (LQ, number of lordosis/10 mounts x 100). All females were used only once.

Fertility and reproductive performance

This analysis was performed through natural mating. Rats from control (*n*=14) and treated (*n*=15) groups were placed with sexually experienced males (1:1), until 3 sexual cycles (3 estrous), in the beginning of the morning, during a dark period of the cycle. At the end of afternoon, males and females were separated and vaginal smears were collected, in which initial sperm detection was determined to be GD 0. On GD 20 females were killed by decapitation. After collection of the uterus and ovaries, the number of corpora lutea, implantation sites, resorptions, live fetuses and fetal weights were determined. From these results were determined: gestation rate: number of pregnant females/number of inseminated females x 100; fertility potential (efficiency of implantation): implantation sites/corpora lutea x 100; pre-implantation loss rate: number of corpora lutea - number of implantations/number of corpora lutea x 100; post-implantation loss rate: number of implantations - number of live fetuses/number of implantations x 100; sex ratio: number of male fetuses/number of female fetuses x 100.

Statistics

Values are expressed in mean $\pm$ SEM. For comparison of results between the experimental groups, Student's t-test and Mann-Whitney test were utilized. Differences were considered significant when $p<0.05$.

Results

<u>Effects on dams</u>

None of the treated group showed visible signs of toxicity during pregnancy or lactation period. At the dose level tested, DBP treatment had no effects on maternal body weight or body weight gain, during pregnancy and lactation (figure 1).

DBP exposition, from GD 12 to GD 20, provoked an increase in fetuses' body weight, when compared to control group (table 1). All the other reproductive parameters were comparable between groups.

<u>Effects on the female offspring</u>

Figure 2 contains histological sections of fetal ovaries in the control and DBP-treated groups, showing normal aspects. The number of germ cells per fetal ovarian sections unit area was comparable between groups (C=3.37 ± 0.23 x 10^3; T=2.83 ± 0.22 x 10^3).

There was no significant difference between the experimental groups in relation to body weight at birth (C=6.99 ± 0.14; T=7.23 ± 0.14).

The absolute (C=1.52 ± 0.03; T=1.51 ± 0.05) and relative (C=13.69 ± 0.36; T=13.02 ± 0.58) anogenital distance (mm), as well as the number of nipples/areolas (C=12.08 ± 0.05; T=12.06 ± 0.03) did not statistically differ.

Administration of DBP at 100mg/kg/day did not affect the age of puberty onset, determined by the age (days) at vaginal opening (C=35.25 ± 0.28; T=35.64 ± 0.36) and first estrous (C=37.04 ± 0.79; T=37.46 ± 0.48).

Ovary and uterus (with fluid) weights were unaffected by treatment (table 2).

Histopathological analysis of the ovary (figure 3) and uterus (figure 4) did not reveal significant differences between the groups. In the same way, there were no

significant differences between groups in relation to the numbers of corpora lutea, primordial/primary, pre-antral, antral and atretic follicles (table 3).

No statistically differences related to the thickness (μm) of uterine endometrium, in estrous phase, was observed between the control group and DBP-treated groups (C=383.90 $\pm$ 10.76; T=391.26 $\pm$ 22.73).

DBP exposure did not significantly affect serum concentration (ng/mL) of FSH (C=5.95 $\pm$ 1.08; T=7.11 $\pm$ 1.19), LH (C=3.98 $\pm$ 1.03; T=2.52 $\pm$ 0.74) and progesterone (C=4.75 $\pm$ 0.72; T=5.76 $\pm$ 1.61).

Female offspring from control and DBP-treated groups exhibited a normal pattern of estrous cyclicity (table 4).

The lordosis quotient, obtained from sexual behavior test, was not different between experimental groups (median (Q_1-Q_3): C= 100 (90 – 100); T=95 (90 – 100), Mann-Whitney test). In the same way, the reproductive performance was not affected by the treatment with DBP *in utero* and lactation, with exception for the fetal weight, that was increased in the treated group (table 5).

Discussion

Herein we evaluated the reproductive effects of DBP on the female offspring of rats exposed *in utero* and during lactation. During these critical phases of development, the fetuses and newborns appear to be more sensitive than adults to endocrine disruption [33, 34], especially in the male reproductive system [13, 35]. Compared to males, there is little information on DBP toxicity on the female reproductive system [6, 15, 36]. The Centers for Disease Control and Prevention (CDC) found DBP in the bodies of all 289 persons tested. The researchers also discovered that the most vulnerable population,

women of childbearing age whose fetuses are exposed to DBP in the womb, appears to receive the highest exposures [37].

The literature on the effects of DPB administered during gestation is controversial. While some authors report that there are no toxic effects to the dams [20, 38], others described decreased weight gain by mothers and significantly lower body weight in the pups [20, 36, 39].

In the present study DBP had no significant effects on maternal body weight gain during pregnancy and lactation, suggesting that the treatment at a dose of 100mg/kg/day did not provoke maternal toxicity.

In the literature there are various reports showing that different phthalates (DBP, BBP, DIBP) are fetotoxic, as evidenced by the decrease in fetal body weight after administering the compounds to the dams [16, 40-42].

On the contrary, the present work found significant weight gain both in the fetuses obtained from DBP-treated dams and, after the fertility test, in the adult offspring. In these fetuses, quantitative analysis of the ovaries demonstrated no alterations in the number of germ cells/unit area. It is difficult to compare our results with the literature, because different doses and routes of administration were utilized. In this regard, the experiment must be replicated to obtain more conclusive results. However, the body weight of pups at birth was similar between the experimental groups.

AGD and number of nipples are hormonally sensitive developmental measures [20, 43]. In the present study, these parameters were not altered by the treatment. Our results are corroborated by others, showing that oral exposure to DBP had no significant effects on AGD in female pups [6, 20].

Puberty, an event indicated by the age of vaginal opening and first estrus [44, 45] is characterized by rapid physiological changes such as growth spurt and maturation of gonads and the brain. It entails the individual's transition period from a non-reproductive to a reproductive state. The main hormone involved in the regulation of puberty onset is gonadotropin-releasing hormone (GnRH) in the hypothalamus that stimulates release of both LH and FSH from the pituitary [46], which act on gonads [47], leading to an increase of the blood estradiol level. Endocrine disruptor chemicals have been implicated in numerous physiological processes affecting normal reproductive health [48].

[36] verified that females treated with DBP in the diet at doses of 12 mg/kg and 50 mg/kg for two months during the pre-pubertal period presented an evident delay in their vaginal opening and occurrence of first estrus. These results suggest that this chemical presents certain estrogenic activity that may be exerted through the estrogenic receptor, since [49] showed that DBP may enhance the proliferation of breast cancer cells. Administration of DEHP (di-2-ethylhexyl-phthalate), a phthalate structurally related to DBP, by gavage or inhalation exposure, also delays the vaginal opening and first estrus [4, 50].

On the other hand, females treated with DBP at doses of 0.5, 5, 50, 100 or 500 mg/kg, from GD 12 to PND 21 [10] and at a dose of 500 mg/kg, by gavage, beginning on PND 21, did not present alterations in the age of vaginal opening [15]. In the same manner, [6] demonstrated that DBP at the concentrations of 20, 200, 2000 and 10,000 ppm administered through the diet, led to a non-significant decrease in the vaginal opening age. These results corroborate the present study, since no difference in ages of vaginal opening or first estrus was observed between the DBP-treated group and control animals, suggesting absence of treatment effects on puberty onset.

Phthalates can proliferate peroxisomes by activating the peroxisome proliferator-activated receptors (PPARs), which regulate apoptosis, differentiation and the cell cycle in the rat ovary, thus disrupting the timing of growth and differentiation of ovarian follicles [51]. DEHP and DBP are known to cause polycystic ovaries in adult female rats [52], altering estradiol metabolism [53]. Initial studies *in vivo* demonstrated that the ovary was a target site for DEHP and that decreased estradiol production was a primary functional alteration of DEHP exposure [54]. As a result of the lack of ovulations, there was an absence of corpora lutea, and follicles became cystic [52]. [42] treated female rats orally with BBP and reported that, at high doses, ovaries presented significantly decreased weight with hypoplasia of the vesicular follicles. [55], after administering DEHP to female rats, by daily gavage, reported a significant increase in the number of tertiary (with antral formation) atretic follicles at the 405mg/kg/day dose, but no statistically significant differences related to the thickness of uterine epithelium. In the present work, no differences were found in weight or histological evaluation of ovaries and uterus between the DBP-treated group and controls.

Sex steroids influence the growth, function and differentiation of female reproduction organs and make them susceptible to endocrine disruption. During the estrous cycle, fluctuating levels of estrogen and progesterone elicit profound effects on epithelial proliferation and cytodifferentiation [56]. We found no alterations in uterine endometrial height in the rats exposed to DPB *in utero* and during lactation, indicating normal hormonal responsiveness of the female reproductive tract, a finding supported by hormonal results, whereas no difference in LH, FSH and progesterone serum levels between groups was observed. Similarly, [50] and [42] also reported normal LH and FSH serum concentrations after the administration of two phthalates (DEHP and BBP, respectively) to female rats.

No significant changes were detected in estrous cyclicity or reproductive organ weights due to DBP exposure. These data corroborate those of [6], who reported absence of effects on the estrous cycle or ovarian and uterine weights in rats that received DBP through the diet. [15] also found no alterations in the estrous cycle after administering DBP orally to female rats at 500 mg/kg dose. When DEHP was used at doses increasing from 0.015 to 405 mg/kg, no alterations were observed either in the reproductive organ weights, or in estrous cyclicity, at all doses tested [55].

On the other hand, [42] demonstrated that females exposed to BBP, by gavage, at doses of 20, 100 and 500 mg/kg, for two weeks, displayed altered estrous cyclicity and a dose-dependent decrease in ovarian weight. [50] also reported that inhalational treatment with DBP at 25 mg/m^3, increased the number of irregular estrous cycles, indicating disruptive effects in females.

[39] reported that exposure to DBP administered in the diet, at different doses (20, 200, 2,000 and 10,000 ppm), from GD 15 to PND 21, decreased the lordosis quotient in rats, the posture adopted by the female in response to a mounting male during mating. This fact occurs due to an inappropriate expression of some specific genes responsible for sexual differentiation in the hypothalamus of neonatal rats by exposure to DBP. In this present study, the normal lordosis quotient, obtained by the sexual behavior test, suggests that DBP did not interfere with the female brain sexual organization.

In conclusion, DBP, in these experimental conditions, did not disturb the reproductive development or function of female rats.

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Legends of figures

Figure 1. Body weight (g) of rats from control (*n*=9) and DBP-treated (*n*=10) groups. Values expressed as mean $\pm$ S.E.M.

Figure 2. Histopathological aspect of longitudinal sections from rat fetal gonads. (A) Control group. (B) DBP-treated group. The primordial follicles are organized in clusters (asterisks). 20X, HE.

Figure 3. Morphological aspect of longitudinal section from rat ovary. : (A) Control group. (B) DBP-treated group. CL: corpora lutea; arrow: antral follicle; asterisk: pre-antral follicle; v: blood vessels. 10X, HE.

Figure 4. Transverse section of uterus from rats on estrous phase. (A) Control group. (B) DBP-treated group. E: endometrium; M: miometrium; arrows: endometrial glands. 10X, HE.

Table 1. Reproductive parameters of control and DBP-treated groups dams.

Parameters	Control (*n*=9 litters)	Treated (*n*=10 litters)
Final body weight (g)	363.78 ± 21.77	371.56 ± 18.50
Uterus weight+fetuses (g)	51.48 ± 1.94	63.23 ± 9.06
Body weight of fetuses (g)	3.04 ± 0.16	$3.65 \pm 0.17*$
Number of corpora lutea	12.75 ± 1.03	13.00 ± 0.84
Number of implants	12.25 ± 0.75	12.80 ± 0.92
Number of resorptions	1.25 ± 0.63	1.20 ± 0.97
Number of fetuses	11.00 ± 0.58	11.60 ± 1.70
[A]Pre-implantation loss (%)	0 (0 – 3.33)	0 (0 - 0)
[A]Post-implantation loss (%)	7.69 (5.77 – 11.54)	0 (0 – 9.09)

Mann-Whitney Test, *p<0,05. [A] Values are expressed in median (Q_1-Q_3).

Table 2. Body weight and absolute and relative organ weights in the female offspring from control and DBP-treated groups.

Parameters	Control (n=05)	Treated (n=05)
Body weight (g)	202.10 ± 8.23	194.42 ± 8.86
Ovaries (mg)	98.00 ± 13.78	93.60 ± 6.59
Ovaries / body weight (mg/g)	0.48 ± 0.06	0.48 ± 0.02
Uterus + fluid (mg)	345.60 ± 26.70	314.20 ± 17.04
Uterus + fluid / body weight (mg/g)	1.71 ± 0.11	1.62 ± 0.87

Table 3. Ovarian follicles and corpora lutea counting in PND 60 female rats from control and DBP-treated groups.

Structures	Control (*n*=5)	Treated (*n*=5)
Primordial and primary follicles	45.60 ± 11.16	30.40 ± 6.45
Pre-antral follicles	21.20 ± 1.72	26.80 ± 3.71
Antral follicles	24.40 ± 5.45	12.00 ± 2.81
Atretic follicles	22.4 ± 3.08	19.20 ± 2.61
Corpora lutea	18.80 ± 2.06	23.60 ± 2.62

Table 4. Assessment of estrous cycle in the female offspring from control and DBP-treated groups.

Parameters	Control (n=9 litters)	Treated (n=10 litters)
Frequency of proestrous (days)	1.78 ± 0.26	1.94 ± 0.13
Frequency of estrous (days)	4.60 ± 0.19	4.28 ± 0.28
Frequency of metaestrous (days)	2.38 ± 0.29	2.31 ± 0.19
Frequency of diestrous (days)	6.24 ± 0.37	6.46 ± 0.25

Table 5. Fertility test results, accomplished in PND 80 female offspring from control and DBP-treated groups.

Parameters	Control (n=14 litters)	Treated (n=15 litters)
Gestation rate (%)	93.33	100
Final body weight (g)	348.00 ± 9.47	349.76 ± 8.99
Uterus + fetuses weight (g)	52.06 ± 2.59	53.29 ± 3.49
Rat weight - (uterus weight + fetuses)	295.94 ± 8.14	296.47 ± 6.91
Number of fetuses	11.36 ± 0.59	11.00 ± 0.79
Number of implants	12.14 ± 0.84	11.40 ± 0.81
Number of corpora lutea	13.71 ± 0.70	12.87 ± 0.43
[A]Fertility potencial (%)	90.90 (85.71 - 100)	93.33 (88.34 - 100)
[A]Pre-implantational loss (%)	9.09 (0 – 14.29)	6.67 (0 – 11.67)
[A]Post-implantacional loss (%)	0 (0 – 7.92)	0 (0 – 4.17)
Sexual rate	1.30 ± 0.16	1.22 ± 0.17
Fetuses weight (g)	2.83 ± 0.08	3.13 ± 0.03**
Weight of placenta (g)	0.45 ± 0.01	0.44 ± 0.01

Student's t-test (**$p<0,01$).
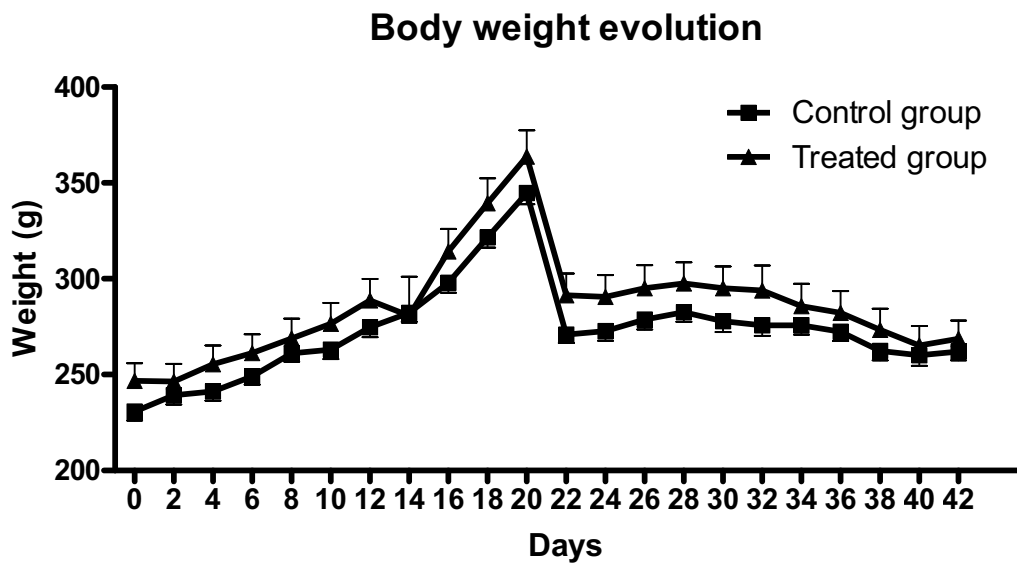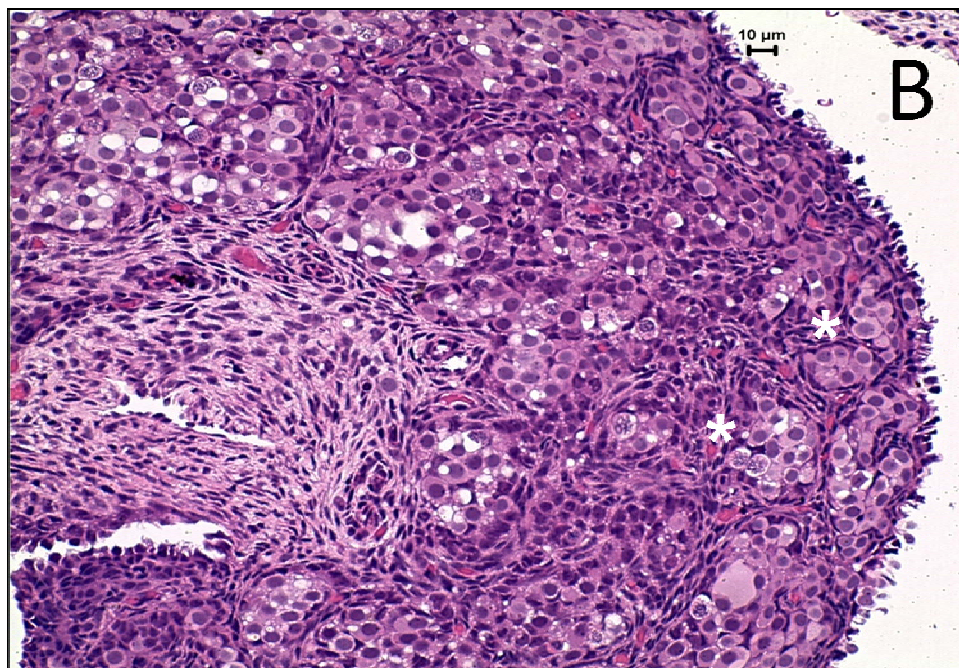
[A]Mann-Whitney test, values expressed as median (Q_1-Q_3).
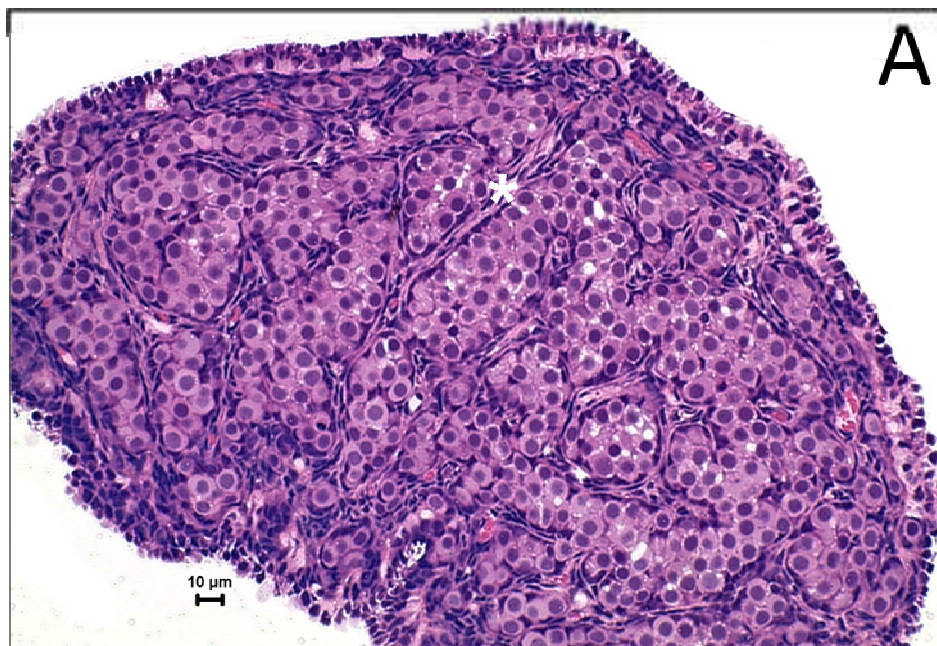
Figure 1
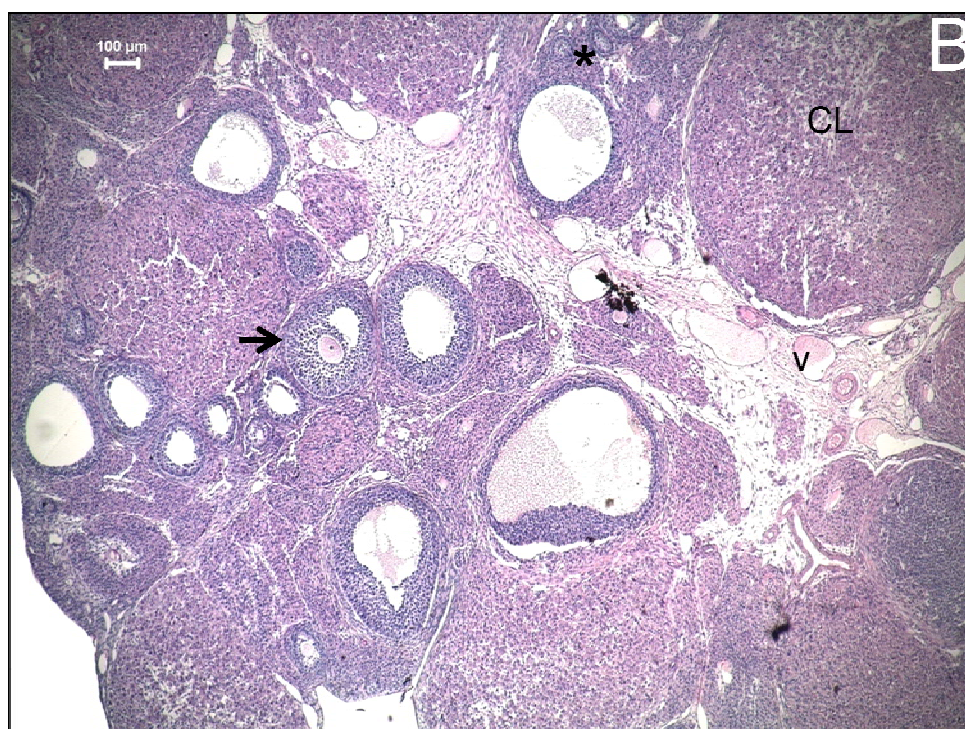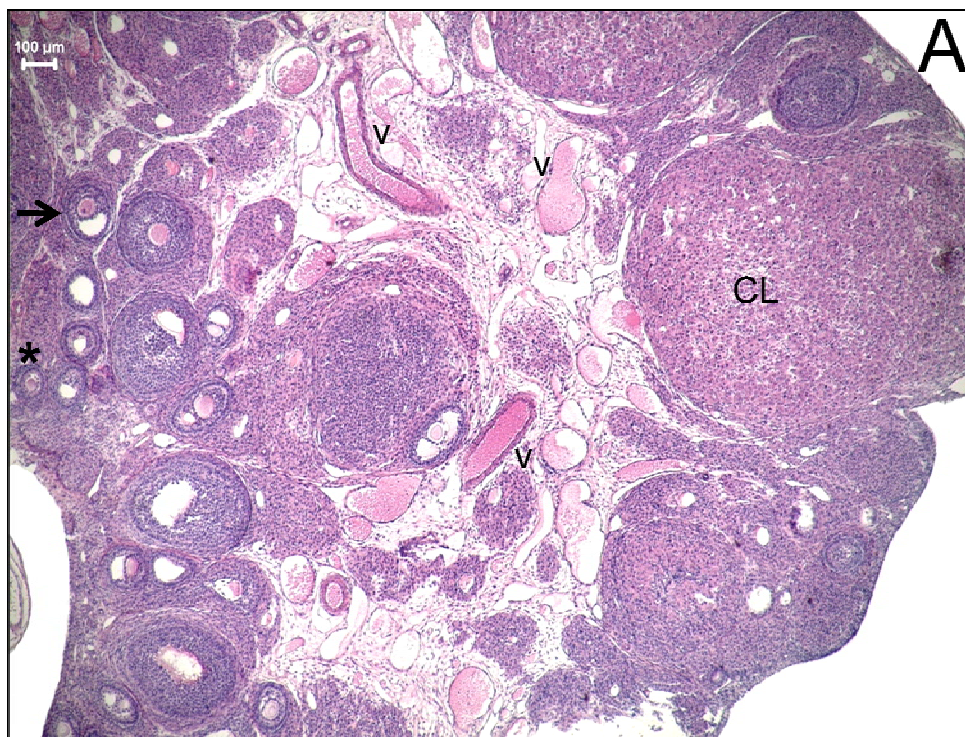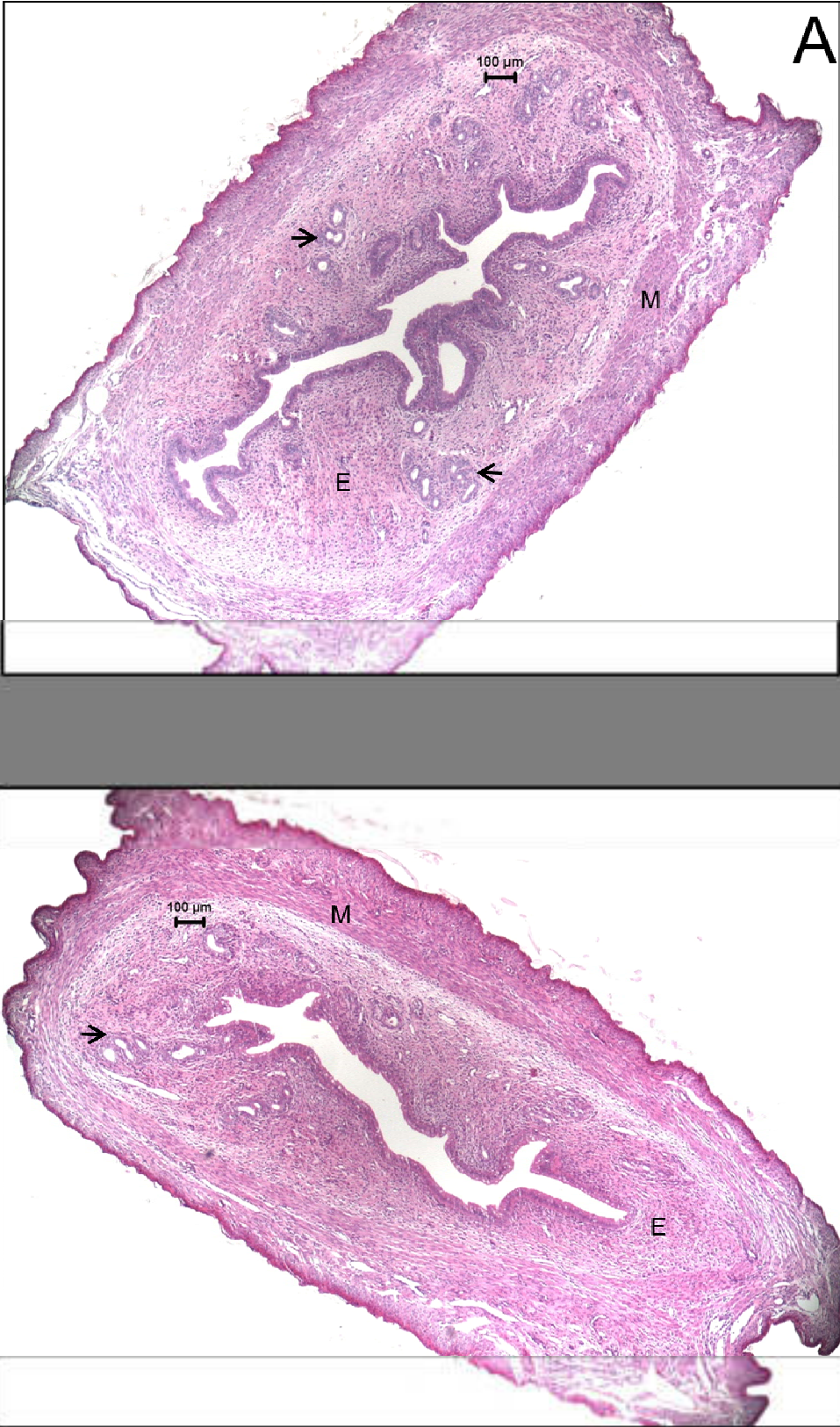

Body weight evolution

Figure 2

Figure 3

Figure 4

Conclusão

Conclui-se que, o DBP, administrado a ratas durante o período intra-uterino e lactacional, na dose de 100/mg/kg/dia, não altera o desenvolvimento nem a função reprodutiva feminina.

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EXPERIMENTAÇÃO ANIMAL

Caixa Postal 510 – 18618-100 – Botucatu, SP fone (014) 3811-6013 fax (014) 3811-3744

CERTIFICADO

Certificamos que o Protocolo nº 60/06-CEEA,
sobre *"FUNÇÃO REPRODUTIVA DE RATAS*
EXPOSTAS AO FTALATO in útero E LACTAÇÃO", sob
a responsabilidade de **WILMA DE GRAVA KEMPINAS**,
está de acordo com os Princípios Éticos na Experimentação
Animal adotado pelo Colégio Brasileiro de Experimentação
Animal (COBEA) e foi aprovado pela *COMISSÃO DE ÉTICA NA*
EXPERIMENTAÇÃO ANIMAL (CEEA), em reunião de 02/03/07.

Botucatu, 02 de março de 2007.

Prof. Dr. MARCELO RAZERA BARUFFI
Presidente - CEEA

NADIA JOVÊNCIO COFRIM
Secretaria - CEEA

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