UNIVESIDADE FEDERAL DO RIO GRANDE – FURG
PROGRAMA DE PÓS GRADUAÇÃO EM
CIÊNCIAS FISIOLÓGICAS :
FISIOLOGIA ANIMAL COMPARADA

EFEITOS DO DESREGULADOR ENDÓCRINO BISFENOL A EM PARÂMETROS REPRODUTIVOS E SISTEMA NERVOSO CENTRAL DE CAMUNDONGOS *Mus musculus* (RODENTIA)

Ana Cristina Kalb

Tese defendida no âmbito do Programa de Pós-Graduação em Ciências Fisiológicas: Fisiologia Animal Comparada como parte dos requisitos para obtenção do título de DOUTOR em Fisiologia Animal Comparada.

Orientador: Pof. Dr. Pablo Elías Martínez

Rio Grande - RS

2012

Dedico este trabalho a todos aqueles que acreditam que a Ciência não é apenas mais um afazer acadêmico, mas um complemento necessário à humanidade.

“Quem passou pela vida em brancas nuvens,
E num plácido repouso adormeceu,
Quem não sentiu o frio da desgraça;
Quem passou pela vida e não sofreu,
Foi espectro de Homem, não foi Homem,
Só passou pela vida, não viveu.”

Martin Luther king

Agradecimentos

Em primeiro lugar à força maior que rege o universo, que guia nossos passos e nossos corações;

Aos meus pais, Max Edmundo e Ivoni, que por uma vida de dedicação, amor e trabalho sempre possibilitaram a suas filhas a oportunidade de realizar sonhos e conquistas;

Ao meu querido avô Evaldo, que mesmo não podendo estar sempre perto, tenho certeza que nós sempre estivemos e estaremos de mãos dadas em todos os desafios;

Ao meu orientador Pablo Martínez, que me acolheu em um momento muito difícil, me deu exemplo de como ser apaixonado pelo que fazemos, e me proporcionou um aprendizado e crescimento únicos.

A minha querida irmã e estagiária Ana Luiza que esteve sempre comigo, em todos os dias de trabalho e da minha vida e que sem o seu auxílio, muito deste trabalho não teria sido concretizado;

Ao meu querido e amado Josencler, que me ensinou a arte de amar, amar a vida, ser uma pessoa melhor e consciente, e que compartilha comigo a paixão e a curiosidade pela Biologia.

A minha querida amiga Mariângela que sempre me tratou com o carinho de uma mãe;

Ao meu querido amigo Vinícius Gonzalez que sempre foi ouvinte nas horas difíceis e coadjuvante nos momentos felizes;

As amigas Camila Dalmolin, Sandra Isabel e Flavinha pela força e carinho, queria muito poder ter vocês sempre por perto;

Ao Reuni pela bolsa cedida;

A todos os professores e funcionários da Fisiologia, por contribuírem de forma direta ou indireta à minha formação;

Ao Curso de Pós-Graduação em Ciências Fisiológicas, onde me foi proporcionado não apenas a oportunidade de realização desse trabalho, mas um ambiente ideal para o meu desenvolvimento.

Sumário

Resumo

Bisfenol A (BPA) é conhecido por ser um desregulador endócrino com conhecida ação no sistema reprodutor e sistema nervoso central. Fêmeas de camundongo com aproximadamente 2 meses foram expostas via gavagem durante o período de amamentação: dois grupos controle (salina 0,9% e azeite de oliva); BPA 300, 900, 3000 (μg/Kg/dia) e a dietilestilbestrol (DES, 650 μg/Kg/dia). Após 21 dias de exposição (período de amamentação, exposição sub-crônica) as mães foram sacrificadas e seus cérebros coletados para posterior análise de parâmetros neuronais. Os machos filhotes foram separados, sua distância ano-genital medida, e após 240 dias sacrificados para avaliação do impacto da transmissão vertical de BPA durante a amamentação em seus testículos, nos quais foram analisados parâmetros espermáticos e estresse oxidativo. Como resultado encontrou-se uma diminuição na distância ano-genital nos animais do tratamento BPA 3000. BPA prejudica severamente os parâmetros espermáticos analisados, tais como: motilidade do espermatozóide, morfologia, funcionalidade mitocondrial, integridade de membrana, de acrossoma e de DNA, nos animais de todos os tratamentos. Nas amostras de cérebro das mães expostas durante o período de lactação, foram analisados o número de astrócitos (GFAP), enzima tirosina hidroxilase (TH), quantidade de Sinaptofisina e a proliferação celular (H3P), nas regiões do Córtex, Estriado e Hipocampo. Através da imunohistoquímica, como resultados observamos uma diminuição do número de astrócitos em todas as regiões e diminuição de área de fluorescência de TH na região CA3 do hipocampo no tratamento BPA300; No tratamento BPA900, observou-se o decréscimo no número de astrócitos, aumento na proliferação celular e diminuição do processo de formação sináptica na região CA3 do hipocampo. A perda da função sináptica também se observou no córtex para este tratamento. Os resultados gerais mostram proliferação celular induzida por BPA, mas

também reportam uma perda na quantidade de astrócitos, concentração de TH e de densidade sináptica. Podemos concluir que BPA administrado indiretamente nos filhotes causou um prejuízo nos parâmetros espermáticos. Quando administrado diretamente nas mães por um período curto, mostrou-se como um potencial indutor de doenças neurodegenerativas.

1. Introdução

O estrogênio é um hormônio esteróide, é produzidos principalmente nos ovários, corpo lúteo e placenta. Adicionalmente, outros tecidos incluindo fígado, glândula adrenal, mamas, tecido adiposo, e tecido nervoso contribuem para uma pequena parcela de hormônio produzido (Judd & Fournet, 1994). Seu mecanismo de ação clássico ocorre pela ligação aos seus receptores intracelulares a tecido/espécie específicos. Esses receptores pertencem à superfamília de receptores hormonais nucleares que ao formarem o complexo hormônio-receptor passam a ativar diferentes fatores de transcrição gênica. Já foram identificados três tipos de receptores para estrógenos, o receptor de estrógeno alfa (REα), o receptor de estrógeno beta (REβ) e o receptor de estrógeno gama (REγ) (Matsushima et al. 2007; Taylor et al. 2009). Nos estrógenos o complexo hormônio-receptor pode regular a expressão gênica sem se ligar diretamente ao DNA por modular a função de outras classes de fatores de transcrição através da interação proteína-proteína no núcleo (Gottlicher et al. 1998). Fatores de transcrição como Fos/Jun (AP1- elemento de resposta) ou SP-1 são alguns exemplos de elementos de resposta a estrógenos de ação genômica independente (Schreihofer et al. 2001). Um terceiro mecanismo, também sem ação genômica, onde os elementos de resposta a estrógenos estão associados a membrana celular (Deecher et al. 2003). Estrógenos se ligam ao recetor de estrógeno acoplado a proteina G (GPR30), o qual é uma proteína trans-membrana, levando a uma resposta da tradução relativamente rápida e transitória, para estrógenos. Esta via de sinalização não genômica é largamente observada na imediata estimulação de células e tecidos por estrógenos. (Qiu et al. 2003)

A síntese de hormônios esteróides envolve principalmente citocromos P450, sendo limitante a primeira enzima a citocromo P450 desmolase (CYP11) que converte o colesterol em pregnenolona. Isto acontece a nível mitocondrial e para o ingresso do

colesterol na mitocôndria é fator limitante a presença da proteína carregadora StaR (Proteína reguladora aguda da esteroidogênese). Entretanto, modificações periféricas são possíveis, assim os andrógenos podem ser convertidos em 3-dihidrotestosterona pela ação da enzima 5α-reductase e permitir a masculinização da genitália externa e outras estruturas. E ainda, os andrógenos podem se, perifericamente, transformados em estrógenos pela ação da enzima aromatase. Os efeitos dos andrógenos ocorrem principalmente através dos receptores de andrógenos (AR) e os estrógenos atuam por meio de três receptores (REα, β e γ). (Lindberg et al. 2002).

Esses receptores de estrógenos apresentam diferentes expressões, dependentes do tipo de tecido alvo, com a espécie envolvida e com a fase da vida do indivíduo, como por exemplo, o receptor de estrogênio alfa é expresso no útero, no fígado (Kuiper et al. 1997), nos rins (Brunette e Leclerc, 2002), no sistema cardiovascular, nas glândulas mamárias, no sistema nervoso, em células de câncer de mama (Cousen et al. 1997; Shearman et al. 2003;). E o receptor de estrogênio beta é expresso nas células da granulosa do folículo ovariano (Kuiper et al. 1997), no tecido ósseo, em núcleos do sistema nervoso central (Taylor et al. 2009; Figtree et al. 2009). Os estrógenos podem atuar através da via genômica (receptores nucleares) ou por vias não-genômicas (receptores de membrana), os quais são amplamente expressos no sistema nervoso central (SNC), incluindo o hipocampo, córtex e estriado e tem uma importante influência direta e indireta em funções cognitivas superiores (Prange, Kiel & Rune , 2006; Brann *et al.* 2007).

Assim, o hormônio esteróide (como também glicocorticóides, mineralocorticóides, androgênios e progesteronas), ao entrar em contato com célula alvo, atravessa sua membrana plasmática e interage com seus receptores intracelulares específicos, formando o complexo hormônio-receptor (Nadal et al. 2001; Norman et al.

2004). Após sofrer o processo de dimerização, esse complexo, então, desloca-se para o núcleo, atravessando pelo poro nuclear, onde atua como cofator de transcrição, no nucleoplasma, através da ligação a regiões específicas do ácido desoxirribonucléico (DNA), chamados de elementos responsivos ao estrogênio, o que por fim resulta na regulação da expressão de genes-alvo (Silberger e Magleby, 1999).

Além desses receptores (REα e REβ), o receptor de estrogênio gama (REγ), que foi inicialmente identificado no tecido testicular, nos rins, e no miocárdio humano, pode ser expresso no tecido muscular esquelético e na retina (Giguere et al. 1988). Também pode ser encontrado em tecidos humanos fetais como na placenta, no cérebro, no coração, nos rins, nos pulmões e na musculatura esquelética, cujo mecanismo de ação pode estar relacionado com a diferenciação e a maturação desses tecidos em indivíduos adultos (Heard et al. 2000).

Os hormônios esteróides sintetizados no sistema nervoso (SN) de vertebrados a partir do colesterol são conhecidos como neuroesteróides. Os hormônios esteróides também podem ser sintetizados em glândulas esteroidogênicas periféricas como, por exemplo, ovários e testículos, e regular funções neuronais importantes durante o desenvolvimento do indivíduo que persistem nos vertebrados até a idade adulta (Chen et al. 2009; Joseph et al. 2009). Hormônios esteróides periféricos, devido a sua característica de lipossolubilidade, podem atravessar a barreira hemato-encefálica atuando no tecido cerebral através de receptores intracelulares que regulam a transcrição de genes específicos (Shao et al. 2012)

Células da glia estão envolvidas na formação de neuroesteróides e no metabolismo do cérebro. Ambos astrócitos e oligodendrócitos podem ser considerados locais primários de síntese de pregnenolona, o passo inicial da neuroesteroidogênese. Há evidências de presença de enzimas esteroidogênicas como a citocromo P450 no

córtex cerebelar nas células de Purkinje, (Haraguchi *et al.* 2011), desta forma, com a presença de grande quantidade de estrógenos é observado um aumento no crescimento dendrítico, espinogênese e sinaptogênese nestas células (Price *et al.* 2000).

Outro papel importante dos estrógenos está relacionado a promoção de neurogênese. Como exemplo de vias relacionadas podemos citar a fosforilação da glicogênio sintase kinase 3 β. Os estrógenos também podem interagir com fatores de crescimento como o fator de crescimento semelhante a insulina 1 (IGF-1) e o fator neurotrófico derivado do cérebro (BDNF) (Garcia-Segura et al. 2006; Scharfman et al. 2006; Scharfman et al. 2012).

Os esteróides gonadais podem atuar no cérebro e influenciar comportamentos reprodutivos em vertebrados como cópupa, corte, comportamento materno. Por exemplo, algumas regiões do cérebro, que são responsáveis por controlar grande variedade de comportamentos reprodutivos, contêm uma grande quantidade de células que concentram andrógenos (Roselli e Resko, 1997; Tsutsui et al. 2000).

1.1 Desreguladores endócrinos

Nos últimos anos evidenciou-se que muitos produtos químicos, presentes no meio ambiente, podem interferir com as ações fisiológicas dos hormônios endógenos. Estas substâncias podem ser agonistas de receptores e interferir (imitando ou apenas ocupando) com hormônios endógenos e, por isso, foram chamados de desreguladores endócrinos (Darbre, 2006b). Os desreguladores endócrinos, que além de serem encontrados no meio ambiente, podem também ser provenientes de produtos sintéticos, podem atuar no genoma celular como agonistas ou antagonistas dos receptores de esteróides.

Dentre os efeitos causados pela alteração hormonal, os disruptores endócrinos podem alterar a função reprodutiva e causar feminilização por ligação com receptores

de estrogênio ou androgênio (Waring e Harris, 2005; Tabb e Blumberg, 2006) e também interferir com o crescimento mamário, a lactação e predispor a doenças uterinas como fibroses e endometriose (Mclachlan et al. 2006). Muitos disruptores endócrinos podem se ligar a receptores tireoidianos e desregular o sistema neuroendócrino (Waring e Harris, 2005; Whitehead e Rice, 2006). Os disruptores endócrinos podem ainda possuir outros mecanismos de ação e, quando não agem no genoma, podem alterar a síntese enzimática de subprodutos hormonais, causando alteração na função imune alteração comportamental e da memória (Waring e Harris, 2005; Whitehead e Rice, 2006).

Muitos estudos concentram-se na avaliação da interferência dos disruptores endócrinos durante o período pré-natal e pós-natal inicial, pois estas fases de crescimento caracterizam-se pelo rápido crescimento e pela grande dependência de ações hormonais. Perturbações no sistema endócrino durante estes períodos podem causar, tardiamente, alterações anatômicas, fisiológicas, comportamentais e até mesmo predispor ao desenvolvimento de doenças (Vandenberg, 2004; Dickerson e Gore, 2007). Durante o desenvolvimento intra-uterino podem determinar crescimento intra-uterino restrito bem como alteração na maturação do cérebro e das gônadas (Schoeters et al. 2008). Tardiamente podem causar puberdade precoce e aumento da incidência de cânceres como o câncer vaginal e o câncer de próstata (Vandenberg, 2004; Dickerson e Gore, 2007).

O estrogênio endógeno, mimetizado por disruptores endócrinos, possui papel crucial na diferenciação sexual de estruturas do sistema nervoso central, controlando algumas funções neuroendócrinas, cognitivas e comportamentais como, por exemplo, a ativação de receptores de estrógenos no hipotálamo estimulando o comportamento maternal logo após o parto. Durante o período pré-natal, o estradiol é responsável pelo

tipo de organização do cérebro em machos e em fêmeas (Wilson, et al. 2000; Champagne, et al. 2001).

1.2 Bisfenol A

A molécula de bisphenol A (4, 4'-dihidroxi-2, 2-difenilpropano) (BPA) possui dois anéis fenólicos ligados por uma ponte com duas ligações metila.

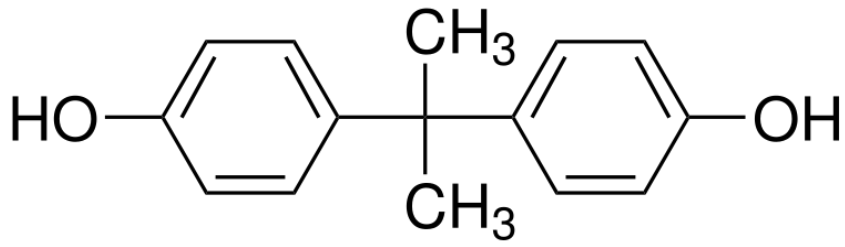


Figura 1. Molécula de Bisfenol A.

O Bisfenol A (BPA) é um monômero de plástico usado na manufatura de muitos produtos químicos incluindo resinas epóxi e policarbonatos. É encontrado em materiais usados como embalagem de alimentos, garrafas pet, mamadeiras, entre outros. Sua estrutura consiste de dois anéis de fenol insaturados com pouca homologia estrutural com o estradiol (E2), mas é semelhante ao dietiletilbestrol (DES), ao hexestriol e ao componente bisfenólico do tamoxifeno (Jonathan et al. 1998). A atividade estrogênica do BPA foi descoberta ao acaso. Pesquisadores da Universidade de Stanford identificaram uma proteína ligadora de estrogênio em levedura e, posteriormente, estudaram a existência de um ligante endógeno acoplado a esta proteína. Depois do primeiro relato de que a levedura produzia E2 (Feldman et al. 1984), esses autores verificaram que a atividade estrogênica não era proveniente da levedura, mas sim do meio de cultura preparado com água autoclavada em frasco de policarbonato (Krishnan, et al. 1993).

Por causa da estabilidade superior, flexibilidade e resistência, as resinas epoxi-BPA são utilizadas em vários produtos, como camadas de revestimento interno de

latas de alimentos, complexos dentários para obturações e embalagens de remédios (Brotons et al, 1995). Sua liberação no ambiente é possível quando a polimerização é incompleta ou através de hidrolização causada por altas temperaturas (Krishnan et al, 1993; Feldman et al, 1984). A partir do revestimento das latas de alimentos, foi detectada liberação de BPA em concentrações na faixa de 0,004 a 0,023mg/kg de alimento (Feldman et al, 1984).

BPA administrado oralmente entra no organismo e é metabolizado primariamente no intestino e fígado, é convertido pelo sistema de detoxificação no fígado, pela citocromo P450 monooxigenase (Enzima de fase I) a 3-hidroxibisfenol A (3-OH-BPA), que pode se ligar a molécula de DNA ou macromoléculas através da formação de BPA-*o*-semiquinona ou BPA-*o*-quinona. A molécula de 3-OH-BPA, em reação de autoxidação, forma o radical ânion superóxido (Nakagawa and Moore 2000).

BPA é metabolizado em fase II de detoxificação, principalmente pela reação de conjugação pela UDP- glucoronosil transferase com o ácido glucurônico em ratos, camundongos, macacos e humanos. Formando BPA-monoglucoronide (BPA-gluc) formado é o maior metabólito produzido de BPA e é fisiologicamente inativo, não tendo afinidade pelos receptores de estrógeno ou atividade estrogênica (Matthews *et al.* 2001; Kurebayashi *et al.* 2003). BPA-gluc é excretado predominantemente pela via biliar nas fezes e urina (Völkel et al. 2002; Kurebayashi et al. 2003).

BPA pode produzir efeitos em mamíferos a partir dos primeiros estágios de vida. Essa exposição precoce pode afetar o desenvolvimento de células (como algumas do sistema imune), órgão, tecidos e sistemas, assim como a produção de hormônios por órgãos específicos. Seus efeitos podem persistir durante toda a vida do indivíduo e ter impacto em sua prole. Precisa ser levado em conta o período crítico de desenvolvimento de cada órgão e sua janela vulnerável de desenvolvimento (Rogers et al. 2013).

Alguns efeitos tóxicos podem ser observados durante o período perinatal de exposição ao BPA onde podemos considerar sua passagem via placenta para o feto, e durante o período de amamentação, onde uma grande quantidade de BPA é recebida pelo lactente (Pryor et al. 2000; Doerge et al. 2010). Alguns efeitos observados no sistema reprodutivo são diminuição no peso do epidídimo, aumento do peso da próstata (Chitra et al. 2003; Kato et al. 2006), baixa produção de espermatozóides em roedores e primatas, declínio na motilidade, alterações morfologia e dano de DNA (Saradha et al. 2006; Yang et al. 2010; Meeker et al.2010). Podemos relacionar a ação do BPA, alterações em genes envolvidos na esteroidogênese e espermatogênese, o que pode levar a um rompimento na barreira hemato-testicular e diminuição dos níveis de testosterona em ratos (Li et al. 2009; Nakamura et al. 2010). A geração de espécies reativas de oxigênio pela metabolização do BPA pode levar a um quadro de estresse oxidativo causando peroxidação lipídica e diminuição da capacidade antioxidante, o que pode levar a um quadro de patologia tecidual e apoptose via ativação de caspase 3 (Halliwell and Gutteridge, 2007). Também pode aumentar a expressão da enzima aromatase e ter efeito direto, por exemplo, na diminuição da distância ano-genital em machos, quando administradas doses altas de BPA (Quignot et al. 2012).

O estrogênio está relacionado com os processos de formação da memória, possui papel crítico na neurotransmissão do hipocampo associado com espinogênese ou neuroproteção (Gould et al. 1990), regulação da sinaptogênese na região CA1 e plasticidade sináptica (Miyagawa et al, 2007). Recentemente Miyagawa (2007) e colaboradores demonstraram que a disrupção no desenvolvimento de neurônios dopaminérgicos e também em outras neurotransmissões pode ser causada pela exposição de ratos pré-natal e neonatal ao BPA. Estes mesmos autores sugerem que altas doses de BPA causam uma piora na memória de ratos neste período. Por exemplo,

estudo usando imunohistoquímica, em animais expostos ao BPA neste mesmo período, demonstram um decréscimo nas fibras colinérgicas nas regiões CA1, CA2 e CA3 no hipocampo, como marcador foi usada a proteína acetiltransferase semelhante a colina imunoreativa (ChAT-IR). A função colinérgica no hipocampo é importante no aprendizado e na memória e pode ser afetada pela ação do BPA. A produção de espécies reativas de oxigênio induzida por BPA está relacionada a ativação de caspase 3 e conseqüente sinalização para apoptose de células da glia em diversas regiões do sistema nervoso central (Obata and Kubota, 2000).

O BPA, além de possuir atividade semelhante ao estrogênio, também pode se assemelhar ao hormônio da tireóide, este quando em baixas concentrações durante o desenvolvimento do cérebro e de outros órgãos, pode causar disrupção na atividade das enzimas do metabolismo central de acetilcolina (Donahue et al. 2004), sua ação ocorre pela supressão da atividade transcricional por inibição competitiva com a triiodotironina (T3) ao receptor de hormônio tireoidiano (TRα1 e TRβ1) (Suna et al. 2009).

Além dos efeitos no sistema reprodutor, sistema nervoso e tireóide, BPA pode afetar profundamente o funcionamento das ilhotas de Langerhans, a unidade endócrina do pâncreas. Combinado com o aumento da glicose, BPA promove a conversão de fibroblastos em adipócitos, possui efeito estimulatório as células β do pâncreas para uma maior produção de insulina, podendo ser através da estimulação pelo influxo de Ca^{2+} (Nadal et al. 2009).

Sendo assim, uma aavaliação da ação de um desruptor endócrino nos estágios iniciais de vida após o nascimento é de fundamental importância para o conhecimento sobre o desenvolvimento do indivíduo.

2. Objetivo Geral

Verificar o efeito da exposição durante a amamentação ao Bisfenol A sobre aspectos morfológicos e bioquímicos dos testículos dos filhotes e imunohistoquímica do sistema nervoso central das mães em camundongos.

2.1 Objetivos Específicos

1. Verificar efeito da exposição pós-natal ao Bisfenol A sobre parâmetros espermáticos de camundongos machos expostos durante o período de amamentação;

2. Verificar efeito da exposição pós-natal ao Bisfenol A sobre parâmetros de estresse oxidativo em testículos de camundongos machos expostos durante o período de amamentação;

3. Determinar o efeito da exposição pós-natal ao Bisfenol A sobre a imunohistoquímica do córtex cerebral, núcleo estriado e hipocampo em camundongos durante o período de lactação;

4. Verificar o efeito da exposição pós-natal ao Bisfenol A sobre a marcação imunohistoquímica da enzima tirosina hidroxilase no córtex cerebral, núcleo estriado e hipocampo em camundongos;

5. Verificar o efeito da exposição pós-natal ao Bisfenol A sobre a marcação imunohistoquímica da proteína fibrilar ácida (GFAP) no córtex cerebral, núcleo estriado e hipocampo em camundongos;

6. Verificar o efeito da exposição pós-natal ao Bisfenol A sobre a marcação imunohistoquímica de Sinaptofisina no córtex cerebral, núcleo estriado e hipocampo em camundongos;

7. Verificar o efeito da exposição pós-natal ao Bisfenol A sobre a marcação imunohistoquímica de Histona-3-Fosfato no córtex cerebral, núcleo estriado e hipocampo em camundongos;

Artigo 1

Revista : Reproductive Toxicology

(Fator de impacto: 3,22)

Sperm impairment in male mice caused by maternal transference of bisphenol A during lactation

Ana Cristina Kalb[a], Ana Luiza Kalb[b], Tainã Figueiredo Cardoso[c], Carine Dahl Corcini[c], Antonio Sergio Varela Junior[b], Pablo Elías Martínez[a, b, *]

[a] Programa de Pós-Graduação em Ciências Fisiológicas: Fisiologia Animal Comparada, Universidade Federal do Rio Grande, Rio Grande, RS, Brazil

[b] Instituto de Ciências Biológicas; Universidade Federal do Rio Grande, Rio Grande, RS, Brazil

[c] REPROPEL- Faculdade de Veterinária; Universidade Federal de Pelotas, Pelotas, RS, Brazil

* Corresponding author. Tel./fax: +55 53 32336848.

E-mail address: pabloeliasm@gmail.com (P.E. Martínez).

Abstract

The effect of bisphenol A (BPA) on the sperm quality of mammals has been investigated. However in mouse lack extensive studies on sperm parameters associated with testicular histology and oxidative stress. In this study mother mice were exposed to BPA via gavage: two negative control groups: a 0.9% saline group and an olive oil (vehicle) group; three BPA treatments: 300, 900 and 3000 groups (300, 900 and 3000 µg/kg/day); and a positive control group (diethylstilbestrol-DES 650 µg/kg/day). Mouse pups were exposed to BPA while nursing from their mothers. At eight months old, male mice were killed by cervical dislocation. The anogenital distance at weaning (21 days) decreased significantly in the BPA 3000 group. BPA significantly impaired several sperm parameters (motility; morphology; mitochondrial functionality; membrane, acrosome and DNA integrity) and also induced histological testicular and oxidative damage. Taken together, these results showed that exposure to BPA causes extensive sperm impairment in mice.

Keyword: Bisphenol A; anogenital distance; spermatozoa membrane integrity; acrosome integrity; spermatozoa DNA integrity; mitochondrial functionality.

Highlights

1. Bisphenol A (BPA) is a ubiquitous endocrine disrupter;
2. We exposed mouse pups to BPA through lactation;
3. BPA impaired reproductive and testicular oxidative-stress parameters;

1. Introduction

In recent years, bisphenol A [BPA, 2,2-bis-(4-hydroxyphenyl)propane] has been widely used by industry to produce synthetic manufactured products such as resin epoxy and plastic polycarbonates. BPA can be found in many end products, including dental sealants, coatings for food cans, lining for metal cans, polyvinyl chloride, and medical equipment, among others [1,2]. BPA is released into the environment through sewage-treatment effluent, via hydrolysis from plastics, or from natural degradation of polycarbonate plastics exposed to heat, acid or alkaline condition. BPA has received heightened attention in the last decade because of its ubiquitous presence and because it is an endocrine disruptor [3].

Endocrine-disrupting chemicals (ED) are biologically active compounds that can mimic or antagonize the effects of endogenous hormones, causing many diseases through systemic deleterious effects [4]. Endocrine-disrupting effects of BPA have been reported in a number of animal models. In mammals, Zoeller *et al*. (2005) [5] observed lower body-weight gain in female rats exposed during pregnancy and lactation, as well as an increase in T4 concentration in their pups, harming the developing brains. Ropero *et al*. (2008) [6] observed that BPA treatment in mice (100 μg/kg/day for 4 days) disrupted function in pancreatic β-cells, producing insulin resistance.

While some toxic effects from BPA have been noted in adult animals, greater attention has been paid to exposures during the perinatal period. This period altered organizational programming and can confer increased susceptibility for diseases later in life. The early postnatal period is also critical in the development of rodent reproductive tracts [7,8]. Doerge *et al*. (2010) [9] reported significant effects from lactational transfer

of BPA suggest high potency and this fact can be related with BPA toxicity levels on target tissue during the critical perinatal period affecting babies and fetuses.

Regarding the effects of BPA on the reproductive system, increased prostate weight, decreased epididymis weight [10, 11] and lower sperm production were also reported in adult rodents and primates [12, 13]. In humans, Meeker *et al*. (2010) [14] found declines in sperm concentration, motility and morphology, and increased DNA damage in sperm. Particularly in the neonatal period, BPA-induced effects can also appear as alterations of gonadal organogenesis or function [15, 16]. For example, some alterations are evident in genes involved in spermatogenesis and steroidogenesis, through epigenetic effects that can result in disruption of the blood-testis barrier in rats [17] and decrease plasmatic and testicular testosterone levels, reducing the expression of the steroidogenic enzymes and cholesterol carrier protein in Leydig cells [18]. Prins *et al*. (2011) [19] also showed increased susceptibility to prostate carcinogenesis in rat pups that received BPA during the post-natal period (3, 5 and 21 days).

Pathological conditions caused by BPA may be related to the generation of reactive oxygen species derived from its metabolization, affecting reproductive and sexual characteristics by disturbing redox control systems [20]. Metabolization by the phase I cytochrome P450 enzyme family (CYPs) causes free-radical generation as superoxide anion ($O_2^{-\bullet}$) via metabolic redox cycling between its quinone and hydroquinone forms. Importantly, the following step in BPA detoxification involves its conjugation with glucuronic acid by phase II enzyme UDP-glucuronosyltransferase, which is not fully expressed in the neonatal period in mammals [21].

It is well-established that BPA exposure at early ages can impair the development of the reproductive system. Nevertheless, the effects of BPA administered through vertical transmission in the postnatal period (i.e. during lactation) on a number of important

sperm variables have not been fully investigated. In the present work, we demonstrated that BPA can permanently impair the reproductive function of the male mice even when some of the reproductive structures are formed, thus reinforcing the importance of the oral exposure in this period. For this, several sperm variables were analyzed and testicular histopathologies as well as key markers of the oxidative status of the testicular tissue were registered.

2. Material and Methods

2.1 Animals and experimental protocol

All of the procedures involving animal subjects were reviewed and approved by the Animal Ethics Committee of the Universidade Federal do Rio Grande-FURG, Rio Grande, Rio Grande do Sul (Approval number: P006/2011). Healthy Swiss albino mice obtained from the Central Animal Facility of the Universidade Federal do Rio Grande-FURG, maintained by random breeding, were housed in standard polystyrene cages at 23°C and 12-h light/12-h dark cycle, with water and a soybean-free diet *ad libitum* (Nuvilab CR-1 NUVITAL, Jundiaí, SP, Brazil). Swiss albino mouse was chosen due to its wide use in toxicological studies, many involving the effects of BPA on the reproductive system of mammals. After the acclimatization period of one week, animals (8 weeks old) were grouped in cages (5 females + 1 male) for random mating. The presence of a vaginal plug was checked twice daily to confirm mating. All the females that presented a vaginal plug were considered pregnant. Each pregnant female was placed in a separate cage to give birth. The litter size chosen was about 10 pups with similar numbers of male and female. From the birth of the pups to the end of the

lactation period (21 days), 6 treatment groups of 5 dams each received 200 µL/day of one of the following solutions via gavage: two control groups, a 0.9% saline (Saline) and an olive oil (vehicle) group; three BPA groups: BPA 300 - 300 µg/kg/day; BPA 900 - 900 µg/kg/day; BPA 3000 - 3000 µg/kg/day; and a positive control group DES (diethylstilbestrol) 650 µg/kg/day. The dose of DES used was chosen for the purpose of producing detectable alterations in testis morphology, since low doses do not induce toxic effects in the reproductive system [7]. After the lactation period, the weanling pups were sexed, the anogenital distance was measured, and the male pups were separated in cages according to litter. Female pups are used for other study. The number of male pups for each treatment was as follow: Saline - n=14; Olive oil – n=16; BPA300 – n=18; BPA 900 – n=14; BPA 3000 – n=17 and DES – n=12. At eight months old they were killed by cervical dislocation [22]. The testes and prostate were removed by laparotomy and weighted , and the epididymis tail and part of the vas deferens were isolated and placed in a Petri dish (35 mm diameter; Corning) filled with 500 µL of Sigma-M2 medium with HEPES (10 mM). For semen collection (n=10 per treatment), the selected structures were disrupted with the aid of hypodermic needles (30 G) [23]. The remaining testicle tissue was immediately dissected out, weighed, and stored at -80°C for biochemical analysis (n=5 per treatment) or placed in 4% paraformaldehyde for histological analysis (n=6 per treatment). For all of the analysis, the individuals were selected randomly to avoid the litter effect.

2.2 Semen Quality

The testes were removed by laparotomy, and the epididymis tail and part of the vas deferens were isolated and placed in a Petri dish (35 mm diameter; Corning) filled with 500 µL of Sigma-M2 medium with HEPES (10 mM). For semen collection, the selected

structures were disrupted with the aid of hypodermic needles (30 G) [23]. The remaining testicle tissue was immediately dissected out, weighed, and stored at -80°C for biochemical analysis or placed in 4% paraformaldehyde for histological analysis.

Sperm quality evaluations were done after incubation of samples for 10 min at 37ºC in M2 medium. Sperm motility was evaluated by putting 10 µL of sperm in a slide covered with a coverslip, using phase-contrast microscopy at 200 x both pre-heated at 37° C (BX 41 Olympus América, Inc., São Paulo, SP, Brazil) [23], always by the same trained technician. Sperm morphology was determined as described by counting 200 cells with phase contrast microscopy at 1000 x [24]. The evaluations of sperm membrane and acrosome integrity was carried out with an epifluorescence microscope (Olympus BX 51, América INC, São Paulo - Brazil), with filter wave length of 450-520 nm. Sperm membrane integrity was evaluated using carboxyfluorescein diacetate (CFDA; C5041) and propidium iodide (PI; P4170) [25] at 400 x. In each slide, 200 cells were counted and classified as intact and functional cell membrane (green fluorescence) or not intact or functional cell membrane (red fluorescence or simultaneous red and green fluorescence). Acrosome integrity was evaluated using FITC-PNA (L7381) by counting 200 cells in dry slides. Acrosomes were classified as intact, when presented red fluorescence and normal conformation, or not intact, when presented green fluorescence or no fluorescence and conformation distinct that from normal spermatozoa [26]. Mitochondrial functionality was evaluated after incubation of a 10 µL sperm sample with a 40 µL rhodamine 123 solution (13 µM), at 20 °C for 10 min. Sperm with positive rhodamine staining (green fluorescence) were considered as having functional mitochondria. Conversely, nonfunctional mitochondria were characterized by negative rhodamine staining (sperm with no fluorescence) [27]. The rate of mitochondrial functionality was determined by the proportion of sperm emitting green fluorescence

compared with total sperm (green or no fluorescence). Sperm DNA integrity (Sperm Chromatin Structure Assay) was evaluated after putting a 45 µL sperm sample in 50 µL TNE (0.01 M Tris-HCl; 0.15 M NaCl; 0.001 M EDTA; pH 7.2). After 30 sec, 200 µL of TritonX-100 solution (1%) was added and, 30 sec later, 50 µL of acridine orange was added (2 mg/mL in deionized H2O). The evaluation was done after 5 min, without exceeding 1 min of slide exposure [28]. Sperm with green fluorescence were considered as having intact DNA, whereas those with red or orange fluorescence were considered as having denatured DNA. The rate of DNA integrity was determined by the proportion of sperm emitting green fluorescence compared with the total number of sperm (green, red, or orange fluorescence) [28]. Assessments of mitochondrial function, membrane integrity and DNA were performed in an epifluorescent microscope (Olympus BX51®, America INC. São Paulo - Brazil) with 5mL of solution with sperm on slides undercover slip (18x18mm), evaluating 200 cells per sample. The rates were expressed as the percentage of viable cells/functional on the total cells evaluated.

2.3 Histology

The testicular tissue fixed in 4% paraformaldehyde was dehydrated in increasing concentrations of ethanol and subsequently embedded in Paraplast X-TRA (Sigma P3808). Sections 6 µm thick were cut with a rotary microtome (Leica RM 2255) and stained with Hematoxylin-Eosin [29]. Histological examination was performed using a light microscope (Olympus BX 51) with a high-resolution digital camera (Olympus DP 72).

2.4 Measurement of total antioxidant capacity

The total antioxidant capacity against peroxyl radicals (ROO•) was determined according to Amado *et al*. (2009) [30]. Aliquots of testis homogenates were placed in a medium containing 30 mM HEPES (pH 7.2), 200 mM KCl, 1 mM $MgCl_2$ and 40 μM of the fluorogenic compound 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA; Invitrogen) in the presence or absence of 2,2′-azobis 2 methylpropionamidine dihydrochloride (ABAP; 4 mM; Aldrich), which generates ROO• by thermal decomposition at 37° C. Fluorescence was considered as a measure of ROS production and was read in a spectrofluorimeter equipped with a microplate reader (Victor 2; Perkin-Elmer) at wavelengths of 485 and 530 nm for excitation and emission, respectively. Total fluorescence generation was calculated by integrating the fluorescence units (FU) over the period of the measurement. The results were expressed as area difference of FU x min in the same sample with and without ABAP addition, and standardized to the ROS area without ABAP (background area). The relative difference between the ROS area with and without ABAP was considered a measure of the antioxidant competence of the testis, where area difference is inversely proportional to the antioxidant capacity [30].

2.5 Determination of glutamate cysteine ligase (GCL) activity and glutathione (GSH) concentration

GCL activity and GSH levels in the sample testis were determined according to White *et al.* (2003) [31]. This method employs the reaction of naphthalene dicarboxaldehyde (NDA) with GSH or γ-glutamylcysteine (γ-GC) to form fluorescent cyclic products. A reaction solution (25 μl) with 400 mM Tris-HCl, 40 mM ATP, 20 mM glutamate, 2.0 mM EDTA, 20 mM sodium borate, 2 mM serine and 40 mM MgCl was prepared just before the assay, to prevent ATP degradation. After

addition of testis homogenates (25 µl), the plate was incubated at room temperature for 60 min and the on stopped by adding 50 µl of 5-sulfosalicylic acid (SSA, 200 mM). After protein precipitation, the plate was centrifuged for 5 min at 2,000 × g and 20 µl of supernatant from each well was transferred to a white plate, an NDA solution was added to all wells, and after 30 min of incubation, the fluorescence intensity of the NDA-GSH (or NDA-ᵹ -GC) complex was read at excitation and emission wavelengths of 485 and 530 nm, respectively (Victor 2, Perkin-Elmer).

2.6 Measurement of lipid peroxidation

Determination of lipid peroxides was performed through estimation of the malondialdehyde content in testis homogenates, employing the thiobarbituric acid-reactive substances (TBARS) fluorimetric method, according to Oakes and Van der Kraak (2003) [32]. Aliquots of sample extracts (10 µl) were added to a reaction solution containing 150 µl of 20% acetic acid, 150 µl of thiobarbituric acid (0.8%), 50 µl of Milli-Q water and 20 µl of sodium dodecyl sulfate (SDS, 8.1%). This mixture was heated at 95 ºC for 30 min for derivatization. Following cooling for 10 min, 100 µl of Milli-Q water and 500 µl of n-butanol were added. After centrifugation (3,000 x g for 10 min at 15 ºC), 150 µl of the organic phase was placed in a microplate reader and the fluorescence recorded with wavelengths of 520 and 580 nm for excitation and emission, respectively. Concentration of TBARS (nM/mg of wet tissue) was calculated, employing a standard curve of tetramethoxypropane (TMP, Acros Organics) as MDA equivalent.

3. Statistical analysis

Data were expressed as mean ± SEM. Once the assumptions of homogeneity and normality of variance were verified, one-way ANOVA was first performed between litters for each treatment, to exclude litter effects. Once litters from the same treatment did not present statistical differences, statistical analysis was performed between treatments by means of one-way ANOVA followed by Tukey post hoc test. The analysis of prostate weight was performed through Kruskal-Wallis non-parametric ANOVA followed by a Mann-Whitney test. The significance level adopted was 5% for all of the cases.

4. Results

The litters exposed to the positive control (DES) presented a serious impairment in their development and grew slowly. The development of the reproductive organs was incomplete and many animals did not possess testis. For this reason, the few litters that presented testis in the DES treatment were used for histopathological analysis, and biochemical measurements as well as sperm parameters were not done in this treatment.

4.1 Body weight gain, anogenital distance (AGD), testis and postate weight

With respect to daily body weight gain over 180 days, mice treated with BPA 3000 had a significantly ($p<0.05$) lower weight gain (Fig. 1A). The anogenital distance (Fig. 1B) was measured using a caliper at weaning (21 days). The results for anogenital distance were expressed with respect to the body weight [anogenital distance (mm)/ body weight (g) at 21 days]. Exposure to BPA 3000 μg/kg/day resulted in a significant decrease ($p<0.05$) in the AGD compared to the control groups. The DES group was not considered for AGD measure due to the delay caused on the development, since male

and female litters were not sufficiently developed to show differences in the external genitalia at 21 days. With respect to testis weight on 180 days, the DES treatment had a significantly ($p<0.05$) lower weight compared to Control and BPA 900 groups (Fig. 2). Did not differences weren't observed on prostate weight.

4.2 Sperm parameters

4.2.1 Sperm Motility

BPA significantly ($p<0.05$) impaired the sperm motility (Fig. 3A). Sperm motility for BPA 300 (57.00±5.15%); BPA 900 (48.00±5.27%) and BPA 3000 (27.00±5.97%) was less than for Saline (88.00±1.33%) and Olive oil (83.00±2.13%). Sperm from the mice whose mothers were exposed to BPA 3000 showed the highest motility impairment.

4.2.2 Normal Sperm Morphology

Figure 3B shows the effects of the BPA exposure on the spermatozoa morphology. The BPA treatments significantly ($p<0.05$) lowered the number of spermatozoa with normal morphology compared to the control. The percentages of normal-appearing sperm obtained for each treatment were: BPA 300 (33.00±1.83%); BPA 900 (33.90±1.39%); BPA 3000 (37.00±0.89%); Saline (46.10±2.82%) and Olive oil (47.80±2.39%).

4.2.3 Spermatozoa Membrane Integrity

The BPA treatments significantly decreased ($p<0.05$) the spermatozoa membrane integrity (Fig. 3C). The percentages of spermatozoa with normal membrane integrity

were: 40.80±7.19%, 26.40±4.07% and 3.10±0.52% for BPA 300, BPA 900 and BPA 3000, respectively. The membrane integrity was lower in the BPA treatments than in the controls, Saline (94.30±1.22%) and Olive oil (92.80±1.33%).

4.2.4 Sperm Acrosome Integrity

Acrosome integrity (Fig. 3D) was significantly impaired ($p<0.05$) in the BPA 3000 (16.00±5.81%) and BPA 900 (52.10±6.51%) treatments compared to the controls, Saline (92.20±2.13%) and Olive oil (94.70±1.41%) and to BPA 300 (94.70±3.30%). Again, the BPA exposure elicited dose response, with no damage at the lowest concentration (300 μg/kg/day).

4.2.5 Sperm DNA Integrity

The data for the spermatozoa DNA integrity (Fig. 3E) showed a significant decrease ($p<0.05$) in all the BPA treatments. The two higher BPA concentrations, BPA 900 and BPA 3000, showed a more pronounced DNA-impairment effect, with percentages of 16.08±3.00 and 17.03±8.14%, respectively, and BPA 300 showed an intermediate effect (57.40±4.73%), compared with the control treatments, Saline (89.80±2.19%) and Olive oil (88.00±1.87%).

4.2.6 Mitochondrial Functionality

Figure 3F shows the data for mitochondrial functionality of the spermatozoa. BPA treatments significantly impaired ($p<0.05$) the mitochondrial functionality, with the most damage when the mothers received BPA 3000 (8.50±2.11%). Administration of BPA 300 (64.40±3.16%) and BPA 900 (51.50±5.39%) also impaired mitochondrial function more than Saline (97.30±0.94%) and Olive oil (94.70±3.30%).

4.3 Total Antioxidant Capacity

The total antioxidant capacity, measured as Relative Area Difference (RAD) of the testis (Fig. 4) was impaired ($p<0.05$) in the BPA 900 (6.89±0.53 RAD) and the BPA 3000 (6.90±0.21 RAD) treatments, compared to the control group (2.57±0.12 RAD). The effect of the BPA 300 (3.4±0.6 RAD) treatment was similar to those of saline, BPA 900 and BPA 3000.

4.4 Lipid peroxidation, GSH and GCL

The different BPA treatments did not modulate ($p>0.05$) the levels of MDA, GSH or the GCL activity in the mice testis (Table 1), compared with the corresponding control groups.

4.5 Testicular Histopathology

Figure 5 shows the histopathological alterations in the mouse testis. The Saline, Olive oil, BPA 300 and BPA 900 treatments (Fig. 5 A, B, C and D, respectively) did not produce any histopathological alterations in the testicular tissue. However, tissue from pups of mice exposed to BPA 3000 and DES (Fig. 5 E and F, respectively) showed important pathologies. In BPA 3000, 67% of the treated animals showed testicular pathologies, and testicular degeneration was observed in one animal. The BPA 3000 treatment induced complete aplasia in some seminiferous tubules. The tubules adjacent to the aplastic site showed complete spermatogenesis and excessive quantities of spermatogonia and spermatocytes, in relation to the total observed numbers of spermatids and sperm. The testes of animals treated with DES showed extensive aplasia, and the lack of spermatozoa and the small size of the testicles prevented further study.

5. Discussion

In the present study, we found several impairments in the mouse testicular function caused by vertical exposure to BPA. It is important to stress that these findings were obtained by employing doses that are below the lowest observed effect level – LOEL, which is 5 mg/kg/day [33]. Moreover, once the mouse litters received BPA only by lactation, and taking into account the loss due to metabolization and slow rate of excretion (enterohepatic circulation) by the mothers, it is likely that the observed effects were triggered by amounts of BPA slightly lower than those offered to the mothers (0.3, 0.9 and 3.0 mg/kg/day during 21 days) [34].

It is particularly important because at post-natal days (PND) 1-21, the newborn mouse, similarly to the human pre-term fetuses, has an immature (very permeable) small intestine [35], and therefore is vulnerable to endocrine disruptors. Since the data concerning the actual human exposure levels to BPA are very scarce and hard to predict, exposure by lactation becomes a matter of concern for public health [36].

With some exceptions [37,38], most of the toxic effects of BPA on the development of the reproductive system (in both acute and chronic exposures) are observed within a few days or weeks post-exposure [10, 39,40]. We investigated the later consequences of vertical transmission of BPA during lactation in male mice at the adulthood (8 months-aged - PND 240) and registered the permanent damages. Gestation and lactation are a sensitive and vulnerable “window” in the perinatal period to endocrine disruptors because differentiation and organ development is occurring. The postnatal stage encompasses the crucial periods of lactation where occurs of development of external genitalia including the prostate, the Sertoli cells number is getting fixed and the formation of blood-testis barrier is happening [41].

Although several endocrine-disrupting effects are well-established, there are some controversies concerning BPA toxicity due to the differences in the dose and mode of exposure. For example, some authors have registered that BPA administered by drinking water (0.01, 0.1, 1 or 10 ppm) [42] or subcutaneous injection (10 μg/kg/day during 3 days) [43] caused no effect on the reproductive organs of rats. On the other hand, a considerable number of studies have shown reproductive impairments associated with BPA. Chitra *et al*. (2003) [10] exposed 45-day-old rats to different BPA concentrations (0.2, 2 and 20 μg/kg/day for 45 days) and reported significant decreases in epididymal sperm motility and sperm counts, such effects may be associated to the inhibition of spermatogenesis, decreased numbers of elongated spermatids, and reduced steroidogenic enzyme activity. Salian *et al*. (2009) [44] found that male offspring exposed to low doses of BPA (1.2 – 2.4 μg/kg/day) in the uterus and in the neonatal period resulted in reduced efficiency in daily sperm production, motility and morphology. Other studies that investigated the BPA effects through the lactation period of rats showed that BPA caused significant increase in mammary tumors in female offspring [7,8,39], decrease in the anogenital distance and other reproductive changes in male offspring [45].

In the present study, it was found a general decrease in sperm quality resulting from maternal BPA exposure (Fig. 3). Concerning DNA fragmentation and mitochondrial dysfunction, these processes are related to apoptosis and in the seminiferous tubules its occurrence is a normal finding in spermatogenesis and testicular homeostasis. However, the two main paths that lead to apoptosis – extrinsic (via Fas/FasL) and intrinsic (mitochondrial pathway) – are apparently involved in abnormal testicular apoptosis induced by exposure to BPA via gavage (160 or 480 mg/kg/day) [46]. We observed that BPA decreased sperm motility, and this may be due

to the effects of BPA on the mitochondrial function, that also affect ATP generation and thus the motility of sperm (Fig. 3 A and F). Salian *et al.* (2009) [44], observed similar results in rats, were that BPA induced declined on sperm counts and motility and decrease in the levels of LH, FSH, testosterone and estrogen can be a possible explanation.

The effect of BPA on mitochondrial functionality may occur both through its involvement in the intrinsic pathway of apoptosis, considering its ability to generate reactive oxygen species (ROS), or by direct action in mitochondrial estrogen receptors (ERs), taking into account the role of estrogen and ERs in the regulation of the mitochondrial respiratory chain [47]. On the other hand, these changes in the pituitary gonadotrophins and steroidogenesis may be mediated by oxidative stress in the central nervous system, adenohypophysis and testicle. As observed by Chitra *et al*. (2003) [10] in rats and Kabuto *et al*. (2004) [15] in mice, BPA produces short or medium-term oxidative damage in the brain, liver, kidney and testes. We studied the long-term effect of BPA in testes of mice, and observed a significant ($p<0.05$) decrease in the total antioxidant capacity in the treatments with BPA 900 and BPA 3000 (Fig. 4). The testicles and the organisms had ample time for homeostatic mechanisms to compensate for the effects of BPA exposure during lactation, but at 8 months of age, the male mice still showed changes in their antioxidant capacity.

Oxidative stress, hormonal changes and apoptosis all can be inducing significant changes in the testes. In fact, we observed significant changes in the testicular histology in mice after the exposure to BPA 3000 (Fig. 5), including aplasia in the seminiferous tubules and testicular degeneration. Hutanu (2011) [48] and Takahashi (2001) [49] reported similar results, including disruption of the integrity of the hematotesticular barrier and decrease in spermatogenesis in the high-dose BPA group (200 µg/kg/day).

From the same morphological pattern of response, we can also predict the mechanism of action of BPA suspected of interfering with testosterone action or changes in the male reproductive tract occurred during the perinatal window.

The histological testicular pathologies (Fig. 5), the spermatic impairment (Fig. 3), and the disturbance in oxidative stress (Fig. 4) observed in male mice exposed to BPA during the lactation period suggest that BPA could have a long and lasting adverse effects on the sperm quality of these offspring during adulthood.

The pups from dams exposed to BPA 3000 showed reduced anogenital distance (Fig. 1). Androgens are very important to the male characters on development, (i.e. in the seminiferous tubules, testosterone acts on spermatogenesis indirectly through Sertoli cells to maintain spermatogenesis, germ cell development and ano-genital distance). Our results are in agreement with those of Kobayashi *et al*. (2002) and Fujii *et al.* (2001) [50, 51], who found that AGD decreases in boys born from mothers exposed to BPA, indicating the potential role of BPA as an antiandrogen. However, exposure of pregnant rats to 4 and 40 mg/kg/day of BPA from gestation day (GD) 6 and PND 20 had no effect on the reproductive parameters evaluated (including AGD) [50]. On the other hand, Gupta (2000) [51], for male mice exposed to BPA (50 µg/kg/day) during gestation (GD 16-18), observed increased prostatic size, decreased epididymal weight and increased anogenital distance. Similarly, but in females, Tyl *et al*. (2002) [37] observed an AGD increase and lower body weight. Differences in the concentrations of BPA exposure in the study of Gupta (2000) [52] and those used here, do not fully explain the contrasting AGDs found in male mice. Furthermore, the observed decrease in AGDs in human boys [45] argues that this measure can be of biological and/or toxicological interest. In our work the decrease in the anogenital distance in the highest BPA treatment could be due that serum conjugated-to-deconjugated estrogen ratios and its

estrogenic activity, and can be decreased or the aromatase expression was increased [53]. Exposure to ED in early life leads to permanent changes in reproductive anatomy, histology and physiology [7,11,15,19].

In summary, the present study demonstrates that the exposure of mice to environmentally relevant doses of BPA during the early period results in long-term adverse effects on the sperm quality and the importance of to make a screening the most of sperm parameters. These findings should raise concerns for humans and vertebrates in general, because BPA may cause changes in the redox status, reducing AGD, spermatic impairment and its harmful effects manifest throughout life.

6. Conflict of interest

The author declared there is no conflict of interest.

7. References

[1] Thomson BM, Grounds PR. Bisphenol A in canned foods in New Zealand: exposure assessment. Food Addit Contam 2005;22:65–72.

[2] Benuchour N, Aris A. Toxic effects of low doses of bisphenol-A on human placenta cells. Toxicol Appl Pharmacol 2009;241:322–8.

[3] Quitmeyer A, Roberts R. Babies, bottles, and bisphenol A: the story of a scientist-mother. PLoS Biol 2007;5:1399–402.

[4] Swedenborg, E, Pongratz I, Gustafsson JA. Endocrine disruptors targeting ERbeta function. Int J Androl 2010;33:288-97.

[5] Zoeller RT, Bansal R, Parris C. Bisphenol-A, an environmental contaminant that acts as a thyroid hormone receptor antagonist in vitro, increases serum thyroxine, and alters RC3/neurogranin expression in the developing rat brain. Endocrinology 2005;146:607-12.

[6] Ropero AB, Alonso-Magdalena P, García-García E, Ripoll C, Fuentes E, Nadal A. Bisphenol-A disruption of the endocrine pancreas and blood glucose homeostasis. Internat J Androl 2008;31:194-200.

[7] Newbold RR, Jefferson WN, Padilla-banks E, Haseman J. Developmental exposure to diethylstilbestrol (DES) alters uterine response to estrogens in prepubescent mice: low versus high dose effects. Reprod Toxicol 2004;18:399-406.

[8] Pryor JL, Hughes C, Foster W, Hales BF, Robaire B. Critical windows of exposure for children's health: the reproductive system in animals and humans. Environ Health Perspect 2000;108:491–503.

[9] Doerge DR, Vanlandingham M, Twaddle NC, Delclos KB. Lactational transfer of bisphenolA in Sprague – Dawley rats. Toxicol Lett 2010;199:372-6.

[10] Chitra, KC, Latchoumycandane C, Mathur PP. Induction of oxidative stress by bisphenol A in the epididymal sperm of rats. Toxicology 2003;185:119-27.

[11] Kato H, Furuhashi T, Tanaka M, Katsu Y, Watanabe H, Ohta Y, Iguchi T. Effects of bisphenol A given neonatally on reproductive functions of male rats. Reprod Toxicol 2006;22:20-9.

[12] Saradha B, Mathur PP. Effect of environmental contaminants on male reproduction. Environ Toxicol Pharmacol 2006;21:34-41.

[13] YangYJ, Lee SY, Kim KY, Hong YP. Acute Testis Toxicity of Bisphenol A Diglycidyl Ether in Sprague-Dawley Rats. J Prev Med Public Health 2010;43:131-7.

[14] Meeker JD, Ehrlich S, Toth TL, Wright DL, Calafat AM, Trisini AT, Ye X, Hauser R. Semen quality and sperm DNA damage in relation to urinary bisphenol A among men from an infertility clinic. Reprod Toxicol 2010;30: 532-9.

[15] Kabuto H, Amakawa M, Shishibori T. Exposure to bisphenol A during embryonic/fetal life and infancy increases oxidative injury and causes underdevelopment of the brain and testis in mice. Life Sci 2004;74:2931-40.

[16] Braniste V, Jouault A, Gaultier E, Polizzi A, Buisson-Brenac C, Leveque M, Martin PG, Theodorou V, Fioramonti J, Houdeau E. Impact of oral bisphenol A at reference doses on intestinal barrier function and sex differences after perinatal exposure in rats. Proc Natl Acad Sci USA 2010;107:448-53.

[17] Li MW, Mruk DD, Lee WM, Cheng Y. Disruption of the blood-testis barrier integrity by bisphenol A in vitro: Is this a suitable model for studying blood-testis barrier dynamics? Int J Biochem Cell Biol 2009;41:2302-14.

[18] Nakamura D, Yanagiba Y, Duan Z, Ito Y, Okamura A, Asaeda N, Tagawa Y, Li C, Taya K, Zhang SY, Naito H, Ramdhan DH, Kamijima M, Nakajima T.. Bisphenol A may cause testosterone reduction by adversely affecting both testis and pituitary systems similar to estradiol. Toxicol Lett 2010;194:16-25.

[19] Prins GS, Ye SH, Birch L, Ho SM, Kannan, K. Serum bisphenol A pharmacokinetics and prostate neoplastic responses following oral and subcutaneous exposures in neonatal Sprague-Dawley rats. Reprod Toxicol 2011;31:1-9.

[20] Bindhumol V, Chitra KC, Mathur PP. Bisphenol A induces reactive oxygen species generation in the liver of male rats. Toxicology 2003;188:117-24.

[21] Pritchett JJ, Kuester RK, Sipes IG. Metabolism of bisphenol A in primary cultured hepatocytes from mice, rats, and humans. Drug Metab Dispos 2002;30:1180–5.

[22] Hogan B, Constantini F, Lacy E. Manipulating the mouse embryo: a laboratory manual. Cold Spring Harbor Laboratory 1986;331.

[23] Sztein JM, Farley JS, Mobraaten LE. In vitro fertilization with cryopreserved inbred mouse sperm. Biol Reprod 2000;63:1774-80.

[24] Tayama K, Fujita H, Takahashi H, Nagasawa A, Yano N, Yuzawa K, Ogata A. Measuring mouse sperm parameters using a particle counter and sperm quality analyzer: a simple and inexpensive method. Reprod Toxicol 2006;22:92–101.

[25] Harrison RAP, Vickers SE. Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. J Reprod Fertil 1990;88:343-52.

[26] Jiménez I, González-Márquez H, Ortiz R, Herrera JA, Garcií A, Betancourt M, Fierro R. Changes in the distribution of lectin receptors during capacitation and acrosome reaction in boar spermatozoa. Theriogenology 2003;59:1171-80.

[27] Johnson LV, Walsh ML, Chen LB. Localization of mitochondria in living cells with Rhodamine 123. Proc Natl Acad Sci USA 1980;77:990-4.

[28] Evenson D, Jost L. Sperm chromatin structure assay is useful for fertility assessment. Methods Cell Sci 2000;22:169-89.

[29] Carson FL, Hladik C. Histotechnology: A Self-Instructional Text. 3rd Edition. American Society for Clinical Pathology Press 2009:409p.

[30] Amado LL, Garcia ML, Ramos PB, Freitas RF, Zafalon B, Ferreira JLR, Yunes JS, Monserrat JM. A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: Application to evaluate microcystins toxicity. Sci Total Environ 2009;407:2115-23.

[31] White CC, Viernes H, Kreysa CM, Botta D, Kavanagh TJ. Fluorescence-based microtiter plate assay for glutamate-cysteine ligase activity. Anal Biochem 2003; 318:175-80.

[32] Oakes KD, Van der Kraak GJ. Utility of TBARS assay in detecting oxidative stress in white sucker (Catostomus commersoni) populations exposed to pulp mill effluent. Aquat Toxicol 2003;63:447-63.

[33] EFSA European Food Safety Authority. Scientific opinion on Bisphenol A: evaluation of a study investigating its neurodevelopmental toxicity, review of recent scientific literature on its toxicity and advice on the Danish risk assessment of Bisphenol A. EFSA J 2010;8:1829.

[34] Snyder RW, Maness SC, Gaido KW, Welsch F, Sumner SC, Fennell TR. Metabolism and disposition of bisphenolA in female rats.ToxicolApplPharmacol 2000;168:225-34.

[35] Drozdowski LA, Clandinin T, Thomson AB. Ontogeny, growth and development of the small intestine: Understanding pediatric gastroenterology. World J Gastroenterol 2010;16:787-99.

[36] Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV. Human exposure to bisphenol A (BPA). Reprod Toxicol 2007; 24:139-177.

[37] Tyl RW, Myers CB, Marr MC, Thomas BF, Keimowitz AR, Brine DR, Veselica MM, Fail PA, Chang TY, Seely JC, Joiner RL, Butala JH, Dimond SS, Cagen SZ, Shiotsuka RN, Stropp GD, Waechter JM. Three-generation reproductive toxicity study of dietary bisphenol A in CD Sprague-Dawley rats. Toxicol Sci 2002;68:121-46.

[38] Doshi T, Mehta SS, Dighe V, Balasinor N, Vanage G. Hypermethylation of estrogen receptor promoter region in adult testis of rats exposed neonatally to bisphenol A. Toxicology 2011;289:74-82.

[39] Doerge DR, Vanlandingham M, Twaddle NC, Delclos KB. Lactational transfer of bisphenolA in Sprague – Dawley rats. Toxicol Lett 2010;199:372-6.

[40] D'Cruz SC, Jubendradass R, Jayakanthan M, Rani SJ, Mathur PP. Bisphenol A impairs insulin signaling and glucose homeostasis and decreases steroidogenesis in rat testis: an in vivo and in silico study. Food Chem Toxicol 2012;50:1124-33.

[41] Salian S, Doshi T, Vanage G. Perinatal exposure of rats to Bisphenol A affects fertility of male offspring—An overview. Reprod Toxicol 2011; 31: 359-362.

[42] Cagen SZ, WaechterJr JM, Dimond SS, Breslin WJ, Butala JH, Jekat FW,Joimer RL, Shiotsuka RN, Veenstra GE, Harris LR. Normal reproductive organ development in Wistar rats exposed to Bisphenol A in the drinking water. Regul Toxicol Pharmacol 1999;30:130-9.

[43] Vandenberg LN, Maffini M V, Sonnenschein C, Rubin BS, Soto AM. Bisphenol-A and the great divide: A review of controversies in the field of endocrine disruption. Endocr Rev 2009;30:75–95.

[44] Salian S, Doshi T, Vanage G. Perinatal exposure of rats to Bisphenol A affects the fertility of male offspring. Life Sci 2009;85:742–52.

[45] Miao M, Yuan W, He Y, Zhou Z, Wang J, Gao E, Li G, Li DK. In utero exposure to bisphenol-A and anogenital distance of male offspring. Birth Defects Research A Clin Molec Teratol 2011;91:867-72.

[46] Wang Q, Zhao XF, Ji YL, Wang H, Liu P, Zhang C, Zhang Y, Xu DX. Mitochondrial signaling pathway is also involved in bisphenol A induced germ cell apoptosis in testes. Toxicol Lett 2010;199:129-35.

[47] Chen JQ, Cammarata PR, Baines CP, Yager JD. Regulation of mitochondrial respiratory chain biogenesis by estrogens/estrogen receptors and physiological, pathological and pharmacological implications. Biochim Biophys Acta 2009;1793: 1540-70.

[48] Hutanu D. Experimental investigations reagarding [sic] the effects of bisphenol A in adult mice spermatogenesis. Annals RSCB 2011;16:74-8.

[49] Takahashi O, Oishi S. Testicular toxicity of dietary 2,2-bis(4-hydroxyphenyl) propane (bisphenol A) in F344 rats. Arch Toxicol 2001;75:42-51.

[50] Kobayashi K, Miyagawa M, Wang RS, Sekiguchi S, Suda M, Honma T. Effects of in Utero and Lactational Exposure to Bisphenol A on Somatic Growth and Anogenital Distance in F1 Rat Offspring. Ind Health 2002;40:375-81.

[51] Ema M, Fujii S, Furukawa M, Kiguchi M, Ikka T, Harazono A. Rat two-generation reproductive toxicity study of bisphenol A. Reprod Toxicol 2001;15:505-23.

[52] Gupta C. Reproductive malformation of the male offspring following maternal exposure to estrogenic chemicals. Proc Soc Exp Biol Med 2000;224:61–8.

[53] Quignot N, Arnaud M, Robidel F, Lecomte A, Barouki R, Lemazurier E. Characterization of endocrine-disrupting chemicals based on hormonal balance disruption in male and female adult rats. Reprod Toxicol 2012; 33:339- 352.

Captions

Fig. 1. Effects of Bisphenol A on daily body weight gain for 180 days (A) and anogenital distance at weaning (21 days old) in male mice (B), according to the treatment administered to their mothers during lactation. Saline (0.9% saline; n=14); Olive oil (Olive oil – vehicle; n=16); BPA300 (BPA 300 µg/kg/day; n=18); BPA 900 (BPA 900 µg/kg/day; n=14); (BPA 3000 µg/kg/day; n=17) DES (DES 650 µg/kg/day; n=12). Data are expressed as mean ± SEM. Bars indicated with the same letter are not significant at the 5% probability level.

Fig. 2. Effects of Bisphenol A on testis weight (A) and prostate weight in male mice (B), according to the treatment administered to their mothers during lactation. Saline (0.9% saline; n=14); Olive oil (Olive oil – vehicle; n=16); BPA300 (BPA 300 µg/kg/day; n=18); BPA 900 (BPA 900 µg/kg/day; n=14); (BPA 3000 µg/kg/day; n=17); DES (DES 650 µg/kg/day; n=12). Data are expressed as mean ± SEM. Bars indicated with the same letter are not significant at the 5% probability level.

Fig. 3. Effects of Bisphenol A on sperm (A) Motility (%); (B) Normal Morphology (%); (C) Membrane Integrity (%); (D) Acrosomal Integrity (%); (E) DNA Integrity (%); and (F) Mitochondrial Functionality (%), in 8-month-old male mice according to the treatment administered to the mothers during lactation. Saline (0.9% saline; n=10); Olive oil (Olive oil – vehicle; n=10); BPA 300 (BPA 300 µg/kg/day; n=10); BPA 900 (BPA 900 µg/kg/day; n=10); BPA 3000 (BPA 3000 µg/kg/day; n=10). Data are

expressed as mean ± SEM. Bars indicated with the same letter are not significant at the 5% probability level.

Fig. 4. Effects of Bisphenol A on Total antioxidant capacity in the testis (Relative area difference) in 8-month-old male mice, according to the treatment administered to the mothers during lactation. Saline (saline 0.9%; n=5); BPA 300 (BPA 300 µg/kg/day; n=5); BPA 900 (BPA 900 µg/kg/day; n= 5); BPA 3000 (BPA 3000 µg/kg/day; n= 5). Data are expressed as mean ± SEM. Bars indicated with the same letter are not significant at the 5% probability level.

Fig. 5. Testicular photomicrographs (H & E x100) showing the effects of Bisphenol A in 8-month-old male mice, according to the treatment administered to the mothers during lactation. A) Saline (n=6); B) Olive oil*;* C) BPA 300 (BPA 300 µg/kg/day; n=6); D) BPA 900 (BPA 900 µg/kg/day; n=6); E) BPA 3000 (BPA 3000 µg/kg/day; n=6)*;* F) DES (DES 650 µg/kg/day; n=6).

Table 1: Effects of Bisphenol A on MDA concentration (nmol/mg of wet tissue), GCL activity (mmol/mg protein/min) and concentration of GSH (mmol/mg protein) in the testis in 8-month-old male mice, according to the treatment administered to the mothers during lactation. Saline (saline 0.9%; n=5); Olive oil – vehicle (n=5) BPA 300 (BPA 300 µg/kg/day; n=5); BPA 900 (BPA 900 µg/kg/day; n= 5); BPA 3000 (BPA 3000 µg/kg/day; n= 5).

Figure 1

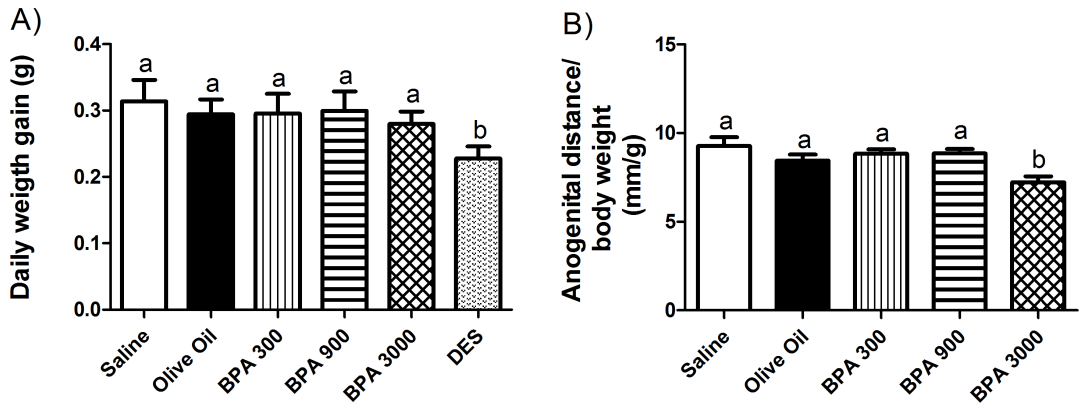

A)
Daily weigth gain (g)
0.4
0.3
0.2
0.1
0.0
a
a
a
a
a
b
Saline
Olive Oil
BPA 300
BPA 900
BPA 3000
DES
B)
Anogenital distance/ body weight (mm/g)
15
10
5
0
a
a
a
a
b
Saline
Olive Oil
BPA 300
BPA 900
BPA 3000

Figure 2

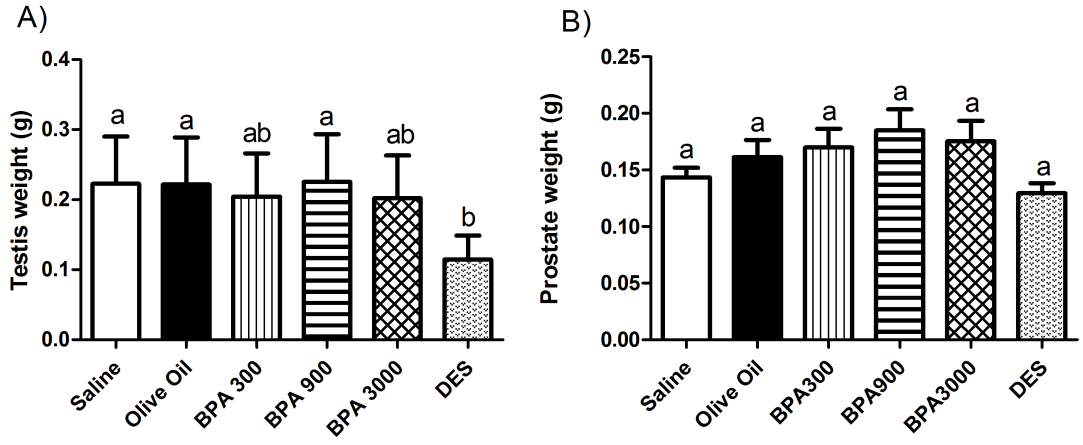

A)
Testis weight (g)
0.4
0.3
0.2
0.1
0.0
a
a
ab
a
ab
b
Saline
Olive Oil
BPA 300
BPA 900
BPA 3000
DES
B)
Prostate weight (g)
0.25
0.20
0.15
0.10
0.05
0.00
a
a
a
a
a
a
Saline
Olive Oil
BPA300
BPA900
BPA3000
DES

Figure 3

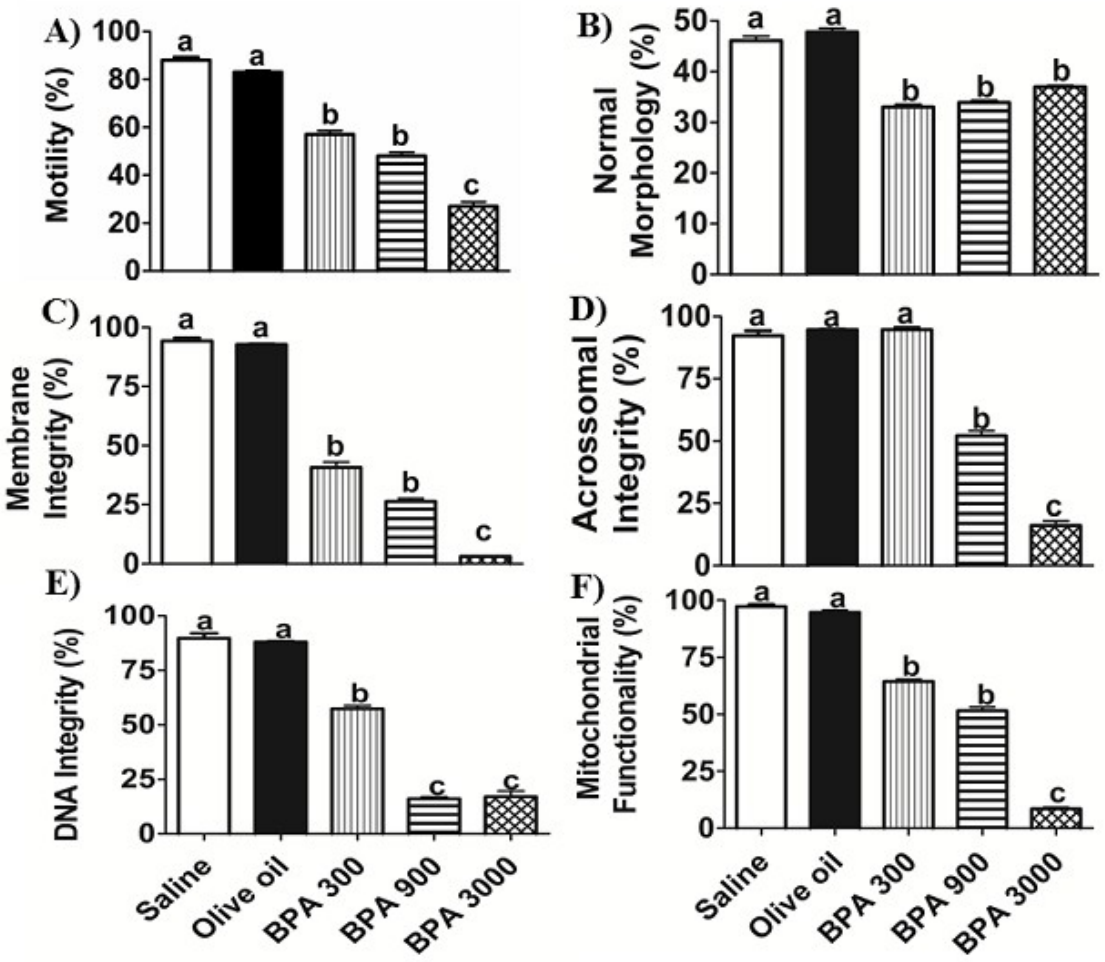

A)
Motility (%)
100
80
60
40
20
0
a
a
b
b
c
B)
Normal Morphology (%)
50
40
30
20
10
0
a
a
b
b
b
C)
Membrane Integrity (%)
100
75
50
25
0
a
a
b
b
c
D)
Acrossomal Integrity (%)
100
75
50
25
0
a
a
a
b
c
E)
DNA Integrity (%)
100
75
50
25
0
a
a
b
c
c
F)
Mitochondrial Functionality (%)
100
75
50
25
0
a
a
b
b
c
Saline
Olive oil
BPA 300
BPA 900
BPA 3000
Saline
Olive oil
BPA 300
BPA 900
BPA 3000

Figure 4

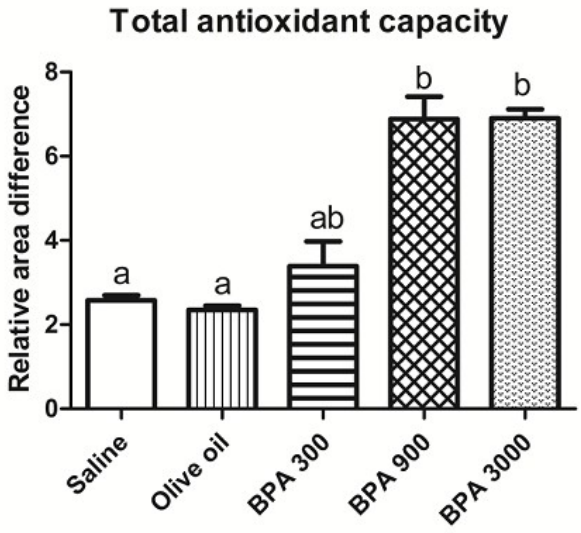

Total antioxidant capacity
Relative area difference
8
6
4
2
0
a
a
ab
b
b
Saline
Olive oil
BPA 300
BPA 900
BPA 3000

Figure 5

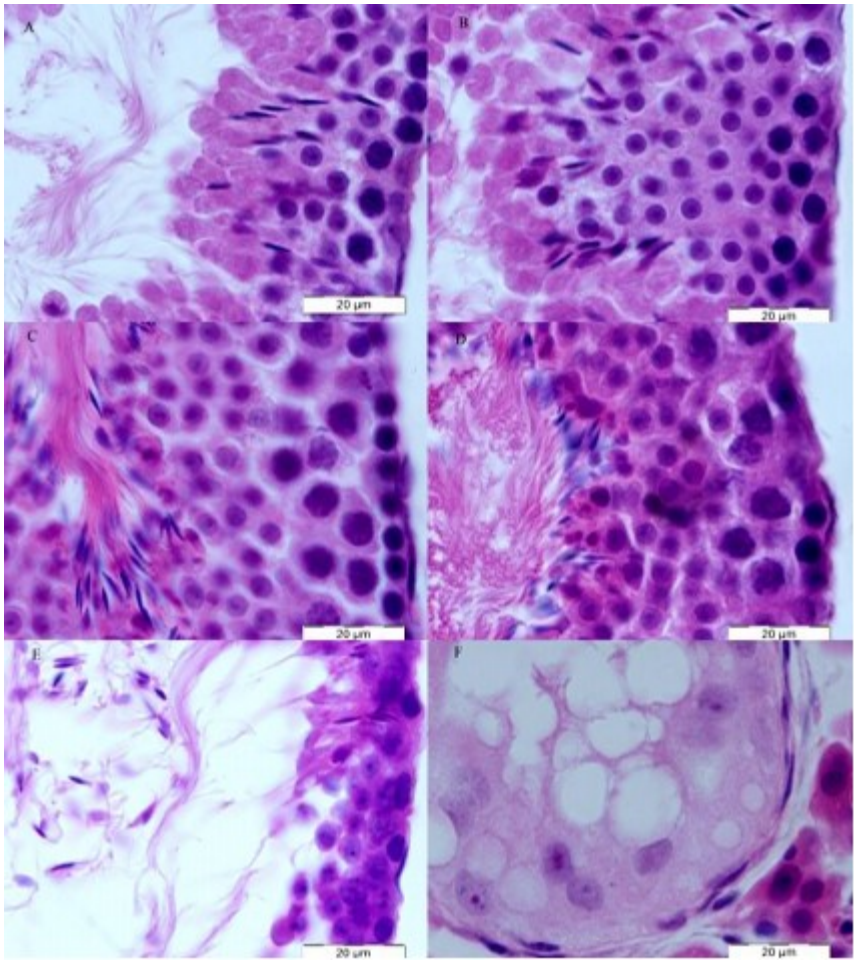


Table 1

	Oxidative Stress Parameters		
Treatment	**TBARS**	**CGL**	**GSH**
Saline	0.39±0.017	2170.81±491.28	4386.48±570.83
Olive oil	0.40±0.01	2251±300.1	4103±304.5
BPA 300	0.40±0.058	2019.89±219.91	5524.44±462.26
BPA 900	0.33±0.074	3041.64±629.94	4070.91±355.58
BPA 3000	0.37±0.063	2771.26±630.00	4984.63±393.45

Data are expressed as mean ± SEM. No significant differences were observed.

Artigo 2

Revista : Toxicology Letters

(Fator de impacto: 3,23)

Effect of BPA on the central nervous system in lactating females

Ana Cristina Kalb[a], Ana Luiza Kalb[b], Gabriela Hollmann[c], Flavia Conde Kneip[d], Antonio Sergio Varela Junior[b], Silvana Allodi[c]; Pablo Elías Martínez[a, b, *]

[a] Programa de Pós-Graduação em Ciências Fisiológicas: Fisiologia Animal Comparada, Universidade Federal do Rio Grande, Rio Grande, RS, Brazil

[b] Instituto de Ciências Biológicas; Universidade Federal do Rio Grande, Rio Grande, RS, Brazil

[c] Laboratório de Biofísica; Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil

[d] Instituto de Física e Matemática; Universidade Federal do Rio Grande, Rio Grande, RS, Brazil

* Corresponding author. Tel./fax: +55 53 32336848.

E-mail address: pabloeliasm@gmail.com (P.E. Martínez).

Abstract

Bisphenol A (BPA) is a known endocrine disruptor that impairs brain function. We employed immunohistochemistry tools to evaluate the effects in many regions of the brain of lactant female mice exposed sub-chronically (21 days) to BPA (300, 900 and 3000 µg/kg/day) via gavage. The parameters observed were labels for: astrocytes - glial acidic fibrillary protein (GFAP), cell proliferation - histone-3-phosphate (H3P), synapse density synaptophisin (SYP), and catecholamine synthesis - tyrosine hydroxilase (TH). The results showed that 300 µg/kg/day BPA decreased the number of astrocytes in all brain regions and diminished TH fluorescence in the striatum and CA3 region of the hippocampus. The dose of 900 µg/kg/day BPA also decreased the number of astrocytes in the CA3 region and increased cell proliferation in the hippocampus, however both in the cortex and in the CA3 region this treatment influenced synaptogenesis, reducing the process. The overall results show that cell proliferation occurred in the brain of the female mice but there were also losses induced by BPA on the number of astrocytes, in TH concentration and synaptic density. These findings reinforce the potential implication of BPA exposure in neurodegenerative diseases.

Keywords: bisphenol A; endocrine disruptor; brain;

Highlights

Bisphenol A (BPA) is a ubiquitous endocrine disrupter;

We exposed mouse mothers to BPA through lactation;

BPA impaired nervous central system parameters;

1. Introduction

Since the decade of 1950, the synthetic xenoestrogen, Bisphenol A (BPA), has been employed in the manufacturing of some plastics with a range of uses, including dental prosthesis and sealants and polycarbonate cans used to preserve foods and items as baby bottles (Suzuki et al., 2000; Brede et al., 2003).

Previous studies regarding the biological and toxicological effects of BPA on the human body have focused on its estrogenic action. This is because BPA seems to mimic some estradiol-induced biological effects, such as sperm toxicity in humans (Hauser et al., 2005) and fertility and fecundity decrease in mice (Cabaton et al., 2011). In addition to the effects on reproductive tissues, BPA is likely to alter the morphological and functional properties of neurons and astrocytes in the central nervous system (CNS). Astrocytes, which may be identified by the intermediate filament glial fibrillary acidic protein (GFAP) immunolabeling, play a key role in brain neurotransmission and express estrogen receptors. BPA inhibits estradiol-induced hippocampal synaptogenesis (MacLusky et al., 2005) and in doses of 1 pM or 1 μM) also amplifies the response of dopaminergic neurons and astrocytes (Miyatake et al. 2006).

One important marker for dopamine is tyrosine hydroxylase (TH), which catalyzes the enzymatic conversion of L-tyrosine to L-3, 4-dihydroxyphenylalanine (L-dopa), the first step in the biosynthesis of chatecolamines. Xu et al. (2011) examined the rapid effect of BPA, with a dose of 500 μg/kg on a single administration in the hippocampus, on passive avoidance memory and *N*-methyl-D-aspartate receptor (NMDAR) expression in the developing hippocampus of rats at the age of postnatal day 18. The authors observed that BPA or estradiol benzoate (EB) rapidly extended the latency to step down from the platform 1 h after footshock and increased the

phosphorylation levels of NR1, NR2B (subunits of NMDAR), and mitogen-activated extracellular signal-regulated kinase (ERK) in hippocampus within 1 h.

BPA interference in spine synapse formation has profound implications. Leranth et al. (2008) observed, under a relative low exposure level (50 μg/Kg/day) to nonhuman primates, a complete abrogation of the synaptogenic response to estradiol. Because sex steroids are widely thought to play critical roles in higher brain activities, such as cognition and mood through modulation of the structural and functional synaptic plasticity, it is important to investigate the consequences of BPA exposure. BPA can also antagonize spine synapse formation induced by estrogens and testosterone in limbic brain areas of gonadectomized female and male rats. One important marker to synaptogenesis is synaptophysin that can be found on the cytoplasmic surface of clear pre-synaptic microvesicles of neurons. (Li et al., 2004; Neil et al., 2005; Liu et al., 2008).

Phosphorylated histone H3 at serine 10 is a marker of cell proliferation. Neuronal proliferation is well established in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus show constitutive neurogenesis under physiological (Sulllivan et al., 2007) and pathological conditions (Nakayama et al., 2010). However, the central dogma of non-renewal of cortical neurons was first called into question in 1962 by Altman in a study that shows evidence of adult-born neurons in the human cortex (Altman, 1962). Posteriorly Gould et al. (1999) recorded in primates, proliferation of neurons in the prefrontal, inferior temporal and posterior parietal cortex. The new neurons may originate in the subventricular zone and then migrate to the neocortex. These neurons are added during adulthood, and may play important role in associative cortical functions (Gould, et al., 1999).

Different physiological conditions such as aging, estrous cycle, pregnancy and lactation can affect rodent neurogenesis (Pawluski et al., 2009). During the period of maternity, there is an increase in neural plasticity, especially due to the induction of maternal behavior and lactation. The hippocampus, although not traditionally associated with this condition, has been hypothesized to be involved in the maternal behavior and motherhood (Kinsley et al., 2006; Pawluski and Galea, 2006). As it is known, reproductive conditions are affected by steroid hormones and prolactin (Pawluski et al., 2009; Larsen and Gratan, 2012). A short time exposure to estradiol time increases neurogenesis in the hippocampus, whereas long-term exposure shows the opposite effect (Galea, 2008). The 17β-estradiol and BPA were also reported to affect neurogenesis of stem and progenitor cells from telencephalum of rat embryos (Okada et al., 2010).

The present study aimed to investigate the effects of BPA in adult female nursing mice exposed sub-chronically, since there are few studies that investigate this particular condition. Studies evaluating the effects of BPA on the CNS during lactation is an innovative research. Therefore, we studied the effects of exposure to BPA on enzymes and cell markers in different regions of the CNS in nursing female mice. For this purpose, it was employed a immunohistochemical approach to estimate the number of astrocytes, cellular proliferation, tyrosine hydroxylase and synaptophysin fluorescent area, which are important parameters of brain function.

2. Materials and methods

2.1 Animals

All procedures involving animal subjects were reviewed and approved by the Animal Ethics Committee of the Universidade Federal do Rio Grande (FURG), Rio Grande, Rio Grande do Sul (Approval number: P006/2011). Healthy Swiss albino mice obtained from the Central Animal Facility - FURG, maintained by random breeding, were housed in standard polystyrene cages at 23°C and 12-h light/12-h dark cycle, with water and a soybean-free diet *ad libitum* (Nuvilab CR-1 NUVITAL, Jundiaí, SP, Brazil). After the acclimation period of one week, the animals (8 weeks old) were grouped in cages (5 females + 1 male each) for random mating. The presence of the vaginal plug was verified twice a day for mating confirmation and, if positive, the females were considered pregnant. Pregnant females were placed into cages to give birth, and the pups were maintained with its dams from the birth day to the end of the lactation period (21 days). During this period, 5 treatment groups of 5 dams each received 200 µL/day of the following solutions, via gavage: saline 0.9% to the control group (SALINE); 300 µg/w/day BPA (BPA 300); 900 µg/w/day BPA (BPA 900); 3000 µg/w/day BPA (BPA 3000) and 650 µg/w/day diethylstilbestrol (DES) to the positive control (DES). At the day 21 after birth, the pups were separated for further analyses, and the dams (sub-chronic exposure) were sacrificed by cervical dislocation (Hogan et al., 1986). The brain tissue was dissected and pieces not larger than 0.5 cm were promptly submerged into formaldehyde 4% freshly prepared from paraformaldehyde, for fixation during 24 h. After, the pieces were dehydrated in a graded series of ethanol up to 100%, cleared in xylene and embedded in paraplast X-TRA (Sigma P3808). Six µm thick sections of the

striatum, cortex and hippocampus were obtained with a rotary microtome (Leica RM 2255) and mounted on poli-L-lisine-coated slides.

2.2 Immunohistochemistry

For the immunohistochemistry (IHC) assays, the brain sections were re-hydrated in solutions with decreasing concentrations of ethanol. The slides were washed three times, 10 min each, in a washing solution (PBS/Triton X-100 0.3%). The antigenic recovery was performed with sodium citrate buffer 0.01% (pH 6.0) during 45 min at 95-98ºC. This step was employed to optimize the binding of the antibody to its respective specific epitope. After, the unspecific binding sites of the tissue were blocked with PBS/BSA 3% and PBS/NGS 10% during 2 h. The slides were then incubated overnight at 4ºC with the primary antibody solutions (diluted 1:100 in PBS 0.1 M). The primary antibodies employed were: monoclonal anti-GFAP produced in mouse (G3893, Sigma-Aldrich), policlonal anti-TH produced in rabbit (T8700, Sigma-Aldrich), policlonal anti- H3P serine 10, produced in rabbit (sc8656, Santa Cruz), and policlonal anti-SYP produced in goat (sc7568, Santa Cruz). After the primary antibody incubation, the slides were washed again with the washing solution (three times, 10 min each) and incubated for 2 h at room temperature with the following secondary antibodies: CY3 (C2181, Sigma-Aldrich, anti-mouse IgG); CY3 anti-rabbit IgG (C2306, Sigma); Alexa-Fluor 488 anti-rabbit IgG (A11094, Invitrogen) and CY3 anti-goat IgG (C2821, Sigma-Aldrich). After, the slides were washed again with the washing solution (three times, 10 min), incubated during 1 min with 4,6-diamidino-2 phenylindole (DAPI) (D9542, Sigma-Aldrich), and mounted with aqueous medium mount (Fluoromount, Ref F4680, Sigma-Aldrich). The structures were observed under a Zeiss Axioskop 2 Plus epifluorescence microscope equipped with a color CCD camera (Media Cybernetics,

model Evolution MP). The fluorescent areas were analyzed using the IMAGE J program.

3. Statistical analysis

Data were expressed as mean ± SEM. Once the assumptions of homogeneity and normality of variance were verified, statistical analysis was performed between treatments by means of one-way ANOVA. Post hoc comparisons were performed using the Tukey test or orthogonal contrasts. The significant level adopted was 5% for all.

4. Results

4.1 GFAP marker

Representative images of astrocytes labeled with GFAP in the cortex, striatum and hippocampus treated with five treatments are shown in Figures 1, 2 and 3. The labeled astrocytes were counted and the difference of numbers of astrocytes in the cortex, striatum and hippocampus (CA3) are shown in Figure 4 (A, B and C). The In the cortex we observed a significant decrease ($p<0.05$) of astrocytes in the BPA 300 and BPA 900 groups, as well as in the DES–treated animals when compared with the control (Saline = 81±47; BPA 300 = 28±16; BPA 900 = 46±26; BPA 3000 = 68±39; DES = 44±20). In the striatum, the BPA 300 and DES treatments resulted in significantly decreased number of astrocytes ($p<0.05$) (BPA 300 = 8±4 and DES = 8±4) when compared with the control group (Saline = 35±20, BPA 900 = 11±6 and BPA 3000 =15±9). However, BPA 900 and BPA 3000 groups showed a not significant difference of number of astrocytes. The number of astrocytes in CA3 (Fig.4, C) region was impaired ($p<0.05$) in the BPA 300 (16±9) and in the BPA 900 (24±14) treatments when compared to the control group Saline (63±36). The BPA 3000 (39±23) and DES (45±20) treatments did not statistically differentiate ($p>0.05$) either to the control or the

other treatments, but a slight decrease in astrocytes number with respect to the control group can be noted.

4.2 Tyrosine Hydroxylase

Representative images of the striatum and hippocampus (CA3) labeled with TH are shown in Figures 5 and 6. Figure 7 (A, B and C) shows the positive cells / fluorescent area (μm^2) in the cortex, striatum and hippocampus (CA3).The different BPA and DES treatments did not affect the fluorescent area of the cortex (Fig. 5 A) when compared with the corresponding control groups (Saline = 0.04269± 0.02465; BPA 300 = 0.02670± 0.01542; BPA 900 = 0.03720± 0.02148; BPA 3000 = 0.03218± 0.01858; DES = 0.02507± 0.01448). The BPA 3000 (0.0137±0.0079) and DES (0.0098±0.0056) treatments significantly decreased ($p<0.05$) the fluorescence area (Fig. 5 B) in the striatum, when compared with all the other groups (Saline = 0.0277±0.0160; BPA 300 = 0.0192±0.0111; BPA 900 = 0.0180±0.0104). The data of fluorescent area from CA3 region (Fig. 5 C) showed a significantly decrease in the BPA 3000 ($p<0.05$) (Saline = 0.04458±0.02574; BPA 300 = 0.03710±0.02142; BPA 900 = 0.02842±0.01641; BPA 3000 = 0.01953±0.01128; DES = 0.02779±0.01605).

4.3 Synaptophysin

Representative image of synaptophysin labeling in the pre-frontal cortex is shown in Figure 8. Figure 9 (A, B and C) shows the differences in the fluorescent area occupied by synaptophysin positive cells in the cortex, striatum and hippocampus (CA3) , respectively. In the cortex (Fig 9 A) the BPA 900 treatment showed a significant decrease (0.02596±0.01079) ($p<0.05$) when compared with the saline treatment (Saline = 0.04853±0.00176) however the other tresatments were not significantly different from the control (BPA 300 = 0.05172±0.00256; BPA 3000 = 0.03354±0.00892; DES = 0.03060±0.00314). In the striatum (Fig 9 B) no difference

was observed between the treatments, however in the hippocampus (CA3) (Fig 9 C) BPA 900 significantly decreased ($p<0.05$) the synaptophysin fluorescent area when compared only to s aline (Saline = 0.03341±0.00585; BPA 300 = 0.02815±0.00839; BPA 900 = 0.01403±0.00741; BPA 3000 = 0.02115±0.00316; DES = 0.01527±0.00281).

4.4 Histone 3 Phosphate

The immunoreactions for H3P in the pre-frontal cortex pre-frontal is shown in Figure 10. Figure 11 (A, B, C and D) shows the differences in the number of the labeled cellsin the pre-frontal ortex , somato-sensory cortex, striatum and hippocampus (CA3) , respectively. The pre-frontal cortex showed moremore labeled cells ($p<0.05$) in the DES treatment (21.0±0.57) thanany other group (Saline = 1.33±0.33; BPA 300 = 0.33±0.33; BPA 900 = 4.33±0.88; BPA 3000 = 4.0±1.0), and less labeled cells ($p<0.05$) after the BPA 300 than the other treatments, except the control (Fig 11 A). The somato-sensory cortex (Fig 11 B) displayed less 3HP labeled cells ($p<0.05$) after the BPA 300 treatment (9.66±1.20) than the positive control DES (34.66±0.33). Both BPA 300 and DES treatmenst were significantly different than the other treatments (Saline = 16±5; BPA 900 = 33.66±16.37; BPA 3000 = 17.66±0.88). In the striatum (Fig 11C) the BPA 3000 treatment decreased significantly ($p<0.05$) the number of cells marked with H3P (1.0±0.0) and the DES treatment resulted in significantly ($p<0.05$) more labeled cells (22.66±6.56) when compared with all the other treatments (Saline = 6±1; BPA 300 = 4.66±4.17; BPA 900 = 6.33±1.66). In the hippocampus (Fig 10 D), the BPA 900 treatment raised the number of3HP marked cells (14.33±1.45) when compared with the cells treated with saline (saline = 9±0.57; BPA 300 = 7.66±3.28; BPA 3000 = 6.0±1.0; DES = 12.67±3.67).

5. Discussion

In thist study, we observed differences in the brain of female mice exposed during 21 days (lactation period) to BPA (300, 900 and 3000 µg/kg/day) when compared with the controls. These doses were below the No Observed Adverse Effect Level (NOAEL) = 50mg/kg/b.w./day in rats (Tyl *et al.*, 2002), which is no biologically or statistically significant increase in the frequency or severity of any adverse effects in the exposed population when compared to its appropriate control. In addition to the effects on reproductive tissues and thyroid gland, BPA is likely to alter the morphological and functional properties of neural cells in the central nervous system (CNS). It was reported that the exposure to BPA in female rats during pregnancy and lactation resulted in behavioral changes in dams, thus indicating that BPA altered neural circuits (Della Seta et al., 2005). Here, we investigated the BPA effects in three distinct regions of the brain: hippocampus, striatum and cortex. In these regions were evaluated GFAP, TH, Synaptophysin and H3P.

Astrocytes are among of the most important target cells for Estradiol (E2) and express nuclear and membrane estrogen receptors during development and in the adult brain. These glial cells modulate synaptic transmissions and respond to neurotransmitters released within the synapse by generating elevations in intracellular Ca^{2+} concentration (Fellin and Carmignoto, 2004). In our study, in the striatum and cortex areas (Figure 4), BPA 300 reduced the number of $GFAP^{+}$ cells when compared with the control. In the striatum, DES also decreased ($P<0.05$) the number of astrocytes. In the hipppocampus (CA3) BPA 300 and 900 decrease ($P<0.05$) $GFAP^{+}$ cells (Figure 4). BPA typically induces a non-monotonic response. However, the results suggested that BPA effects can be mediated by its estrogenic activity.

Miyatake et al. (2006) administrated low doses of BPA *in vitro* on midbrain glial cellular cultures (1fM to 1μM), and showed increase in $GFAP^+$ astrocytes demonstrating that the dopamine-induced Ca^{2+} responses in mixed cultures of neurons and astrocytes were significantly enhanced by the treatment with BPA. Jang et al (2012) did not observe GFAP increase in female mice *in vivo*. We observed that the exposure to the lowest BPA concentration (resulted in the lowest number of astrocytes when compared with the control group, in all the brain regions studied. Therefore, another explanation could be related with BPA-induced production of reactive oxygen species (ROS) activating the caspase-3 pathway, which is a marker of neuronal cell death (Obata and Kubota, 2000).

In our study, fluorescence of TH (rate-limiting enzyme for the catecholamines synthesis) decreased by treatment with BPA in the Striatum and Hippocampus (CA3) regions (Figure 7). It was observed in both regions that fluorescence in the BPA 3000 treatment significantly decreased ($P<0.05$) when compared to control. Ishido et al. (2007) exposed pups to 600μg/day and demonstrated that BPA-induced alterations in the dopamine (DA) system which can be pre-synaptic, affecting DA synthesis, DAT expression, and DA release and turnover in midbrain. Honma et al. (2006) observed that in female rats exposed perinatally (GD 6 to PND 20) to BPA (4 and 40mg/kg/day) the levels of DA increased significantly in the group receiving 4mg/kg/day in hippocampus, and no significant differences were observed in the cortex and striatum regions in all treatments. Our results showed a decrease of TH in the hippocampus and striatum and coincidentally not changes in the cortex (Figure 7).

BPA activates both of the major types of ERs (ERα and ERβ) in the anterior pituitary to modify estrogenic activity. Consequently, BPA may regulate the transcriptional activity of genes targeted by the estrogen system, including

dopaminergic genes (Ishido et al. 2007). Pre-natal BPA exposure decreased the number of TH neurons observed in the rostral periventricular preoptic area in female rat offspring in low BPA concentration and can be reflected on changes in the behavioral activities in adult age (Sun et al. 2002). However, in our work the TH alterations could be observed in high BPA concentrations in nursing female mice and were recorded in specific brain areas. Similar results were found by Honma et al. (2006) whose observed changes in dopamine and its metabolites in pups as well as dams. Furthermore, it has been reported that BPA stimulates •OH generation in rat striatum (Obata and Kubota, 2000). Thus, it is most likely that the toxicity of BPA can be attributed to the degeneration of dopaminergic neurons via oxidative stress effects. Nevertheless, we could not eliminate the possibility that the estrogenicity of BPA should, at least in part, be involved in this process. Ishido et al. (2007) demonstrated that the BPA oral administration (exposed pups to 600μg/day) caused hyperactivity on first time and with a prolonged administration (7 weeks) caused a large reduction of immunoreactivity for TH in the midbrain This shows that BPA can lead to decreased levels on tyrosine hydroxylase enzyme and the number of dopaminergic neurons. In our work, we found similar results in different brain locals, an accentuate decrease on tyrosine hydroxylase in the striatum and hippocampus.

BPA inhibits estradiol-induced hippocampal synaptogenesis (Mac Lusky et al., 2005), alters dopamine responsiveness in neurons and astrocytes and suppresses the estradiol-induced enhancement of long-term potentiation in the hippocampal CA1 area (Mukai et al., 2006). Synaptophysin is a suitable indicator of synaptic density. In our study synaptophysin^{+} area was decreased after using the highest dose in the cortex and hippocampus (CA3) (Figure 10). These findings were similar to those reported by Yokosuka et al. (2008) who administrated in hypothalamic cell culture 1μM of BPA and

this suppressed synaptic density (Synapsin^{+} area, and different synaptic marker, but with similar function). On the other hand, a single exposure to BPA (0.32, 3.2 or 4.8 mg/kg) in male and female mice (PND 10) resulted at 5 months old in an increase in the level of synaptophysin (Viberg and Lee, 2012).

Iwakura et al. (2010) demonstrated that hypothalamic cell culture from fetuses treated with 100 nM BPA increased the Synapsin I, but the change was not accompanied by the increase in protein or mRNA expression levels ERs. The authors postulated that BPA, similar to E2, induced effects on Synapsin I mainly through a non genomic pathway. Alyea and Watson (2009) have demonstrated that low levels of xenoestrogens including BPA act via membrane bound receptors (G protein coupled receptor 30 - GPR30) but not via intracellular ones. Our exposure to BPA in female lactating mice was subchronic, thus difficulting the comparison with other studies. However, in general the results are consistent with the labeling observed for GFAP and TH.

H3P phosphorilated in serine 10 is an indicative of mitosis and meiosis. We employed H3P as a labeling for cellular proliferation. The neurogenesis is influenced by hormonal condition. In the postpartum period, Pawluski et al. (2009) observed increase in neurogenesis in the lateral ventricle zone (SVZ) and gyrus dentatus of hippocampus. Estrogen and progesterone increased the neurogenesis whereas chronic E2 expositions result in decreasing of this process (Galea, 2008).

In physiological condition, a study with adult macaques employing BrdU showed increased neurogenesis in the neocortex and gyrus dentatus (Gould et al., 2001). Additionally, in adult rats thymidine - H^3 as used as proliferation markershowed that neurogenesis occurred in the neocortex, mesencephalon and diencephalon (Altman, 1962). In our study, neurogenesis was decreased after BPA 300 treatment in the pre-frontal cortex and after BPA 3000 treatment in the striatum. tTheseresults indicate

that BPA may have anti-neurogenic effects. Kim et al. (2012) and Jang et al. (2012) observed that neurogenesis in the hippocampal region was decreased by BPA in high concentrations (20 mg/kg) and increased at low concentrations (1mg/kg), respectively.

The exposure to BPA (200μg/kg) in pregnant female mice resulted in increase in neurogenesis of its brood (Komada et al., 2012). In our results on cortex pre-frontal and striatum it was observed beneficial effects of DES in terms of cellular proliferation. This indicates the proliferative sensitivity to high concentrations of DES in these regions, although BPA showed no effect on hippocampus and cortex somato-sensory, however, the proliferation was visually higher in this region. In the assessment of cellular proliferation in the hippocampus it was included the gyrus dentatus and it was observed an increase on proliferation in the BPA 900 when compared to the control. The synaptophysin labeling was lowin CA3 after the treatment of BPA 900 and proliferation in the same concentration in the hippocampus including gyrus dentatus was high. The important proliferation observed in adult females during lactation can be positively affected by this condition although other studies would be carried out to confirm these findings.

In summary, cell proliferation occurred in the nervous system of adult female mice but there were also losses induced by BPA or DES on the number of astrocytes, in tyrosine hydroxylase concentration and synaptic density. This shows the potential implication of BPA in neurodegenerative diseases.

6. Conflict of interest

The author declared there is no conflict of interest.

7. Acknowledgements

This study was supported by a grant from the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS Proc. No. 1013533– Edital PqG) to the

Postgraduate Program in Physiological Sciences Post-graduate Program of the Universidade Federal do Rio Grande. Ana Cristina Kalb received an undergraduate fellowship from the Brazilian agency CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and Ana Luiza Kalb received a scientific initiation grant from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico).

8. References

Alyea, R.A.,Watson, C.S. 2009. Differential regulation of dopamine transporter function and location by low concentrations of environmental estrogens and 17beta stradiol. Environ Health Perspect. 117,778–783.

Brede C., Fjeldal, P., Skjevrak I., Herikstad H. 2003. Increased migration levels of bisphenol A from polycarbonate baby bottles after dishwashing, boiling and brushing, Food Addit. Contam. 20, 684–689.

Della Seta, D., Minder, I., Dessì-Fulgheri, F., Farabollini, F. 2005. Bisphenol-A exposure during pregnancy and lactation affects maternal behavior in rats. Brain Res Bull. 65(3), 255-60.

Fellin, T., Carmignoto, G. 2004. Neurone-to-astrocyte signalling in the brain represents a distinct multifunctional unit. J Physiol. 559, 3–15.

Galea, L.A. 2008. Gonadal hormone modulation of neurogenesis in the dentate gyrus of adult male and female rodents. Brain Res Rev. 57(2), 332-41.

Gould, E., Vail, N., Wagers, M., Gross, C.G. 2000**.** Adult-generated hippocampal and neocortical neurons in macaques have a transient existence PNAS. 98(19), 10910–10917.

Gould, E., Reeves, A.J., Graziano, M.S., Gross, C.G. 1999. Neurogenesis in the neocortex of adult primates. Science 286, 548–552.

Hauser, R., Williams, P., Altshul, L.A.M. 2005. Evidence of Interaction between Polychlorinated Biphenyls and Phthalates in Relation to Human Sperm Motility. EnvironHealth. 113(4), 425-430.

Honma, T., Miyagawa, M., Suda, M., Wang, R.S., Kobayashi, K., Sekiguchi, S. 2006. Effects of perinatal exposure to bisphenol A on brain neurotransmitters in female rat offspring. Ind Health. 44,510–24.

Ishido M, Yonemoto J, Morita M. 2007. Mesencephalic neurodegeneration in the orally administered bisphenol A-caused hyperactive rats. Toxicol Lett. 173, 66–72.

Jang, Y.J., Park, H.R., Kim, T.H., Yang, W.J., Lee, J.J., Choi, S.Y., Lee, E., Park, J.H., Kim, H.P., Kim, H.S., Lee, J. 2012. High dose bisphenol A impairs hippocampal neurogenesis in female mice across generations. Toxicol. 296, 73– 82.

Kim, M.E., Park, H.R., Gong, E.J., Choi, S.Y., Kim, H.S., Lee, J., 2011. Exposure to bisphenol A appears to impair hippocampal neurogenesis and spatial learning and memory. Food Chem Toxicol**.** 49, 3383–3389.

Kinsley, C.H., Trainer, R., Stafisso-Sandoz, P. Quadros, L.K., Marcus, C., Hearon, E.A., Meyer, N., Hester, M., Morgan, F.J., Kozub, K.G. 2006. Motherhood and the hormones of pregnancy modify concentrations of hippocampal neuronal dendritic spines. Horm. Behav. 49, 131–142.

Komada, M., Asai, Y., Moric, M., Matsuki, M., Sato, M., Nagao, T. 2012. Maternal bisphenol A oral dosing relates to the acceleration of neurogenesis in the developing neocortex of mouse fetuses. Toxicol. 295, 31– 38.

Larsen, C.M., Grattan, D.R. 2012. Prolactin, neurogenesis, and maternal behaviors. Brain Behav Immun. 26(2), 201-9.

Leranth, C., Hajszan, T., Szigeti-buck, K., Bober, J., Maclusky, N.J. 2008. Bisphenol A prevents the synaptogenic response to estradiol in hippocampus and prefrontal cortex of ovariectomized nonhuman primates. PNAS, 105(16), 14187–14191.

Li, C., Brake, W.G., Romeo, R.D., Dunlop, J.C., Gordon, M., Buzescu, R. 2004. Estrogen alters hippocampal dendritic spine shape and enhances synaptic protein immunoreactivity and spatial memory in female mice. PNAS. 101(7), 2185-2190.

Liu, F., Day, M., Muniz, L., Bitran, D., Arias, Grauer, G.Z. 2008. Activation of estrogen receptor-b regulates hippocampal synaptic plasticity and improves memory. Nat neurosc. 11(3), 334-343.

MacLusky, N.J., Hajszan, T., Leranth, C. 2005. The environmental estrogen bisphenol A inhibits estradiol-induced hippocampal synaptogenesis. Environ. Health Perspect. 113, 675–679.

Makiko, D.M., Makoto, Y., TakashiShiga, C., Ritsuko, O. 2010. In vitro effects of bisphenol A on developing hypothalamic neurons. Toxicol. 272, 52–58.

Miyatake, M., Miyagawa, K., Mizuo, K., Narita, M., Suzuki, T. 2006. Dynamic changes in dopaminergic neurotransmission induced by a low concentration of bisphenol-A in neurones and astrocytes. J. Neuroendocrinol. 18, 434-444.

Mukai, H., Takata, N., Ishii, H.T., Tanabe, N., Hojo, Y., Furukawa, A., Kimoto, T., Kawato, S. 2006. Hippocampal synthesis of estrogens and androgens which are paracrine modulators of synaptic plasticity: synaptocrinology. Neurosc. 138, 757–764.

Nakayama, D., Matsuyama, T., Ishibashi-Ueda, H., Nakagomi, T., Kasahara, Y., Hirose, H., Kikuchi-Taura, A., Stern, D.M., Mori, H., Taguchi, A. 2010. Injury-induced neural stem/progenitor cells in post-stroke human cerebral cortex. Eur J Neurosci. 31(1), 90-8.

Neil, J., MacLusky, Hajszan, T., Leranth C. 2005. The Environmental Estrogen Bisphenol A Inhibits Estradiol-Induced Hippocampal Synaptogenesis. Environ Health Perspect, 113(6), 675-679.

Nicolas, J., Cabaton, P.R., Wadia, B.S., Rubin, D.Z., Schaeberle, C.M., Sonnenschein, C.A. 2011. Perinatal Exposure to Environmentally Relevant Levels of Bisphenol A Decreases Fertility and Fecundity in CD-1 Mice. Environ Health. 4, 547-552.

Obata, T., Kubota, S. 2000. Formation of hydroxyl radicals by environmental estrogen like chemicals in rat striatum. Neurosci Lett. 296, 42–44.

Okada, M., Makino, A., Nakajima, M., Okuyama, S., Furukawa, S., Furukawa, Y. 2010. Estrogen Stimulates Proliferation and Differentiation of Neural Stem/Progenitor Cells through Different Signal Transduction Pathways. Int J Mol Sci. 11(10), 4114-23.

Pawluski, J.L., Galea, L.A. 2006. Hippocampal morphology is differentially affected by reproductive experience in the mother. J. Neurobiol. 66, 71–81.

Pawluski, J.L., Brummelte, S., Barha, C.K., Crozier, T. M., Galea, L.A.M. 2009. Effects of steroid hormones on neurogenesis in the hippocampus of the adult female rodent during the estrous cycle, pregnancy, lactation and aging. Front Neuroendocrinol. 30, 343–357.

Qiu, J., Ronnekleiv, O.K., Kelly, M.J. 2008. Modulation of hypothalamic neuronal activity through a novel G-protein-coupled estrogen membrane receptor. Steroids. 73,985–991.

Roepke, T.A., Qiu, J., Bosch, M.A., Rønnekleiv, O.K., Kelly, M.J. 2009. Crosstalk between membrane initiated and nuclear initiated oestrogen signaling in the hypothalamus. J.Neuroendocrinol. 21,263–270.

Sullivan, J.M., Benton, D.C., Beltz, B. 2007. Adult Neurogenesis: A Common Strategy Across Diverse Species. J Comp Neurol. 500(3), 574–584.

Suzuki K., Ishikawa K., Sugiyama K., Furuta H., Nishimura F. 2000. Content and release of bisphenol A from polycarbonate dental products. Dent. Mater. J. 19, 389–395.

Sun, Y., Nakashima, M.N., Takahashi, M., Kuroda, N., Nakashima, K., 2002. Determination of bisphenol A in rat brain by microdialysis and column switching high-performance liquid chromatography with fluorescence detection. Biomed. Chromatogr. 16, 319–326.

Tyl, R.W., Myers, C.B., Marr, M.C., Thomas, B.F., Keimowitz, A.R., Brine, D.R., Veselica, M.M., Fail, P.A., Chang, T.Y., Seely, J.C. 2002. Three-generation reproductive toxicity study of dietary bisphenol A in CD Sprague-Dawley rats. Toxicol. Sci. 68, 121–146.

Yokosuka, M., Ohtani-kaneko, R., Yamashita, K., Muraoka, D., Kuroda, Y., Watanabe, C. 2008. Estrogen and environmental estrogenic chemicals exert developmental efects on rat hypothalamic neurons and glias. J Comp Neurol. 22, 1-9.

Viberg, H., Lee, I. 2012. A single exposure to bisphenol A alters the levels of important neuroproteins in adult male and female mice. NeuroToxicol. 33, 1390–1395.

Xu, X., Li, T., Luo, Q., Hong, X., Xie, L., Tian, D. 2011. Bisphenol-A rapidly enhanced passive avoidance memory and phosphorylation of NMDA receptor subunits in hippocampus of young rats. Toxicol Appl Pharm. *255*, 221-228.

Captions

Figure 1. **Immunofluoresce analisys of glial fibrilary acid protein (GFAP) in cortex in female mice (n=3 and 13 weeks old).** 1: DAPI-labelling (blue), a nuclear marker, 2: GFAP (red), astrocyte cells marker and 3: merged both images to each marker and treatment. A: Saline; B: BPA 300µg/Kg./day (BPA 300); C: BPA 900µg//Kg./day (BPA 900); D: 3000µg//Kg./day (BPA 3000) and E: DES 650µg//Kg./day (DES). White arrows indicate astrocytes. (Scale bar = 20µm).

Figure 2. **Immunofluoresce analisys of glial fibrilary acid protein (GFAP) in striatum in female mice (n=3 and 13 weeks old).** 1: DAPI-labelling (blue), a nuclear marker, 2: GFAP (red), astrocyte cells marker and 3: merged both images to each marker and treatment. A: Saline; B: BPA 300µg/Kg./day (BPA 300); C: BPA 900µg//Kg./day (BPA 900); D: 3000µg//Kg./day (BPA 3000 and E: DES 650µg//Kg./day (DES). White arrows indicate astrocytes. (Scale bar = 20µm).

Figure 3. **Immunofluoresce analisys of glial fibrilary acid protein (GFAP) in hippocampus (CA3) in female mice (n=3 and 13 weeks old).** 1: DAPI-labelling (blue), a nuclear marker, 2: GFAP (red), astrocyte cells marker and 3: merged both images to each marker and treatment. A: Saline; B: BPA 300µg/Kg./day (BPA 300); C: BPA 900µg//Kg./day (BPA 900); D: 3000µg//Kg./day (BPA 3000) and E: DES 650µg/Kg./day (DES). White arrows indicate astrocytes. (Scale bar = 20µm).

Figure 4. (A), (B) and (C) shows the differences in female mice (n= 3 and 13 week old) on astrocytes number on Cortex, Striatum and hippocampus (CA3) regions from brain, respectively. Saline (0.9% saline); BPA300 (BPA 300 µg/kg/day); BPA 900 (BPA 900 µg/kg/day); (BPA 3000 µg/kg/day) DES (DES 650 µg/kg/day). Data are expressed as mean ± SEM. Bars indicated with the same letter are not significant at the 5% probability level.

Figure 5. **Immunofluoresce analisys of tyrosine hydroxylase (TH) in striatum in female mice (n=3 and 13 weeks old).** 1: DAPI-labelling (blue), a nuclear marker, 2: TH (red), TH positive cells marker and 3: merged both images to each marker and treatment. A: Saline; B: BPA 300µg/Kg./day (BPA 300); C: BPA 900µg//Kg./day (BPA 900); D: 3000µg//Kg./day (BPA 3000) and E: DES 650µg//Kg./day (DES). White arrows indicate TH positive cells marker. (Scale bar = 20µm).

Figure 6. **Immunofluoresce analisys of tyrosine hydroxylase (TH) in hippocampus (CA3) in female mice (n=3 and 13 weeks old).** 1: DAPI-labelling (blue), a nuclear marker, 2: TH (red), TH positive cells marker and 3: merged both images to each marker and treatment. A: Saline; B: BPA 300µg/Kg./day (BPA 300); C: BPA 900µg//Kg./day (BPA 900); D: 3000µg//Kg./day (BPA 3000) and E: DES 650µg//Kg./day (DES). White arrows indicate TH positive cells marker. (Scale bar = 20µm).

Figure 7. (A), (B) and (C) shows Tyrosine hydroxylase positive cells in female mice (n= 3 and 13 week old) on Cortex, Striatum and hippocampus (CA3) regions from brain, respectively. Saline (0.9% saline); BPA300 (BPA 300 µg/kg/day); BPA 900 (BPA 900

µg/kg/day); (BPA 3000 µg/kg/day) DES (DES 650 µg/kg/day). Data are expressed as mean ± SEM. Bars indicated with the same letter are not significant at the 5% probability level.

Figure 8. **Immunofluoresce analisys of Synaptophysin in cortex in female mice (n=3 and 13 weeks old).** 1: DAPI-labelling (blue), a nuclear marker, 2: Synaptophysin (red), Synaptophysin positive cells marker and 3: merged both images to each marker and treatment. A: Saline; B: BPA 300µg/Kg./day (BPA 300); C: BPA 900µg//Kg./day (BPA 900); D: 3000µg//Kg./day (BPA 3000) and E: DES 650µg//Kg./day (DES). White arrows indicate synatophysin cells marker. (Scale bar = 20µm).

Figure 9. (A), (B) and (C) shows Synaptophysin positive cells in female mice (n= 3 and 13 week old) on Cortex, Striatum and hippocampus (CA3) regions from brain. Saline (0.9% saline); BPA300 (BPA 300 µg/kg/day); BPA 900 (BPA 900 µg/kg/day); (BPA 3000 µg/kg/day) DES (DES 650 µg/kg/day). Data are expressed as mean ± SEM. Bars indicated with the same letter are not significant at the 5% probability level.

Figure 10. **Immunofluoresce analisys of histone 3 phosphate (H3P) in cortex pre-frontal in female mice (n=3 and 13 weeks old).** 1: DAPI-labelling (blue), a nuclear marker, 2: Allexa 488 (green), H3P cells marker and 3: merged both images to each marker and tratment. A: Saline; B: BPA 300µg/Kg./day (BPA 300); C: BPA 900µg//Kg./day (BPA 900); D: 3000µg//Kg./day (BPA 3000) and E: DES 650µg//Kg./day (DES). White arrows indicate histone 3 phosphate. (Scale bar = 20µm).

Figure 11. (A), (B), (C) and (D) shows number of histone 3 phosphate (H3P) in female mice (n= 3 and 13 week old) on Cortex pre-frontal, Cortex somato-sensory, Striatum and hippocampus (CA3) regions from brain. Saline (0.9% saline); BPA300 (BPA 300 µg/kg/day); BPA 900 (BPA 900 µg/kg/day); (BPA 3000 µg/kg/day) DES (DES 650 µg/kg/day). Data are expressed as mean ± SEM. Bars indicated with the same letter are not significant at the 5% probability level.

Figure 1

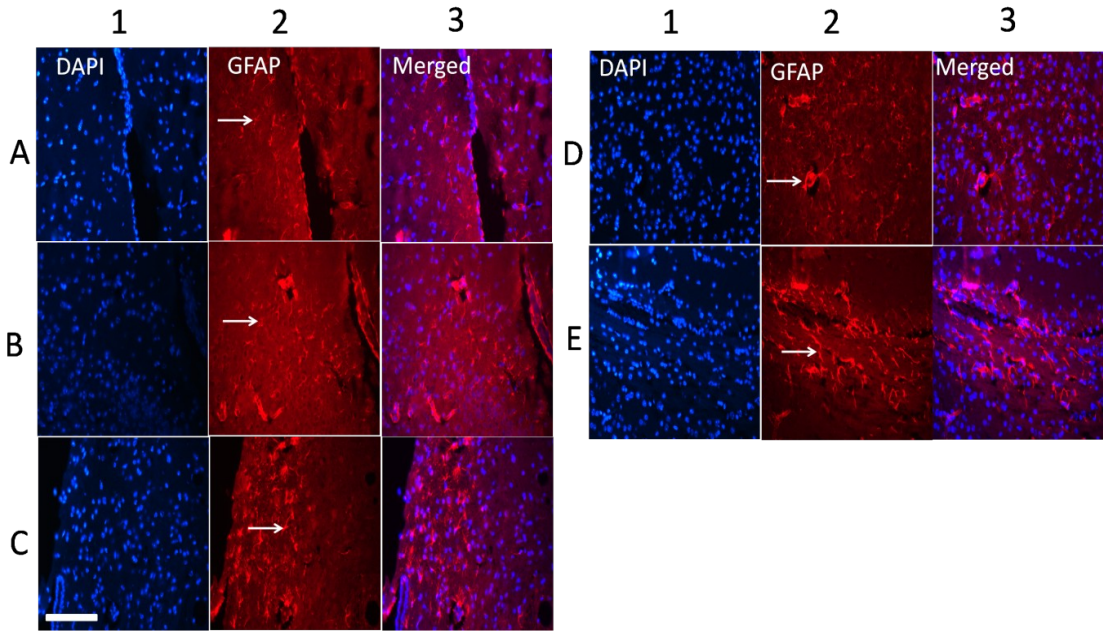


Figure 2

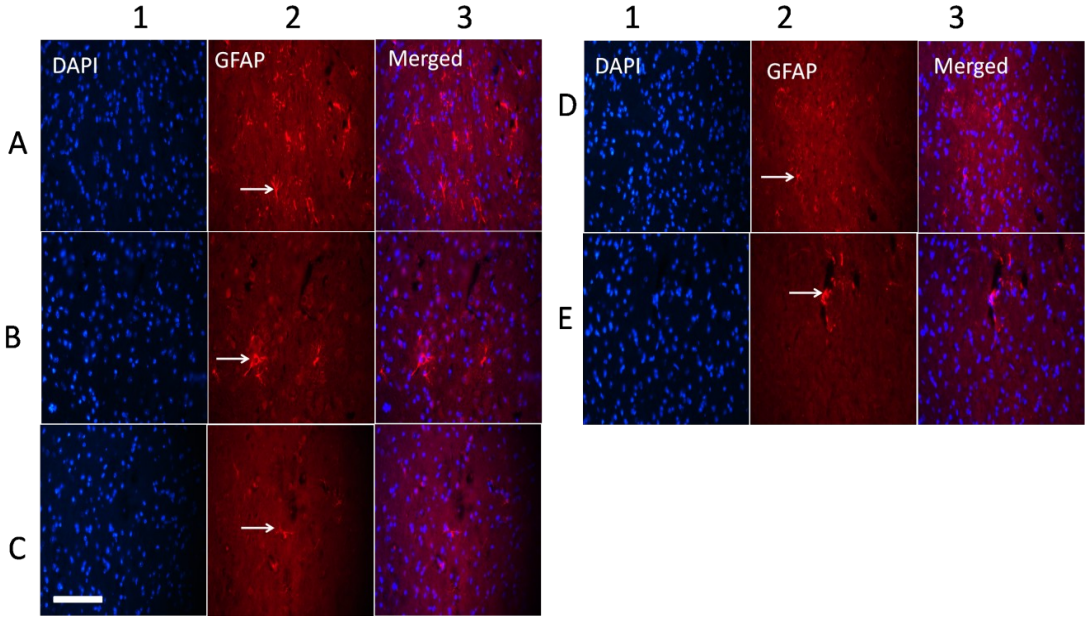


Figure 3

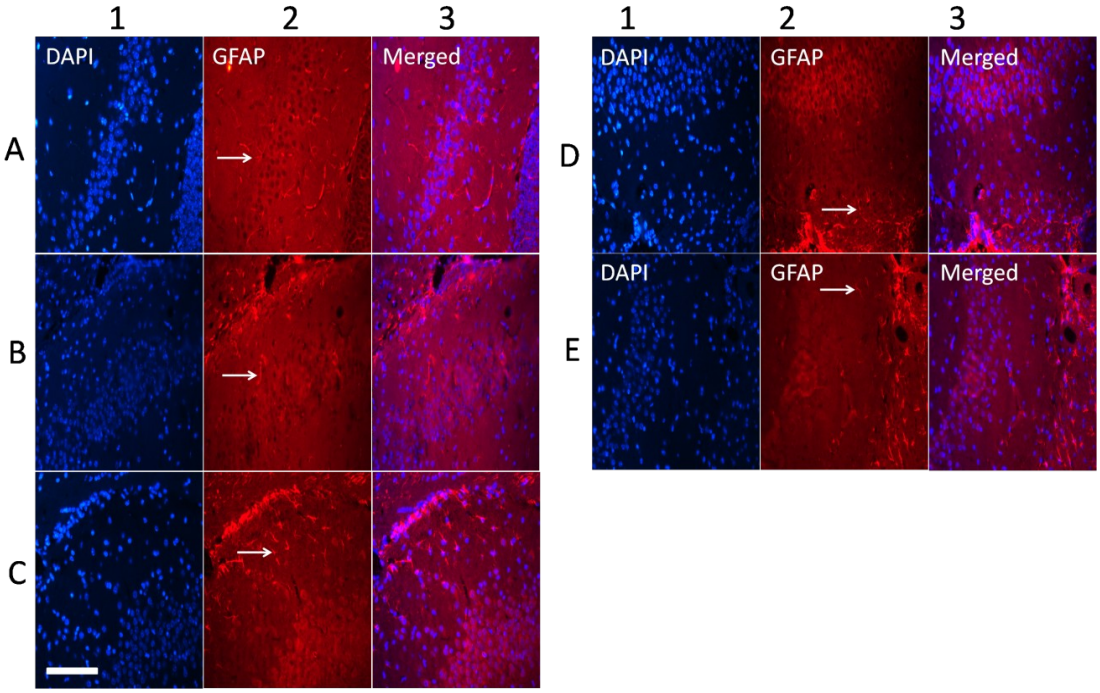


Figure 4

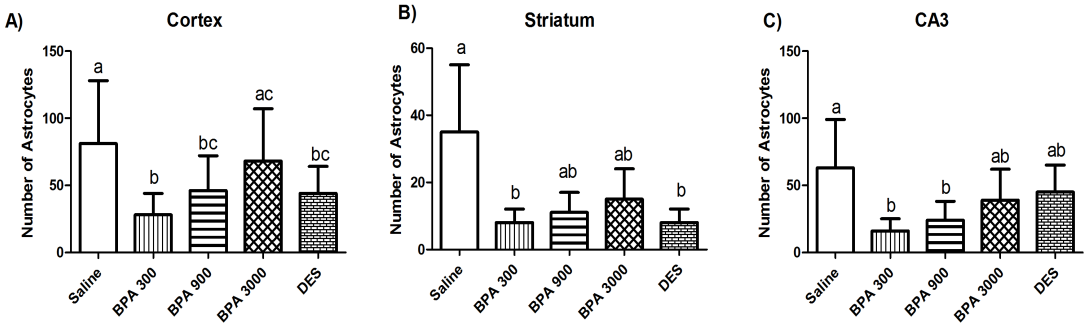

A)
Cortex
Number of Astrocytes
150
100
50
0
a
b
bc
ac
bc
Saline
BPA 300
BPA 900
BPA 3000
DES
B)
Striatum
Number of Astrocytes
60
40
20
0
a
b
ab
ab
b
Saline
BPA 300
BPA 900
BPA 3000
DES
C)
CA3
Number of Astrocytes
150
100
50
0
a
b
b
ab
ab
Saline
BPA 300
BPA 900
BPA 3000
DES

Figure 5

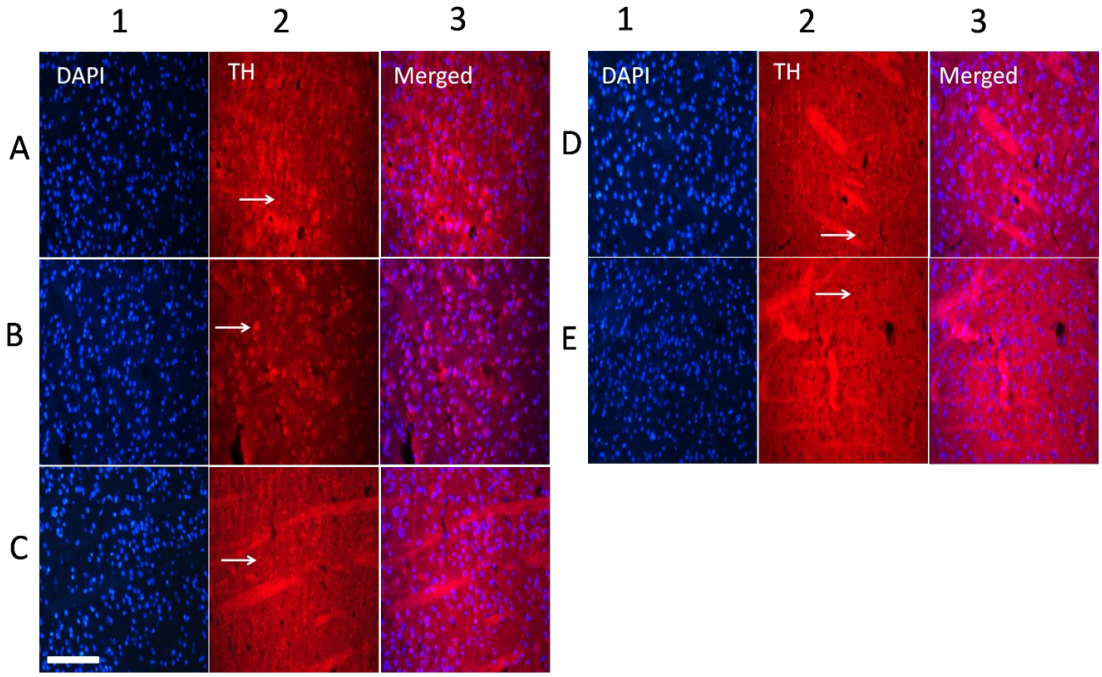


Figure 6

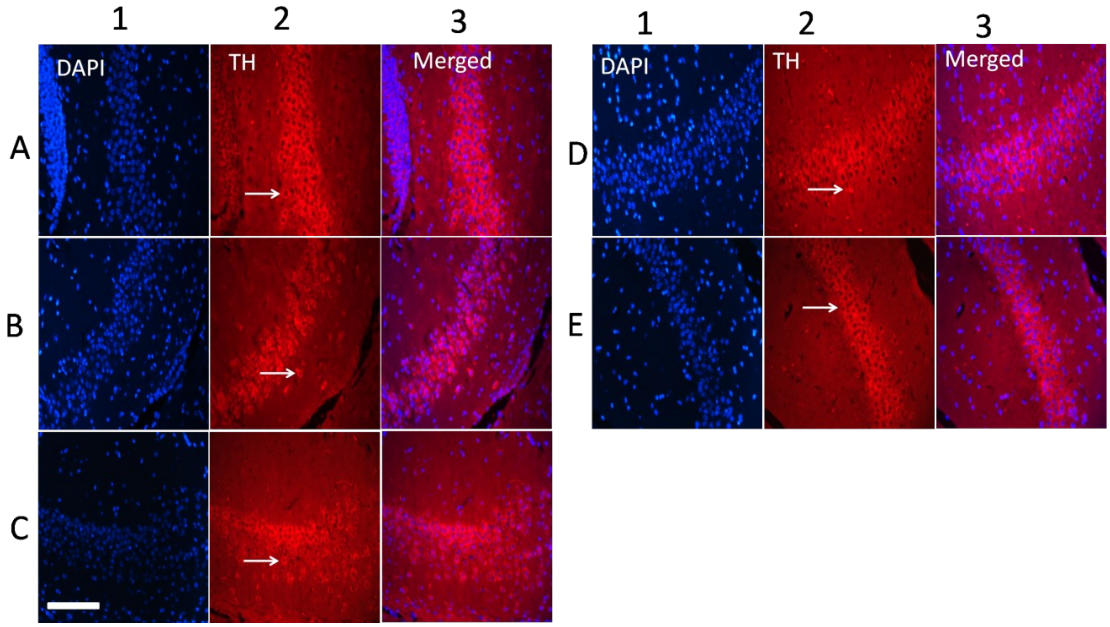


Figure 7

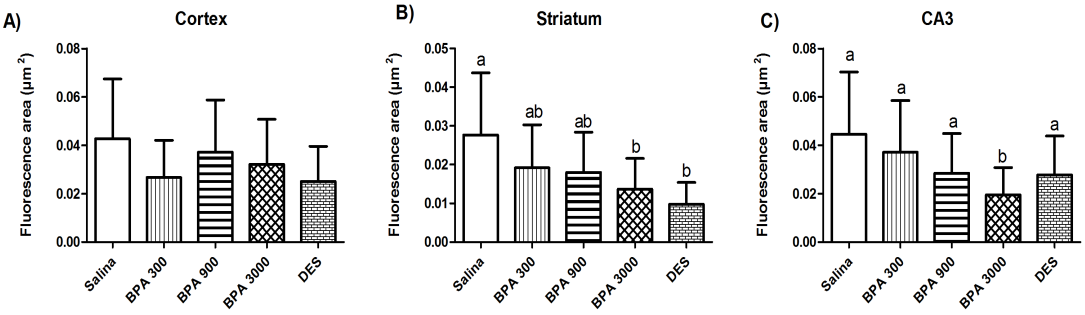

A)
Cortex
Fluorescence area (µm 2)
0.08
0.06
0.04
0.02
0.00
Salina
BPA 300
BPA 900
BPA 3000
DES
B)
Striatum
Fluorescence area (µm 2)
0.05
0.04
0.03
0.02
0.01
0.00
a
ab
ab
b
b
Salina
BPA 300
BPA 900
BPA 3000
DES
C)
CA3
Fluorescence area (µm 2)
0.08
0.06
0.04
0.02
0.00
a
a
a
b
a
Salina
BPA 300
BPA 900
BPA 3000
DES

Figure 8

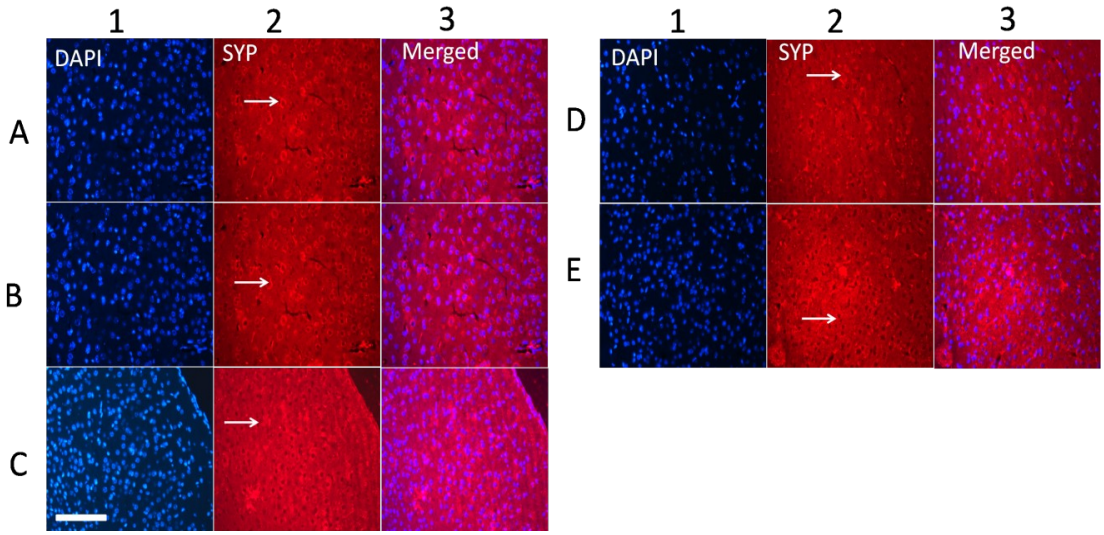


Figure 9

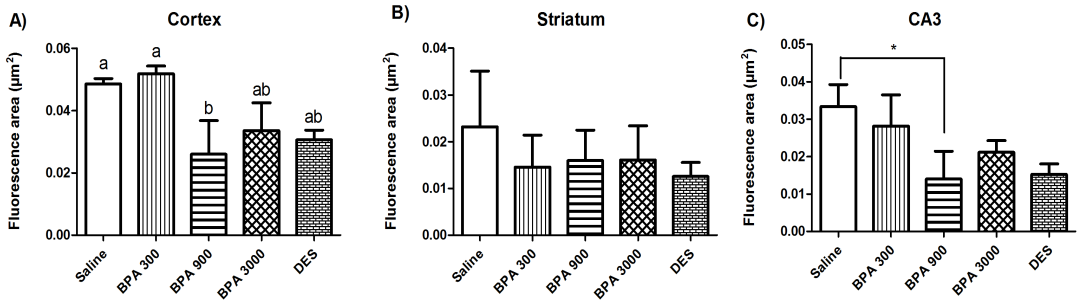

A)
Cortex
Fluorescence area (µm²)
0.06
0.04
0.02
0.00
a
a
b
ab
ab
Saline
BPA 300
BPA 900
BPA 3000
DES
B)
Striatum
Fluorescence area (µm²)
0.04
0.03
0.02
0.01
0.00
Saline
BPA 300
BPA 900
BPA 3000
DES
C)
CA3
Fluorescence area (µm²)
0.05
0.04
0.03
0.02
0.01
0.00
*
Saline
BPA 300
BPA 900
BPA 3000
DES

Figure 10

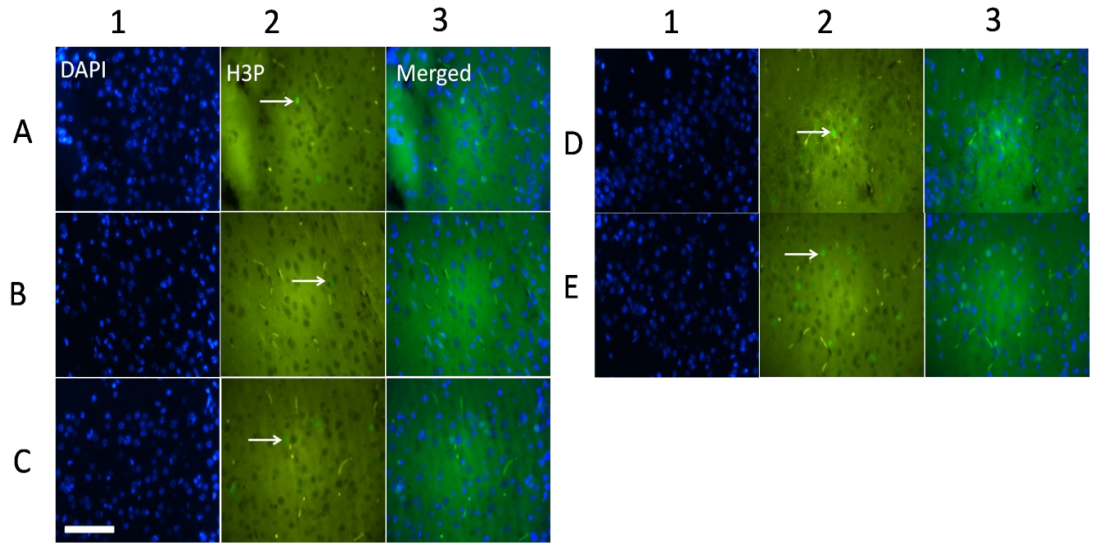
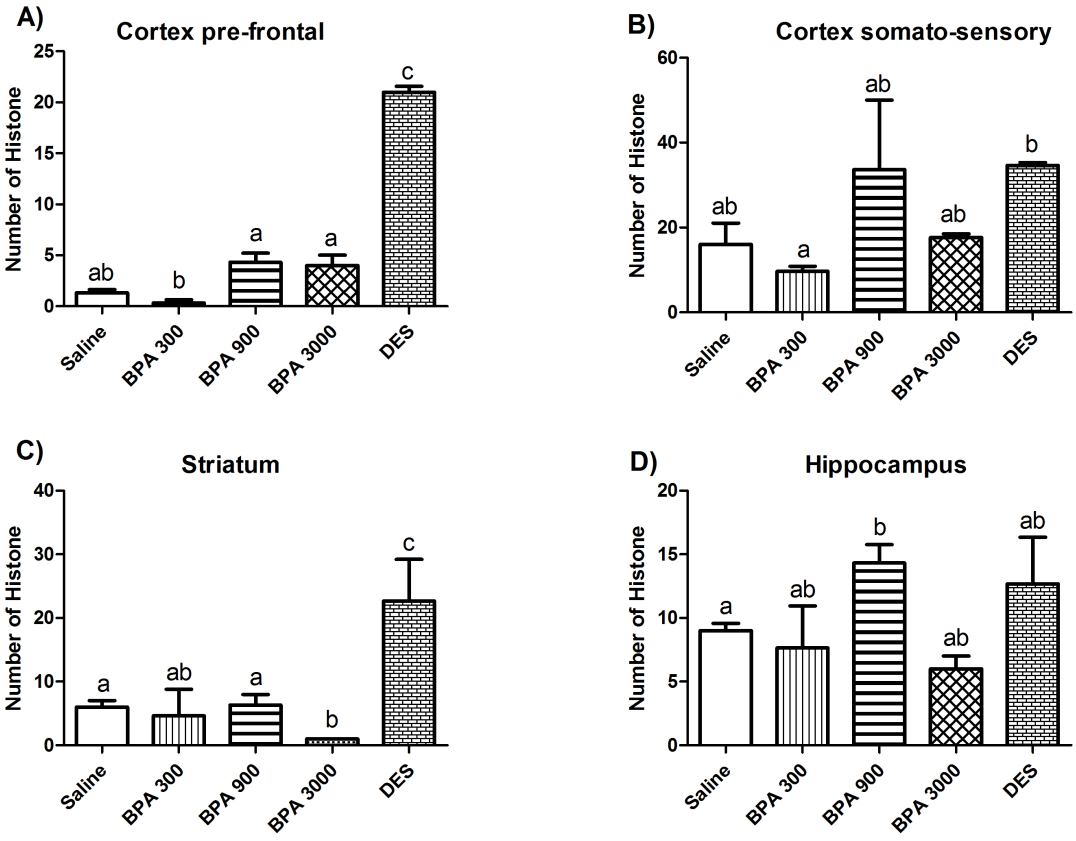


Figure 11


A)
Cortex pre-frontal
Number of Histone
25
20
15
10
5
0
ab
b
a
a
c
Saline
BPA 300
BPA 900
BPA 3000
DES
B)
Cortex somato-sensory
Number of Histone
60
40
20
0
ab
a
ab
ab
b
Saline
BPA 300
BPA 900
BPA 3000
DES
C)
Striatum
Number of Histone
40
30
20
10
0
a
ab
a
b
c
Saline
BPA 300
BPA 900
BPA 3000
DES
D)
Hippocampus
Number of Histone
20
15
10
5
0
a
ab
b
ab
ab
Saline
BPA 300
BPA 900
BPA 3000
DES

Conclusões

BPA quando administrado por via indireta (transmissão vertical) pode induzir a danos em parâmetros espermáticos como motilidade do espermatozóide, morfologia, funcionalidade mitocondrial, integridade de membrana, de acrossoma e de DNA.

Por diversas vias, dentre elas estresse oxidativo, o BPA pode causar alterações morfológicas nos testículos dos animais. Também causa diminuição da distância ano-genital destes animais.

Nas fêmeas que receberam BPA diretamente, o impacto nos parâmetros analisados (número de astrócitos, quantidade da enzima tirosina hidroxilase, densidade sináptica e proliferação celular), deixou evidente o papel deste tóxico na possível indução de doenças neurodegenerativas.

Anexo 1

Normas da revista Reproductive Toxicology

REPRODUCTIVE TOXICOLOGY

Article structure

Subdivision - numbered sections
Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods
Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Theory/calculation
A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

Results
Results should be clear and concise.

Discussion
This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Essential title page information

- ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
- ***Author names and affiliations.*** Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
- ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.**
- ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.**The abstract should contain no more than 150 words.**

Graphical abstract
A Graphical abstract is optional and should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. See http://www.elsevier.com/graphicalabstracts for examples.
Authors can make use of Elsevier's Illustration and Enhancement service to ensure the best presentation of their images also in accordance with all technical requirements: Illustration Service.

Highlights
Highlights are a short collection of bullet points that convey the core findings of the article. Highlights are optional and should be submitted in a separate file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See http://www.elsevier.com/highlights for examples.

Keywords
Immediately after the abstract, provide a maximum of 8 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, "and", "of"). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations
Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements
Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Database linking
Elsevier aims at connecting online articles with external databases which are useful in their respective research communities. If your article contains relevant unique identifiers or accession numbers (bioinformatics) linking to information on entities (genes, proteins, diseases, etc.) or structures deposited in public databases, then please indicate those entities according to the standard explained below.
Authors should explicitly mention the *database abbreviation (as mentioned below) together with the actual database number*, bearing in mind that an error in a letter or number can result in a dead link in the online version of the article.
Please use the following format: **Database ID: xxxx**
Links can be provided in your online article to the following databases (examples of citations are given in parentheses):
- ASTM: ASTM Standards Database (ASTM ID: G63)
- CCDC: Cambridge Crystallographic Data Centre (CCDC ID: AI631510)
- GenBank: Genetic sequence database at the National Center for Biotechnology Information (NCBI) (GenBank ID: BA123456)
- GEO: Gene Expression Omnibus (GEO ID: GSE27196; GEO ID: GPL5366; GEO ID: GSM9853)
- MI: EMBL-EBI OLS Molecular Interaction Ontology (MI ID: 0218)
- MINT: Molecular INTeractions database (MINT ID: 6166710)
- NCBI Taxonomy: NCBI Taxonomy Browser (NCBI Taxonomy ID: 48184)
- NCT: ClinicalTrials.gov (NCT ID: NCT00222573)
- OMIM: Online Mendelian Inheritance in Man (OMIM ID: 601240)
- PDB: Worldwide Protein Data Bank (PDB ID: 1TUP)
- TAIR: The Arabidopsis Information Resource database (TAIR ID: AT1G01020)
- UniProt: Universal Protein Resource Knowledgebase (UniProt ID: Q9H0H5)

Math formulae

Present simple formulae in the line of normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Table footnotes

Indicate each footnote in a table with a superscript lowercase letter.

Artwork

Electronic artwork

General points

- Make sure you use uniform lettering and sizing of your original artwork.
- Save text in illustrations as 'graphics' or enclose the font.
- Only use the following fonts in your illustrations: Arial, Courier, Times, Symbol.
- Number the illustrations according to their sequence in the text.
- Use a logical naming convention for your artwork files.
- Provide captions to illustrations separately.
- Produce images near to the desired size of the printed version.
- Submit each figure as a separate file.

A detailed guide on electronic artwork is available on our website:
http://www.elsevier.com/artworkinstructions
You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

Regardless of the application used, when your electronic artwork is finalised, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS: Vector drawings. Embed the font or save the text as 'graphics'.
TIFF: Color or grayscale photographs (halftones): always use a minimum of 300 dpi.
TIFF: Bitmapped line drawings: use a minimum of 1000 dpi.
TIFF: Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.
If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is'.
Please do not:

- Supply files that are optimised for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low;
- Supply files that are too low in resolution;
- Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF, EPS or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article.** Please indicate your preference for color: in print or on the Web only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.
Please note: Because of technical complications which can arise by converting color figures to 'gray scale' (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software

This journal has standard templates available in key reference management packages EndNote (http://www.endnote.com/support/enstyles.asp) and Reference Manager (http://refman.com/support/rmstyles.asp). Using plug-ins to wordprocessing packages, authors only need to select the appropriate journal template when preparing their article and the list of references and citations to these will be formatted according to the journal style which is described below.

Reference style

Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.
List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.
Examples:
Reference to a journal publication:
[1] Van der Geer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. J Sci Commun 2010;163:51–9.
Reference to a book:
[2] Strunk Jr W, White EB. The elements of style. 4th ed. New York: Longman; 2000.
Reference to a chapter in an edited book:
[3] Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, Smith RZ, editors. Introduction to the electronic age, New York: E-Publishing Inc; 2009, p. 281–304.
Note shortened form for last page number. e.g., 51–9, and that for more than 6 authors the first 6 should be listed followed by 'et al.' For further details you are referred to 'Uniform Requirements for Manuscripts submitted to Biomedical Journals' (J Am Med Assoc 1997;277:927–34) (see also http://www.nlm.nih.gov/bsd/uniform_requirements.html).

Journal abbreviations source

Journal names should be abbreviated according to
Index Medicus journal abbreviations: http://www.nlm.nih.gov/tsd/serials/lji.html;
List of title word abbreviations: http://www.issn.org/2-22661-LTWA-online.php;
CAS (Chemical Abstracts Service): http://www.cas.org/sent.html.

Video data

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 50 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages at http://www.elsevier.com/artworkinstructions. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

Supplementary data

Elsevier accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, high-resolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. In order to ensure that your submitted material is directly usable, please provide the data in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. For more detailed instructions please visit our artwork instruction pages at http://www.elsevier.com/artworkinstructions.

Anexo 2

Normas da revista Toxicology Letters

TOXICOLOGY LETTERS
Official Journal of EUROTOX
ELSEVIER

Article structure

Manuscripts should be typewritten, 1.5-spaced. In general, manuscripts should not be longer than 10 printed pages (approx. 15, 1.5-spaced pages).

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Theory/calculation

A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

- ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
- ***Author names and affiliations.*** Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
- ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.**
- ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract
A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself. **The Abstract should contain approx. 150 - 200 Words.**

Graphical abstract
A Graphical abstract is optional and should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. See http://www.elsevier.com/graphicalabstracts for examples.
Authors can make use of Elsevier's Illustration and Enhancement service to ensure the best presentation of their images also in accordance with all technical requirements: Illustration Service.

Highlights
Highlights are a short collection of bullet points that convey the core findings of the article. Highlights are optional and should be submitted in a separate file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See http://www.elsevier.com/highlights for examples.

Keywords
Immediately after the abstract, provide a minimum of 3, maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, "and", "of"). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations
Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements
Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Database linking
Elsevier aims at connecting online articles with external databases which are useful in their respective research communities. If your article contains relevant unique identifiers or accession numbers (bioinformatics) linking to information on entities (genes, proteins, diseases, etc.) or structures deposited in public databases, then please indicate those entities according to the standard explained below.
Authors should explicitly mention the *database abbreviation (as mentioned below) together with the actual database number*, bearing in mind that an error in a letter or number can result in a dead link in the online version of the article.
Please use the following format: **Database ID: xxxx**
Links can be provided in your online article to the following databases (examples of citations are given in parentheses):
- ASTM: ASTM Standards Database (ASTM ID: G63)
- CCDC: Cambridge Crystallographic Data Centre (CCDC ID: AI631510)
- GenBank: Genetic sequence database at the National Center for Biotechnology Information (NCBI) (GenBank ID: BA123456)
- GEO: Gene Expression Omnibus (GEO ID: GSE27196; GEO ID: GPL5366; GEO ID: GSM9853)
- MI: EMBL-EBI OLS Molecular Interaction Ontology (MI ID: 0218)
- MINT: Molecular INTeractions database (MINT ID: 6166710)
- NCBI Taxonomy: NCBI Taxonomy Browser (NCBI Taxonomy ID: 48184)
- NCT: ClinicalTrials.gov (NCT ID: NCT00222573)

• OMIM: Online Mendelian Inheritance in Man (OMIM ID: 601240)
• PDB: Worldwide Protein Data Bank (PDB ID: 1TUP)
• TAIR: The Arabidopsis Information Resource database (TAIR ID: AT1G01020)
• UniProt: Universal Protein Resource Knowledgebase (UniProt ID: Q9H0H5)

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Table footnotes

Indicate each footnote in a table with a superscript lowercase letter.

Artwork

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.
• Save text in illustrations as 'graphics' or enclose the font.
• Only use the following fonts in your illustrations: Arial, Courier, Times, Symbol.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Produce images near to the desired size of the printed version.
• Submit each figure as a separate file.

A detailed guide on electronic artwork is available on our website:
http://www.elsevier.com/artworkinstructions
You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

Regardless of the application used, when your electronic artwork is finalised, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS: Vector drawings. Embed the font or save the text as 'graphics'.
TIFF: Color or grayscale photographs (halftones): always use a minimum of 300 dpi.
TIFF: Bitmapped line drawings: use a minimum of 1000 dpi.
TIFF: Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.
If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is'.

Please do not:

• Supply files that are optimised for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF, EPS or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article.** Please indicate your preference for color: in print or on the Web only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.
Please note: Because of technical complications which can arise by converting color figures to 'gray scale' (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Text graphics
Text graphics may be embedded in the text at the appropriate position. Further, high-resolution graphics files must be provided separately whether or not the graphics are embedded. See further under Electronic artwork.

Tables

Tables should be numbered with Arabic numbers and bear a short descriptive title. Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References

Citation in text
Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references
As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue
Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software
This journal has standard templates available in key reference management packages EndNote (http://www.endnote.com/support/enstyles.asp) and Reference Manager (http://refman.com/support/rmstyles.asp). Using plug-ins to wordprocessing packages, authors only need to select the appropriate journal template when preparing their article and the list of references and citations to these will be formatted according to the journal style which is described below.

Reference style
Text: All citations in the text should refer to:
1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year of publication;
2. *Two authors:* both authors' names and the year of publication;
3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication.
Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.
Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....'
List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples:
Reference to a journal publication:
Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.
Reference to a book:
Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.
Reference to a chapter in an edited book:
Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

Video data

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 50 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages at http://www.elsevier.com/artworkinstructions. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

Supplementary data

Elsevier accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, high-resolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. In order to ensure that your submitted material is directly usable, please provide the data in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. For more detailed instructions please visit our artwork instruction pages at http://www.elsevier.com/artworkinstructions.

Referências

Brann, D.W., Dhandapani, K., Wakade, C. Neurotrophic and neuroprotective actions of estrogen: basic mechanisms and clinical implications. *Steroids*. 2007. 72: 381-405.

Brotons, J.A., Olea-Serrano, M.F., Villalobos, M., Pedraza, V., Olea, N. Xenoestrogens released from lacquer coatings in food cans. *Environ Health Perspect*.1995. 103:608-12.

Brunette, M.G., Leclerc, M. Renal action of progesterone: effect on calcium reabsorption. *Mol Cell Endocrinol.* 2002. 30(194):183-90.

Champagne, F., Diorio, J.S., Sharma, M.J. Naturally occur- ring variations in maternal behavior in the rat are associated with differences in estrogen-inducible central oxytocin receptors. *Proc. Natl. Acad. Sci. U.S.A*. 2001. 98: 12736–12741.

Chen, J., Cammarata, P.R., Baines, C.P., Yager, J.D. Regulation of mitochondrial respiratory chain biogenesis by estrogens/estrogen receptors and physiological, pathological and pharmacological implications. *Bioch Bioph Acta*. 2009.1793:1540-1570.

Chitra, K,C., Latchoumycandane, C., Mathur, P.P. Induction of oxidative stress by bisphenol A in the epididymal sperm of rats. *Toxicol.* 2003.185:119-27.

Couse, J.F., Lindzey, J., Grandien, K. Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. *Endocrinology* 1997. 138: 4613-21.

Darbre, P. D. Preface. *Best. Pract. Res. Clin. Endonol. Metab*. v.20, n.1, p.vii-x, 2006b.

Deecher, D.C., Swiggard, P., Frail, D.E. Characterization of a membrane-associated estrogen receptor in a rat hypothalamic cell line (D12). *Endocrine.* 2003. 22: 211-23.

Doerge, D.R., Vanlandingham, M., Twaddle, N.C., Delclos, K.B. Lactational transfer of bisphenolA in Sprague – Dawley rats. *Toxicol Lett.* 2010.199:372-6.

Donahue, D.A., Dougherty, E.J., Meserve, L.A. Influence of a combination of two tetrachloro biphenyl congeners (PCB47;PCB77) on thyroid status, cholineacetyltransferase (ChAT) activity, and short and long-term memory in 30 day old Sprague Dawley rats. *Toxicol*. 2004. 203:99–107.

Feldman, D., Tokes, L., Stathis, P.A. Identification of 17b-estradiol as the estrogenic substance in Saccharomyces cerevisiae. *Proc Natl Acad Sci USA*. 1984. 81:4722-6.

Feldman, D. Estrogens from plastic - are we being exposed? *Endocrinol*. 1997.138:1777-9.

Figtree, G.A., Noonan, J.E., Bhindi, R. Estrogen receptor polymorphisms: significance to human physiology, disease and therapy. *Recent Pat DNA Gene Seq.* 2009. 3: 164-71.

Garcia-Segura, L.M., Sanz, A., Mendez, P. Cross-talk between IGF-I and estradiol in the brain: focus on neuroprotection. *Neuroendocrinol.* 2006. 84: 275-9.

Giguere, V. Identification of a new class of steroid hormone receptors. *Nature.* 1988.331:919–948.

Gottlicher, M., Heck, S., Herrlich, P. Transcriptional cross-talk, the second mode of steroid hormone receptor action. *J Mol Med.* 1998. 76: 480-9

Gould, E., Woolley, C.S., Frankfurt, M., McEwen, B.S. Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood. *J.Neurosci.* 1990.101:286–1291,

Halliwell, B. and Gutteridge, J.M. *Free radicals in biology and medicine*. New York: Oxford University Press ,2007.

Haraguchi, S., Katsunori, S., Hanako, S., Shin-ichiro, H., Kazuyoshi, T. Estradiol Promotes Purkinje Dendritic Growth, Spinogenesis, and Synaptogenesis During Neonatal Life by Inducing the Expression of BDNF. *Cerebellum.* 2012. 11:416-417.

Heard, D.J. Human ERRγ, a third member of the estrogen receptor-related receptor (ERR) subfamily of orphan nuclear receptors: tissue-specific isoforms are expressed during development and in the adult. *Mol. Endocrinol.* 2000.14: 382–392.

Jonathan, N. B., Steinmetz, R. Xenoestrogens: the emerging story of bisphenol A. *Trends Endocrinol Metab.*1998. 9:124-8.

Joseph, A., Hess, R.A., Schaeffer, D.J. Absence of Estrogen Receptor Alpha Leads to Physiological Alterations in the Mouse Epididymis and Consequent Defects in Sperm Function. *Biol of Reprod.* 2010. 957:948-957.

Kato, H., Furuhashi, T., Tanaka, M., Katsu, Y., Watanabe, H., Ohta, Y., Iguchi, T. Effects of bisphenol A given neonatally on reproductive functions of male rats. *Reprod Toxicol.* 2006. 22:20-9.

Kazuyoshi, T., Kazuyoshi, U., MarikoUsui, H. Novel brain function: biosynthesis and actions of neurosteroids in Neurons. Neurosc Res. 2000. 36:261–273.

Kim, J.C., Shin, H.C., Cha, S.W., Koh, W.S., Chung, M.K., Han, S.S. Evaluation of developmental toxicity in rats exposed to the environ- mental estrogen bisphenol A during pregnancy. *Life Sci.* 2001. 69:2611–2625.

Krishnan, A.V., Stathis, P., Permuth, S.F., Tokes, L., Feldman, D. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinol*.1993.132:2279-86.

Kurebayashi, H., Betsui, H., Ohno,Y. Disposition of a lowdose of 14C-bisphenol A in male rats and its main biliary excretion as BPA glucuronide. *Toxicol. Sci*. 2003. 73: 17–25.

Kuiper, G.G. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alfa and beta. *Endocrinol.* 1997. 138: 863–870.

Li, M.W., Mruk, D.D., Lee, W.M., Cheng, Y. Disruption of the blood-testis barrier integrity by bisphenol A in vitro: Is this a suitable model for studying blood-testis barrier dynamics? *Int J Biochem Cell Biol.* 2009. 41:2302-14.

Lindberg, M.K., Weihua, Z., Andersson, N.S., Movérare, H. Estrogen receptor speci city for the effects of estrogen in ovariectomized mice. *J Endocrinol*. 2002. 167-178.

McLachlan, J. A., Simpson, E., Martin, M. Endocrine disrupters and female reproductive health. Best. Pract. Res. *Clin. Endocrinol. Metab.* 2006. 20(1): 63-75,

Matsushima, A., Kakuta, Y., Teramoto, T., Koshiba, T., Liu, X., Okada, H., Tokunaga, T., Kawabata, S., Kimura, M., Shimohigashi, Y. "Structural evidence for endocrine disruptor bisphenol A binding to human nuclear receptor ERR gamma". J. *Biochem.* 2007. 142(4): 517–24

Matthews, J.B., Twomey, K., Zacharewski, T.R. In vitro and in vivo interactions of bisphenol A and its metabolite, bisphenol A glucuronide, with estrogen receptors alpha and beta. *Chem. Res. Toxicol.* 2001. 14:149–157.

Meeker, J.D., Ehrlich, S., Toth, T.L., Wright, D.L., Calafat, A.M., Trisini, A.T., Ye, X., Hauser, R. Semen quality and sperm DNA damage in relation to urinary bisphenol A among men from an infertility clinic. *Reprod Toxicol*. 2010. 30: 532-9.

Miyatake, M., Miyagawa, K., Mizuo, K., Narita, M., Suzuki, T. Dynamic changes in dopaminergic neurotransmission induced by alow concentration of bisphenol-A in neurons and astrocytes. *J.Neuroendocrinol.* 2006. 18: 434–444.

Miyagawa, K., Narita, M., Narita, M., Akama, H., Suzuk, T. Memory impairment associated with a dysfunction of the hippocampal cholinergic system induced by prenatal and neonatal exposures to bisphenol-A. *Neurosc Lett.* 2007. 418:236–241.

Nadal, A., Alonso-Magdalena, P., Soriano, S., Quesada, I., Ropero, A. The pancreatic β-cell as a target of estrogens and xenoestrogens: implications for blood glucose homeostasis and diabetes. *Molec Cell Endocrinol.* 2009. 304: 63-68

Nadal, A. The estrogen trinity: membrane, cytosolic, and nuclear effects. *News Physiol Sci.* 2001.16: 251–255.

Nakamura, D., Yanagiba, Y., Duan, Z., Ito, Y., Okamura, A., Asaeda, N., Tagawa, Y., Li, C., Taya, K., Zhang, S.Y., Naito, H., Ramdhan, D.H., Kamijima, M., Nakajima, T. Bisphenol A may cause testosterone reduction by adversely affecting both testis and pituitary systems similar to estradiol. *Toxicol Lett.* 2010. 194:16-25.

Nakagawa, Y. and Tayama, S. Metabolism and cytotoxicity of bisphenol A and other bisphenols in isolated rat hepatocytes. *Arch Toxicol.* 2000. 74: 99-105.

Norman, A.W. Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model. Nature Rev. 2004. 3: 27–40.

Obata,T., Kubota,S. Formation of hydroxyl radicals by environmental estrogen like chemicals in rat striatum. *Neurosci.Lett*. 2000. 296, 42–44.

Ogiue-Ikedaa, M., Tanabe, N., Mukai, H., Hojoa, Y., Murakami, G. Rapid modulation of synaptic plasticity by estrogens as well as endocrine disrupters in hippocampal neurons. *Brain Res.* 2008. 57: 363 – 375.

Prange-Kiel, J., Rune, G.M. Direct and indirect effects of estrogen on rat hippocampus. *Neurosc.* 2006. 138:765–772.

Price, R.H., Handa, R.J. Expression of estrogen receptor-β protein and mRNA in the cerebellum of the rat. *Neurosci Lett.* 2000. 288:115–8.

Judd, H.L, Fournet, N. Changes of ovarian hormonal function with aging. *Exp Gerontol.* 1994. 29: 285-98.

Pryor, J.L., Hughes, C., Foster, W., Hales, B.F., Robaire, B. Critical windows of exposure for children's health: the reproductive system in animals and humans. *Environ Health Perspect.* 2000. 108:491–503.

Qiu, J., Bosch, M.A., Tobias, S.C. Rapid signaling of estrogen in hypothalamic neurons involves a novel G-protein-coupled estrogen receptor that activates protein kinase C. J *Neurosci*. 2003. 23: 9529- 40.

Quignot, N., Arnaud, M., Robidel, F., Lecomte, A., Barouki, R., Lemazurier, E. Characterization of endocrine-disrupting chemicals based on hormonal balance disruption in male and female adult rats. *Reprod Toxicol*. 2012. 33:339- 352.

Rogers, J.A., Metz, L. Review: Endocrine disrupting chemicals and immune responses: A focus on bisphenol-A and its potential mechanisms. *Molec Immun.* 2013. 53: 421– 430

Roselli, C. e Resko J. Sex Differences in Androgen-regulated Expression of Cytochrome P450 Aromatase in the Rat Brain. *Steroid Bioch Mol Biol.* 1997. 61(3-6): 365-374.

Saradha, B., Mathur, P.P. Effect of environmental contaminants on male reproduction. *Environ Toxicol Pharmacol.* 2006. 21:34-41.

Scharfman, H.E., MacLusky, N.J. Estrogen and brain-derived neurotrophic factor (BDNF) in hippocampus: complexity of steroid hormone-growth factor interactions in the adult CNS. *Front Neuroendocrinol.* 2006. 27: 415-35.

SCHARFMAN, H.E. *Neuroscience*, 2012. Em impressão.

Schreihofer, D.A., Resnick, E.M., Lin, V.Y. Ligand-independent activation of pituitary ER: on PKA-stimulated dependence pathways. *Endocrinol.* 2001. 142: 3361-8.

Shao, B., Cheng, Y., Jin, K. Estrogen, Neuroprotection and Neurogenesis after Ischemic Stroke. *DNA Seq*. 2012:188-198.

Shearman, A.M. Association between estrogen receptor alpha gene variation and cardiovascular disease. *JAMA.* 2003. 290: 2263–70.

Silberger, S.D., Magleby, K.L. Beting the odds with big K. *Science.* 1999. 285:1859–1860.

Suna, H.B., Shenb, O.X., Wang, X.R., Zhoua, L., Zhena, X.C. Anti-thyroid hormone activity of bisphenol A, tetrabromobisphenol A and tetrachlorobisphenol A in an improved reporter gene assay. *Toxicol in Vitro.* 2009. 23(5):950-4.

Tabb, M.M., Blumberg, B. New modes of action for endocrine-disrupting chemicals. *Mol. Endocrinol.* 2006. 20(3):475-482.

Taylor, S. E. Oestrogen receptor splice variants in the pathogenesis of disease. *Cancer Lett.* 2009.15.

Yang, Y.J., Lee, S.Y., Kim, K.Y., Hong, Y.P. Acute Testis Toxicity of Bisphenol A Diglycidyl Ether in Sprague-Dawley Rats. J Prev Med Public Health 2010. 43:131-7.

Waring, R.H., Harris, R.M. Endocrine disrupters: a human risk? *Mol. Cell. Endocrinol.* 2005. 244(1-2):2-9.

Whitehaed, S. A., Rice, S. Endocrine-disrupting chemicals as modulators of sex steroid synthesis. Best. Pract. Res. *Clin. Endocrinol. Metab.* 2006. 20(1):45-61.

Wilson, T.A., Garner, S.C., Anderson, J.J.B. Dietary protein source and serum estradiol concentrations of rats during pregnancy, lactation and post-lactation, *Nutr. Res.* 2000. 20:1735–1747.

Völkel, W., Colnot, T., Csanady, G.A., Filser, J.G., Dekant,W. Metabolism and kinetics of bisphenol A in humans at low doses following oral administration. *Chem. Res.Toxicol.* 2002.15:1281–1287.