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Efeito do Bisfenol-A na esteroidogênese e defesa antioxidante em *Danio rerio*: análises moleculares, bioquímicas e histológicas

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Sumário

RESUMO

O Bisfenol-A (BPA) é um químico utilizado na fabricação de policarbonatos e resinas epóxi e é considerado um desregulador endócrino para diversas classes de vertebrados. Entre as alterações causadas pelo BPA está a queda de fertilidade e espermatozóides defeituosos em mamíferos. Além dos efeitos como desregulador endócrino, o BPA tem sido apontado em estudos recentes como um indutor de estresse oxidativo. O estudo da ação de baixas doses deste composto é mais bem estabelecido em mamíferos do que em peixes, e portanto, a presente tese teve como objetivo a investigação dessa ação no peixe *Danio rerio*. Os animais foram expostos a 2, 10 e 100 μg/L de BPA por 24 horas, 96 horas e 14 dias e a partir daí, foram realizadas análises moleculares, bioquímicas e histológicas. As análises moleculares e bioquímicas demonstraram alterações na expressão e atividade nas enzimas antioxidantes Superóxido dismutase-1 (SOD-1), Catalase (CAT) e Glutationa-S-transferase (GST) em animais expostos ao BPA por 24 horas. O gene *park7*, que codifica para a proteína reguladora antioxidante DJ-1 apresentou uma indução de até 15 vezes em relação ao grupo controle. As alterações em genes relacionados à reprodução surgiram mais tardiamente, nos animais expostos por 96 horas. As análises histológicas demonstraram principalmente danos nas células de Sertoli, que são responsáveis pelo suporte e nutrição das células germinativas. De acordo com os resultados, o presente estudo pôde concluir que além de afetar os aspectos relacionados à reprodução, o BPA é capaz de alterar importantes enzimas do sistema antioxidante, provavelmente tornando as células mais vulneráveis ao estresse oxidativo.

Palavras-chave: Bisfenol-A, Danio rerio, Defesa antioxidante, Via esteroidogênica, Reprodução

ABSTRACT

Bisphenol-A (BPA) is a chemical used for production of polycarbonates and epoxy resins and is considered an endocrine disrupter for various classes of vertebrates. Among the changes caused by BPA is fertility decline and defective sperm in mammals. In addition to the effects as an endocrine disrupter, BPA has been demonstrated in recent studies as an inducer of oxidative stress. The study of the action of low doses of this compound is best established in mammals than in fish, and therefore this thesis aimed to research this action in *Danio rerio* fish. The animals were exposed to 2, 10 and 100 µg/L of BPA for 24 hours, 96 hours and 14 days and thereafter were held molecular, biochemical and histological analysis. The molecular and biochemical analyzes showed changes in expression and activity in antioxidant enzyme superoxide dismutase-1 (SOD-1), Catalase (CAT), and glutathione-S-transferase (GST) in animals exposed for 24 hours to BPA. The *park7* gene encoding the regulatory protein DJ-1 antioxidant showed an induction up to 15 times compared to the control group. Changes in genes related to reproduction emerged later, in animals exposed for 96 hours. Histological analysis showed mainly damage Sertoli cells, which are responsible for support and nutrition of germ cells. According to the results, this study could conclude that in addition to affecting the aspects related to reproduction, BPA can alter important enzymes of the antioxidant system, probably making the most vulnerable cells to oxidative stress.

Keywords: Bisphenol-A, *Danio rerio*, antioxidant defense, steroidogenic pathway, reproduction

INTRODUÇÃO GERAL

Durante séculos, a sociedade tem observado que os fatores ambientais podem influenciar a saúde tanto de um indivíduo quanto da população como um todo (Vandenberg et al., 2015). Dentre esses fatores estão os contaminantes ambientais, e desde o final da Segunda Guerra Mundial, momento em que ocorreu a revolução química, diversos desses compostos provenientes de indústrias, atividade agrícola, etc. têm sua liberação intensificada no ambiente, afetando diversos hábitats e populações (Carson, 1962). Entretanto, apenas no início dos anos 60 a humanidade tornou-se consciente dos potenciais efeitos em longo prazo destes produtos e seus riscos para os ecossistemas aquáticos e terrestres.

Os primeiros relatos sobre o efeito de químicos no ambiente e nos seres vivos foram através do livro *Silent Spring*, de Rachel Carson, analisando principalmente os impactos do pesticida diclorodifeniltricloroetano (DDT) sobre a vida de animais silvestres e humanos (Carson, 1962). A partir daí, iniciaram-se diversos estudos relacionando contaminantes ambientais a alterações na vida selvagem e ao aparecimento de doenças (Giger et al., 1984; Billa e Dezotti, 2007; Vandenberg et al., 2015).

Em seu livro publicado em 1962, a ecologista Rachel Carson questionou a segurança de um grande número de substâncias químicas, e centrou-se principalmente nos efeitos dos pesticidas sobre as espécies não alvo. Apesar de diversas evidências serem recolhidas por Carson, a questão chave, se a exposição a químicos pode influenciar o aparecimento de doenças, continua sendo debatida (Lamb et al., 2014; Zoeller et al., 2014). Uma importante evidência de que substâncias químicas poderiam perturbar a vida selvagem foi observadaem 1960,quando diversas alterações em ninhos de gaivotas, nos Grandes Lagos da América do Norte foram registradas. Essas

alterações se apresentaram principalmente como anormalidades comportamentais e cascas dos ovos frágeis, as quais foram mais tarde ligadas à presença de altos níveis do pesticida DDT naquela área (Birkett e Lester, 2003; Lintelman et al., 2003). Outro fato importante ocorreu também na década de 60 quando gestantes utilizaram o medicamento dietilbestrol (DES) com suposta ação antiabortiva e, aproximadamente vinte anos depois foram observados tipos raros de cânceres nas filhas e filhos das mulheres usuárias do DES. Estas patologias tardias foram relacionadas com a passagem desse composto de mãe para o feto (Thomas, 1996). Esse é um efeito bem documentado em humanos onde a exposição da mãe a um contaminante, causa efeito na sua descendência (Bila e Dezotti, 2007). Esses primeiros achados foram fundamentais para que se iniciasse uma série de pesquisas que culminaram na constatação de que existiam vários desses compostos no ambiente afetando as populações de diferentes formas (Crain et al., 2007; Vandenberg et al., 2009). O destino final da maioria destes contaminantes é o ambiente aquático, devido a descargas diretas ou a processos hidrológicos e atmosféricos, portanto organismos viventes em rios, lagos e oceanos são constantemente alvo de diversos estudos relacionados a esses químicos, também chamados de xenobióticos (Hahn e Stegeman, 1994). Entre as substâncias naturais e antropogênicas que podem perturbar a fisiologia dos organismos, podem ser destacadas as substâncias que afetam o sistema endócrino, porque essa perturbação pode conduzir a efeitos negativos sobre o crescimento, o desenvolvimento, o comportamento e a reprodução (Daughton e Ternes, 1999). Os químicos que interferem na função endócrina, afetando a reprodução e outros aspectos da fisiologia hormonal, foram denominados “Desreguladores Endócrinos” (Wingspread Conference Center, Racine, Wisconsin, 1991).

1.Desreguladores Endócrinos

São definidos como desreguladores endócrinos "agentes exógenos que interferem com a síntese, secreção, transporte, ligação, ação ou eliminação dos hormônios naturais, os quais são responsáveis pela manutenção da homeostase, reprodução, desenvolvimento e/ou comportamento". O termo foi proposto em 1991, em uma conferência, pela pesquisadora Theo Colborn (Wingspread Conference Center, Racine, Wisconsin, 1991). O sistema endócrino regula diversos aspectos do desenvolvimento, do metabolismo e processos reprodutivos, incluindo o desenvolvimento embrionário, formação gonadal, diferenciação sexual, crescimento e digestão. Assim, compostos com capacidade de desregulação endócrina podem afetar estes processos por qualquer ligação ou bloqueio de receptores hormonais, desencadeando ou prevenindo as respostas hormonais (Markey et al., 2001; Witorsch, 2002; Hotchkiss et al., 2008). Além da definição original dada por Theo Colborn, existem diversas outras, entre elas a mais recente e mais simplificada utilizada pela Sociedade de Endocrinologia, que caracteriza um desregulador endócrino como "um químico exógeno, ou uma mistura de produtos químicos, que interferem com qualquer aspecto da ação de um hormônio" (Zoeller et al., 2012).

Os compostos denominados desreguladores endócrinos podem afetar diferentes glândulas endócrinas e, portanto diferentes grupos hormonais como, por exemplo, hormônios da tireóide, hormônios relacionados ao estresse, hormônio do crescimento e hormônios reprodutivos. Esses últimos têm recebido nas últimas décadas atenção especial já que muitos contaminantes têm se mostrado capazes de modificar as quantidades dos hormônios sexuais e consequentemente provocarem fenótipos alterados, como mudanças na diferenciação sexual, infertilidade e feminização (Vandenberg et al.,2009).

Entre os desreguladores endócrinos relacionados à reprodução, muitas pesquisas nas décadas passadas identificaram uma crescente lista de contaminantes ambientais que perturbamesses processos em muitos organismos, principalmente através interação direta com receptores de hormônios esteróides (EPA, 2007). Um bem documentado exemplo de desbalanço endócrino causado por um contaminante na vida selvagem ainda é a masculinização em fêmeas de gastrópodes (imposex) causada pelo tributilestanho (TBT) (Santos et al., 2005). O efeito de masculinização do TBT também é conhecido em peixes (Mcallister e Kime, 2003; Shimasaki et al., 2003; Santos et al., 2006).

Alguns desreguladores endócrinos influenciam o metabolismo de hormônios esteróides atuando como ativadores ou repressores de enzimas chave, podendo alterar diretamente a expressão do gene que codifica para essas enzimas (Hampl et al., 2016). Entre os alvos de diversos desreguladores endócrinos, tem se dado particular atenção a enzima aromatase, não apenas em humanos e roedores, mas também em peixes, onde a alteração da aromatase tem afetado a reprodução (Bonefeld-Jorgensen et al., 2007, Chenschenko et al., 2008).

A interferência dos poluentes na regulação endócrina tem gerado crescente interesse público, uma vez que os efeitos têm sido observados tanto em humanos quanto em outros animais. Esses efeitos, de uma forma geral, incluem queda de fertilidade (Petersen et al., 1998), câncer de mama (Wolff et al., 2000) puberdade precoce (Honma et al., 2001) ou puberdade tardia (Faqi et al., 1998), e anormalidades gonadais (Roos et al., 2001; Cook et al., 2003). Entre os contaminantes que atuam sobre a via esteroidogênica e através de receptores de esteróides estão diversos ftalatos, pesticidas, e o Bisfenol-A, que é foco do presente estudo.

1.1.Bisfenol A (BPA)

O BPA é uma substância química de origem industrial, utilizado em uma vasta gama de produtos de consumo, incluindo as guarnições de alimento, papéis térmicos, latasde bebida, plásticos de policarbonato, brinquedos, equipamentos médicos, entre outros (Vandenberg et al., 2007; Geens et al., 2012a, 2012b). O BPA possui uma ampla produção mundial, onde na Europa, por exemplo, quatro empresas fabricam um montante total de 700.000 toneladas/ ano de BPA em seis locais de produção (European Commission,2003). A molécula de BPA (4, 4' –dihidroxi-2, 2-difenilpropano) é constituída de dois anéis fenólicos ligados por uma ponte com duas ligações metila (Fig.1) e esse composto é liberado no ambiente em altas quantidades, sendo o ambiente aquático afetado pela presença do mesmo (Sodré et al., 2010; Huang et al., 2012). O BPA em condições ambientais se encontra em estado sólido, porém se dissolve relativamente bem em água, sendo sua solubilidade de 120–300 mg/L. A contaminação de águas superficiais por BPA pode ocorrer através de uma variedade de rotas, incluindo as operações industriais, lixiviação e a eliminação e tratamento de dejetos humanos (Petrovic et al., 2004). Já a contaminação das águas subterrâneas é menos direta, com fontes conhecidas incluindo aterros, fossas sépticas, misturas com águas superficiais, etc. (Lapworth et al., 2012). Em ambientes aeróbios, como a maioria dos rios, o BPA tem uma meia vida ambiental de 4,5 a 4,7 dias (Cousinset al.,2002), sendo essa degradação realizada principalmente por bactérias (Kang e Ha, 2002).

Embora o BPA seja degradado quase que prontamente por microorganismos, a sua presença no ambiente como fragmento de policarbonato cria uma fonte não pontual de lixiviação lenta, sendo assim, esse contaminante é considerado a maioria dos detritos antropogênicos encontrados em bacias hidrográficas e ambientes marinhos do mundo (Crain et al., 2007).

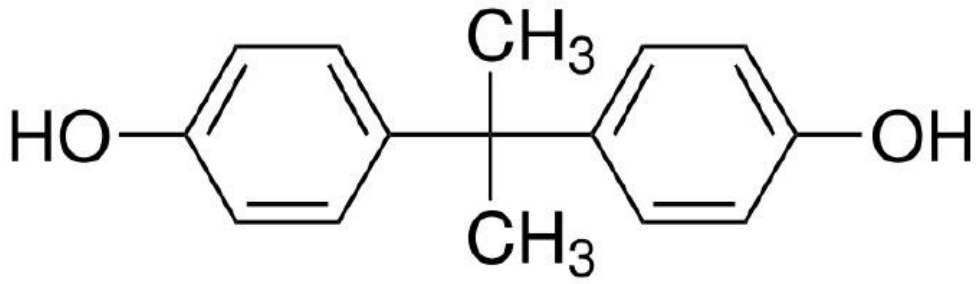

Fig. 1 Estrutura química da molécula de Bisfenol-A (BPA).

No Brasil, investigações recentes demonstraram que o BPA foi o xenoestrógeno mais detectado em águas de rios, águas de esgotos e até em água da rede pública, onde foram registradas concentrações que oscilaram entre 25–84 ng/L (Sodré et al., 2010). Em geral, as concentrações de BPA estão abaixo de 1 µg/L em ambientes aquáticos menos contaminados (Huang et al., 2012; Yamazi et al., 2015). Entretanto, alguns estudos também demonstram o BPA em concentrações mais altas, acima de 21 µg/L em rios e lagos mais poluídos (Crain et al., 2007) e em alguns lugares próximos a zonas industriais com liberação de BPA as concentrações podem chegar a centenas de microgramas por litro (Careghini et al., 2014; Fukazawa et al., 2001). As concentrações de BPA observadas nos oceanos e estuários são relativamente baixas em comparação com alguns sistemas de água doce. No entanto, a lixiviação do BPA pode ser uma preocupação em locais marinhos onde resíduos de plástico se acumulam, já que o BPA lixivia mais rapidamente em água marinha do que em sistemas de água doce (Sajiki e Yonekuni, 2003; Crain et al., 2007) e a degradação microbiana pode ocorrer mais lentamente (Kang e Kondo, 2005).

A utilização de BPA em embalagens em contato com alimentos está permitida na União Europeia mediante a diretiva 2002/72/CE, no qual se estabelece um limite máximo de ingestão diária tolerável de BPA para o homem de 0,05 mg/kg/dia

*(*European Food Safety Authority, 2008) e um limite de migração específico para os alimentos de 0,6 mg/g. No Brasil, segundo a ANVISA, esse limite de migração específico é 3 mg/kg. Em relação à presença de BPA nos rios, a legislação brasileira não contempla limite para esse composto nas águas, o que dificulta ainda mais o controle da quantidade dessas substâncias e de suas ações em organismos aquáticos.

A molécula de BPA é considerada componente estrogênico já que seus dois anéis de benzeno e dois substituintes OH, auxiliam o ajuste no local de ligação do receptor de estrógeno (ER) (Vandenberg et al., 2009). Assim, o BPA atua ligando-se a receptores de estrógeno, apesar de também possuir diversas outras atividades como desregulador químico mediadas via múltiplos caminhos moleculares (Le et al., 2008). Assim como muitos contaminantes considerados desreguladores endócrinos, o BPA é uma substância que pode atuar tanto a nível organizacional para alterar permanentemente a estrutura de órgãos (ex. gônadas) quanto a nível ativacional, causando uma mudança nos sistemas organizados normalmente (ex. gônadas e fígado) (Crain et al., 1998).

Além de ser considerado um agonista de receptores de ERs, se ligando aos ERs nucleares com 0,1- 0,01% da afinidade do estradiol pelos mesmos receptores (Wetherill et al., 2007), o BPA também pode antagonizar ações de estrogênios, principalmente em órgãos como cérebro e útero (Leranth et al., 2008 a, 2008b). Ainda, esse composto tem a capacidade de se vincular a outros receptores esteróides e não esteróides tais como o receptor de andrógeno (AR) (Lee et al.,2003, Wetherill et al., 2002, Xu et al.,2005) e receptor de hormônios tiroideanos (TR) (Zoeller et al., 2005).

Por fim, o BPA é capaz de afetar a expressão de importantes enzimas do metabolismo de esteróides, como por exemplo a aromatase (Castro et al., 2013). A função da enzima aromatase é a conversão de andrógenos em estrógenos e uma alta

atividade dessa enzima, portanto pode acarretar processos de feminilização ou infertilidade (Vandenberg et al., 2015; Crain et al., 2007).

Em relação aos efeitos do BPA nos primeiros estágios do desenvolvimento, exposição a concentrações de 500 a 2.000 µg/L de BPA em um curto espaço de tempo provocam diversos efeitos em peixes como curvatura da coluna vertebral, edema pericárdico, atraso no desenvolvimento e defeitos em otólitos (Alexander et al.,1998; Duan et al., 2012; Fei et al., 2010). Já nas menores concentraçoes de BPA, eventos organizacionais podem ser interrompidos como é observado na alteração da determinaçao sexual em *Oryzias latipes* (Kang et al., 2002; 2007) e peixe-zebra (Draschikova et al., 2005).

Expressão gênica por microarranjo demonstram a mudança significativa que o BPA promove em milhares de genes nas gônadas de uma série de modelos vertebrados de laboratório (Bredhult et al., 2009; Hermeier et al., 2009; Duan et al., 2010). Além disso, modificações epigenéticas como metilação do DNA, estão extremamente relacionadas com variações ambientais e ultimamente associadas à presença de BPA (Dolinoy et al., 2010). Em células epiteliais mamárias a exposição ao BPA foi encontrada suprimindo a apoptose através da indução de hipermetilação na região promotora do gene *bcl2/11* (Fernandez et al., 2012).

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

Kurebayashi et al., 2003). Além do efeito do BPA como desregulador endócrino, diversos estudos têm demonstrado que esse composto pode causar estresse oxidativo, pois gera espécies reativas de oxigênio (ROS) no cérebro, fígado, rins, testículos e epidídimo, e reduz a função mitocondrial em mamíferos (Chitra et al., 2003; Nakagawa e Toyama, 2000).

2. Estrutura reprodutiva em peixes

A maioria dos peixes teleósteos é ovípara, mas a viviparidade está presente em alguns grupos, e sua fertilização pode ser externa ou interna nos casos dos animais vivíparos. Cada espécie possui um período de desova bem regulado tanto por fatores ambientais (sazonalidade, fotoperíodo, temperatura) quanto pela regulação hormonal (Norris, 2007).

Em relação aos machos, a organização do tecido testicular nos peixes é diferente da organização do tecido testicular nos mamíferos. No caso dos mamíferos, as células germinativas estão organizadas em túbulos, que vão desembocar no epidídimo. Nesses túbulos, as células mais indiferenciadas estão na região mais externa (*ex.*espermatogônias) enquanto os espermatozóides estão na luz dos túbulos. Já nos peixes teleósteos a organização é um pouco mais simplificada e esse tecido testicular encontra-se organizado em câmaras/bolsas/cistos testiculares onde estão localizados agrupamento de células constituintes de uma mesma fase da espermatogênese, associado a uma ou duas células de Sertoli (Norris, 2007; Shultz et al., 2010).

A espermatogênese é um processo específico de proliferação e diferenciação de células germinativas, que ocorre nos testículos e é controlada basicamente por atividade endócrina e mediadores das junções celulares das células de Sertoli (Fiorini et al., 2004). Durante a espermatogênese, organelas citoplasmáticas são organizadas em

regiões específicas. Nos espermatozóides maduros, uma alta quantidade de mitocôndrias constitui um sítio de atividade energética para os movimentos ciliares, e dessa forma, tóxicos podem alterar o metabolismo energético dessa região e diminuir a fertilidade espermática. O estresse oxidativo induzido por tóxicos, por exemplo, é uma das causas mais comuns de dano espermático em diversos grupos animais (Wisniewski et al., 2015).

O funcionamento normal da espermatogênese é dependente de um ambiente hormonal bem orquestrado. Assim, o desenvolvimento normal do sistema reprodutivo e a programação comportamental em adultos são dependentes da correta exposição (concentração e tempo) aos hormônios esteróides, especialmente estrogênio e testosterona (Robinson, 2006; Schulz et al., 2009).

2.1. A via esteroidogênica

Os hormônios esteróides exercem sua ação na célula-alvo através de receptores intracelulares ou de membrana. O mecanismo genômico clássico empregando receptores intracelulares (nucleares) consiste na regulação – estimulação ou repressão – de genes particulares. O processo individual inicia com a ligação do hormônio ao receptor, seguido pela translocação do complexo receptor-esteróide para o núcleo, a sua ligação a elementos de resposta ao estrógeno e início da transcrição. Entretanto, esse processo necessita de uma série de fatores nucleares, coativadores ou repressores (Shibata et al.,1997; Ratman et al., 2013). Estes hormônios, juntamente com outros tipos hormônios, genes, fatores de crescimento e neurotransmissores desempenham um papel importante no desenvolvimento e função do cérebro e do sistema nervoso em geral (Hampl et al., 2016).

Os hormônios esteróides são sintetizados a partir do colesterol, através de uma série de reações enzimáticas, onde participam principalmente enzimas da família citocromo P450 (Norris, 2007). As enzimas responsáveis pela síntese dos hormônios esteróides sexuais estão representadas na Fig. 2. A produção de esteróides ocorre em órgãos esteroidogênicos clássicos, tais como o córtex adrenal, testículo, ovário e placenta, no caso de mamíferos. Estes órgãos são capazes, portanto, de converter colesterol em pregnenolona pela enzima P450 a partir da clivagem da cadeia lateral do colesterol (P450scc), no interior da mitocôndria, e a partir da pregnenolona, produzir os outros esteróides ativos, utilizando a maquinaria enzimática específica da via esteroidogênica (Ueyama et al., 2002).

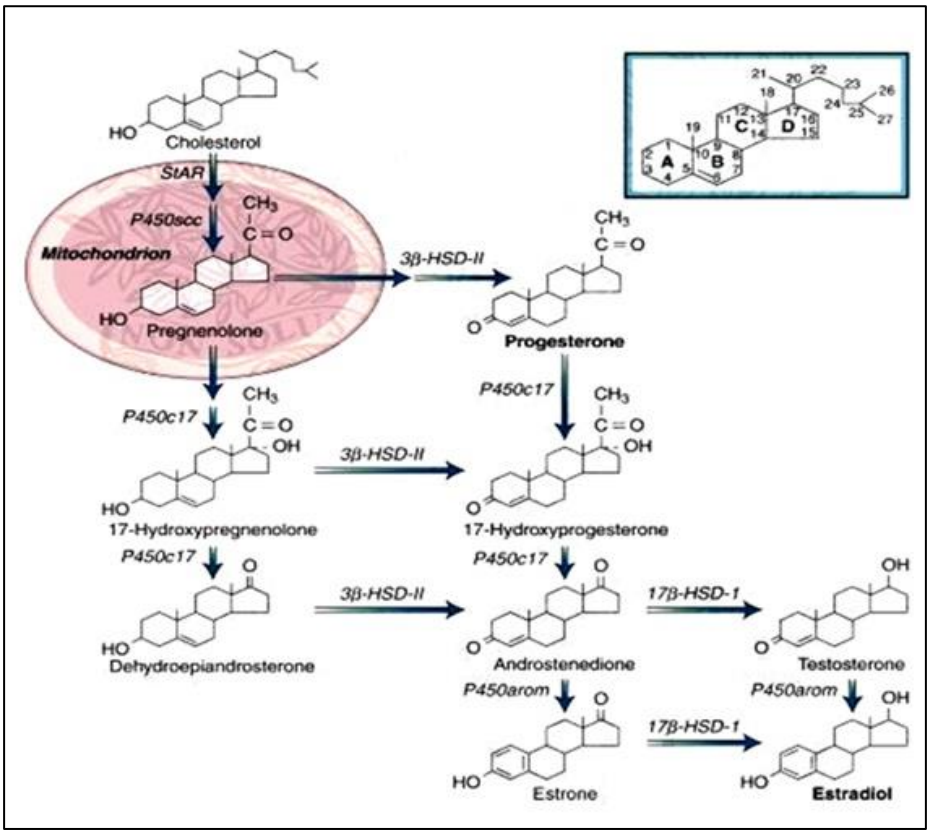

Fig. 2 Representação da via de produção de hormônios esteróides sexuais. StAR- proteína reguladora aguda da esteroidogênese; P450scc – desmolase; P450c17 – 17 alfa hidroxilase; p450aro – aromatase (Kronenberg et al., 2011)

2.2. Reprodução e BPA

Organismos expostos ao BPA e outros plastificantes têm sido associados a mudanças reprodutivas permanentes em nível organizacional como, por exemplo, alterações nas características de dimorfismo sexual (Evans et al., 2004), comportamento sexual (Pimenova et al., 2010) e também à alterações de nível ativational, ou seja temporárias e reversíveis, como por exemplo, a proliferação de peroxissomos (Wilkinson e Lamb, 1999) e expressão de proteínas testiculares (Sobarzo et al., 2009).

Quanto ao mecanismo de ação, efeitos deletérios do BPA no eixo de hormônios esteróides sexuais têm sido bem caracterizados, principalmente em mamíferos (Mathieu-Denoncourt et al., 2015). Estudos demonstraram que uma dose de 2 ng de BPA por grama de peso corporal aumentou a expressão de receptores de estrogênio ERα e ERβ em camundongos recém-nascidos (Kawai et al., 2007) enquanto apenas ERβ foi regulada positivamente pelo BPA em outros estudos com roedores (Akingbemi et al., 2004; Phrakonkham et al., 2008). O BPA também pode alterar a via esteroidogênica atuando na modificação da expressão de alguns genes importantes que codificam para proteínas dessa via (Saili et al., 2013).

Outros genes, proteínas e hormônios relacionados à esteroidogênese têm sido alterados após a exposição de BPA, incluindo progesterona, receptor de progesterona (PR) e vitelogenina (VGT), proteína precursora de gema de ovo essencial no crescimento do ovário e nutrição dos embriões (Mathieu-Denoncourt et al., 2015; Vandenberg et al., 2015). Em peixes, por exemplo, a exposição ao BPA aumentou a expressão de mRNA codificante para a VTG em peixes truta arco-íris e peixe-zebra (Aluruet al., 2010; Segner et al, 2003).

Em roedores e peixes, BPA reduziu significativamente o peso dos testículos e a qualidade dos espermatozóides (Sakaue et al., 2001; Sohoni et al., 2001; Lahnsteiner et al., 2005). A contagem de espermatozóides também foi reduzida em exposição do barrigudinho *Poecilia reticulata* a baixas doses de BPA (Haubruge et al., 2000). No peixe *Oryzias latipes*, exposição a 800 µg/L de BPA promoveu uma redução da fecundidade e fertilidade desses peixes, além de um desbalanço na razão sexual nos animais expostos em período embrionário (Kang et al., 2002). Por fim, o BPA quando administrado em altas doses também pode afetar a diferenciação sexual em peixes. Foi observado em experimentos com peixe-zebra, que a exposição a doses altas de BPA culmina em um desenvolvimento gonadal anormal e em alterações na razão sexual, com tendência à feminilização (Drastichova et al., 2005).

Sabe-se, porém, que níveis ambientalmente relevantes também podem contribuir para o aparecimento de condições intersexuais. Por exemplo, trabalhos com barbo imaturo (*Barbus sp.*) coletados a jusante de uma fonte de poluição por BPA apresentavam uma alta incidência de intersexualidade, enquanto barbos intersexuais não foram observados a montante dessa fonte de poluição (Vigano et al., 2006). Entretanto, dados de laboratório sugerem que o BPA sozinho não é capaz de induzir o efeito de intersexualidade em peixes, tendo possivelmente esse efeito a participação de demais contribuintes, caracterizando a mistura de substâncias presentes no ambiente (Kang et al., 2002).

3. Sistema de defesa antioxidante em peixes

As espécies reativas de oxigênio abrangem o ânion superóxido (O_2^-), peróxido de hidrogênio (H_2O_2), e radicais hidroxilas (HO^-), que estão envolvidas em diferentes vias de sinalização celular, incluindo apoptose, mas também estão implicados no

desenvolvimento de patologias, produzindo dano em vários componentes celulares como lipídios insaturados, proteínas e ácidos nucléicos (Sies, 1993).

A situação de dano oxidativo, pode se dar, entre outros fatores, pela presença de xenobióticos nos organismos. Águas residuais, por exemplo, contém uma grande variedade de poluentes orgânicos e metálicos, incluindo pesticidas, hidrocarbonetos aromáticos, dibenzofuranos, compostos estrogênicos e muitos metais, sendo a maioria dessas substâncias, agentes oxidantes (Avci et al., 2005).

A defesa dos organismos contra danos oxidativos pode ser não enzimática, através de substâncias antioxidantes, como algumas vitaminas, ácido úrico, glutationa e carotenóides. Além disso, existe em todas as classes de vertebrados e em invertebrados um sistema de defesa antioxidante enzimático, onde diversas enzimas antioxidantes, entre elas superóxido dismutase (SOD) e catalase (CAT), atuam impedindo a cascata de reações oxidantes, interceptando e inativando os compostos reativos de oxigênio intermediários. As enzimas antioxidantes são, portanto, cruciais no esforço de neutralizar a toxicidade de oxigênio quando o fornecimento de outros compostos antioxidantes são escassos ou esgotados (Ahmad, 1995).

3.1 A via DJ-1/NRF2

Na produção de ROS e dano celular induzido por BPA a proteína DJ-1 tem importante papel em mamíferos (Ooe et al., 2005). Essa proteína atua na regulação antioxidante, anti-apoptótica e citoprotetora, modulando a transcrição de genes-chave (Khale et al., 2009; Wilson, 2011). A DJ-1 regula a liberação do fator eritróide nuclear de transcrição 2 (Nrf2), que por sua vez induz a expressão de diversos genes antioxidantes, se ligando aos chamados elementos de resposta eletrofílicos (EprE) no DNA (Malhotra et al., 2008). Sob condições normais, o sistema EpRE- Nrf2 mantém a

proteína Nrf2 em baixos níveis no citoplasma. Isso é conseguido pela síntese e degradação constitutiva de Nrf2. A Proteína repressora Keap1, que está presente principalmente no citoplasma, regula fortemente esse processo (Zhang e Hannick, 2003).

Em células expostas à indutores de estresse oxidativo, foi observado um aumento da presença de uma forma mitocondrial oxidada da DJ-1, que perde assim sua capacidade reguladora antioxidante levando à diminuição da expressão de NRF2, e conseqüente diminuição na expressão de genes antioxidantes (Fig. 3) (Kinumi et al., 2004; Mitsumoto et al., 2001). O Nrf-2 é membro da família de fatores de transcrição *cap'n'collar,* com zíper de leucina, e responsável pela expressão constitutiva e induzível de genes controlados pelo elemento de resposta EprE (ou ARE) como por exemplo quinona oxidoredutase-1 (NQO1), heme oxigenase-1 (HO1), tioredoxinas (TRX), glutationa-S-transferase (GST), superóxidos dismutase (SODs), glutationa redutase (GR), glutationa peroxidase (GPX) e outras enzimas de fase I, II e III (Thimulappa et al., 2002). A DJ-1 é expressa em vários tecidos, e homólogos de DJ-1 são expressos desde microrganismos a mamíferos, inclusive no peixe zebra *Danio rerio* (Khale et al., 2009).

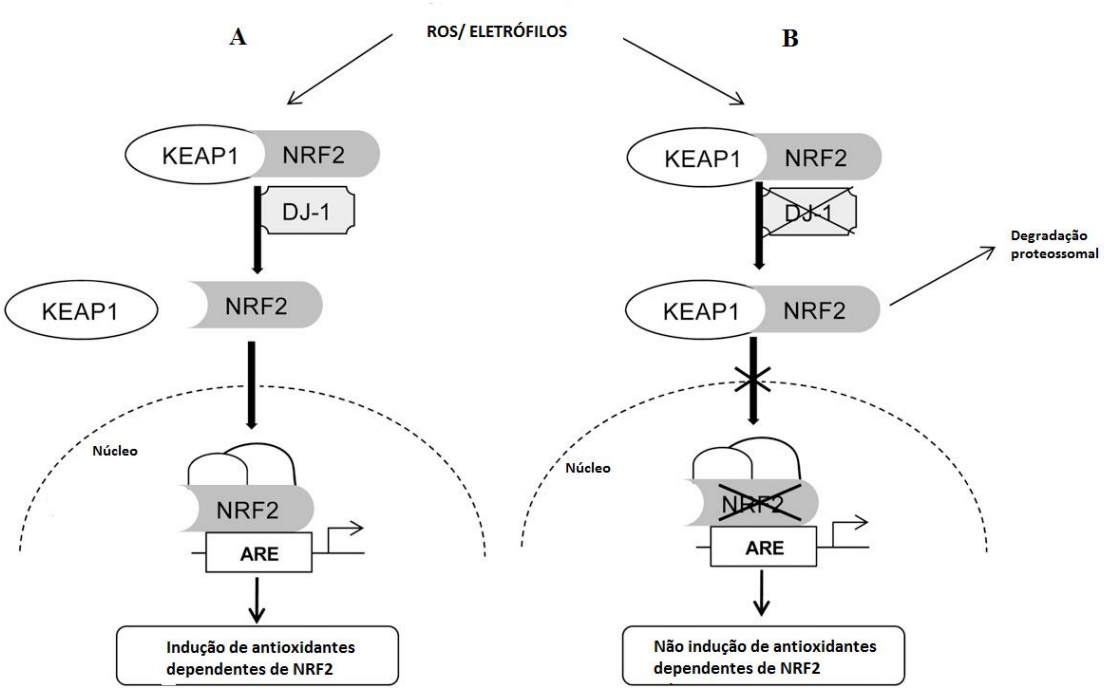

Fig.3 Esquema adaptado de Malhotra et al. (2008) demonstrado a ação da DJ-1 em situação fisiológica de geração de ROS (A), e frente a uma indução forte de estresse oxidativo, que leva a oxidação/inativação da proteína DJ-1 (B). ROS = Espécies Reativas de Oxigênio. ARE=elemento de resposta antioxidante.

3.2.Sistema de defesa antioxidante e BPA

Conhecimentos e avanços recentes dos estudos de toxicidade oxidativa por xenobióticos em organismos aquáticos oferecem um campo fértil para estudos de toxicologia aquática (Livingstone, 1998). Muitos contaminantes ambientais têm sido relatados por perturbar o equilíbrio pró-oxidante/antioxidante de células, induzindo assim o estresse oxidativo (Bindhumol et al., 2003). Nos últimos anos, uma série de estudos ecotoxicológicos investigou danos em parâmetros de estresse oxidativo em várias espécies de peixes expostos a metais e poluentes orgânicos (Valvanidis 2006; Tao et al*.*, 2016).

Em relação ao BPA, o mecanismo de ação desse composto na geração de estresse oxidativo não é totalmente claro, mas fígados de ratos expostos por 30 dias a

doses baixas (0,2, 2 e 20 µg/Kg/dia) de BPA demonstraram uma diminuição na atividade de importantes enzimas antioxidantes como SOD-1, CAT, GPX e GR (Bindhumol et al., 2003). O BPA também foi demonstrado com potencial para induzir a produção de ROS no esperma epididimal dos ratos expostos a doses baixas (Chitra et al., 2003). Já em peixes, foi observado que larvas de *Gobiocypris rarus* expostas ao BPA por sete dias às concentrações de 1.225 e 1.000 µg/L apresentaram um aumento na produção de H_2O_2, indução de estresse oxidativo e imunossupressão (Tao et al., 2016). Além disso, mamíferos expostos a doses baixas de BPA apresentaram alterações tanto na expressão quanto na quantidade de proteína DJ-1 no cérebro, demonstrando a relação desse composto com a regulação de vias relacionadas à defesa antioxidante nesses animas (Ooe et al., 2005).

4. MODELO EXPERIMENTAL

O peixe-zebra é um modelo experimental amplamente usado em laboratório, devido a seu tamanho pequeno, manutenção fácil e barata, desenvolvimento rápido, entre outros. A fertilização é externa e os embriões são de fácil manipulação e monitoramento. Em peixe-zebra o desenvolvimento inicial das gônadas é como ovários, a diferenciação inicia nos machos aproximadamente 5 - 7 semanas após a eclosão, se desenvolvendo como testículos normais aproximadamente no terceiro mês. A determinação genética do sexo é desconhecida, mas a maturidade sexual geralmente é relacionada com o tamanho do animal. A ovulação depende da exposição das fêmeas a ferormônios gonadais dos machos e apresentam comportamentos de cortejo específicos. A eclosão acontece aproximadamente 72 horas após a fertilização, mas pode ser afetada pela incidência de luz (48-72 horas) (Spence et al., 2007).

Esse modelo experimental tem origem no Sudeste da Ásia e foi introduzido como um organismo modelo genético por George Streisinger no final dos anos 1960 (Streisinger et al., 1981; Bhat, 2003). O genoma do peixe-zebra está completamente sequenciado e os genes de peixe-zebra partilham uma homologia de 60-80% com os genes humanos, mais uma importante característica para estudos sistêmicos toxicológicos que envolvem expressão gênica (Esch et al.,2012). Além disso, devido ao seu pequeno tamanho, a avaliação patológica de baixo custo eficiente de todos os principais órgãos pode ser realizada em um número limitado de lâminas.

5. JUSTIFICATIVA

Os estudos recentes relacionando desreguladores endócrinos e peixes têm sido focados no desenvolvimento embrionário ou reversão sexual frente à exposição a esses compostos, geralmente utilizando doses relativamente altas. No presente estudo, utilizamos concentrações encontradas no ambiente e analisamos aspectos moleculares referentes às duas vias de extrema importância para o organismo, a via esteroidogênica e a do sistema de defesa antioxidante enzimático. Trabalhos relacionando o efeito do BPA em doses ambientais no sistema de defesa antioxidante ainda são escassos e poucos dados têm sido obtidos principalmente em organismos aquáticos. Dessa forma, escolhemos o peixe *Danio rerio* como modelo para a obtenção de resultados que possam esclarecer parcialmente alguns aspectos do impacto que concentrações já encontradas no ambiente podem trazer aos peixes.

OBJETIVO GERAL

Avaliar aspectos reprodutivos e relacionados à defesa antioxidante em *Danio rerio* expostos a concentrações ambientalmente relevantes de BPA.

Objetivos Específicos

- Avaliar a expressão de um grupo de genes relacionados à enzimas atuantes na via esteroidogênica e na reprodução de *Danio rerio* expostos ao BPA de forma aguda e subcrônica.

- Avaliar a expressão de um grupo de genes relacionados à enzimas atuantes no sistema de defesa antioxidante e detoxificação de fase II em *Danio rerio* expostos ao BPA de forma aguda e subcrônica.

- Avaliar a expressão do gene que codifica para a proteína reguladora de defesa antioxidante DJ-1 em *Danio rerio* expostos ao BPA de forma aguda e subcrônica.

- Verificar respostas bioquímicas relacionadas com as defesas antioxidantes e atividades enzimáticas em *Danio rerio* expostos ao BPA de forma subcrônica.

- Analisar aspectos histopatológicos nos testículos de *Danio rerio* expostos diferentes de concentrações de BPA um tempo considerado crônico.

- Analisar a morfologia ultraestrutural espermática de *Danio rerio* expostos à diferentes de concentrações de BPA em um tempo considerado crônico.

Capítulo I

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BPA effects on steroidogenesis and antioxidant defense in zebrafish, *Danio rerio*

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Abstract

Bisphenol-A (BPA) is a chemical widely used in the industry, and considered an endocrine disrupter for various animal groups, but recent studies has been seen as a causative agent of oxidative stress by decreasing the enzymatic antioxidant defenses. The action of BPA as a pro-oxidant has been well described in mammals and suggested that this compound acts by repressing the expression of regulatory pathways of antioxidant genes, such as the DJ-1/Nrf2 pathway. In present study, zebrafish *Danio rerio* were exposed to concentrations of 2, 10 and 100 μg/L of BPA for 24 and 96 hours and gene expression and biochemical parameters related to reproduction and antioxidant defense were observed. Regarding gene expression, we performed a expression of genes related to steroidogenic pathway components and enzymes of antioxidant defense system. The results demonstrated a down-regulation of genes related to antioxidant defense, as superoxide dismutase-1 (*SOD-1*), and catalase (*CAT*), in animals exposed to BPA for 24 hours. In contrast, the gene coding for glutathione S-transferase (*GST*) enzyme was induced in 24 hours (5 fold induction). The *park7* gene was induced significantly in 24 and 96 hours (15 and 7 fold induction, respectively), indicating their participation in action of BPA on the cell, as occurs in mammals. Changes in genes related to steroidogenic pathway emerged later, in animals exposed for 96 hours. Important genes as *Cyp19a1*, *Cyp19a2*, *StAR* and *Vtg2* were organ-dependent induced. It was also observed biochemical responses of decreased total antioxidant capacity in animal brain, and changes in the activity of SOD-1, CAT and GST enzyme in various organs. Thus, the present study found that environmentally concentrations of BPA induce significant molecular and biochemical changes of time-dependent and tissue-dependent in zebrafish.

Keywords: Antioxidant defense, Bisphenol-A, Steroidogenesis, Zebrafish.

1. Introduction

Bisphenol-A (BPA: 4,4'-dihydroxy-2,2-diphenylpropane) is a carbon-based synthetic chemical, commonly used in the manufacture of epoxy resins and polycarbonates, which displays endocrine disrupter activity *in vitro* and *in vivo* studies (Keum et al., 2010). This compound is considered a pseudo-persistent chemical, since despite its short half life, is ubiquitous in the environment due to continuous release (Oehlmann et al., 2009). The production of materials containing BPA has grown exponentially. In 2003, the production of BPA was 3.2 million tonnes (Tsai, 2006), with approximately one third manufactured in the United States (US National Institute of Health, 2008). In 2011, the global production of BPA reached approximately 4.4 million tones (Merchant Research ans Consulting ltd, 2016). Recent studies have shown that the action of BPA as an endocrine disrupter is mainly by binding to estrogen receptors (Crain et al., 2007; Suzawa and Ingraham, 2008; Vandenberg et al., 2015), although also have several other disruptor activities, mediated via multiple molecular pathways, including the change in transcription factors and gene expression (Le et al., 2008). BPA was seen altering both expression and activity of important enzymes related steroidogenic pathway, as aromatase (*Cyp19* gene) and 5α- reductase (*Srd5a* gene) in mammals (Suzawa and Ingraham, 2008).

In addition to the effect as an endocrine disrupter, several studies have suggested that this compound may cause oxidative stress, increasing the amount of reactive oxygen species (ROS) in the brain, liver, kidney, testis and epididymis, and reducing mitochondrial function in mammals (Nakagawa and Toyama, 2000; Chitra et al., 2003). Oxidative stress is defined as an imbalance between production and depletion of reactive oxygen species (ROS). Increased ROS generation is a product of imbalance on the components responsible for eliminating excess ROS or the result of

dysfunction in the complexes mitochondrial respiratory chain (CRM). These processes are also involved steroid hormones (Chen et al., 2009). This situation may occur when the organisms are exposed to environmental contaminants such as metals, pesticides and oil (Valvanidis et al., 2006). The protection to oxidative damage may be non-enzymatic, by antioxidants such as some vitamins, uric acid, glutathione and carotenoids. Furthermore, there is in all vertebrates and invertebrates animals an enzymatic antioxidant defense system, where several antioxidant enzymes including superoxide dismutase (SOD) and catalase (CAT), act preventing the cascade of oxidative reactions. The antioxidant enzymes are crucial in efforts to counteract the toxicity of oxygen in situations of high ROS production (Ahmad, 1995). Furthermore, in order to prevent situations of high amount of reactive oxygen species in the cell environment, there are several regulatory proteins that act activating the system antioxidant response, such as nuclear erythroid transcription factor - 2 (Nrf-2) and cytoplasm regulatory protein DJ-1. The protein DJ-1 is expressed in several tissues, highly conserved among vertebrates, which is homologs are expressed from microorganisms to mammals, including zebrafish *Danio rerio* (Bai et al., 2006; Khale et al., 2009). In zebrafish, DJ-1 also seems to act as redox-reactive and neuroprotective agent, regulating the action of antioxidant enzymes and exerting an anti-apoptotic role (Bai et al, 2006; Baulac et al., 2009). DJ-1 protein may act as a scavenger or stabilize the transcription factor NRF2, master regulator of antioxidant response (Clements et al., 2006).

Most of the studies related to BPA treat their effects as endocrine disrupter and its consequences for reproduction, however, studies related to the oxidizing effect of this compound is low (Toyama and Nakagawa, 2000; Bindhumol et al. 2003; D'Cruz et al, 2011). *In vivo* data suggest that BPA is able to induce oxidative damage in liver

repeated exposure (Bindhumol et al., 2003). Furthermore, *in vitro* assays demonstrated that pro-oxidant BPA effect was also observed, but at higher doses (Ooe et al., 2005).

Finally, mammals studies have shown that exposure to BPA can lower the activity of DJ-1 protein, decreasing the antioxidant response of organisms and causing an intense cellular oxidative stress situation (Ooe et al., 2005).

Considering this scenario, the objective of this study is to analyze expression of genes related to reproduction and antioxidant defense in a fish for toxicological studies, the zebrafish *Danio rerio*. Along with molecular analysis, biochemical analysis of antioxidant capacity and enzymatic activity will help in understanding the BPA primary mechanisms of action in freshwater fish exposed to environmental concentrations.

2. Material and methods

2.1. Animal exposure and sampling protocol

Adult animals were maintaned from Institute of Biological Sciences, Federal University of Rio Grande, Rio Grande, RS, Brazil. The fish were kept at 28ºC and photoperiod 14C: 10D, and fed twice daily with a commercial feed. The commercial feed (Supervit®) is estrogenic and antioxidant components (Vitamine E and C, glutathione) free. Adults *Danio rerio* with sexual maturity (4 months) were used for exposure to BPA in independent experiments. The animals were exposed to BPA in concentrations of 2, 10 and 100 μg/L, and a control group, only the solvent BPA used in the experiment, 0.01% ethanol. No quantification of the compound was carried out, since daily water renewal and replacement 50% of BPA. After the exposures, the animals were anesthetized with tricaine solution (500 mg/L) and testis, liver, brain, and gut were collected to the molecular and biochemical analyses.

2.2. RNA extraction and cDNA synthesis

Total RNA was isolated from various tissues of each exposure group (n=10) using reagent TRIzol (Invitrogen, Brazil), following the manufacturer's recommendations. The extracted RNA was treated with DNAse I (Invitrogen, Brazil). The amount total of RNA was determined with a Fluorometer Qubit and a Quant-iT RNA BR Assay Kit (Invitrogen, Brazil), and the RNA integrity was assessed by electrophoresis with agarose 1% gel. For cDNA synthesis, the total RNA was reverse transcribed using the reverse transcription kit High-Capacity cDNA (Applied Biosystems, Brazil), following the manufacturers' protocols.

2.3. Gene expression

Gene expression experiments were performed by real time qPCR (7500 Real Time System, Applied Biosystems). Each sample was analyzed in duplicate. Specific primers were designed for each gene analyzed using the Primer-BLAST tool from GenBank. The *beta-actin* and *18S* were used as housekeeping genes. In this study, the housekeeping genes were tested using geNorm VBA applet for Microsoft Excel (Vandesompele et al., 2002).The genes analyzed were *DJ-1, SOD-1*, *SOD-2*, *CAT*, *GST*, *GCLc* (antioxidant genes) *StAR*, *Cyp19a1*, *Cyp19a2*, *Vtg2* (genes related to synthesis and action of reproductive hormones) (Table 1). The results were analyzed to statistical software REST 2009 (Pffafl et al., 2009)

2.4. Total antioxidant capacity (ACAP)

Total antioxidant capacity against peroxyl radicals was determinedaccording to Amado et al. (2009). Homogenates aliquots of tissues (testes, brain, liver and gut) were placed in a medium containing 30 mM HEPES (pH 7.2), 200 mM KCl, 1 mM $MgCl_2$ and 40 μM fluorogenic compound 2 ', diacetate 7'- dichlorofluorescein (H_2DCFDA,

Invitrogen) in the presence or absence of 2,2'-azobis 2-methylpropionamidine dihydrochloride (ABAP; 4 mM, Aldrich), which generatesperoxyl radicals by thermal decomposition at 37°C. Fluorescence was considered as a measure of ROS and was read on a spectrophotometer equipped with a microplate reader (Victor 2, Perkin-Elmer) at wavelengths of 485 and 530 nm for excitation and emission, respectively. The total yield of fluorescence was calculated by integrating the fluorescence units (FU) over the measurement period. The relative difference between the area of ROS with or without ABAP has been regarded as a measure of the antioxidant powers of the tissues, where the area difference is inversely proportional to the antioxidant capacity (Amado et al., 2009).

2.5. Enzyme Assays

The fish organs were homogenized (1:4 w/v) at 4°C in buffer containing 5 mM EDTA, 250 mM sucrose, Tris-base 100 mM, 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma) and the pH adjusted. The homogenates were centrifuged at 1,000 g for 20 min at 4°C and the resulting supernatants were centrifuged again at 10,000 g for 45 min at 4°C. The obtained supernatant was collected and stored at -70°C. Six samples were used for biochemical determinations for each experimental group.

The activity of catalase enzyme (CAT) was analyzed following the protocol established by Beutler (1975), determining the initial rate of decomposition of $H_2O_2$to 240 nm. The results were expressed in CAT units, where one unit is the amount of enzyme which hydrolyzes 1 mol of H_2O_2 per minute per mg protein at 30°C and pH 8.0.Superoxide dismutase (SOD) was measured by the method described by McCord and Fridovich (1969). The enzymatic activity was expressed as a SOD unit, where one unit is definedas the amount of enzyme required to inhibit 50% reduction of

cytochrome C per minute per mg protein at 25 ° C and pH 7.8. The glutathione-S-transferase (GST) was determined by monitoring at 340nm, the formation of a conjugate between 1 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) (Habig and Jakoby, 1981). The results were expressed as GST units, where one unit is defined as the amount of enzyme conjugate CDNB 1 mol per minute per mg of protein at 25°C and pH 7.40. In all the enzymatic assays, the reagents were supplied by Sigma.

2.6. Statistical analysis

The biochemical data are presented as mean ± SE. Statistical analyses were performed using one-way ANOVA ($P<0.05$), with normality and homogeneity of variance requirements previously tested. When there was a significant difference, the Tukey post test was applied. For molecular data was used the statiscal software *REST 2009.*

3. Results and discussion

The chemical estrogenic effects study still requires a greater understanding of the molecular pathways related to their mechanisms of toxicity, particularly with regard to gene expression and cell signaling (Kiyama et al., 2014). Endocrine disrupters have challenged the classical toxicological assumptions related to the dose-response relationship, the action at different stages of life and the chemical mixtures impact (Vogel, 2004). The exposure to these chemicals, besides disturb the reproduction, has been reported to increase oxidative stress , thereby disturbing the balance pro /antioxidant components on the cells (Morgan et al., 2014). Studies have associated BPA with redox status changes in various organs of mammals, especially brain, contributing to the progression of certain diseases, including neurodegenerative diseases (Suzuki et al., 2003; Jones and Miller, 2008).

The biochemical marker Vitellogenin (Vtg), an egg yolk precursor essential for the development of eggs and embryos is produced in the liver of females during oocyte maturation. The expression of this protein in male fish has been considered estrogenic disruptor endocrine exposure marker (Soffker and Tyler, 2012). The *Vtg2* gene was induced in testis and liver of animals when exposed to 96 hours (Fig. 2AB). Studies relating gene expression of vitellogenin and endocrine disruptors in fish are scarce, more common direct measurements of protein in animal liver. Li et al. (2016) reported a 125% increase in the amount of this protein in *Oryzias latipes* liver, though in much larger doses than those used in this study. Moreover, it has been shown that the VGT synthesis in male fathead minnow *Promelas pimephales* and Japanese medaka. *O. latipes* was induced by 160 µg / L BPA for 3 days (Sohoni et al., 2001), and 100 µg / L for 70 days (Tabata et al., 2004).

Studies with mammals has shown that BPA has the ability to change the enzymatic antioxidant defense system, making it generally less active and therefore generating oxidative stress in the brain, liver, kidney, testicles, and other organs (Chitra et al., 2003; Kabuto et al. 2003; D'Cruz et al, 2011). In zebrafish embryos, exposure to BPA and nonylfenol also led to changes related to oxidative stress and antioxidant parameters (Wu et al., 2011).

In the present study, adult zebrafish exposed to BPA for 24 hours demonstrated a decrease in the expression and activity of SOD-1 and CAT enzymes,in organs such as the testis, liver and brain (Fig. 1ABCD, 5BC and 7B). The decrease in SOD and CAT expression have also been observed in several studies in mammals exposed to similar concentrations of BPA and may be related in part to inactivate regulatory antioxidant proteins such as DJ-1 present in high concentrations in the brain (Ge et al., 2014; Morgan et al, 2014). Already in animals exposed to BPA for 96 hours, the SOD

expression remained reduced compared to control, in liver, brain and gut (Fig 2BCD) and the CAT expression was reduced only in liver (Fig 2B). Regarding the activity of these enzymes in 96 hours CAT showed no significant difference compared to control, while SOD happens to be induced in the liver in animals exposed to the highest BPA concentration (Fig. 6B). This response of SOD and CAT may be associated with compensatory regulatory mechanisms, making the expression of these genes remain low but the proteins activity is recovered over time and the activity of enzymes and even induced, such SOD, in an attempt to compensate for the decrease in gene expression.

The total antioxidant capacity was lower in 24 hours in the brain and liver of animals (Figs. 3BC) and at 96 hours just in the brain (Fig. 4C) indicating that indeed the BPA is able to decrease the antioxidant response in fish.The decrease of antioxidant capacity demonstrating a failure of the primary antioxidant system to act against free radicals, as occurs in mammals exposed to similar doses of BPA, causing an increase in ROS generation (Chitra et al., 2003 Korouma et al., 2014).

The GSTα1 gene expression was induced in 24 hours (Fig. 1B) and the GST activity was induced in 24 and 96 hours in the liver and brain of animals (Figs. 10B and 9BC). Increased GST activity has also been observed in Japanese medaka*Oryzias latipes* exposed to high concentrations of BPA (Li et al., 2016) and probably acts to offset the reduced activity of other antioxidant enzymes. Furthermore, the enzyme GST is known as a major detoxifying agent related to BPA, at least in mammals (Tanabe et al, 2012; Tamiselvan et al, 2014.). This study demonstrated that expression of the gene encoding this enzyme also appears to be a reliable biomarker of exposure to relatively low doses of BPA.

In addition, expression of the gene related to biotransformation CYP1A was induced in 24 and 96 hours in the testis and liver of exposed animals (Figs. 1AB, 2B). The relation of the BPA presence and modifications in detoxifying genes such as *CYP1A* still is not clear, however it is known that in mice liver, the presence of endocrine disruptors with a similar mechanism of action to BPA alters significantly specific activity of cytochromes P450 (Hanioka et al., 1998). BPA has been shown to modulate the expression of different genes of the cytochrome P450 family (Lee et al., 2006) and, unlike the present study, reducing the *CYP1A* expression at *in vitro* experiments with higher doses of the compound (Jeong et al., 2000).The liver was a highly responsive organ in the expression responses and antioxidant enzymes activity, such in other studies with fish (Buhler and Williams, 1988; Li et al., 2016). This organ is generally known as the main site of detoxification of environmental contaminants. Antioxidants levels (constitutive or induced) in fish are tissue-specific and are generally higher in the liver than in other tissues (Buhler and Williams, 1988).

The *park7* gene, that encoding the DJ-1 protein, was induced in the brain at 24 hours and 96 hours, with fold induction of 15 and 7, respectively (Figs. 1C and 2C). The DJ-1 protein is highly conserved and has a high basal expression and activity in the brain of zebrafish, but is also active in other organs, to a lesser extent (Bai et al., 2006). DJ-1 regulates the antioxidant activity in the cell because has the function of releasing to the Nrf2 transcription factor, which induces genes as *SOD-1*, *CAT* and *GSTα1*. Studies with rats demonstrate that BPA is capable of inactivating the DJ-1 protein, through molecular mechanisms remain unclear. This may explain the increased of gene expression in zebrafish exposed, in order to compensate the decline of the protein. Despite having high activity in the brain, the DJ-1 protein is also active in other organs, such liver and brain (Bai et al, 2006; Yanagida et al., 2009). Thus, the activity of this

protein could be somehow influencing the reduction of antioxidant enzyme activity in others studied organs, but more studies are needed to confirm this hypothesis.

BPA has already been observed by altering the expression of various genes related to reproduction in all classes of vertebrates (Suzawa and Ingraham, 2008). Liu et al (2012) observed effects of BPA concentration dependent on the expression of genes encoding steroidogenic enzymes such as *StAR*, *CYP11A1*, *3βHSD*, *CYP17A1* and *CYP19a1*inrare minnow *Gobiocypris rarus* gonads.

The Cyp19 gene encodes Aromatase (CYP19 enzyme, cytochrome P450arom), which has an important and essential role in synthesis of steroids, catalyzing the transformation of androgens into estrogens (Huang and Leunga, 2009; Hinfray et al., 2006). Different to mammals, many teleost fish, including zebrafish, have two of the CYP19 gene isoforms in their genomes (*cyp19a1*e *cyp19a2*), thus producing two different cDNAs are expressed in the brain and gonads (Callard et al., 2001; and Linney Lassiter, 2007). The *Cyp19a1* gene is expressed predominantly in the gonads, and the *Cyp19a2* is predominant in the brain (Hinfray et al., 2006, Cheshenko et al., 2008).In this study the *Cyp19a1* and *Cyp19a2* genes were induced in the gonads and brain, respectively (Figs 1BC and 2BC) corroborating studies that demonstrate their difference in basal and inductive expression in different tissues (Suzawa and Ingraham, 2008, Chenschenko et al., 2009).

Another gene altered by exposure to BPA in organs such gut, liver and testis was the StAR gene (Figs. 2AB and 1D). The steroidogenic acute regulatory protein is an important target for endocrine disruptors and is responsible for cholesterol trafficking, precursor of steroids into the mitochondria (Hampl et al., 2016). The expression of StAR gene can be altered by a variety of endocrine disruptors (Caron et

al, 1998; Clark et al., 2007, Kumar et al., 2008) but higher levels than those used in this study.

In general, steroidogenic genes are considered targets of estrogenic endocrine disruptors, since in its promoter region are present responsive targets to estrogen receptors (Suzawa and Ingraham, 2008; Cheschenko et al., 2009). Thus, the mechanism of action by which BPA alters the expression of genes related to steroidogenic pathway may be via direct interaction of nuclear estrogen receptors. These receptors finally binding to estrogen response elements in DNA, for example, as shown in studies with fish and mammals (Kuiper et al., 1998; Suzawa and Ingraham, 2008).

The BPA concentrations which the animals were more responsive both related to reproduction as the antioxidant defense were 10 and 100μg/L both environmentally relevant and present in polluted sites throughout the world (Crain et al., 2007). Although more studies are needed, this study shows some potential molecular biomarkers for acute and subchronics exposures at relatively low concentrations.

In addition, the relationship between time-dependent gene expression and activity of proteins after exposure to endocrine disrupting contaminants have been little explored, so the present study showed an interesting relationship between the selected groups of genes and response in relation to the exposure times, where the reproductive genes was later responsive that the antioxidant genes.

4. Conclusion

BPA is able to alter the expression of important genes related to both the steroidogenic pathway as the antioxidant defense system, and change biochemical aspects related to cell antioxidant defense in environmentally relevant concentrations.

5. References

Ahmad, S., 1995.Oxidative stress and antioxidant defenses in biology.Chapman e Hall, New York.

Bai, Q., Mullett, S.J., Garver, J.A. Hinkle, D., Burton, E., 2006. Zebrafish DJ-1 is evolutionarily conserved and expressed indopaminergic neurons. Brain Res. 1113,33–44.

Baulac, S., Lu, H., Strahle, J., Yang, T., 2009.Increased DJ-1 expression under oxidative stress and in Alzheimer's disease brains. Mol. Neurodegener. 4, 12.

Bindhumol, V., Chitra, K. C., Mathur, P. P., 2003. Bisphenol A induces reactive oxygen species generation in the liver of male rats. Toxicology. 188, 117–124.

Callard G., Tchoudakova A., Kishida M., Word E., 2001. Differential tissue distribution, developmental programing, estrogen regulation and promoter characteristics of *cyp19* genes in teleost fish. J. Ster. Biochem. Mol. Biol. 79, 305–314.

Chen, J.Q., Cammarata, P.R., Baines, C.P. Yager, J., 2009. Regulation of mitochondrial respiratory chain biogenesis by estrogens/estrogen receptors and physiological, pathological and pharmacological implications. Biochem.Biophys.Acta. 10, 1540-1570.

Cheshenko K., Pakdel F., Sener H. Kah, O., Eggen, R., 2008. Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. Gen. Comp. Endocrinol. 155, 31–62.

Chitra, K.C., Latchoumycandane, C., Mathur, P.P., 2003. Induction of oxidative stress by bisphenol A in the epididymal sperm of rats. Toxicology. 185, 119–127.

Clements, C. M., McNally, R. S., Conti, B., 2006. DJ-1, a cancer and Parkinson's disease-associated protein, stabilizes the antioxidant transcriptional master regulator Nrf2. Proc. Natl. Acad. Sci. USA 103, 15091–15096.

D'Cruz, S.C., Jubendradass, R., Mathur, P.P., 2011. Bisphenol A Induces Oxidative Stress and Decreases Levels of Insulin Receptor substrate 2 and Glucose Transporter 8 in Rat Testis. Reprod. Sci. 19, 163-172.

Forlano, P., Schlinger, B., Bass, A., 2006. Brain aromatase: New lessons from non-mammalian model systems. Front. Neuroendocrinology. 27, 247–274.

Ge.,L., Chen, Z., Liu, H., Zhang, K., Su, Q., Ma, X., Huang, H., Zhao, Z.,Wang, Y., Giesy, J., Du, J., Wang, H., 2014. Signaling related with biphasic effects of bisphenol A (BPA) on Sertoli cell proliferation: A comparative proteomic analysis. Biochim. Biophys. Acta. 1840, 2663-2673.

Hanioka, N., Jinno, H., Nishimura, T., Ando, M., 1998. Supression of male-specific cytochrome P450 isoforms by bisphenol A in rat liver. Arch. Toxicol. 72, 387-394.

Hinfray N., Palluel O., Turies C. Cousin, C., Porcher J., Brion, F., 2006. Brain and Gonadal Aromatase as Potential Targets of Endocrine Disrupting Chemicals in a Model Species, the Zebrafish (*Danio rerio*). Environ. Toxicol. 21, 332- 337.

Huang H., Leunga L., 2009. Bisphenol A downregulates CYP19 transcription in JEG-3 cells. Toxicol.Lett. 189, 248–252.

Jeong, H.G., Kim, J., Choi, C., 2000. Down-Regulation of Murine *Cyp1a-1* in Mouse Hepatoma Hepa-1c1c7 Cells by Bisphenol A. Biochem. Bioph. Res. Co. 277, 594-598.

Jones, D., Miller, G., 2008. The effects of environmental neurotoxicants on the dopaminergic system: A possible role in drug addiction. Biochem. Pharmacol. 76, 569-581.

Kabuto, H., Hasuike, S., Minagawa, N., Shishibori, T., 2003. Effects of bisphenol A on the metabolisms of active oxygen species in mouse tissues. Environ. Res.93, 31–35.

Keum,Y.S., Lee, H.R., Park, H.W. Kim, J., 2010. Biodegradation of bisphenolA and its halogenated analogues by Cunning hamella elegans ATCC36112. Biodegradation. 21, 989–997.

Kiyama, R., Zhu, Y., Kawaguchi, K., Iitake, N., Wada-Kiyama, Y., Young, S., 2014. Estrogen-responsive genes for environmental studies. Environ. Technol. Innov. 1-2, 16-28.

Kourouma, A., Peng, D., Chao, Q. Lopez, Y., Changjiang, L.,Chengmin, W., Wenjuan., F., Suqin, Q., Tingting, W., Kedi, Y., 2014.Bisphenol A induced reactive oxygen species (ROS) in the liver and affect epididymal semen quality in adults Sprague-Dawley rats. J. Toxicol. Environ. Health Sci. 6, 103–112.

Kuiper G.G., Lemmen J.G., Carlsson B., Corton, J., Safe, J., Van der sag, P., Van der burg., B., Gustafssonn, J., 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta.Endocrinology. 139, 4252–4263.

Lassiter C., Linney E. 2007. Embryonic Expression and Steroid Regulation of Brain Aromatase *cyp19a1b* in Zebrafish (*Danio rerio*). Zebrafish. 4, 1.

Lee, Y., Seo, J., Kim, I., Yoon, Y., Lee, J., 2006. Endocrine disrupting chemicals (bisphenol A, 4-nonylphenol, 4-tert-octylphenol) modulate expression of two distinct cytochrome P450 aromatase genes differently in gender types of the hermaphroditic fish *Rivulus marmoratus*. Biochem. Bioph. Res. Co. 345, 894-903.

Li, D., Chen, K., Cao, J., Chen, H., Li, L., Cadergreen, N., Xie, H., Xie, L., 2016. The chronic effects of lignin-derived bisphenol and bisphenol A inJapanese medaka *Oryzias latipes*. Aquat. Toxicol. 170, 199-207.

Lopéz-Cervantes, J., Paseiro-Losada, P., 2003. Determination of bisphenol A in, and its migration from, PVC stretch film used for food packaging. F. Addit.Contam. 20, 596–606.

Merchant Research and Consulting ltd, 2016 (http://mcgroup.co.uk/news/20131108/)

Mouriec K., Lareyre J.J., Tong S.K., Page, Y., Vaillant C., Pellegrini, E., Pakdel, F., Chung, B.C., Kah, O., Anglade, I., 2009. Early regulation of brain aromatase (*cyp19a1b*) by estrogen receptors during zebrafish development. Develop. Din. 238, 2641-2651.

Morgan, A.M., El- Ballal, S.S., El-Bialy, B.E., El-Borai, N.B., 2014. Studies on the potential protective effect of cinnamon against bisphenol A- and octylphenol-induced oxidative stress in male albino rats. Toxicol. Rep. 1, 92-101.

Nakagawa, Y., Toyama, S., 2000. Metabolism and cytotoxicity of bisphenol A and other bisphenols in isolated rat hepatocytes. Arch.Toxicol. 74, 99–105.

National Institute of Health, 2008.NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol-A.National Toxicology Program, US Department of Health and Human Services.Center for the Evaluation of Risks to Human Reproduction (CERHR-NTP), Washington D.C.

Oehlmann, J., Schulte-Oehlmann, U., Kloas, W., Jagnytsch, O., Lutz, I., Kush, K.O., Wollenberger, K.L., Santos, E., Paull, G., Van Lok, K., Tyler, C., 2009.A critical analysis of the biological impacts of plasticizers on wildlife.Phil. Trans. R. Soc. B 364, 2047-2062.

Ooe, H.,Taira, T.,Iguchi-Ariga, S.M., Ariga, H., 2005. Induction of reactive oxygen species by bisphenol A and abrogation of bisphenol A-induced cell injury by DJ-1.Toxicol.Sci.88, 114-26.

Sies, H. 1993. Strategies of antioxidant defense. European J. of Biochem. 215, 213-219.

Spence, R., Gerlach, G., Lawrence, C., Smith, C., 2007. The behaviour and ecology of the zebrafish, *Danio rerio*. Biol. Rev. 83, 13–34.

Suzawa, M., Ingraham, H.A., 2008. The herbicide atrazine activates endocrine gene networks via non-steroidal NR5A nuclear receptors in fish and mammalian cells. Plos one. 2117, 3-5.

Suzuki, T., Mizuo, K., Nakazawa, H., Funae, Y., Fushiki, S., Fukushima, S., Shirai, T., Narita, M., 2003 Prenatal and neonatal exposure to bisphenol-A enhances the central dopamine D1 receptormediated action in mice: enhancement of the methamphetamine-induced abuse state. Neuroscience. 117, 639–44.

Tan, B.L.L., Mustafa, A.M., 2003. Leaching of bisphenol A from new and old babies' bottles, and new babies' feeding teats. Asia Pac. J. Public.Health. 15, 118–123.

Tong, S, Chung, B.,2003. Analysis of zebrafish *cyp19* promoters. J. Ster. Biochem, Mol. Biol. 86, 381–386.

Tsai, W.T., 2006. Human health risk on environmental exposure to bisphenol-A: a review. J. Environ. Sci. Health C. 24, 225-255.

Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullos, M., 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environ- mental pollutants. Ecotoxicol. Environ. Saf. 64, 178–189.

Vogel, J.M., 2004. Tunnel vision: the regulation of endocrine disruptors. Policy Sci. 37, 3-4.

Vom Saal, F., Hughes, C., 2005. An extensive new literature concerning low-dose effects of bisphenol A shows the need for a new risk assessment. Environ. Health Perspect. 113, 926-933.

Wu, M., Xu,H., Shen,Y. Qiu, W., Wang, M., 2011. Oxidative stress in zebrafish embryos induced by short-term exposure to bisphenol A, nonylphenol, and their mixture. Environ. Toxicol. Chem. 30, 2335–2341.

Xu, S.Y., Zhang, H., He, P.J., Shao, L.M., 2011. Leaching behaviour of bisphenol A from municipal solid waste under landfill environment. Environ. Technol. 32, 1269-1277.

Zhang, J., Wei, X.L., Chen, L.P. Chen, N., Li, Y., Wang, W., Wang, H., 2013.Sequence analysis and expression differentiation of chemokine receptor CXCR4b among three populations of *Megalobrama amblycephala*.Dev. Comp. Immunol. 40, 195–201.

Captions

Table 1 Primers employed in the qPCR reactions.

Figure 1. Expression of genes related to steroidogenic pathway and antioxidant defense (n=8) in testis, liver, brain and gut of *Danio rerio* exposed to BPA for 24 hours. The results are given by subtracting the CT (Cycle Threshold) from the normalizing gene by the CT of the target gene. The relative expression results are normalized by the values of *beta-actin*and EF1α gene expression and the control group is represented by the line of the x axis (1). Asterisks represent significant differences in relation to control group (ANOVA – $p< 0.05$ – Tukey).

Figure 2. Expression of genes related to steroidogenic pathway and antioxidant defense (n=8) in testis, liver, brain and gut of *Danio rerio* exposed to BPA for 96 hours. The results are given by subtracting the CT (Cycle Threshold) from the normalizing gene by the CT of the target gene. The relative expression results are normalized by the values of *beta-actin*and EF1α gene expression and the control group is represented by the line of the x axis (1). Asterisks represent significant differences in relation to control group (ANOVA – $p< 0.05$ – Tukey).

Figure 3. Effects of Bisphenol A on Total antioxidant capacity in testis, liver, brain and gut of *Danio rerio* exposed for 24 hours (1/ Relative area difference).Significant difference ($p < 0.05$) between controland exposed animals is indicated by different letters.

Figure 4. Effects of Bisphenol A on Total antioxidant capacity in testis, liver, brain and gut of *Danio rerio* exposed for 96 hours (1/ Relative area difference). Significant difference ($p < 0.05$) between controland exposed animals is indicated by different letters.

Figure 5. Superoxide dismutase (SOD) activities in testis, liver, brain and gut of *Danio rerio* exposed to BPA for 24 hours. The results are referred as SODunits (amount of sample inhibiting cytochrome c reduction by 50% under thespecified conditions of the assay). Significant difference ($p < 0.05$) between controland exposed animals is indicated by different letters.

Figure 6. Superoxide dismutase (SOD) activities in testis, liver, brain and gut of *Danio rerio* exposed to BPA for 96 hours. The results are referred as SODunits (amount of sample inhibiting cytochrome c reduction by 50% under thespecified conditions of the assay). Significant difference ($p < 0.05$) between controland exposed animals is indicated by different letters.

Figure 7. Catalase (CAT) activities in testis, liver, brain and gut of *Danio rerio* exposed to BPA for 24 hours. The results are referred as CAT units (amount of enzyme able to decompose 1 mmol ofH_2O_2 per minute per milligram of protein). Significant difference ($p < 0.05$) between controland exposed animals is indicated by different letters.

Figure 8. Catalase (CAT) activities in testis, liver, brain and gut of *Danio rerio* exposed to BPA for 96 hours. The results are referred as CAT units (amount of enzyme able to decompose 1 mmol ofH2O2 per minute per milligram of protein). Significant difference ($p < 0.05$) between controland exposed animals is indicated by different letters.

Figure 9. Glutathione S-transferase (GST) activities in testis, liver, brain and gut of *Danio rerio* exposed to BPA for 24 hours. The results are referred as GST units (enzyme amount needed toconjugate 1 mmol of CDNB per minute and per milligram of

total proteins). Significant difference ($p < 0.05$) between controland exposed animals is indicated by different letters.

Figure 10. Glutathione S-transferase (GST) activities in testis, liver, brain and gut of *Danio rerio* exposed to BPA for 24 hours. The results are referred as GST units (enzyme amount needed toconjugate 1 mmol of CDNB per minute and per milligram of total proteins). Significant difference ($p < 0.05$) between controland expoded animals is indicated by different letters.

Table 1

Primer name	Primer sequence 5' – 3' forward	Primer sequence 5' – 3' reverse	Efficiency
B-actin	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC	F 100.0 – R 96.0
18S	AGGGACAAGTGGCGTTCAGC	GCAGGGTAGGCACACGT GA	F 99.0- R100.0
SOD-1	TGGAACCCCACTAGTCAATT	TTGGCGTTGCCACCGGAT	F 100.0 – R 100.0
SOD-2	TGGCAAAGGGTGA	CACCGCCATTGGGTGACAGA	F 90.8- R 99.7
CAT	AACCTGCTGCAGAAACCGTG	AACCCTGTGCTGGGGTACACTG	F 99.9-R 100.0
GSTα1	AGGTCCCTTTGGTGGAGATT	TCCTTGTTTTCAGCCGGTCCT	F 94.4 – R 98.8
GCLc	GGTGGCAAGCCGGATCACAT	TGATGCTGCAAGCCTGAAGG	F 100.0 – R 98.0
CYP1A	GCATAACGATACGTTCGATAAGGA	GCTCCGAATAGGTCATTGACGA	F 99.9 – R 100.0
Park7	TGCCCTCCTGGTCCTTCAACA	AGCCTGGAGGACGCACACAA	F 96.6 – R 98.6
Cyp19a1	ATTGAAGCCGTGCTGCTGC	TCCACAGCGATTGTCCTGAGC	F 100.0 – R 98.5
Cyp19a2	TGTGTGAGCGCCACCAACAG	CGATGCAGCCGAGGAGATTG	F 98.2 R 91.3
sTAR	AGGCTGTGCCGGAGATTG	CAGATGTCCCGTGGT	F 100.0 – R 97.2
Vtg2	TCATGGGTGCTGCCTGCTGA	ACAGCGCAGCATAGGCTCAA	F 100.0 – R 92.0

Figure 1

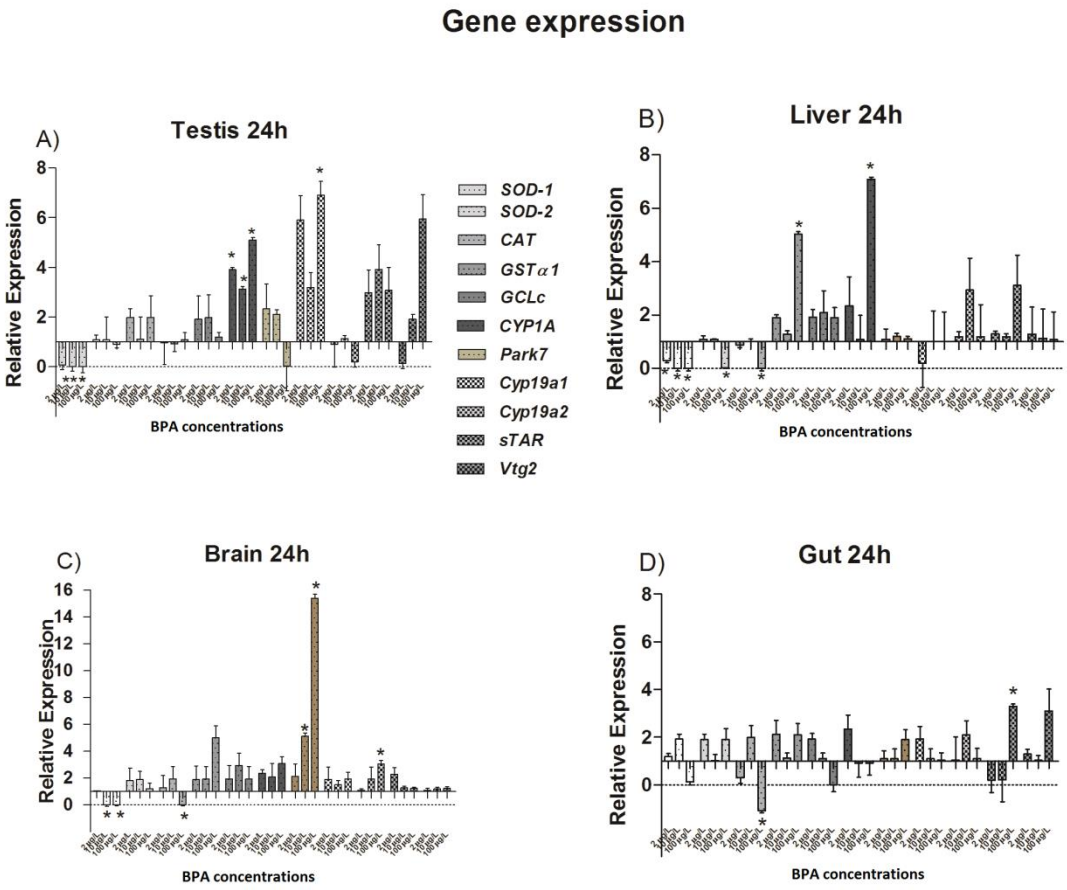

Gene expression
A)
Testis 24h
B)
Liver 24h
C)
Brain 24h
D)
Gut 24h
Relative Expression
BPA concentrations
SOD-1
SOD-2
CAT
GSTα1
GCLc
CYP1A
Park7
Cyp19a1
Cyp19a2
sTAR
Vtg2

Figure 2

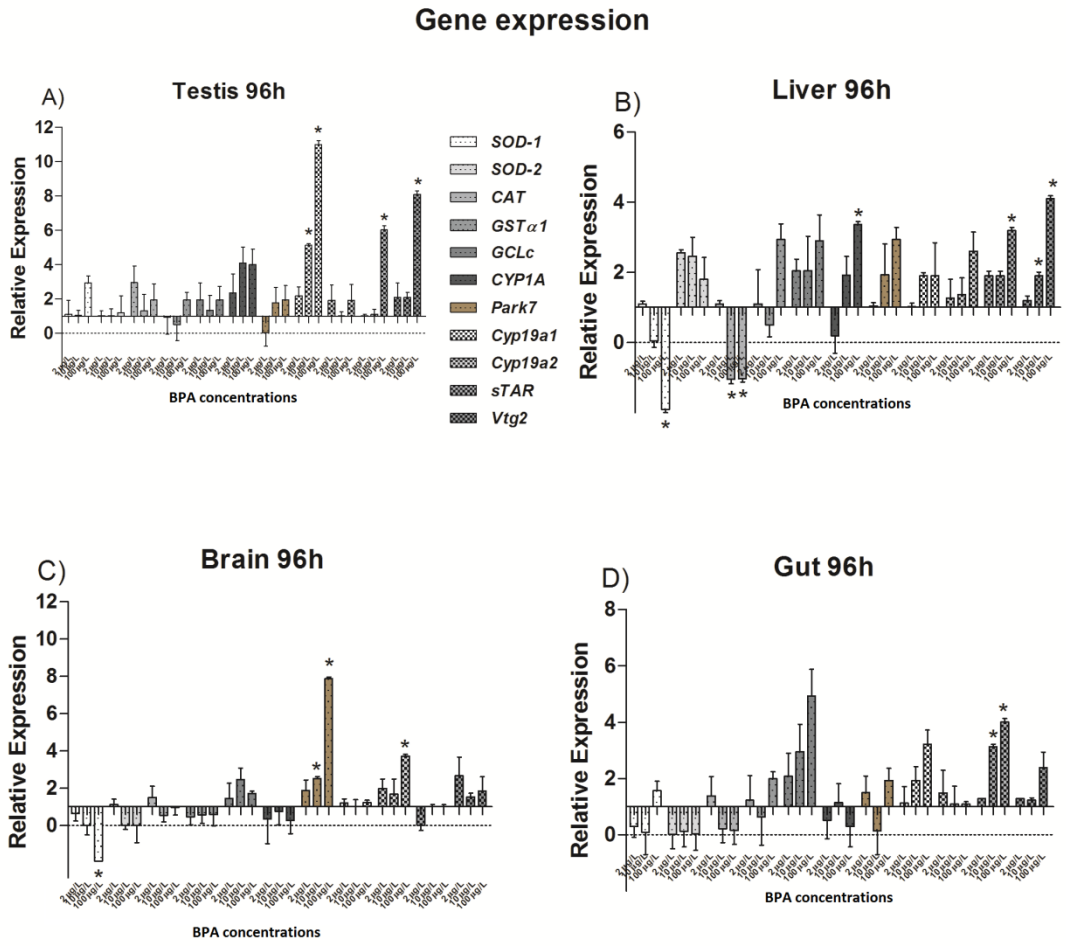

Gene expression
A)
Testis 96h
B)
Liver 96h
C)
Brain 96h
D)
Gut 96h
Relative Expression
BPA concentrations
SOD-1
SOD-2
CAT
GSTα1
GCLc
CYP1A
Park7
Cyp19a1
Cyp19a2
sTAR
Vtg2

Figure 3

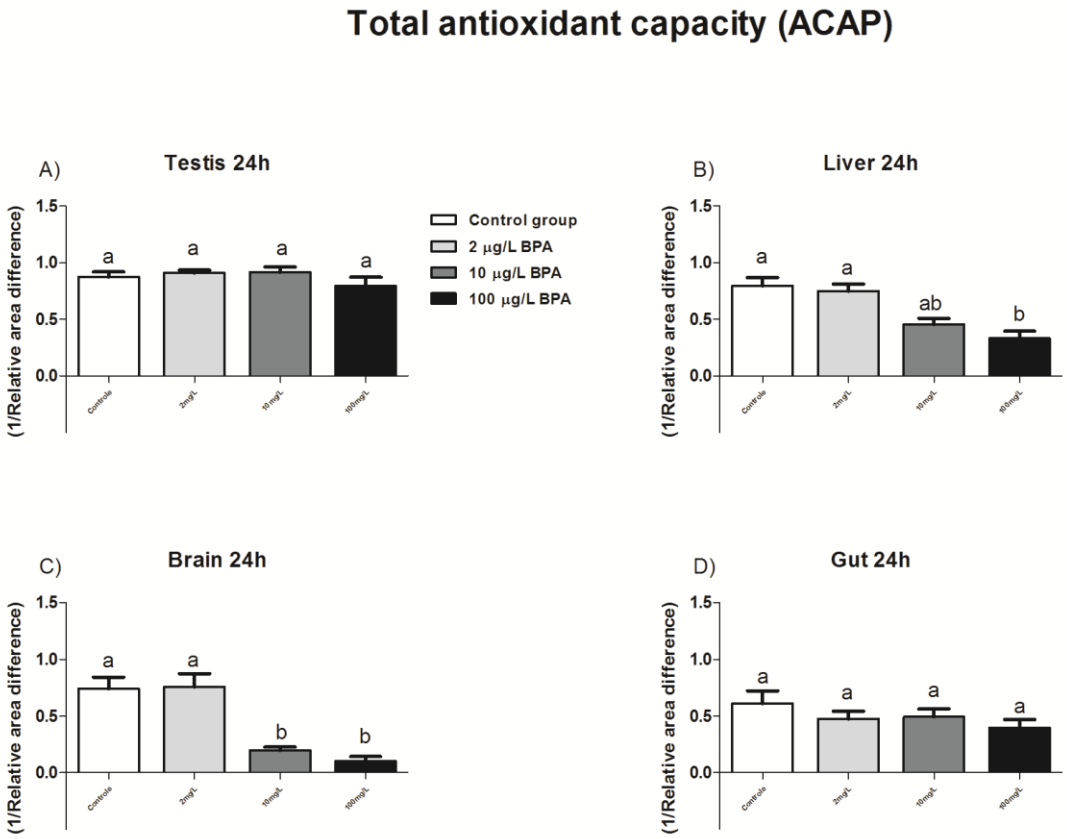

Total antioxidant capacity (ACAP)
A)
Testis 24h
B)
Liver 24h
C)
Brain 24h
D)
Gut 24h
(1/Relative area difference)
Control group
2 µg/L BPA
10 µg/L BPA
100 µg/L BPA
a
a
a
a
a
a
ab
b
a
a
b
b
a
a
a
a

Figure 4

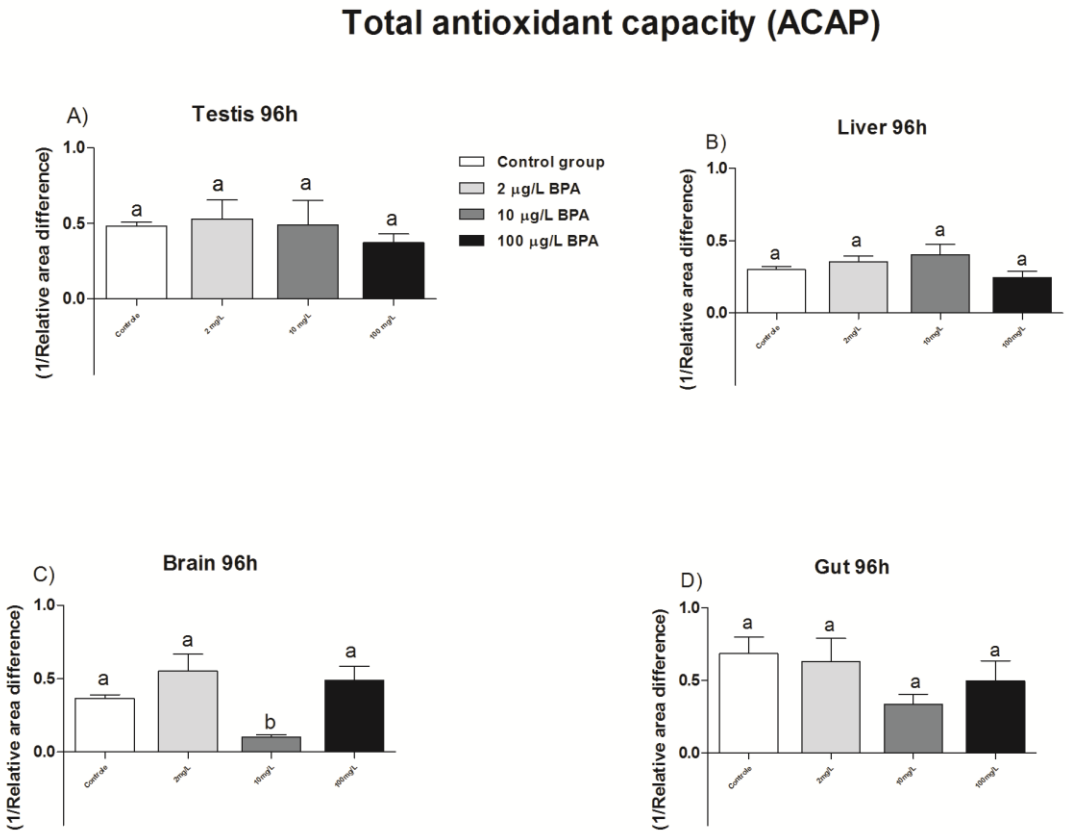

Total antioxidant capacity (ACAP)
A)
Testis 96h
(1/Relative area difference)
1.0
0.5
0.0
a
a
a
a
Control group
2 µg/L BPA
10 µg/L BPA
100 µg/L BPA
B)
Liver 96h
(1/Relative area difference)
1.0
0.5
0.0
a
a
a
a
C)
Brain 96h
(1/Relative area difference)
1.0
0.5
0.0
a
a
b
a
D)
Gut 96h
(1/Relative area difference)
1.0
0.5
0.0
a
a
a
a

Figure 5

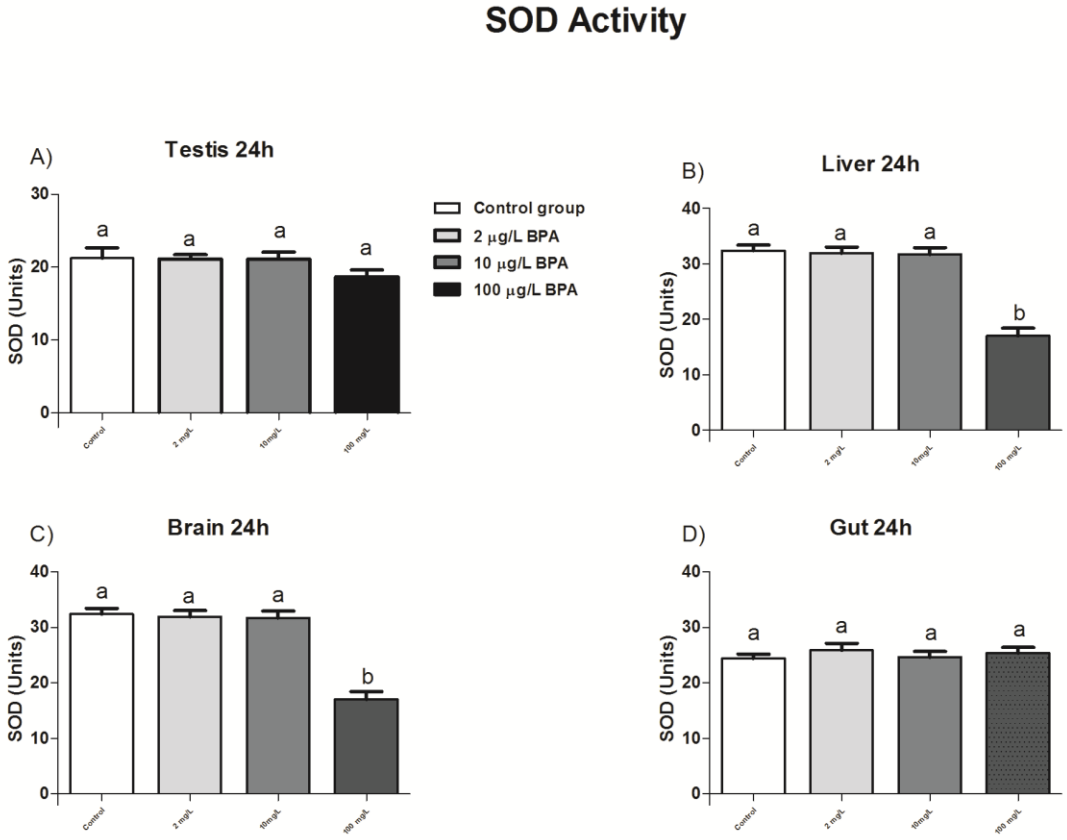

SOD Activity
A)
Testis 24h
B)
Liver 24h
C)
Brain 24h
D)
Gut 24h
SOD (Units)
Control group
2 µg/L BPA
10 µg/L BPA
100 µg/L BPA
a
b
Control
2 mg/L
10mg/L
100 mg/L

Figure 6

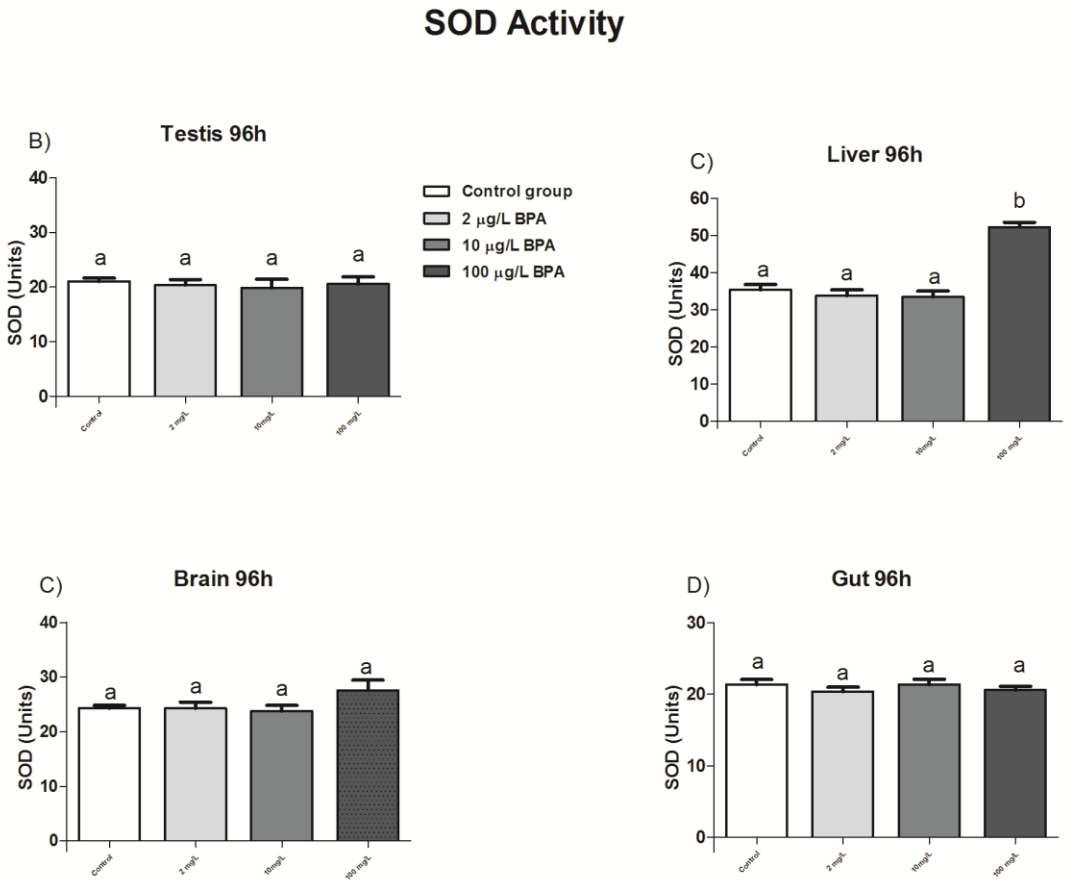

SOD Activity
B)
Testis 96h
SOD (Units)
Control group
2 µg/L BPA
10 µg/L BPA
100 µg/L BPA
C)
Liver 96h
C)
Brain 96h
D)
Gut 96h

Figure 7

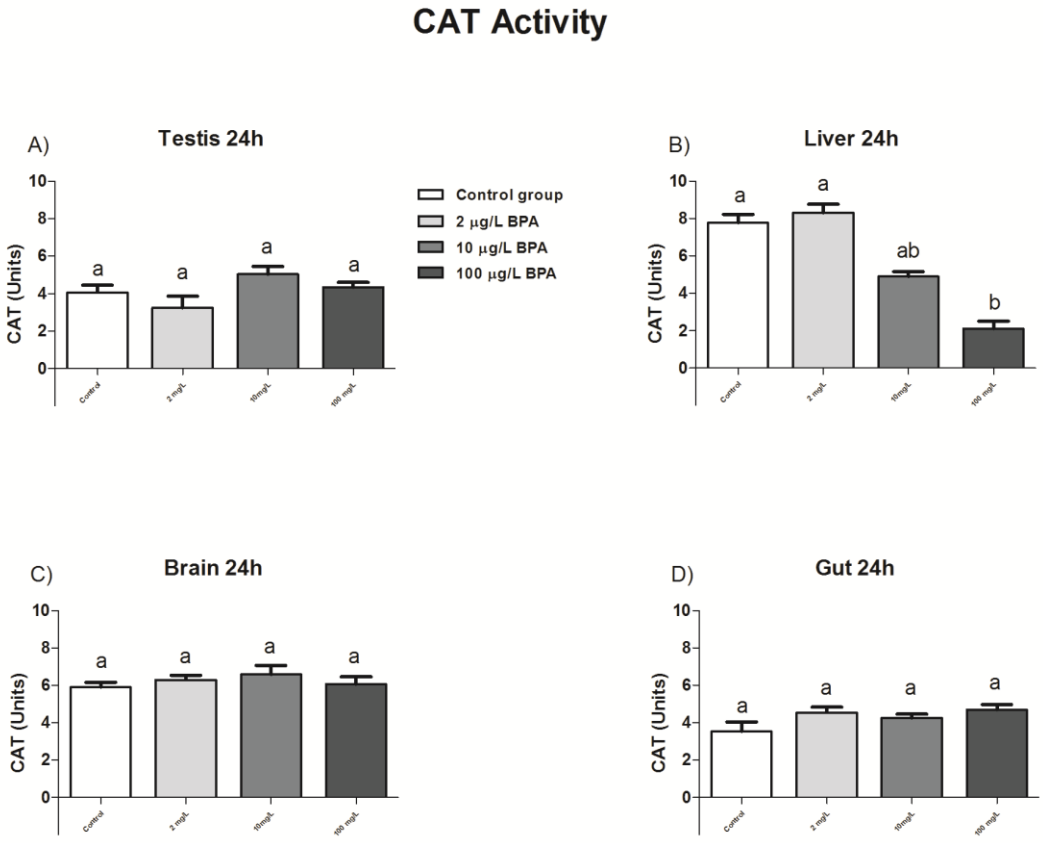

CAT Activity
A)
Testis 24h
B)
Liver 24h
C)
Brain 24h
D)
Gut 24h
CAT (Units)
Control group
2 µg/L BPA
10 µg/L BPA
100 µg/L BPA
a
ab
b

Figure 8

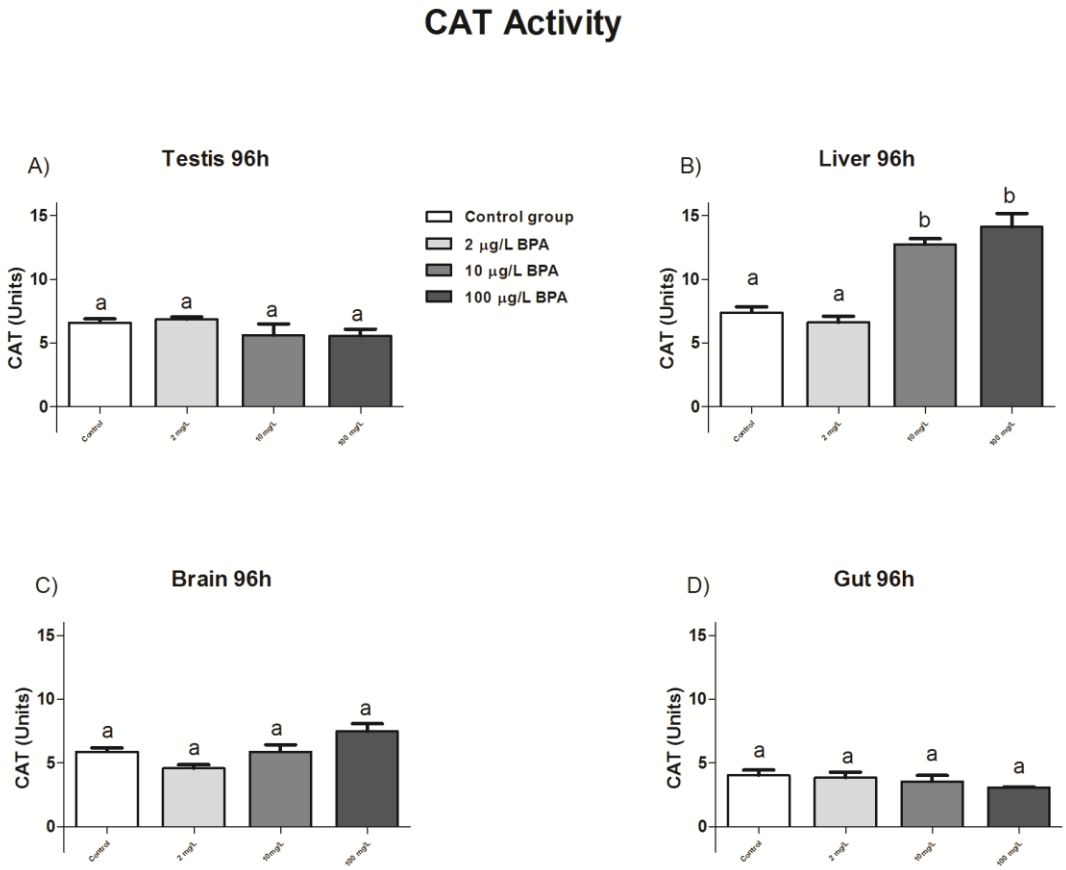

CAT Activity
A)
Testis 96h
B)
Liver 96h
C)
Brain 96h
D)
Gut 96h
CAT (Units)
Control group
2 µg/L BPA
10 µg/L BPA
100 µg/L BPA
a
b

Figure 9

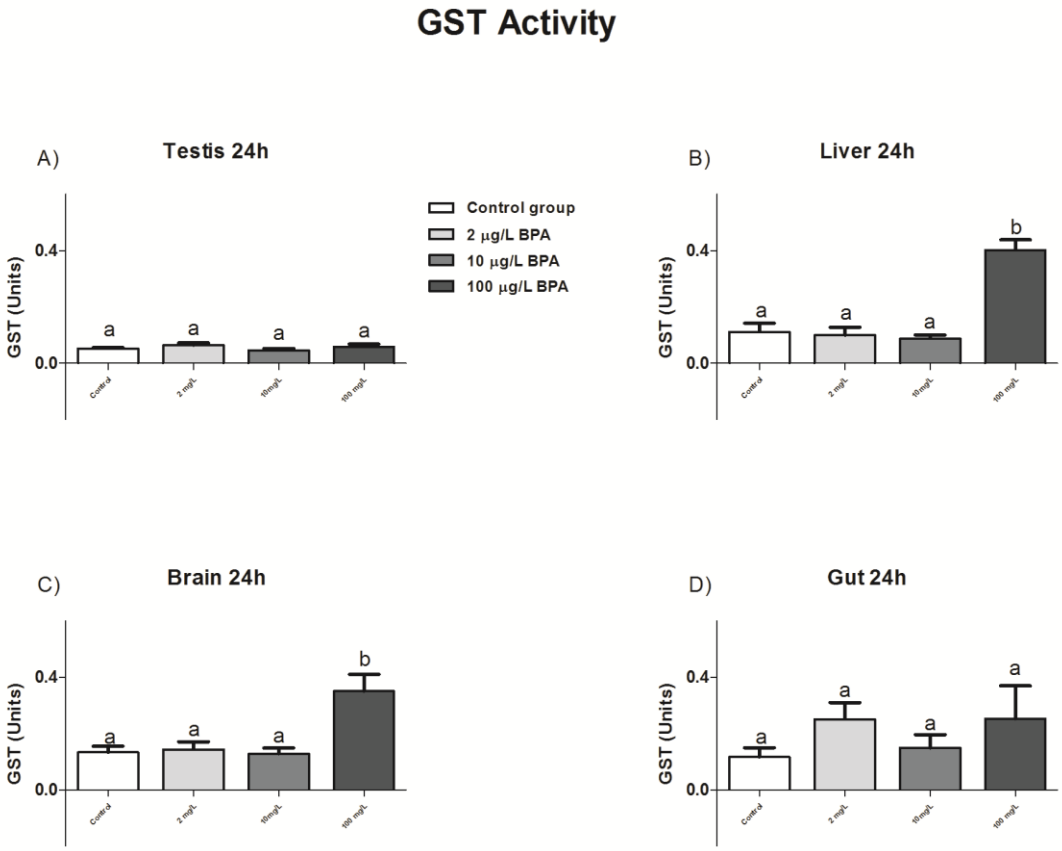
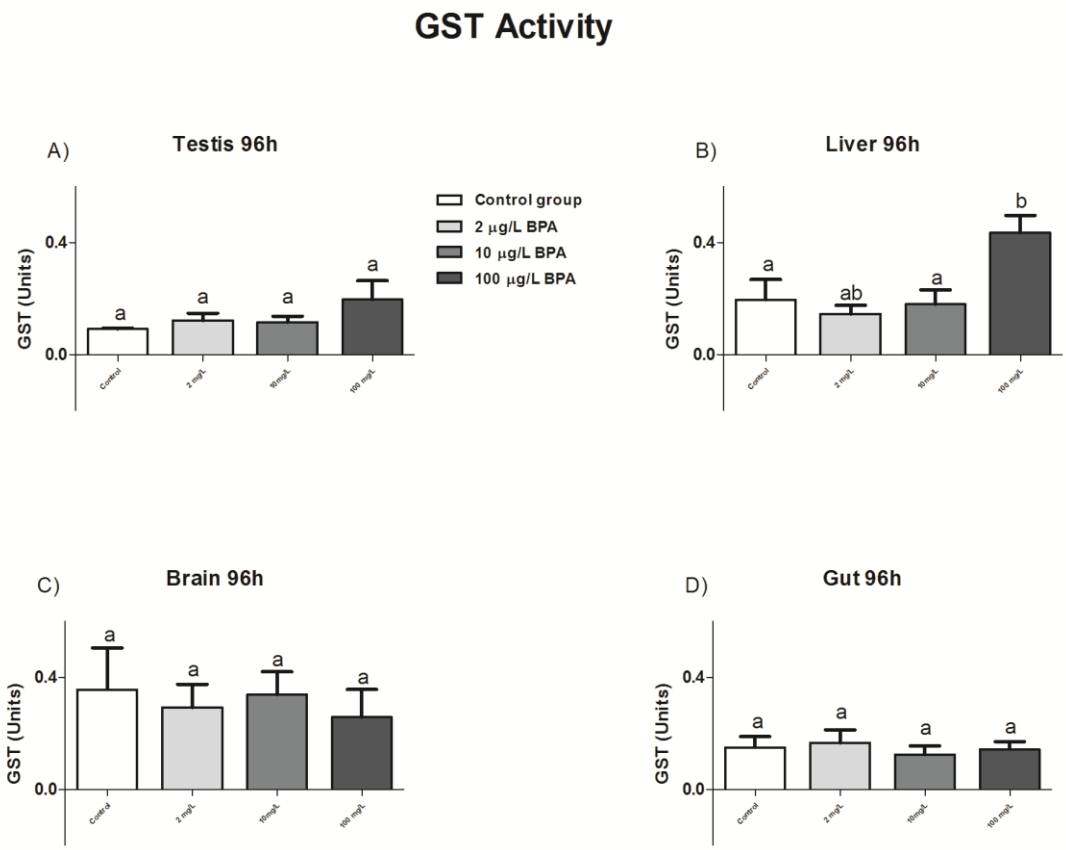

GST Activity
A)
Testis 24h
B)
Liver 24h
C)
Brain 24h
D)
Gut 24h
GST (Units)
0.4
0.0
a
b
Control group
2 µg/L BPA
10 µg/L BPA
100 µg/L BPA

Figure 10


GST Activity
A)
Testis 96h
B)
Liver 96h
C)
Brain 96h
D)
Gut 96h
GST (Units)
0.4
0.0
a
ab
b
Control group
2 µg/L BPA
10 µg/L BPA
100 µg/L BPA

Capítulo II

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Structural and ultrastructural analysis of gonadal histopathology zebrafish adults exposed to Bisphenol A

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Abstract

Bisphenol-A (BPA) is a chemical used as monomer for polycarbonates and epoxy resins production, and its production is major worldwide. This compound is considered an endocrine disrupter for various classes of vertebrates, but its effects in some specific groups such as fish, still are poorly studied. To check the BPA effects on spermatogenesis was chose a fish model extremely used in toxicological studies, the zebrafish (*Danio rerio*). In order to analyze tissue and cellular structure after chronic exposure to BPA (14 days), we expose the males at three concentrations: 2, 10 and 100 μg/L of BPA, and a negative control group. The first two concentrations (2 and 10 μg/L BPA) can be found in natural environments contaminated with BPA. After exposure, the males were anesthetized and their testes removed for further analysis in optical and electron microscope. Additionally, water samples were stored for further quantification of BPA by LC-MS/MS. The lowest concentration used showed no visible and significant differences from the control group in cell and tissue morphology of the testis. In contrast, the 10 and 100 μg/L concentrations showed defects that are potentially related damage to Sertoli cells, which play a central role in spermatogenesis. The changes include presence of immature cells, changes in *crossing-over*, break testicular wall and damages in Sertoli cells and germ cells. Moreover, in higher concentration, was possible to observe a malformation in a high number of sperm. This study therefore shown that chronic exposure to environmental concentrations of BPA was able to change various structural parameters of sperm cells of zebrafish, probably via damage to Sertoli cells.

Keywords: Bisphenol-A, Histopatology, Reproduction, Sertoli cells, Zebrafish

1.Introduction

Bisphenol A (BPA) is an important industrial chemical, widely used in the manufacture of polycarbonate, epoxy resins, chemical papers, dental sealants, etc. (Bermudez et al., 2010; Riu et al., 2011). This compound is one of the chemicals with highest volume of world production and persists in residual wastewater through incomplete polymerization or gradual release products containing BPA (Biedermann et al., 2010; Vandenberg et al., 2010; Zhang et al., 2013). Recent studies have demonstrated that BPA is an endocrine disrupter for various vertebrate classes (Vandenberg et al., 2010, 2015;Bandhari et al, 2015) and exposure in humans may be partly responsible for the increase in reproductive male disorders classified as testicular syndromes, including cryptorchidism, testicular cancer and low adulthood sperm production (Lucas et al., 2009). Its known that BPA can act interacting with reproductive hormones such as estradiol and testosterone, causing an imbalance in normal production of these hormones and consequent reproductive changes in various animal groups (Cheschenko et al., 2005; Suzawa and Ingraham, 2008; Vandenberg et al., 2009). BPA binds to estrogen receptors, interacting primarily with the ERα and ERβ receptors with greater affinity for the ERβ (Le et al., 2008; Suzawa and Ingraham, 2008; Vandenberg et al., 2009). Additionally can also bind to the androgen receptor (AR), present both activities, estrogenic and anti-androgenic (Bondesson et al., 2009).

Regarding the BPA effectsin fish, these include steroidogenic genes transcription activation, vitellogenin (Vtg) induction in males, impaired development and sex ratio, among others (Crane et al., 2007). Zebrafish exposed to BPA during the embryonic period had their differentiation/altered ratio associated with gonadal ontogeny. The fishes were fed diets containing 17β estradiol or BPA and were abnormal sex ratios (3,8F:1M) when fed with 100 mg/kg (Drastichova et al., 2005).

This data are mechanically informative, but ecologically irrelevant, since such concentrations are too high and generally not found in the environment (Drastichova et al., 2005).

It is known, however, that environmentally relevant concentrations may also contribute to the intersex conditions. For example, immature barbel (*Barbus sp.*) collected downstream from a source of pollution for BPA had a high incidence of intersex barbs that were observed upstream of the pollution source (Vigano et al., 2006). Whereas high BPA concentrations cause determination / proportion of abnormal sex in fish, amphibians, reptiles and birds, lower concentrations can cause changes in gonadal function, and therefore environmentally relevant concentrations the potential to alter the testicular structure of teleost fish, contributing for intersex conditions and infertility (Crain et al., 2007).

Spermatogenesis is a specific proliferation and differentiation process of germ cells that occurs in the testis, and is controlled primarily by endocrine activity (FSH and LH) and cell juntions mediators (Fiorini et al., 2004). Recent studies with ratsWistar have shown that BPA can affect spermatogenesis, reduce the sperm qualityand affect the hypothalamic-pituitary axis (Wisnieski et al., 2015). Moreover, was seen that BPA can interfere with the expression of junctional proteins of the Sertoli cells in mammals when exposed during the neonatal period, affecting spermatogenesis and generating infertility. Sertoli cells provide nutritional and physical support to the cells precursors of sperm (Fiorini et al., 2004; Salian et al., 2009). However, spermatogenesis studies under the effect of BPA on fish are still relatively scarce.

The zebrafish is considered a model for toxicological studies due to advantages, such as protocol established, small size, easy maintenance among others. Thus, this

paper aims to examine of the histological structure of the zebrafish testis exposed to different concentrations of BPA in a sub chronic time.

2. Material and methods

2.1. Exposure and sampling

The exposure was performed at the laboratory of toxicology and pharmacology of University of Cordoba (Cordoba, Spain). The photoperiod was 16 hours light / 8 hours dark, the temperature of 26±1°C and an oxygen saturation of 60%. The animals were fed twice a day (Supervit ®). The animals were randomly divided into four groups (n=12) in 25 liter tanks: control group and three groups of concentrations of 2, 10 and 100µg/L BPA (Sigma-Aldrich ®). Fish were exposed to BPA for 14 days following the guidelines of OECD (OECD, 1993), through a continuous flow regulated by programmed peristaltic pumps. After two weeks exposure, all animals were euthanized with an overdose of tricaine anesthetic solution (MS-222®, Aldrich® Sigma) at a concentration of 500 mg/L buffered to a concentration of 300 mg/L sodium bicarbonate (Sigma Aldrich®)

2.2. BPA content analysis in water

Three times a week, every water tank was sampled, keeping –20ºC until its analytical verification of BPA concentrations present. Prior to analysis, samples were thawed and subsequently processed with ammonium hydroxide, filtered through 0.22 micron and injected in a volume of 20 µL system LC-MS/MS, with a detection limit of 0.2 to 0.3 µg/L.

2.3 Histopathological analysis: optical and electronic microscopy

2.3.1. Structural study

Testes were fixed with 10% buffered formaldehyde at room temperature, dehydrated in ascending scale of ethanol and embedded in paraffin. The testicular samples of each block (4μm) were stained with hematoxylin/eosin for histopathology. The sections were observed and photographed under an optical microscope.

2.3.2.Ultra structural study

Testes were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer solution (pH 7.4) overnight and subsequently refixed with osmium tetroxide in 0.1 M phosphate buffer solution (pH 7.4) for 30 minutes. After dehydration in ascending scale of alcohols and inclusion in Araldita, the semithin and ultra-thin sections were performed in ultramicrotome LKB. The semithin sections were stained with toluidine blue, while for ultra-thin sections was used stained-double with uranyl acetate and lead citrate. The cuts for the ultra structural study were photographed and analyzed in a transmission electron microscope Philips CM10 (Philips Export BV).

3. Results and discussion

The clearest effects of BPA are the reproductive damages, which is relatively well studied in mammals. BPA acts primarily interacting with estrogen receptors, as an agonist or sometimes antagonist of these receptors, and therefore causing hormonal imbalance and structural changes in the brain, gonads and other organs of exposed animals (EPA, 2005; Crain et al., 2007, Vandenberg et al., 2015). Among the analyzes related to reproductive aspects, effects on spermatogenesis BPA currently seem to be the most sensitive reproductive endpoint in adult fish (Crane et al., 2007).

In this study we analyzed the structure and ultrastructure of zebrafish testis exposed to BPA and histopathological effects were observed mainly at the highest exposure concentrations, 10 and 100 μ/L, being more pronounced at the highest concentration used. BPA was maintained in constant water concentrations according with the data obtained by LC-MS / MS (Table 1). The concentrations used in this study are very similar to those used by different authors in several species of fish (Lindholst et al., 2000; Mandich et al., 2007, Villeneuve et al, 2012.). Coinciding with previously reported data, mortality was inconsiderable (less than 1%) during the period of exposure to BPA (Villeneuve et al., 2012). Furthermore, there were no signs of abnormal behavior during exposure.

Control group animals showed, as expected, a normal testicular structure with sperm cell groups organized into testicular cysts, Sertoli cells with normal appearance and completely viable sperm. The *Danio rerio* testis are paired organs, elongated and located symmetrically in the dorsal region of the celomic cavity between the ventral surface of the swim bladder, the liver and the dorsal part of the lateral. Within this testis, Sertoli cells surround germ cells forming cysts, where the germ wall cell groups develop synchronously and the spermatogenesis process occurs (Rupik et al., 2011). Testicular structure displayed in the control group allowed us to distinguish and characterize the different types and stages of the sperm cells (Fig.1).

The spermatogonia have dispersed chromatin, single nucleolus and many ribosomes in the cytoplasm. Already the primary spermatocytes appear as oval cells and having a structure denominated synaptonemal complex in the nucleus. Spermatids can be observed at different stages of their development, and finally the rounded head spermatozoa are out of the cysts in the lumen of the tubular region.

Testicular structure of animals exposed to 10 and 100 µg/L were found many adipocytes related to disruption of parenchyma, thus featuring a break testicular wall (Fig. 2). Oehlmann et al. (2000) observed cell wall breakage oviducts in molluscs exposed BPA and octylphenol (OP) in similar concentrations to those used in this study. Studies with mammalian demonstrate that several endocrine disrupters can cause damage in testicular membranes, and those caused by hormonal imbalance or for the generation of oxidative stress and lipid peroxidation (Yang et al., 2005; Xi et al., 2013; Jianjie et al., 2016; Kalb et al., 2016).

In primary spermatocytes, an important ultrastructure characteristic is the synaptonemal complex, which is a meiosis-specific structure essential to recombination of homologous chromosomes (*crossing-over*) (Iwai et al., 2005). When the animals were exposed to a concentration of 100 µg/L of BPA did not have the synaptonemal complex in the vast majority of its primary spermatocytes compared to primary spermatocytes in the control group (Fig. 3). This effect may be related to any changes caused directly or indirectly by BPA, possibly preventing the normal operation of crossing-over during meiosis. Studies in mammals have shown that some contaminants such as alcohol, the mycotoxin zearalenone and antimitotic agents can cause structural damage or changes inexpression of genes related to the synaptonemal complex (Obe and Ristow, 1979; Allen et al., 1988; Zatecka et al, 2014). Studies concerning the presence of contaminants and damage in synaptonemal complex in fish, however, are extremely scarce. BPA has been shown to be mutagenic in germ cells and sperm of rats, influencing sperm motility and fertility of the animals (Tiwari and Vanage, 2013).

At concentrations of 10 and 100 µg/L, several tubular parts with abnormal sperm counts were observed, and when these areas were observed in transmission microscopy is shown defective spermatozoa with changes that included high amount of

cytoplasm and atrophied tails (Fig. 4). Likewise, when Fathead minnow (P*imephales promelas*) were exposed to 16 µg/L or higher concentrations of BPA, their testis significantly reduced the number of mature spermatozoa and increased the number of immature sperm in their seminiferous tubules (Sohoni et al., 2001). Furthermore, Guppies (*Poecilia reticulata*) exposed to 294 µg/L BPA for 21 days obtained significantly reduced sperm count (Haubruge et al., 2000). In rats, exposure to BPA reduced sperm production and caused significant damage to acrosome, the plasma membrane and reduced mitochondrial activity (Wisniewski et al., 2015). In addition to the histopathological alterations, decreased motility and velocity of sperm in goldfish exposed to environmentally relevant concentrations of BPA suggests that changes in the structure and sperm function are associated with deficits in steroidogenesis and changes in sperm maturity (Hatef et al., 2010). Although the mechanisms by which they occur all these effects of BPA in sperm quality are not yet fully elucidated, it is suggested that the damage observed in Sertoli cells can be closely related to the changes observed in the epididymis of animals, since these cells they are responsible for nutritional support and much of the hormone synthesis in spermatogenesis fish.

The process cytoplasmatic of Sertoli cells surrounding the germ cells at different stages of development since the beginning of spermatogenesis until the final stages, and bind to these cells through junctional protein complexes (Rupik et al., 2011). Maintenance of these cells in normal operation is extremely important for reproductive success of vertebrates, since it is responsible for providing physical support, perform phagocytosis residual bodies, and the formation of hemotesticular barrier (Griswold and Russel, 1993; Andrade et al., 2001; Rupik et al, 2011.). In this sense, structural analysis in animals exposed to 100 µg/L showed that Sertoli cells appear to be damaged and with little association to spermatic cells. This featuring an

abnormal situation where this cells are not possibly performing its role efficiently (Fig. 5).

Studies in mammals showed that BPA can affect protein cell junction / occlusion Sertoli cells, such as occludins and cadherins, which can cause problems in the production of spermatozoa and therefore effects on fertility of the animals (Fiorini et al., 2004, Salian et al., 2008). It was observed in rats that a low dose of 2.4 µg/Kg was capable of altering the expression of Sertoli cell junction proteins, reducing the expression of proteins such as Cx-43 and increased the expression of N-cadherin and ZO-1, suggesting so that BPA is a potential toxicity Sertoli cells (Salian, et al., 2008). Furthermore, *in vitro* studies demonstrate that exposure to BPA may cause apoptosis in mammalian Sertoli cells (Iida et al., 2003). Experiments with fish suggest that BPA is a toxic compound on the Sertoli cells, acting for example by changing hormone receptors present on these cells (Higaki et al., 2013). Finnaly, related damage to Sertoli cells may have consequences of oxidative stress caused by BPA, as previously observed in studies with ratsthat showed that BPA is probably able to generate intracellular ROS and trigger apoptotic cascades in Sertoli cells (Ge et al, 2014; Qian et al, 2014).

4.Conclusion

This study showed that chronic exposure to environmental concentrations of BPA was able to alter many structural parameters of zebrafish spermatogenesis. These alterations are via damage to Sertoli cells, probably through the generation of oxidative stress or or direct damage to cell junction proteins.

5. References

Allen, J., Gibson, J., Poorman, P. Backer, L.C., Moses, M.J., 1988. Synaptonemal complex damage induced by clastogenic and anti-mitotic chemicals" implications for non-disjunction and aneuploidy. Mut. Res. 202, 313-324.

Andrade, R. F., Bazolli, N., Rizzo, E., Sato, Y., 2001. Continuous gametogenesis in the neotropical freshwater teleost, *Bryconops affinis* (Pisces: Characidae). Tiss. Cell. 33, 524-532.

Agency EP. 2005. Cross-species mode of action information assessment: a case study of bisphenol A. National Center for Environmental Assessment; EPA 600 R05/044.

Bermudez, D.S., Gray Jr., L.E., Wilson, V.S., 2010. Modeling the interaction of binary and ternary mixtures of estradiol with bisphenol A and bisphenol AF in an in vitro estrogen-mediated transcriptional activation assay (T47D-KBluc). Toxicol. Sci. 116, 477–487.

Biedermann, S., Tschudin, P., Grob, K., 2010. Transfer of bisphenol A from thermal printer paper to the skin. Anal.Bioanal. Chem. 398, 571-576.

Bondesson, M., Jonsson, J., Pongratz, I.,Olea, N., Cravedi, J.P., Zalko, D., Hankansson, H., Halldin, K., Di Lorenzo, D., Behl, C., Manthey, D., Balagher, P., Demeneix, P., Fini, J.B., Laudet, V., Gustafsson, J., 2009. A cascade of Effects of Bisphenol A.Reprod. Toxicol.28, 563-567.

Buhler, D.R., Williams, D.E., 1988. Oxidative stress in gill, erythrocytes, liver andkidney of Nile tilapia (*Oreochromis niloticus*) from a polluted site. Aquat.Toxicol. 11, 19–28.

Callard G., Tchoudakova A., Kishida M., Word E., 2001. Differential tissue distribution, developmental programing, estrogen regulation and promoter characteristics of *cyp19* genes in teleost fish. J. Ster. Biochem. Mol. Biol, 79, 305–314.

Cavalli, V., Cattani, D., Heinz Rieg, C.E., Zanatta, L., Bendetti, P., Wilhelm, D., Mena, F., Pessoa, R., Zamoner, A., 2013.Roundup disrupts male reproductive functions by triggering calcium-mediatedcell death in rats testis and Sertoli cells. Free Radic. Biol. Med. 65, 335–346.

Cheshenko K., Pakdel F., Segner H., Kah, O., Eggen., R., 2008. Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. Gen. Comp. Endocrinol. 155, 31–62.

Crane, M., Burton, G.A., Culp, J.M., Greenberg, M., Munkttrick, K., Ribeiro, R., Salazar, M.H., St-Jean., S.D., 2007. Review of aquatic in situ approaches for stressor and effect diagnosis. Integr. Environ. Assess. Manage. 3, 234–245.

Drastichova, J., Svobodova, Z., Groenland, M., Dobsikova, V., Zlabek, D., Weissová, M., 2005. Effect of exposure to bisphenol A on the sex differentiation in zebrafish (*Danio rerio*). Acta. Vet. Brno. 74, 287–291.

Fiorini, C., Tilloy-Ellul, A., Chevalier, S., Chawel, C., Pointis, J., 2004. Sertoli cell junctional proteins as early targets for different classes of reproductive toxicants. Reprod. Toxicol. 18, 413-421.

Forlano P., Schlinger B., Bass A., 2006. Brain aromatase: New lessons from non-mammalian model systems. Front. Neuroendocrinology. 27, 247–274.

Ge, L., Chen, Z., Liu, H., Zhang, K., Su, Q., Xu, M., Huang, H., Zhao, Z., Wang, Y., Giesy, J., Du, J., Wang, H., 2014.Signaling related with biphasic effects of bisphenol A (BPA) on Sertoli cell proliferation: A comparative proteomic analysis. Biochim. Biophys. Acta. 1840, 2663-2673.

Hachfi L., Couvray S., Simide R., Tarnowska, K., Pierre, S., Gaillard, S., Richard, S., Coupé, S., Grillasca, J., Ahuse, N., 2012. Impact of Endocrine Disrupting Chemicals

[EDCs] on Hypothalamic-Pituitary-Gonad-Liver [HPGL] Axis in Fish. Wor. J of Fish Mar. Sci. 4, 14-30.

Hatef, A., Alavi, S., Linhartova, Z., 2010. In vitroeffects of bisphenol A on sperm motility characteristics in *Percafluviatilis L.* (Percidae; Teleostei). J. Appl. Ichthyol. 26, 696–701.

Haubruge E., Petit F., Gage M.J., 2000. Reduced sperm counts in guppies (*Poecilia reticulata*) following exposure to low levels of tributyltin and bisphenolA. Proc. Biol. Sci.1459, 23-33.

Hinfray N., Palluel O., Turies C., Cousin, C., Porcher, J.M., Brion, F., 2006.Brain and Gonadal Aromatase as Potential Targets of Endocrine Disrupting Chemicals in a Model Species, the Zebrafish (*Danio rerio*). Environm. Toxicol. 332, 4-7.

Higaki, S., Koyama, Y., Shimada, M., Ono, Y., Tooyama, I., Fujioka, Y., Sakai, N., Ikeuchi, T., Takada, T., 2013. Response to fish specific reproductive hormones and endocrine disrupting chemicals of a Sertoli cell line expressing endogenous receptors from an endemic cyprinid *Gnathopogoncaerulescens*. Gen. Comp. Endocrinol.191, 65-73.

Huang H. and Leunga L., 2009. Bisphenol A downregulates CYP19 transcription in JEG-3 cells. Toxicol. Lett. 189, 248–252.

Iida, H., Maehara, K., Doiguchi, M., Mõri, T., Yamada, F., 2003. Bisphenol A-induced apoptosis of cultured rat Sertoli cells. Reprod. Toxicol. 17, 457-464.

Iwai, T., Yoshii, A.,Yokota, T., Sakai, C., Hori, H., Kanamori, A., Yamashita, M., 2005. Structural components of the synaptonemal complex, SYCP1 and SYCP3, in the medaka fish *Oryzias latipes*. Experim. Cell. Res. 13, 2528- 2537.

Jianjie, C., Wenjuan, X., Jinling, C., Jie, S., Ruhui, J., Meyan, L., 2016. Fluoride caused thyroid endocrine disruption in male zebrafish (*Danio rerio*). Aq. Toxicol. 171, 48-58.

Kalb, A.C., Kalb, A.L., Cardoso, T.F., Fernandes, C.G., Corcini, C.D., Junior, A.S., Martínez, P.E., 2016.Maternal transfer of bisphenol A during nursing causes sperm impairment in male offspring. Arch. Environ. Contam. Toxicol. 70- 793-801.

Lassiter C. and Linney E., 2007. Embryonic Expression and Steroid Regulation of Brain Aromatase *cyp19a1b* in Zebrafish (*Danio rerio*). Zebrafish 4, 49-57.

Le H., Carlson E., Chua J., Belcher S., 2008.Bisphenol A is released from polycarbonate drinking bottles and mimics the neurotoxic actions of estrogen in developing cerebellar neurons. Toxicol.Lett. 176, 149–156.

Lindholst., C., Wynne, P., Marriott, P., Pedersen, S.N., Bjerregaard, P., 2003. Metabolism of bisphenol A in zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*) in relation to estrogenic response. Comp. Biochem. Phys. C. 135, 169-177.

Liu, S., Qin, F., Wang, H., Wu, T., Zhang, Y., Zheng, Y., Li, H., Wang, Z., 2012.Effects of 17alpha-ethinylestradiol and bisphenolA on steroidogenic messengerribonucleic acid levels in the rare minnow gonads. Aquat.Toxicol. 122, 19–27.

Lucas, B., Fields, C., Hofmann, M.C., 2009. Signaling pathways in spermatogonial stem cells and their disruption by toxicants. Birth Defects Res. C: Embryo Today. 87, 35–42.

Mandich A., Bottero S., Benfenati E., Cevasco, A., Erratico, C., Maggioni, S., Massari, A., Pedemonte, F., Vigano, L., 2007. *In vivo* exposure of carps to graded concentrations of bisphenol A. Gen. Comp. Endocr. 153,15-24.

Molina, A., Lora, A., Blanco, A., Monterde, J., Ayala, N., Moyano, R., 2013.Endocrine-active compound evaluation: Qualitative and quantitative histomorphological assessment of zebrafish gonads after bisphenol-A exposure. Ecotox. Environ. Safe. 88, 155-162.

Mouriec K.,Lareyre J.J., Tong S.K., Le, Y., Vaillant, C., Pellegrini, E., Pakdel, F., Chung, B.C., Kah, O., Anglade, I., 2009. Early Regulation of Brain Aromatase (*cyp19a1b*) by Estrogen Receptors During Zebrafish Development. Develop. Din. 238, 2641-2651.

Obe, G. and Ristow, H., 1979. Mutagenic, cancerogenic and teratogenic effects of alcohol. Mut. Res. 65, 229-259.

OECD (1993) OECD Guidelines for Testing of Chemicals: Fish, Prolonged Toxicity Test: 14-Day-Study. Section 2, Guideline 204.

Oehlmann, J., Bauer, B., Minchin, D., Schulte- Oehlmann, U., Fioroni, P., Markert, B., 1998. Imposex in Nucella lapillus and intersex in Littorina littorea: interspecific comparison of two TBT-induces effects and their geographical uniformity. Hydrobiology. 378, 199–213.

Qian, W., Zhu, J., Mao, C., Liu, J., Wang, Y., Wang, Q., Liu, Y., Gao, R., Xiao, H., Wang, J., 2014. Involvement of CaM- CaMKII-ERK in bisphenol A - induced Sertoli cell apoptosis. Toxicology. 324, 27-34.

Rhee, J.S., Kim, B.M., Lee, C., Yoon, Y., Lee, Y., Lee, J., 2011.Bisphenol Amodulates expression of sex differentiation genes in the self-fertilizing fish *Kryptolebias marmoratus*.Aquat. Toxicol. 104, 218–229.

Riu, A., Grimaldi, M., le Maire, A., Bey, G., Phillips, K., Boulahtory, A., Perdu, E., Zalko, D., Bourguet, W., Balaguer, P., 2011. Peroxisome proliferator-activated receptor c is a target for halogenated analogs of bisphenol A. Environ. Health Perspect. 119, 1227–1232.

Rupik, W., Huszno, J.,Klag, J., 2011.Cellular organisation of the mature testes and stages of spermiogenesis in *Danio rerio* (Cyprinidae; Teleostei)—Structural and ultrastructural studies.Micron.42, 833- 839.

Russell, L.D., Griswold, M.D., 1993. The Sertoli Cell. Cache River Press, Clearwater, FL.

Salian, S., Doshi, T., Vanage, G., 2009. Neonatal exposure of male rats to Bisphenol A impairs fertility and expression of sertoli cell junctional proteins in the testis. Toxicology. 265, 56-67.

Schimdt K., Stachel C., Gowik P., 2008. Development and in house validation of an LC-MS/MS method for the determination of stilbenes and resorcylic acid lactones in bovine urine. Anal.Bioanal. Chem. 391,1199-1210.

Soffker, M., Tyler, C.R., 2012. Endocrine disrupting chemicals and sexual behaviorsin fish—a critical review on effects and possible consequences. Crit. Rev.Toxicol. 42, 653–668.

Sohoni, P., Tyler, C.R., Hurd, K., Caunter, J., Heitheridge, M., Williams, T., Woods, C., Evans, M., Toy, R., Gargas, M., Sunpter, J., 2001. Reproductive effects of long term exposure to bisphenol A in the fathead minnow (*Pimephales promelas*). Environ. Sci. Technol. 35, 2917–2925.

Suzawa, M., Ingraham, H., 2008 The Herbicide Atrazine Activates Endocrine Gene Networks via Non-Steroidal NR5A Nuclear Receptors in Fish and Mammalian Cells. PLoS ONE 3.5,1-11.

Tabata, A., Watanabe, N., Yamamoto, I., Ohnishi, Y., Itoh, M., Kamei, T., Magara, Y.,Terao, Y., 2004. The effect of bisphenol A and chlorinated derivatives of bisphenol A on the level of serum vitellogenin in Japanese medaka (*Oryziaslatipes*). Water Sci. Technol. 50, 125–132.

Tamiselvan, P., Langeswaran, K., Vijayaprakash, S., Kumar, S., Revhaty, R., Balasubramanian, M., 2014. Efficiency of lycopene against reproductive and

developmental toxicity of Bisphenol A in male Sprague Dawley rats. Biomed. Prev. Nutr. 4, 491-498.

Tanabe, N., Yoshino, H., Kimoto, T., Hojo, Y., Ogiue-Ikeda, M., Shimohigashi, Y., Kawato, S., 2012. Nanomolar dose of bisphenol A rapidly modulates spinogenesis in adult hippocampal neurons. Mol. Cell. Biol. 351, 317-325.

Tiwari, D., Vanage, G., 2013. Mutagenic effect of Bisphenol A on adult rat male germ cells and their fertility. Reprod.Toxicol. 40, 60-68.

Tong, S., Chung, B.,2003. Analysis of zebrafish *cyp19* promoters. J. Ster. Biochem. Mol. Biol. 86, 381–386.

Vandenberg L., Maffini M., Sonnenschein C., Rubin, B.S., Soto, A.M., *et al*.,2009. Bisfenol-A and the great divide: A review of controversies in the field of endocrine disruption. End. Rev. 30, 75–95.

Vandenberg, L., Chahoud, I., Heindel, J.J., Padmanabhan, V., Paumgatten, F.J., Schoenfelder, G., 2010. Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A. Environ. Health Perspect. 118, 1055-1070.

Vigano, L., Mandich, A., Banfenati, E., Bertolotti, R., Bottero, S., Porazzi, E., Agradi, E., 2006. Investigating the estrogenic risk along the river Po and its intermediate section. Arch. Environ. Contam. Toxicol. 51, 641–51.

Villaneuve D.L., García-Reyero N., Escalon B.L., Jensen, K.H., Cavallin, J.E., Makynem, E.A., Duhan, E.J., Kahl, M.D., Thomas, L., Perkins, E., Ankley, G.T., 2012. Ecotoxicogenomics to support ecological risk assessment: A case study with bisphenol A in fish. Environ. Sci. Technol. 46, 51-59.

Xi, Y., Li, D., San, W., 2013.Exposure to the endocrine disruptor nonylphenol alters structure and function of thyroid gland in rats. Regul.Pept. 185, 52-26.

Yanagida, T., Tsushima, J., Kitamura, Y., Yagagisawa, D., Takata, K., Shibaike, T., Yamamoto, A., Tanigushi, T., Yasui, H., Taira, T., Morikawa, S., Inubushi, T., Tooyama, I., Ariga, H., 2009.Oxidative stress induction of DJ-1 protein in reactive astrocytes scavenges free radicals and reduces cell injury. Oxid. Med. Cell. Longev. 2, 36-42.

Yang., S., Han, X., Wei, C., Chen, J., Yin, D., 2005. The toxic effects of pentachlorophenol on rat Sertoli cells in vitro. Enviromn.Toxicol. Pharmacol. 20, 182-187.

Wang, J., Liu, X., Wang, H., Liu, X., Duan, J., Xie, S., Zhao, H., 2010. Expression of two cytochrome P450 aromatase genes is regulated by endocrine disrupting chemicals in rare minnow *Gobio cyprisrarus* juveniles. Comp. Biochem. Physiol. Toxicol. Pharmacol. 152, 313–320.

Wisniewski, P., Romano, R.M., Kizys, M., 2015. Adult exposure to bisphenol A (BPA) in Wistar rats reduces sperm quality with disruption of the hypothalamic–pituitary–testicular axis.Toxicology. 329, 1–9.

Zatecka, E., Ded, L., Elzeinova, F., Kubatova, A., Dorosh, A., Margaryan, H., Doslotova, P., Korenkova, V., Hoskova, K., Pernicova, J., 2014. Effect of zearalenone on reproductive parameters and expression of selected testicular genes in mice. Reprod. Toxicol. 45, 20-30.

Zhang, T., Sun, H., Kannan, K., 2013. Blood and urinary bisphenolA concentrations in children, adults, and pregnant women from China: partitioning between blood and urine and maternal and fetal cord blood. Environ. Sci. Technol. 47, 4686-4694.

Captions

Table 1. Bisphenol A nominal and real concentrations (µg/L) in water expressed by mean ± SD.

Figure 1. (A) Light microscope. Bars 50 µm. (B) Ultrastructural observations. Bars 2 µm.(A) Control group: cystic structure of spermatogenesis in teleost, demonstrating sperm cells at different levels of development stages. (B) Control group: ultrastructural view demonstrating cysts in different stages of sperm cells. STC- Sertoli cells; SG- Spermatogonia; PSC- Spermatocyte; SSC- Secondary spermatocyte; SPM- Spermatids; SZ- Spermatozoa.

Figure 2. Histopathological changes in zebrafish testis exposed to 10 and 100 µg/L of bisphenol-A. (A), (C) and (E): Light microscope. Bars, 20 µm. (B), (D) and (F): ultrastructural observations. Bars, 2 µm. (A) Control group: cystic structure associated with Sertoli cells.(B) Control group: ultrastructure of Sertoli cells and few adipocytes (C) 10µg/L BPA: larger number of adipocytes among cystic structures of testicular tissue. (D) 10µg/L BPA: ultrastructure Sertoli cells and adipocytes (E) 100µg/L BPA: impaired cystic structure and higher number of adipocytes, indicating parenchymal disruption (F) 100µg/L BPA: Ultrastructure of damaged Sertoli cells and adipocytes. AD- Adipocytes STC- Sertoli cells; SG- Spermatogonia; SPM- Spermatids; SZ- Spermatozoa.

Figure 3. Histopathological changes in zebrafish testis exposed to 100µg/L of bisphenol-A. (A) and (C): Light microscope. Bars, 20µm. (B) and (D): ultrastructural observations.Bars, 2µm. (A) Control group: structure demonstrating cysts with primary spermatocytes (B) Control group: ultrastructure of primary spermatocytes with presence of synaptonemal complex (C) 100µg/L BPA: structure demonstrating cysts

with primary spermatocytes (D) 100µg/L BPA: ultrastructure of primary spermatocytes with absence of synaptonemal complex. STC- Sertoli cells; SG- Spermatogonia; PSC- Spermatocyte; SN- Synaptonemal complex SSC- Secondary spermatocyte; SPM- Spermatids

Figure 4. Histopathological changes in zebrafish testis exposed to 100µg/L of bisphenol-A. (A) and (C):Light microscope. Bars, 20µm. (B) and (D) ultrastructural observations. Bars, 2µm. (A) Control group: cystic structure demonstrating cysts of spermatids and differentiation into sperm (B) Control Group: ultrastructure of mature spermatozoa (C) 100µg/L BPA: impaired cystic structure containing spermatozoa (D) 100µg/L BPA: ultrastructure of damaged spermatozoa demonstrating high amount of residual cytoplasm. AD- Adipocytes STC- Sertoli cells; SG- Spermatogonia; PSC- Spermatocyte; SZ- Spermatozoa; N = Nucleous; Ce- Centriole; M= Mitochondria; F- Flagellum; C= cytoplasm

Figure 5. Histopathological changes in zebrafish testis exposed to 10 and 100µg/L of bisphenol-A. (A),(C) and (E):Light microscope. Bars, 20 µm. (B),(D) and (F): ultrastructural observations. Bars, 2µm. (A) Control group: cystic structure and Sertoli cells (B) Ultrastructure of Sertoli cell (C) 10µg/L BPA: cystic structure and Sertoli cells (D) 10µg/L BPA: Ultrastructure of Sertoli cell (E) 100µg/L BPA: structure cystic impaired and Sertoli cells among the rare cells sperm cysts (F) 100µg/L BPA: Ultrastructure of damaged Sertoli cells. STC- Sertoli cells; LC- Leydig cells; SG- Spermatogonia; PSC- Spermatocyte;SPM- Spermatids; SZ- Spermatozoa

Table 1

	BPA nominal concentration			
	Control	2 µg/L	10 µg/L	100 µg/L
BPA concentration in water (mean 14 days)	Non-detected	1,5888 ± 0,1188	6, 272 ± 0,8084	70,0875 ± 3,9686

Figure 1

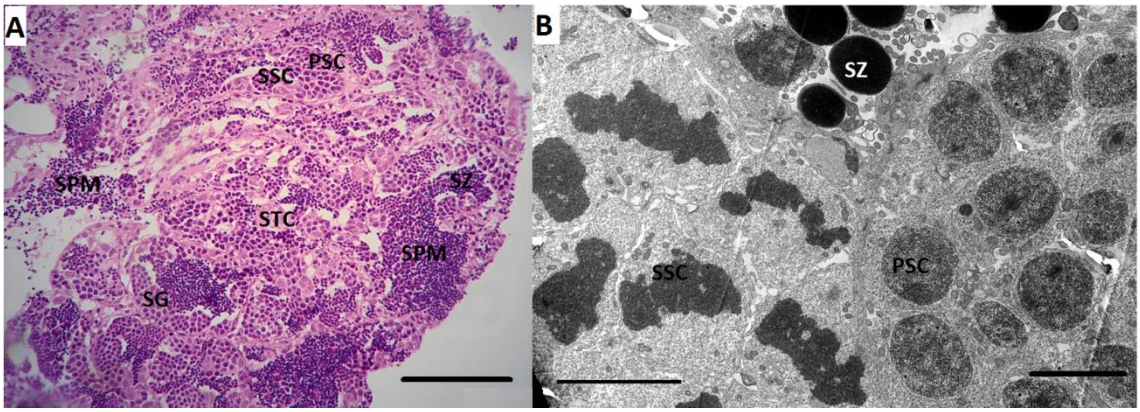

Figure 2

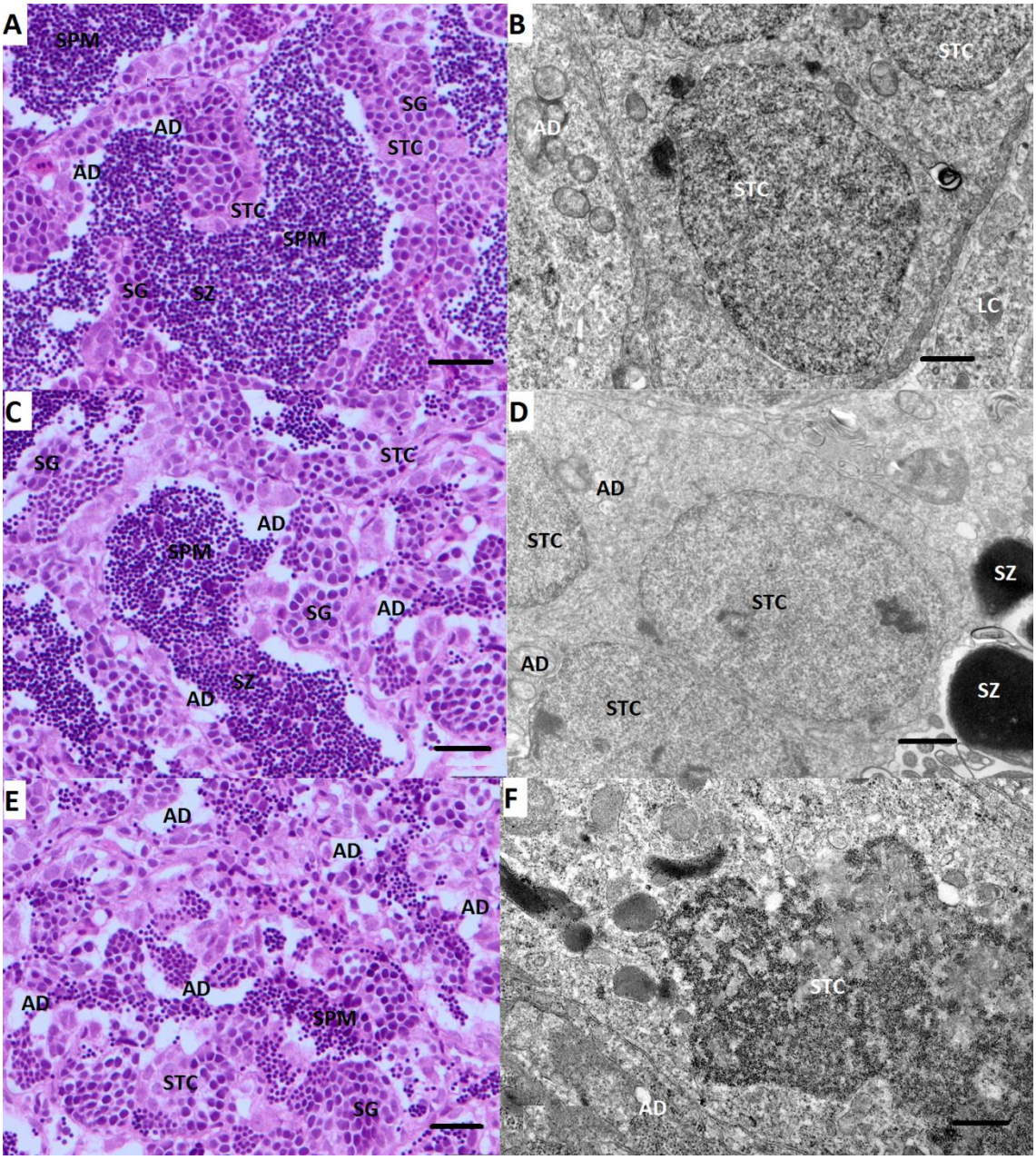
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Figure 3

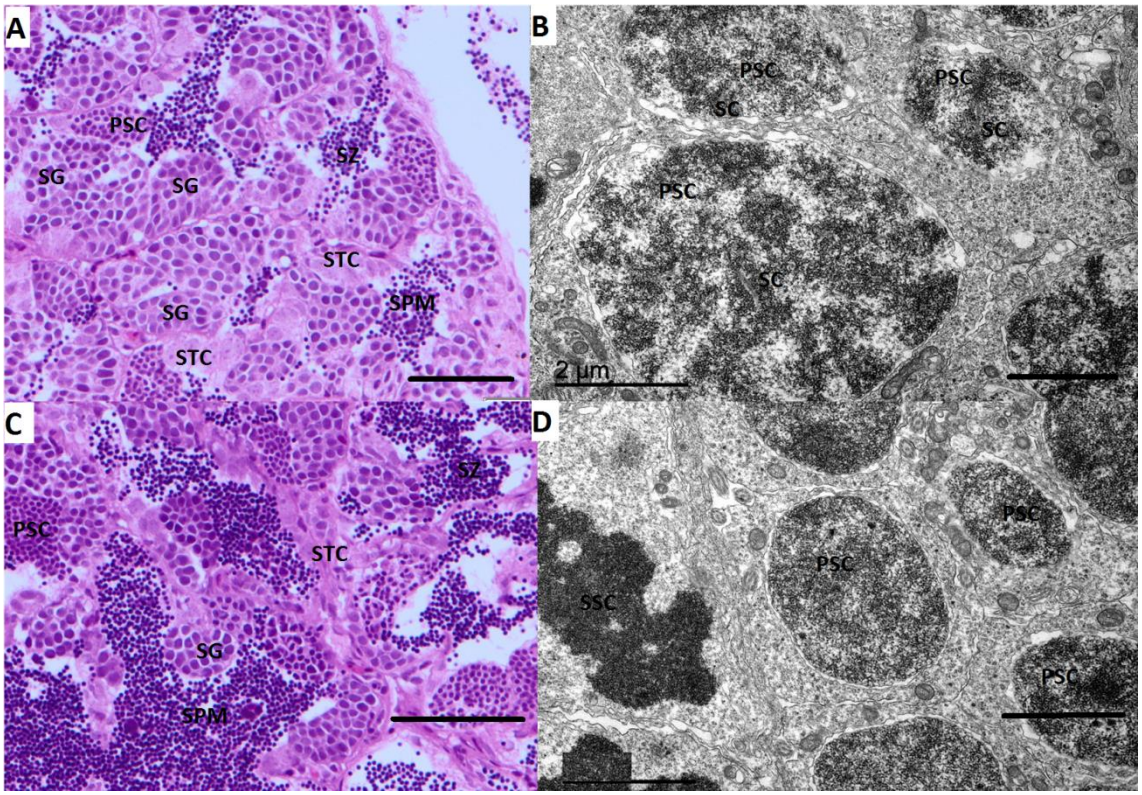


Figure 4

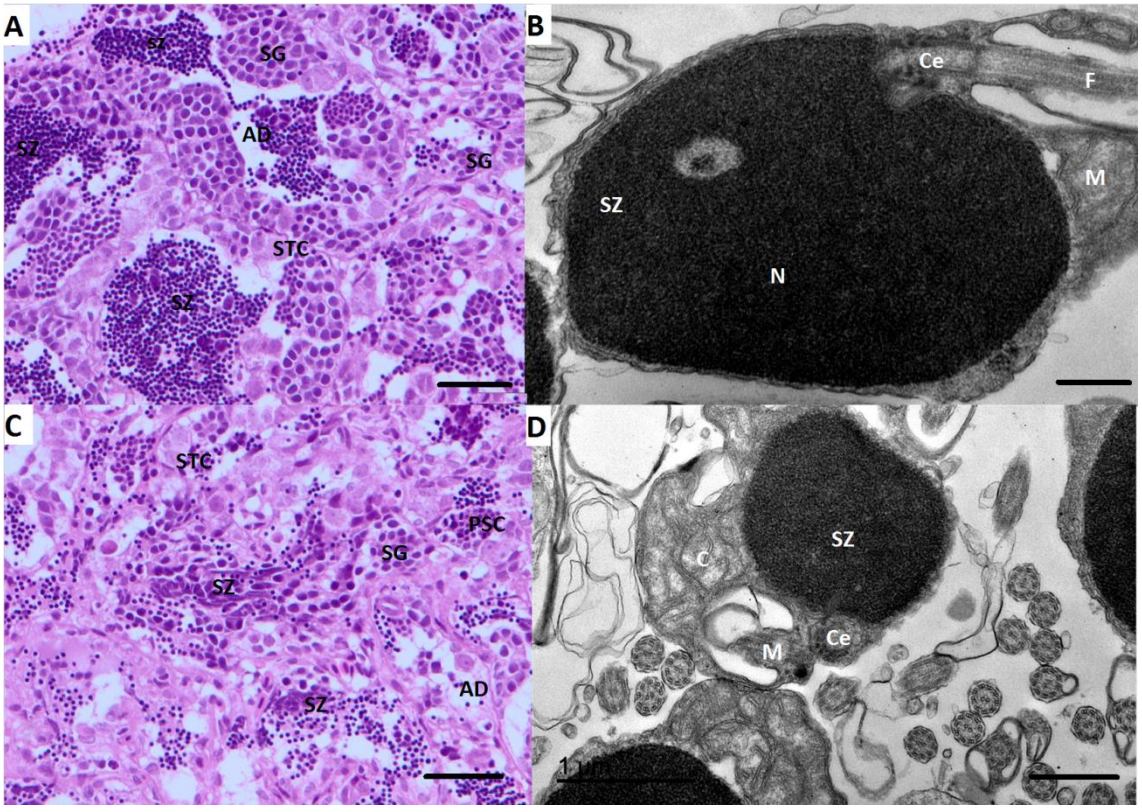
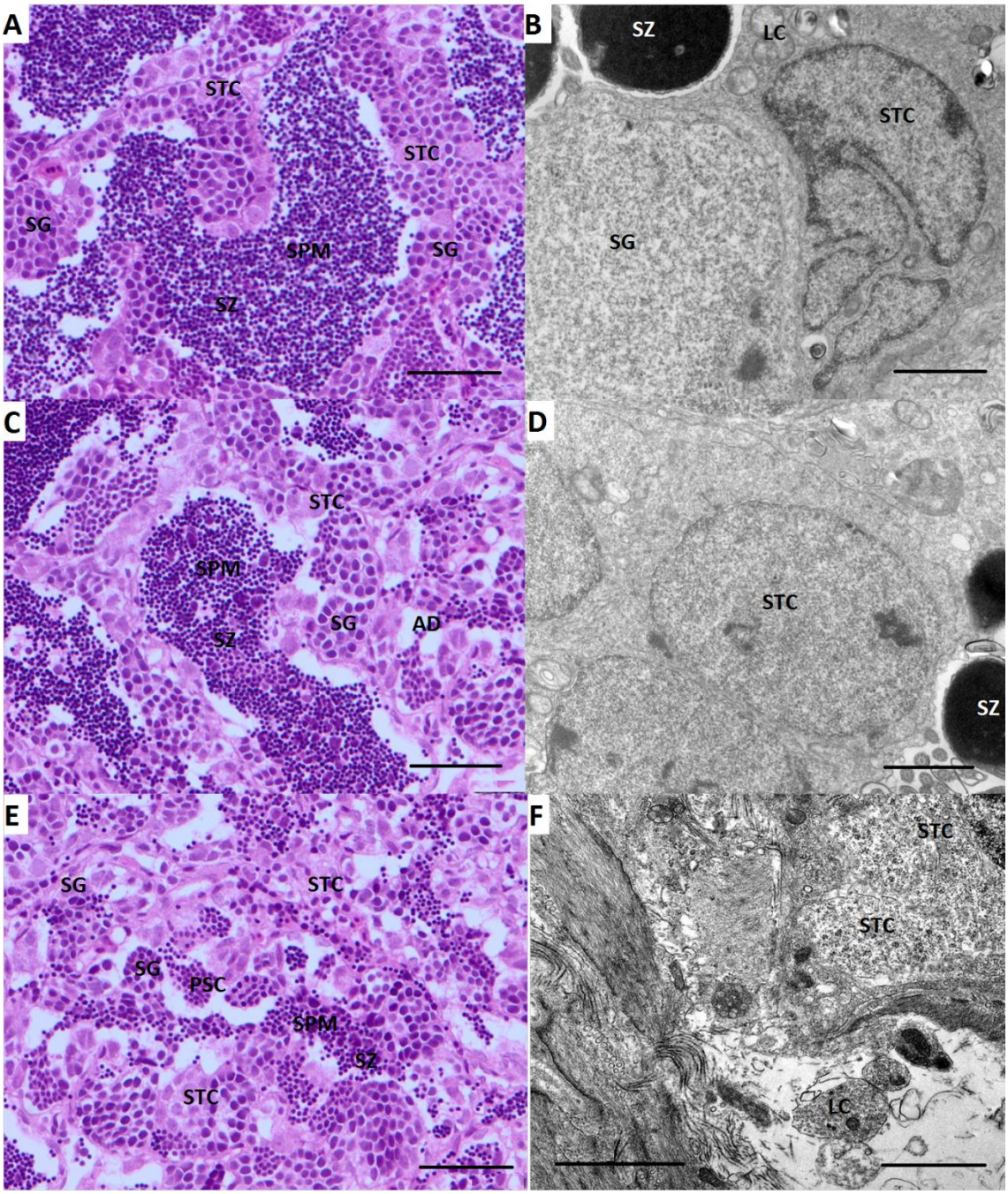


Figure 5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DISCUSSÃO GERAL

A interferência de contaminantes na vida dos animais tem sido bem relatada ao longo das últimas décadas, e estudos demonstram que um número cada vez maior desses químicos estão disponível em quantidades ambientais que são capazes de gerar danos irreversíveis e em todos os grupos animais (Crain et al., 2007). Entre esses contaminantes, os desreguladores endócrinos têm recebido atenção especial por seus diferentes efeitos dependentes de dose, de estágio de vida, tecido, etc. (Vandenberg et al., 2015).

Existe uma grande discussão principalmente nos países desenvolvidos, relacionadas às essas doses e concentrações e aos testes relacionados por organizações nacionais para estabelecer essas doses. Nas últimas décadas, uma série de especialistas concluiu que não há provas suficientes de que as exposições a baixas doses de BPA pode alterar o desenvolvimento de órgãos e sistemas específicos, incluindo o cérebro, a próstata, glândula mamária, entre outros (Chapin et al., 2008; Richter et al., 2007; vom Saal et al., 2007). Apesar de existirem estudos que não conseguem identificar os efeitos de BPA em doses baixas (Delclos et al, 2014; Tyl et al, 2008) muitos destes estudos tem falhas significativas, como poucos exemplares ou ausência de controles adequados, que reduzem dessa forma a sua qualidade informativa. Nesta controvérsia atual em relação às concentrações seguras, pouca atenção tem sido dada à toxicidade a nível molecular e bioquímica, abordada na presente tese.

Muitos estudos com mamíferos relatam que o BPA é capaz de gerar estresse oxidativo nas células desses organismos promovendo principalmente a diminuição das defesas oxidantes e a produção/liberação de espécies reativas de H_2O_2 (Bindhumol et al., 2003; Chitra et al., 2003). Embora as análises moleculares sejam muitas vezes utilizadas para apoiar ou até mesmo substituir resultados bioquímicos, a ligação entre

os efeitos que ocorrem em vários níveis celulares nem sempre é fácil prever e resulta que a expressão do gene e a atividade catalítica da proteína podem ter diferentes tendências e portanto, respostas conflitantes (George et al., 2004; Kammann et al., 2008).

Em relação às respostas relacionadas a enzimas atuantes na via esteroidogênica, a quantidade de trabalhos é maior, principalmente com mamíferos, e demonstra que a análise de expressão quantidade e atividade dessas proteínas podem ser um caminho para o estabelecimento de níveis seguros de BPA em diferentes ambientes (Vandenberg et al., 2015).

Nas últimas décadas, a grande maioria dos estudos relacionando peixes com a ação de desreguladores endócrinos tem focado em exposições a doses altas e efeitos como alteração de prole, infertilidade, reversão sexual e mortalidade. No presente estudo, foram utilizadas concentrações relativamente baixas, encontradas no ambiente, e além das alterações moleculares, também foram demonstrados danos histológicos importantes, o que vai ao encontro a estudos que demonstram ruptura nos tecidos reprodutivos apenas em doses altas, não encontradas no ambiente.

Na presente tese, foi observado uma relação tempo-dependente e tecido-dependente de efeitos do Bisfenol-A em *Danio rerio* expostos à concentrações ambientalmente relevantes, o que indica que diferentes tempos de exposição podem tender a induzir diferentes sistemas, começando com a diminuição das respostas antioxidantes e tardiamente levando a danos no sistema reprodutivo, associado principalmente à esteroidogênese e à produção de gametas. No ambiente, o BPA é continuamente liberado e portanto tanto danos oxidativo quanto endócrinos podem ser perpetuados e causar efeitos mais severos como reversão sexual, infertilidade e apoptose (Crain *et al*., 2007). Entretanto, os resultados aqui demonstrados sugerem que

não é necessária uma exposição contínua ou em altas doses para que sejam alterados sistemas básicos, provavelmente causando uma maior demanda energética e danos histológicos irreversíveis.

CONCLUSÕES

- O BPA foi capaz de alterar a expressão de genes relacionados a defesa antioxidante, biotransformação e regulação antioxidante em *Danio rerio* expostos de forma aguda a concentrações ambientalmente relevantes;
- O BPA induziu a expressão de genes relacionados à via esteroidogênica em *Danio rerio* expostos de forma subcrônica a concentrações ambientalmente relevantes;
- A capacidade antioxidante total foi alterada nos peixes expostos ao BPA, onde os principais tecidos afetados foram fígado e cérebro, como ocorre em mamíferos expostos a baixas doses desse composto;
- A Atividade das enzimas SOD-1, CAT, e GST foi alterada nos tecidos dos animais expostos ao BPA por 24 e 96 horas, demonstrando serem bons biomarcadores para a presença do contaminante na água;
- O BPA foi capaz de induzir danos significativos nas células espermáticas de *Danio rerio* quando expostos a concentrações ambientalmente relevantes de forma crônica, indicando que concentrações presentes no ambiente são capazes de desencadear danos em células de Sertoli, espermatócitos e espermatozóides;
- O presente trabalho demonstrou que concentrações de BPA presentes no ambiente atualmente, são capazes de alterar aspectos moleculares, bioquímicos e histológicos de *Danio rerio*, em estudos *in vivo* com diferentes tempos de exposição.

REFERÊNCIAS

Akingbemi, B.T., Sottas, C.M., Koulova, A.I., *et al*., 2004. Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A isassociated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig cells. Endocrinology. 145, 592–603.

Ahmad, S., 1995.Oxidative stress and antioxidant defenses in biology.Chapman e Hall, New York.

Alexander, H.C., Dill, D.C., Smith, L. *et al*., 1988. Bisphenol A: acute aquatic toxicity. Environ. Toxicol. Chem. 7, 19–26.

Aluru, N., Leatherland, J.F., Vijayan, M.M., 2010. Bisphenol a in oocytes leads to growth suppression and altered stress performance in juvenile rainbow trout. PLoS ONE 5, e10741.

Avci, A., Kacmaz, M., Durak, I., 2005. Peroxidation in muscle and liver tissues from fish in a contaminated river due to a petroleum refinety industry.Ecotoxicol. Environ. Saf. 60, 101–105.

Bhat A., 2003. Diversity and composition of freshwater fishes in river systems of Central Western Ghats, India.Environ. Biol. Fishes. 68, 25–38.

Billa, D.M., Dezotti, M., 2007. Desreguladores endócrinos no meio ambiente: efeitos e consequencias. Química nova. 30, 651-666.

Bindhumol, V., Chitra, K. C., Mathur, P. P., 2003. Bisphenol A induces reactive oxygen species generation in the liver of male rats. Toxicology.188, 117–124.

Birkett, J.W., Lester, J.N., 2003. Endocrine disrupters in Wastewater and sludge treatment process, 1ª ed., IWA Publishing, Lewis Publishers CRC Press, LLC:USA.

Bonefeld-Jorgensen, E.C., Long, M., Hofmeister, M.V. Vinggaard M.A., 2007. Endocrine disrupting potential of bisphenol A, bisphenol A dimethacrylate, 4-

nnonylphenol, and 4-n-octylphenol in vitro: new data and a brief review. Environ. Health Perspect. 115, 69–76.

Bredhult, C., Sahlin, L., Olovsson, M., 2009. Gene expression analysis of human endometrial endothelial cells exposed to bisphenol A. Reprod. Toxicol. 28, 18–25.

Careghini, A., Mastorgio,A.F., Saponaro,S.,Sezenna, E., 2014. Bisphenol A, nonylphenols, benzophenones, and benzotriazoles in soils, ground water, surface water, sediments, and food: a review. Environ. Sci. Pollut. Res. 22, 5711–5741.

Caron, K.M., Soo, S.C., Parker,K.L., 1998. Targeted disruption of StAR provides novel insights into congenital adrenal hyperplasia. Endocr. Res. 24, 827–834.

Carson, R., 1987. Silent Spring: 25th Anniversary Edition. Houghton Mifflin Co., NewYork

Castro, B. Sanchez, P., Torres, J.M. *et al.,* 2013. BisphenolA exposure during adulthood alters expression of aromatase and 5alphareductase isozymes in rat prostate, PLoS One 8 (2) e55905.

Chapin, R.E., Adams, J., Boekelheide, K., Gray Jr., L.E., Hayward, S.W., Lees, P.S., McIntyre, B.S., Portier, K.M., Schnorr, T.M., Selevan, S.G., Vandenbergh, J.G., Woskie, S.R., 2008. NTP-CERHR expert panel report on the reproductive and developmental toxicity of bisphenol A. Birth Defects Res. B Dev. Reprod. Toxicol. 83, 157-395.

Richter, C., Birnbaum, L.S., Farabollini, F., Newbold, R.R., Rubin, B.S., Talsness, C.E., Vandenbergh, J.G., Walser-Kuntz, D.R., vom Saal, F.S., 2007. In vivo effects of bisphenol A in laboratory rodent studies. Reprod. Toxicol. 24, 199-224.

Cheshenko, K., Pakdel, F., Segner, H. *et al.,* 2008.Interference of endocrinedisrupting chemicals with aromatase CYP19 expression or activity, and consequencesfor reproduction of teleost fish. Gen. Comp. Endocrinol. 155, 31–62.

Chitra, K.C., Latchoumycandane, C., Mathur, P.P., 2003. Induction of oxidative stress Bybisphenol A in the epididymal sperm of rats. Toxicology 185, 119–127.

Clark, B.J., Cochrum, R.K., 2007. The steroidogenic acute regulatory protein as a targetof endocrine disruption in male reproduction, Drug Metab.Rev. 39, 353–370.

Cook, P.M., Robbins J.A., Endicott, D.D., *et al*., 2003. Effects of aryl hydrocarbon receptor mediated early life stage toxicity on lake trout populations in lake Ontario during the 20th century. Environ. Sci. Technol. 37, 3864 - 3877.

Cousins I.T., Staples C.A., Klecka G.M., Mackay D., 2002.A multimedia assessment of the environmental fate of bisphenol A. Hum. Ecol. Risk Assess. 8, 1107–35.

Crain, D., AaJr, L.J.G., 1998.Reptiles as models of contaminant-induced endocrine disruption.Anim. Reprod. Sci. 53, 77–86.

Crain, D.A., Eriksen, M., Iguchi, T., *et al.,*2007.An ecological assessment of bisphenol-A: evidence from comparative biology. Reprod.Toxicol. 24, 225–239.

Crane, M., Burton, G.A., Culp, J.M.,*et al.,*2007. Review of aquatic in situ approaches forstressor and effect diagnosis. Integr. Environ. Assess. Manage. 3, 234–245.

Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and personal care products in theenvironment: agents of subtle change? Environ. Health Perspect. 107, 907–938.

Delclos, K.B., Camacho, L., Lewis, S.M., *et al*., 2014. Toxicity evaluation of bisphenol A administered by gavage to Sprague Dawley rats from gestation day 6 through postnatal day 90. Toxicol. Sci. 139, 174-97.

Dolinoy, D.C., Weinhouse, C., Jones, T.R., Rozek, L.S., Jirtle, R.L., 2010.Variable histone modifications at the A(vy) metastable epiallele. Epigenetics 5, 637–644.

Donahue, D.A., Dougherty, E.J., Meserve, L.A., 2004. Influence of a combination of two tetrachloro biphenyl congeners (PCB47; PCB77) on thyroid status, cholineacetyl

transferase (ChAT) activity, and short and long-term memory in 30 day old Sprague Dawley rats. Toxicology.203, 99–107.

Drastichova, J., Svobodova, Z., Groenland, M., *et al*., 2005.Effect of exposure to bisphenol A on the sex differentiation in zebrafish (*Daniorerio*).Acta. Vet. Brno. 74, 287–291.

EPA, 2007. Draft List of Initial Pesticide Active Ingredients and Pesticide Inerts to be364 Considered for Screening under the Federal Food, Drug and Cosmetic Act.

Esch, C., Slieker, R., Wolterbeek, A., *et al*., 2012. Zebrafish as potential model for developmental neurotoxicity testing A mini review. Neurotoxicol.Teratol. 34, 545-553.

European Commission. Risk assessment report of 4,4_-isopropylideden diphenol (BISPHENOL-A). Italy: Ispra; 2003.

European Food Safety Authority.2008.Statement of EFSA on a study associating bisphenol A with medical disorders. The EFSA Journal 838, 1-3.

Fei, X.C., Song, C., Gao, H.W., 2010. Transmembrane transports of acrylamide and bisphenol A and effects on development of zebrafish (*Daniorerio*). J. Hazard. Mater. 184, 81–88.

Evans, N.P., North, T., Dye, S., Sweeney, T., 2004. Differential effects of the endocrinedisrupting compounds bisphenol-A and octylphenol on gonadotropin secretion, in prepubertal ewe lambs. Domest. Anim. Endocrinol. 26, 61–73.

Faqi A.S., Dalsenter P.R., Merker, H.J., Chahoud, I., 1998. Reproductive toxicity and tissue concentrations of low doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin in male offspring rats exposed throughout pregnancy and lactation. Toxicol. Appl. Pharmacol. 150, 383–392.

Fernandez, S.V., Huang, Y., Snider, K.E., *et al*., 2012.Expression and DNA methylation changes in human breast epithelial cells afterbisphenol A exposure. Int. J. Oncol. 41, 369–377.

Fiorini, C., Tilloy-Ellul, A., Chevalier, S., *et al*., 2004.Sertoli cell junctional proteins as early targets for different classes of reproductive toxicants. Reprod. Toxicol. 18,413-421.

Fukazawa,H.,Hoshino,K.,Shiozawa,T.,*et al*.,2001. Identification and quantification of chlorinated bisphenol A in waste water from waste- paper recycling plants. Chemosphere.44, 973–979.

Geens, T., Aerts, D., Berthot, C., *et al*., 2012. A review of dietary and non-dietary exposure to bisphenol-A. Food Chem. Toxicol. 50, 3725-3740.

Geens, T., Goeyens, L., Kannan, K.,*et al*., 2012. Levels of bisphenol-A in thermal paper receipts from Belgium and estimation of human exposure. Sci.Total Environ. 436, 30-33.

George, S., Gubbins, M., MacIntosh, A.,*et al*., 2004. A comparison of pollutant biomarker responses with transcriptional responses in European flounders (*Platicthys flesus*) subjected to estuarine pollution. Mar. Environ. Res. 58, 571–575.

Giger, W., Brunner, P.H., Schaffner, C., 1984.4-Nonylphenol in Sewage Sludge: Acumulation of toxic metabolites from nonionic surfactants. Science.225, 623-625.

Hahn, M.E., Stegeman, J.J., 1994. Regulation of Cytochrome p4501A1 in Teleosts: Sustained induction of CYP1A1 mRNA, protein, and catalytic activity by 2,3,7,8-Tetrachlorodibenzofuran in the marine fish *Stenotomuscrhysops*. Toxicol.and Appl. Pharmacol. 127, 187–198.

Heimeier, R.A., Das, B., Buchholz, D.R., Shi, Y.B., 2009. The xenoestrogen bisphenol A inhibits postembryonic vertebrate development by antagonizing gene regulation by thyroid hormone. Endocrinology. 150, 2964–2973.

Hampl, R., Kubátová, J., Stárca, L., 2016. Steroids and endocrine disruptors—History, recent state of art and open questions. J.of Ster. Biochem.and Mol. Biol. 155, 215-223.

Haubruge, E., Petit, F., Gage, M.J.G. 2015. Reduced sperm counts in guppies (*Poecilia reticulata*) following exposure to low levels of tributyltin and Bisphenol A. Proc. Biol. Sci. 1459, 2333-2337.

Honma, S., Suzuki, A., Buchanan, D.L., *et al*., 2001. Low dose effect of in utero exposure to bisphenol A and diethylstilbestrol on female mouse reproduction. Reprod. Toxicol. 16, 117–122.

Hotchkiss, A.K., Rider, C.V., Blystone, C.R.,*et al*., 2008. Fifteen years after "wingspread"e environmental endocrine disrupters and human and wildlife health: wherewe are today and where we need to go. Toxicol. Sci. 105, 235-259.

Huang, Q., Chao, F., Chen,Y.,*et al*., 2012. Embryonic exposure to low concentration of bisphenol A affects the development of Oryzias mela- stigma larvae. Environ. Sci. Pollut. Res. 19, 2506-2514.

Jiang, L., Zhang, J., Wang, J.J., *et al*., 2013. Sperm, but not oocyte, DNA methylome is inherited by zebrafish early embryos. Cell. 153, 773–784.

Jin, Y., Zheng, S., Fu, Z., 2011. Embryonic exposure to cypermethrin induces apoptosis and immunotoxicity in zebrafish (*Danio rerio*). Fish Shellfish Immunol. 30,1049-1054.

Kahle, P.J., Waak, J., Gasser, T., 2009. DJ-1 and prevention of oxidative stress in Parkinson's disease and other age-related disorders. Free Radic. Biol. Med. 47, 1354-1361.

Kammann, U., Lang, T., Berkau, A.-J., Klempt, M., 2008. Biological effect monitoring indab (*Limanda limanda*) using gene transcript of CYP1A1 or EROD.A comparison. Environ. Sci. Pollut. Res. 15, 600–605.

Kang, J.H., Kondo, F., 2005. Bisphenol-A degradation in seawater is different fromthat in river water. Chemosphere. 60, 1288-1292.

Kang, J., Ha, F.K., 2002.Bisphenol a degradation by bacteria isolated from river water. Arch. Environ. Contam. Toxicol.43, 265–9.

Kang, I.J., Yokota, H., Oshima, Y.,*et al*., 2002. Effects of bisphenol a on the reproduction of Japanese medaka (*Oryziaslatipes*). Environ. Toxicol.Chem. 21, 2394–2400.

Kang, J.H., Asai, D., Katayama, Y., 2007. Bisphenol A in the aquatic environment and its endocrine-disruptive effects on aquatic organisms. Crit. Rev. Toxicol. 37, 607–625.

Kawai, K., Murakami, S., Senba, S., *et al*., 2007. Changes in estrogen receptors a and b expression inthe brain of mice exposed prenatally to bisphenol A. Regul. Toxicol.Pharmacol. 47, 166–170.

Kinumi, T., Kimata, J., Taira, T., *et al*., 2004.Cysteine-106 of DJ-1 is the most sensitive cysteine residue to hydrogen peroxide mediated oxidation in vivo in human umbilical vein endothelial cells. Biochem.Biophys. Res. Commun.317, 722–728.

Kronenberg, H.M., Memed, S., Polonsky, S., Larsen, P.R., 2011. Willians Textbook of Endocrinology. 11 ed.

Kumar, V., Balomajumder, C., Roy, P., 2008. Disruption of LH-induced testosterone biosynthesis in testicular Leydig cells by triclosan: probable mechanism ofaction, Toxicology. 250, 124–131.

Kuiper, G.G., Lemmen, J.G., Carlsson, B., *et al*., 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology.139, 4252 – 4263.

Lahnsteiner, F., Berger, B., Kletz, M., Weismann, T., 2005. Effect of Bisphenol A on maturation and quality of semen and eggs in the brown trout, *Salmo trutta f. fario*. Aquat.Toxicol. 75, 213-224.

Lamb, J.C., Boffetta, P., Foster, W.G., *et al*., 2014. Critical comments on theWHO-UNEP state of the science of endocrine disrupting chemicals - 2012. Regul. Toxicol. Pharmacol. 69, 22-40.

Lapworth, D.J., Baran, N., Stuart, M.E., Ward, R.S., 2012. Emerging organic contaminants in ground water: a review of sources, fate and occurrence. Environ. Pollut. 163, 287–303.

Le H., Carlson E., Chua J., Belcher S.,2008.Bisphenol A is released from polycarbonate drinking bottles and mimics the neurotoxic actions of estrogen in developing cerebellar neurons. Toxicol. Let. 176, 149–156.

Lee, H.J., Chattopadhyay, S., Gong, E.Y.,*et al*., 2003. Antiandrogenic effects of bisphenol A and nonylphenol on the function of androgen receptor.Toxicol. Sci. 75, 40–46.

Leranth, C., Hajszan, T., Szigeti-Buck, K.,*et al*., 2008. Bisphenol Aprevents the synaptogenic response to estradiol in hippocampus and prefrontalcortex of ovariectomized nonhuman primates. Proc. Natl. Acad. Sci. U.S.A. 105,14187–14191.

Leranth, C., Szigeti-Buck, K., Maclusky, N.J., Hajszan, T., 2008. Bisphenol A prevents the synaptogenic response to testosterone in the brain of adult male rats. Endocrinology. 149, 988–994.

Li, G., Chang, H., Xia, W., *et al*., 2014. F0 maternal BPA exposure induced glucose intolerance of F2 generation through DNA methylation change in Gck. Toxicol. Lett. 228, 192–199.

Lintelmann, J., Katayama, A., Kurihara, M.,*et al*., 2003. Endocrine disruptors in the environment (IUPAC Technical Report). Pure Applied Chemistry. 75, 631-681.

Livingstone, D.R., 1998. Organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. Comp. Environ. Physiol. 120, 43–49.

Malhotra, D.,Thimmulappa, R., Acien, A.N.,*et al*., 2008.Decline in NRF2-regulated Antioxidants in Chronic Obstructive Pulmonary Disease Lungs Due to Loss of Its Positive Regulator, DJ-1. J Respir. Crit. Care Med. 178, 592–604.

Mandich, A., Bottero, S., Benfenati, E.,*et al*., 2007. In vivo exposure of carp to graded concentrations of bisphenol A. Gen. Comp. Endocrinol. 153, 15-24.

Mao, Z., Xia, W., Chang, H., *et al*., 2015. Paternal BPA exposure in early life alters Igf2 epigenetic status in sperm and induces pancreatic impairment in rat offspring. Toxicol. Let. 238, 30-38.

Markey, C.M., Michaelson, C.L., Sonnenschein, C., Soto, A.M., 2001. Alkylphenols and bisphenol-A as environmental estrogens. In: Metzler, M. (Ed.), The Handbook of Environmental Chemistry. Part L. Endocrine Disruptors, Part I. M, vol. 3. Springer-Verlag, Berlin.

Mathieu-Denoncourt, J., Wallace, S., Solla, S.R., Langlois, V., 2015. Plasticizer endocrine disruption: Highlighting developmental and reproductive effects in mammals and non-mammalian aquatic species. Gen. Comp. Endocrinol. 219, 74-88.

Mcallister B.G., Kime D.E., 2003. Early life exposure to environmental levels of the aromatase inhibitor tributyltin cause masculinisation and irreversible sperm damage in zebrafish (*Danio rerio*). Aquat.Toxicol.65, 309–16.

Milla, S., Depiereux, S., Kestemont, P., 2011.The effects of estrogenic and androgenic endocrine disruptors on the immune system of fish: a review. Ecotoxicology. 20, 305–319.

Mitsumoto, A., Nakagawa, Y., Takeuchi, A., *et al*., 2001. Oxidized forms of peroxiredoxins and DJ-1 on two-dimensional gels increased in response to sublethal levels of paraquat. Free Radic. Res. 35, 301-310.

Monje, L., Varayoud, V., Munoz-de-Toro, M., *et al*., 2010. Exposure of neonatal female rats to bisphenol A disrupts hypothalamic LHRH pre-Mrna processing and estrogen receptor alpha expression in nuclei controlling estrous cyclicity. Reprod. Toxicol. 30, 625–634.

Nakagawa, Y., Toyama, S., 2000. Metabolism and cytotoxicity of bisphenol A and other bisphenols in isolated rat hepatocytes. Arch.Toxicol.74, 99–105.

Newbold, R.R., Hanson, R.B., Jefferson, W.N.,*et al*., 2000. Proliferative lesions and reproductive tract tumors in male descendants of mice exposed developmentally to diethylstilbestrol. Carcinogenesis. 21, 1355–1363.

Ooe, H., Taira, T., Iguchi-Ariga, S.M., Ariga, H., 2005. Induction of reactive oxygen species by bisphenol A and abrogation of bisphenol A-induced cell injury by DJ-1.Toxicol. Sci. 88, 114-26.

Petersen, P.M., Giwercman, A., Skakkebaek, N.E., Rorth, M., 1998. Gonadal function in men with testicular cancer.Semin.Oncol. 25, 224–233.

Petrovic, M., Eljarrat, E., Lopez De Alda, M.J., Barcelo, D., 2004. Endocrine disrupting

compounds and other emerging contaminants in the environment: a survey on new monitoring strategies and occurrence data. Anal. Bioanal. Chem. 378, 549–562.

Pelayo, S., Oliveira, E., Thienpont, B.,*et al*., 2012. Triiodothyronine-induced changes in the zebrafishtranscriptome during theeleutheroembryonic stage: implications for bisphenol A developmental toxicity.Aquat.Toxicol. 110, 114–122.

Phrakonkham, P., Viengchareun, S.,*et al*., 2008. Dietary xenoestrogens differentially impair 3T3-L1 preadipocyte differentiation and persistently affect leptin synthesis. J. Steroid Biochem. Mol. Biol. 110, 95–103.

Ratman, D., Vanden Berghe, W., Dejager, L., *et al*., 2013. How glucocorticoid receptors modulate the activity of other transcription factors: a scope beyond tethering. Mol. Cell. Endocrinol. 380, 41–54.

Robinson, J., 2006. Prenatal programming of the female reproductive neuroendocrine system by androgens. Reproduction. 132, 539–547.

Roos, A., Greyerz, E., Olsson, M., Sandegren, F., 2001. The otter (*Lutra lutra*) in Sweden population trends in relation to sigma DDT and total PCB concentrations during 1968–99. Environ. Pollut. 111, 457–469.

Safe, S.H., Pallaroni, L., Yoon, K.,*et al*., 2001.Toxicology of environmental estrogens. Reprod. Fertil. Dev.13, 307–315.

Sajiki, J., Yonekubo, J., 2003. Leaching of bisphenol-A (BPA) to seawater from polycarbonate plastic and its degradation by reactive oxygen species.Chemosphere.51, 55-62.

Sakaue, M., Ohsako, S., 2001. Bisphenol-A affects spermatogenesis in the adult rat even at low dose. J. Occup. Health 43, 185–190.

Santos M.M., Castro L.F.C., Vieira M.N.,*et al*., 2005.Newinsights into the mechanism of imposex induction in the dogwhelk *Nucella lapillus*.Comp.Biochem.Physiol. 141, 101–9.

Santos M.M., Micael J., Carvalho A.P., *et al*., 2006.Estrogens counteract the masculinizing effect of tributyltin in zebrafish. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 5, 142-151.

Scippo, M.L., Argiris, C., Weerdt, C.V., *et al*., 2004. Recombinant human estrogen, androgen and progesterone receptors for detection of potential endocrine disruptors. Anal. Bioanal. Chem. 378, 664–669.

Schulz, K.M., Molenda-Figueira, H.A., Sisk, C.L. 2009. Back to the future: the organizational-activational hypothesis adapted to puberty and adolescence. Horm. Behav. 55, 597–604.

Segner, H., Caroll, K., Fenske, M., *et al*., 2003. Identification of endocrine disrupting effects in aquatic vertebrates and invertebrates: report from the European IDEA project. Ecotoxicol. Environ. Saf. 54, 302–314.

Shibata, H., Spencer, T.E., Onate, S.A., *et al*., 1997. Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. Recent Prog. Horm. Res. 52, 141–164.

Sies, H., 1993. Strategies of antioxidant defense. European J. of Biochem. 215, 213-219.

Sobarzo, C.M., Lustig, L., Ponzio, R., *et al*., 2009. Effects of di(2-ethylhexyl) phthalate on gap and tight junction protein expression in the testis of prepubertal rats. Microsc. Res. Tech. 72, 868–877.

Sodré F.F., Pescara I.C., Montagner C.C., Jardim W.F., 2010. Assessing selected estrogens and xenoestrogens in Brazilian surface waters by liquid chromatography tandem mass spectrometry. Microchem. J. 96, 92–98.

Sohoni, P., Tyler, C.R., Hurd, K., *et al*., 2001. Reproductive effects of long term exposure to bisphenol A in the fathead minnow (*Pimephales promelas*). Environ. Sci. Technol. 35, 2917-2925.

Shimasaki Y, Kitano T, Oshima Y.,*et al*., 2003. Tributyltin cause masculinization in fish. Environ. Toxicol. Chem. 22, 141–4.

Skinner, M.K., 2011. Environmental epigenetic transgenerational inheritance and somatic epigenetic mitotic stability.Epigenetics. 6, 838–842.

Spence, R., Gerlach, G., Lawrence, C., Smith, C., 2007. The behaviour and ecology of the zebrafish, *Danio rerio*. Biol. Rev. 83, 13–34.

Strakovsky, R., Wang, H., Engeseth, N., *et al*., 2015. Developmental bisphenol A (BPA) exposure leads to sex-specific modification of hepatic gene expression and epigenome at birth that may exacerbate high-fat diet-induced hepatic steatosis. Toxicol. and Apl. Pharmacol. 284, 101-112

Streisinger, G., Walker C., Dower, N., *et al*., 1981. Production of clones of homozygous diploid zebrafish (*Brachydanio rerio*). Nature.291, 293–6.

Suna, H.B., Shenb, O.X., Wang, X.R., *et al*., 2009. Anti-thyroid hormone activity of bisphenol A, tetrabromobisphenol A and tetrachlorobisphenol A in an improved reporter gene assay. Toxicol in Vitro. 23, 950-4.

Saili, K., Tilton, S., Woth, K., Tanguay, R., 2013. Global gene expression analysis reveals pathway differences between teratogenic and non-teratogenic exposure concentrations of bisphenol A and 17-estradiol in embryonic zebrafish. Repr. Toxicol. 38,89-101.

Tao, S., Zhang, Y., Yuan, C., *et al*., 2016. Oxidative stress and immunotoxic effects of bisphenol A on the larvae of rareminnow *Gobiocypris rarus*. Ecotoxicol. Envirom. Saf. 124, 377–385.

Thimmulappa, R. K., Mai, K. H., Srisuma, S., *et al*., 2002. Identification of Nrf2-regulated genes induced by the chemo preventive agent sulforaphane by oligonucleotide microarray. Cancer. Res. 62, 5196–5203.

Thomas, J.A., 1996. Toxic responses of the reproductive system. In: KLASSEN, C.D. Casarett & Doull´s Toxicology. The basic science of poisons. New York: McGraw-Hill, p. 547-581.

Tyl, R.W., 2009. Basic exploratory research versus guideline-compliant studies usedfor hazard evaluation and risk assessment: bisphenol A as a case study. Environ.Health Perspect. 117, 1644-1651.

Ueyama, T., Shirasawa, N., Numazawa, M., 2002. Gastric parietal cells: potent endocrine role in secreting estrogen as a possible regulator of gastro-hepatic axis. Endocrinology.143, 3162 – 3170.

Valavanidis, A., Vlahogianni, T.,Dassenakus, M., Scoullos, M., 2006. Molecular biomarkers of oxidative stress in aquatic organisms inrelation to toxic environmental pollutants.Ecotoxicol. and Environ.Saf. 64, 178-189.

Vandenberg, L.N., Hauser, R., Marcus, M., *et al*., 2007. Human exposure to bisphenol A (BPA). Reprod.Toxicol. 24, 139-177.

Vandenberg L., Maffini M., Sonnenschein C., *et al*., 2009. Bisfenol-A and the great divide: A review of controversies in the field of endocrine disruption. End. Rev. 30, 75–95.

Vandenberg, L.N., 2014. A path forward in the debate over health impacts ofendocrine disrupting chemicals. Environ. Health. 13, 109-118.

Vandenberg, L., Luthi, D., Quinerly, A., 2015. Plastic bodies in a plastic world: multi-disciplinary approaches to study endocrine disrupting chemicals.J. of Clean.Produc.25, 1-13.

Vigano L., Mandich A., Banfenati E. *et al*., 2006.Investigating the estrogenic risk along the river Po and its intermediate section. Arch. Environ. Contam. Toxicol. 51, 641–651.

Vom Saal, F.S., Akingbemi, B.T., Belcher, S.M., *et al* ., 2007. Chapel Hill bisphenol A expert panel consensus statement: integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure. Reprod. Toxicol. 24, 131-138.

Wetherill, Y.B., Petre, C.E., Monk, K.,*et al*., 2002.TheXenoestrogenbisphenol A induces inappropriate androgen receptor activation andmitogenesis in prostatic adenocarcinoma cells. Mol. Cancer Ther. 1, 515–524.

Wilson, M.A., 2011.The role of cysteine oxidation inDJ-1_function and dysfunction. Antioxid. Redox Signal.15, 111-122.

Wisniewski, P., Romano, R.M., Kizys, M., 2015. Adult exposure to bisphenol A (BPA) in Wistar rats reduces sperm quality with disruption of the hypothalamic–pituitary–testicular axis.Toxicology. 329, 1–9.

Wolff, M.S., Berkowitz, G.S., Brower, S., *et al*., 2000. Organochlorine exposures and breast cancer risk in New York City women. Environ. Res. 84, 151–161.

Xu, L.C., Sun, H., Chen, J.F., *et al*., 2005. Evaluation of androgen receptor transcriptional activities of bisphenol A, octylphenol and nonylphenol in vitro. Toxicology. 216, 2-3, 197-203.

Yamazaki, E., Yamashita, N., Taniyasu, S., *et al*., 2015. Bisphenol A and other bisphenol analogues including BPS and BPF in surface water samples from Japan, China, Korea and India. Ecotoxicol. Environ. Saf. 122, 565–572.

Xu, H., Shao, X., Zhang, Z., *et al*., 2013. Oxidative stress and immune related gene expression following exposure to di-n-butylphthalate and diethylphthalate in zebrafish embryos. Ecotoxicol. Environ. Saf. 93, 39–44.

Zhang, D. D., Hannink, M., 2003. Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. Mol Cell Biol. 23, 8137–8151.

Zoeller, R.T., Bergman, A., Becher., *et al*., 1999. The potential health effects of phthalate esters in children's toys: a review and risk assessment. Regul. Toxicol. Pharmacol. 30, 140–155.

Witorsch, R.J. 2002. Endocrine disruptors: can biological effects and environmental risks be predicted? Regul. Toxicol. Pharmacol. 36, 118-130.