GESSICA DUTRA GONÇALVES

BISPHENOL A (BPA) ALTERA OS NÍVEIS DE TESTOSTERONA EM CÉLULAS DE LEYDIG (TM3) BEM COMO A MORFOLOGIA TESTICULAR E PROSTÁTICA DE CAMUNDONGOS ADULTOS.

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Orientadora: Profa. Dra. Glaura Scantamburlo Alves Fernandes.

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BANCA EXAMINADORA

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Londrina, 23 de Fevereiro de 2017.

Dedico,

Aos meus pais, minha irmã e meu noivo, que sempre me apoiaram em todas as minhas decisões. À minha professora orientadora pela paciência na orientação e incentivo, que tornaram possível a conclusão desta dissertação. E a Deus que tornou possível este momento sempre me dando força para continuar de pé e finalizar mais esta etapa.

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“O que destrói a humanidade: A Política, sem princípios; o Prazer, sem compromisso; a Riqueza, sem trabalho; a Sabedoria, sem caráter; os negócios, sem moral; a Ciência, sem humanidade; a Oração, sem caridade.”

Mahatma Gandhi

GONÇALVES, Gessica Dutra. **Bisphenol A (BPA) Altera os Níveis de Testosterona em Células de Leydig (TM3) bem como a Morfologia Testicular e Prostática de Camundongos Adultos.** 2017. 104 f. Dissertação de Mestrado – Programa de Pós-Graduação em Patologia Experimental Universidade Estadual de Londrina, Londrina, 2017.

RESUMO

O Bisfenol A (BPA) é um desregulador endócrino ainda utilizado na produção de plásticos e resinas de latas para alimentos. O principal mecanismo de ação do BPA é sua ligação nos receptores de estrógenos e andrógenos, levando a alterações em hormônios importantes como a testosterona. O sistema genital masculino é dependente da resposta à ligação de receptores de estrógenos e andrógenos, assim, tecidos como o prostático e testicular além de células de Leydig podem sofrer alterações pela ação do BPA. Portanto o objetivo do presente estudo é avaliar as alterações em tecido de próstata e de testículo *in vivo* e das células de Leydig *in vitro* causadas pela ação do BPA. 60 Camundongos *Mus musculus* foram divididos aleatoriamente em 3 grupos e receberam via gavagem água contendo: DMSO 1% (C), 1µg/kg de BPA (BPA 1µg) ou 10µg/kg de BPA (BPA 10µg) diariamente durante 50 dias. Aos 110 dias de idade, os animais foram anestesiados, pesados, o sangue coletado para avaliação dos níveis plasmáticos de testosterona e em seguida submetidos à eutanásia. As próstatas, os testículos, ductos deferentes e a vesícula seminal foram retiradas e pesadas. As próstatas e os testículos foram utilizados para avaliação dos parâmetros histopatológicos, estereológicos ou morfométricos, e para avaliação do perfil inflamatório (atividade da mieloperoxidade (MPO) e N-Acetil-β-D –Glucosaminidase (NAG) e quantificação das citocinas IL-10, IL-6 e TNF-α). Os testículos foram utilizados para contagem espermática e os espermatozoides do ducto deferente foram obtidos para avaliação da morfologia e motilidade espermática. Nossos resultados demonstraram que a testosterona plasmática foi reduzida nos dois grupos tratados com BPA (BPA 1µg e BPA 10µg). No testículo, o diâmetro dos túbulos seminíferos reduziu apenas no grupo BPA 10µg, contudo o tamanho do epitélio seminífero aumentou nos dois grupos BPA. A contagem espermática foi semelhante em todos os grupos, porém o número de espermatozoides imóveis assim como número de espermatozoides com morfologia anormal foram maiores no grupo BPA 10µg. Este grupo também apresentou uma diminuição da citocina IL-10 com o aumento da atividade de MPO no testículo. Na próstata, BPA induziu alterações morfológicas com redução do compartimento luminal e aumento do compartimento estromal, nas duas doses de BPA. A presença de hiperplasia, metaplasia e células no lúmen prostático foi observada no grupo BPA 10µg, enquanto o grupo BPA 1µg apresentou um aumento de tumefação celular. O grupo BPA 1µg apresentou um aumento na atividade de MPO, entretanto outros parâmetros inflamatórios não foram significativos. Células de Leydig TM3 foram submetidas às concentrações de BPA 0,5 a 500µM para as análises de viabilidade celular e da cinética de crescimento celular em tempo real e as concentrações de BPA 1, 10 e 100µM para avaliação do ciclo celular, presença de células em morte celular (Hoechst 3342), potencial de membrana mitocondrial (rodamina 123), ensaio do cometa e produção de testosterona. Nossos resultados demonstraram que BPA 100 µM reduziu a viabilidade celular e foi capaz de aumentar o número de células em morte celular indicado pelo aumento da população em fase sub-G1 e de células marcadas com Hoechst 3342. A coloração com rodamina 123 mostrou uma diminuição na atividade metabólica mitocondrial apenas na concentração de 100µM de BPA. BPA também levou a redução na produção de testosterona em todas as concentrações testadas. Nós concluímos que o BPA leva a disfunção celular das células de Leydig assim como baixas doses de BPA causa alterações nos tecidos prostáticos e testiculares e induzindo a diminuição na produção de testosterona.

Palavras-chave: Bisfenol A, Células de Leydig, Testículo, Próstata.

GONÇALVES, Gessica Dutra**. Bisphenol a (BPA) Changes Testosterone Levels in Leydig Cells (TM3) as well as the Testicular and Prostatic Morphology of Adult Mice**. 2017. 104 p. Master's Degree Dissertation – Experimental Pathology Post graduation Program – State University of Londrina, Londrina, 2017.

ABSTRACT

Bisphenol a (BPA) is an endocrine disrupter used in the industry in the production of plastics and food can resins. The BPA act in the estrogen and androgens receptors, modifying levels of the main hormones how testosterone. The male genital system is dependent on response to binding of estrogen and androgen receptors, therefore tissues such as the prostatic and testicular as well as Leydig cells may be altered by the action of BPA. The aim of this study is to evaluate alteration of the tissue prostatic and testicular *in vivo and* Leydig cells *in vitro* by action of BPA. 60 *Mus musculus* mice were randomly divided into 3 groups and received via gavage water containing: DMSO 1% (C), 1μg/kg of BPA (BPA 1μg) or 10μg/kg of BPA (BPA 10μg) daily during 50 days. With 110 days old the mice were anesthetized, weighed, blood collected for dosage of plasma testosterone levels, and the animals were submitted to euthanasia. The prostates, testis, vans deferens and vesicle seminal were collected and weighed. The prostates and testis were used to histopathological, stereological or morphometric parameters, as well as evaluate the inflammatory profile (activity of myeloperoxidase and N-Acetyl-β-D-Glucosaminide and quantification of cytokines IL-10, IL-6 and TNF-α). The testis were used for sperm count and the sperm of vas deferens were used in the evaluation of the motility and morphology spermatic. We results demonstrated that the plasm testosterone decreased in the groups of BPA 1μg and 10μg. In the testis, the diameter of seminiferous tubules decreased only in the BPA 10μg group, but the height of the seminiferous epithelium increased in both BPA groups. The spermatic count was similar in all groups, however the number of immobile sperm as well as number of sperm with abnormal morphology were higher in the BPA 10μg group. This group also showed a decrease in IL-10 cytokine with increased MPO activity in the testis. In the prostate, BPA induced morphological alterations as well as reduction of the luminal compartment and increase of the stromal compartment in the two doses of BPA tested. The presence of hyperplasia, metaplasia and cells in the prostatic lumen was observed in the BPA 10μg group and the BPA 1μg group showed an increase in cellular swelling. BPA 1μg group showed an increase in MPO activity, but other inflammatory parameters were not significant. Leydig TM3 cells were submitted at concentrations of the 0.5 μM at 500 μM of BPA for cell viability and real-time cell growth kinetics analyzes. For analysis of cell cycle evaluation, presence of cells in cell death (Hoechst 3342 staining), mitochondrial membrane potential (rhodamine 123 staining), comet assay and testosterone production was used the concentrations of 1μM, 10 μM and 100 μM of BPA. We results demonstrated that the BPA 100 μM decrease the cell viability and increased the cell death indicated by increase sub-G1 phase cell population and number of cells staining by Hoechst 3342. The rhodamine 123 staining showed a decreased in the metabolically active mitochondria in the BPA 100 μM. The BPA decreased the testosterone levels in all concentrations tested. We conclude that BPA leads to cell dysfunction of Leydig cells as well as low doses of BPA causes changes in prostatic and testicular tissues and induces a decrease in testosterone production.

Key words: Bisphenol A, Leydig Cells, Testis, Prostate.

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LISTA DE ABREVIATURAS E SIGLAS

17β-estradiol	Estradiol
AR	Androgen receptor (Receptor de Andrógeno)
ASC1	Co-integrador de sinal ativador 1
ATP	Adenosine Triphosphate (Trifosfato de Adenosina)
BPA	Bifenol A
cAMP	Cyclic adenosine monophosphate (Monofosfato Cíclico De Adenosina)
COX-2	Ciclo-oxigenase-2
AP	Anterior prostate (Lobos Anteriores)
DLP	Dorsolateral prostate (Lobos Dorsolaterais)
VP	Ventral prostate (Lobos Ventrais)
EGF	Epidermal Growth Factor (Fator de Crescimento Epidérmico)
EPA	United States Environmental Protection Agency (Agência De Proteção Ambiental Dos Estados Unidos)
ERK	Extracellular-regulated protein kinases (Proteína Quinases Reguladas Extracelularmente)
ERα	Estrogen receptor alfa (Receptor de Estrógeno alfa)
ERβ	Estrogen receptor beta(Receptor de Estrógeno beta)
F2a	Gene da Prostaglandina
FSH	Follicle Stimulating Hormone (Hormônio Folículo Estimulante)
GnRH	Gonadotropin-releasing hormone (Hormonio Liberador de Gonadotrofina)
LH	Luteinizing Hormone (Hormônio Luteinizante)
IL	Interleucina
IL-1α	Interleucina 1 alfa
IL-1β	Interleucina 1 beta
IC50	Half maximal inhibitory concentration (Média da concentração máxima inibitória)
LOAEL	Lowest observed adverse effect level (Menor Nível de Efeitos Adversos Observados)
NF-κB	Nuclear Factor κB (Fator nuclear κB)
NOAEL	No Observed Adverse Effect Level (Dose Onde Não se Observa Efeito Adverso)

PSA	Specific Prostatic Antigen (Antígeno Prostático Especifico)
RfD	Reference Dose (Dose Referencia)
StAR	Steroidogenic acute regulatory protein (Proteína Reguladora Aguda Esteroidogênica)
TGF-β	Transforming growth factor beta (Fator De Crescimento Transformador Beta)
Th1	Linfócitos T $CD4^+$ com fenótipo 1
Th2	Linfócitos T $CD4^+$ com fenótipo 2
TM3	Linhagem de Células De Leydig de camundongo
TNFR1	Tumor Necrosis Factor Receptor 1 (Receptor do fator de necrose tumoral 1)
TNF-α	Tumor Necrosis Factor alfa (Fator de Necrose Tumoral)

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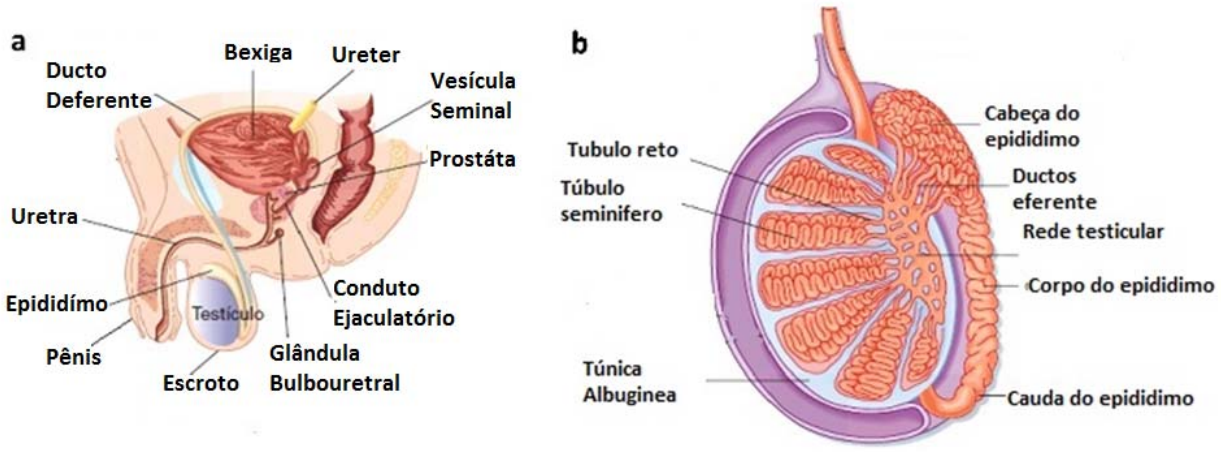
1 INTRODUÇÃO

1.1 Fisiologia do Sistema Genital Masculino

O sistema genital masculino em mamíferos é composto por testículos, epidídimos, ductos deferentes, pênis além das glândulas acessórias: próstata, vesícula seminal e bulbouretrais (Figura 1a) (MOORE; PERSAUD, 2012; ALLAIS-BONNET; PAILHOUX, 2015).

Os testículos são órgãos encontrados em pares no interior do escroto, estão encapsulados por um tecido conjuntivo denso denominado túnica albugínea, e são compostos pelo espaço intersticial e pelos túbulos seminíferos (JUNQUEIRA; CARNEIRO, 2013; KRAWETZ, 2005) (Figura 1b). Os testículos possuem duas principais funções: a produção de gametas (espermatozoides) e a produção de testosterona, essas duas funcionalidades são reguladas pelo sistema nervoso central, principalmente pelos hormônios folículo estimulante (FSH) e luteinizante (LH) por um sistema de *feedback* negativo (AMORY; BREMNER, 2001) (Figura 2).

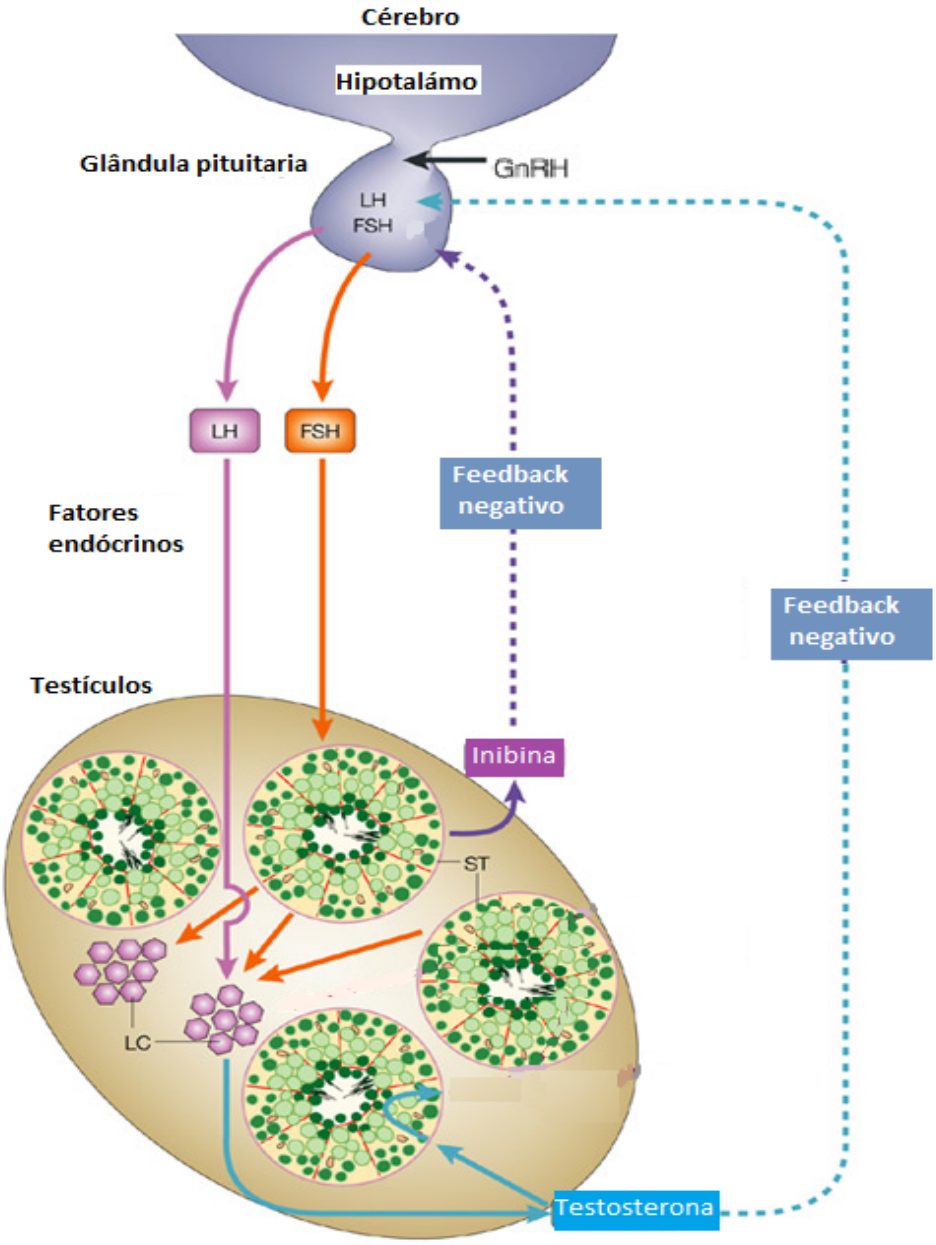
Figura 1 Organização testicular. **a.** Anatomia do sistema reprodutor masculino. **b**.Corte transversal através de um testículo, mostrando a localização dos túbulos seminíferos e os túbulos epididimários. (modificado de BERNE; KOEPPEN; STANTON, 2009; BARRET, et al., 2012).



O espaço intersticial em mamíferos adultos é composto por vasos sanguíneos e linfáticos, nervos, fibroblastos, macrófagos, linfócitos, mastócitos ocasionais e as células de Leydig (PINEAU; DUPAIX ; JEGOU, 1999). As células de Leydig são células produtoras de testosterona (AMORY; BREMNER, 2001). Esse hormônio é produzido por LH ao se ligar aos receptores na membrana plasmática da célula de Leydig (LHR), estimulando a síntese de uma proteína reguladora esteroidogênica (StAR), que iniciará uma cascata de eventos, ao qual o colesterol será convertido em pregnenolona nas mitocôndrias e depois a pregnenolona será transferida para o retículo endoplasmático liso onde ocorrerá a conversão de pregnenolona em testosterona (ZIRKIN;CHEN, 2000; AMORY; BREMNER, 2001). Em adultos, a testosterona

é responsável pela manutenção da barreira hemato-testicular além de contribuir na espermiogênese durante a espermatogênese (Figura 3c) (MRUK;CHENG, 2015).

Figura 2 Ação dos hormônios LH e FSH nos testículos (modificado de COOKE; SAUNDERS, 2002). LH- Hormônio Luteinizante; FSH- Hormônio Folículo estimulante; GnRH- Hormônio liberador de gonadotrofina; ST- Túbulos Seminíferos; LC- Células de Leydig.



Em mamíferos os túbulos seminíferos são considerados a unidade funcional do testículo (COOKE; SAUNDERS, 2002). Os túbulos seminíferos são compostos por células mioides que envolvem o epitélio germinativo e por células de Sertoli e células germinativas

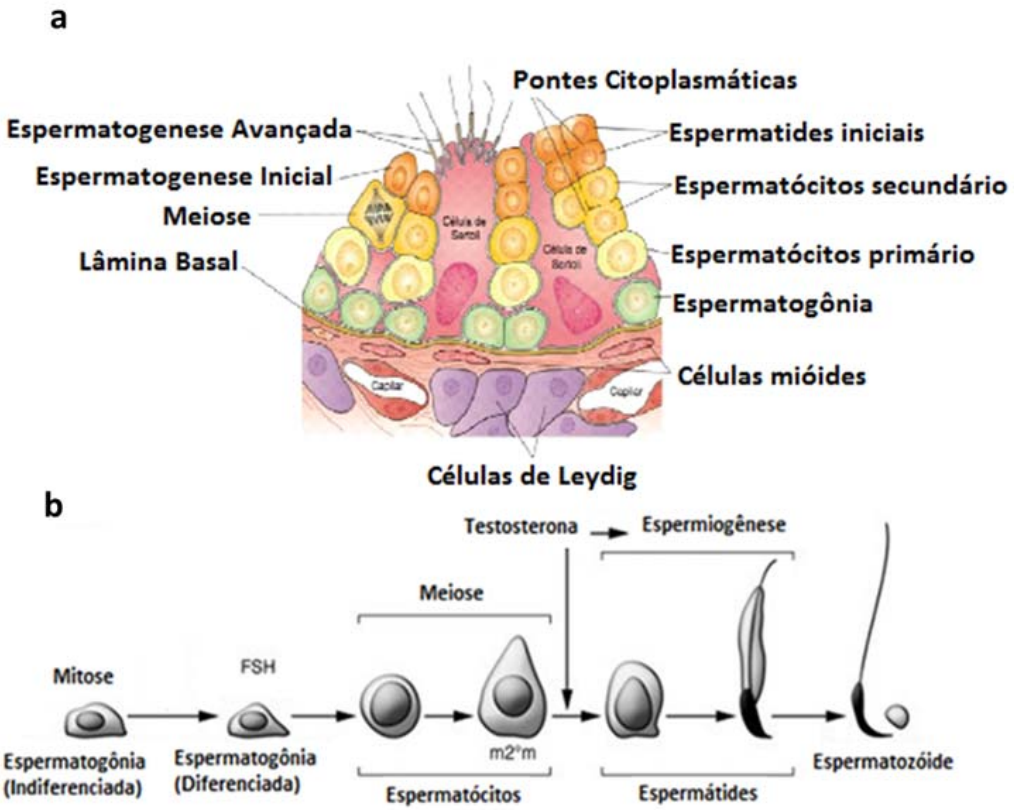
(Figura 3a) (COOKE; SAUNDERS, 2002; MRUK;CHENG, 2015) .

As células de Sertoli são células epiteliais polarizadas que se estendem desde a base do túbulo seminífero até o seu lúmen (Figura 3a) (RATO et al., 2012). Estas células são responsáveis por fornecer energia e apoio nutricional ao desenvolvimento de células germinativas pela secreção de nutrientes ou intermediários metabólicos como aminoácidos, carboidratos, lipídios, vitaminas e íons metálicos além de produzirem lactato. (RATO et al., 2012; MRUK;CHENG, 2015). As células de Sertoli também possuem como funcionalidade formação da barreira hemato-testicular, fagocitose e a secreção de fluido e hormônios importantes para a espermatogênese (FOLEY, 2001). Portanto, alterações nestas células podem resultar no comprometimento da espermatogênese (BOEKELHEID et al., 2005). O hormônio FSH é o principal estimulador do espermatogênese em adultos se ligando a receptores específicos presentes em células de Sertoli (FSHR) (Figura 2). A diminuição deste hormônio em homens adultos leva a redução na produção de espermatozoides e consequente alterações na espermatogênese (AMORY; BREMNER, 2001)

Em um corte transversal de testículos é possível encontrar centenas de túbulos seminíferos que podem estar em estágios diferentes, cada estágio sendo representado por um conjunto específico de células germinativas que estão presentes num único ponto no túbulo ao mesmo tempo (HOGARTH; GRISWOLD, 2010). Dependendo de cada espécie de mamíferos o número de estágios se diferem, em humanos existem apenas 6 estágios, enquanto em camundongos possuem 12 estágios distintos (HESS; FRANCA, 2008). Em média nos mamíferos a duração total da espermatogênese é correspondendo aproximadamente 30 a 78 dias, em camundongos a espermatogênese completa dura em torno de 39-40 dias; em humanos a duração é de aproximadamente 70 dias (HESS; FRANCA, 2008).

Segundo Hess e Franca (2008) “a espermatogênese é a transformação de células espermatogônicas em espermatozoides em um determinado período de tempo dentro dos limites dos túbulos seminíferos no testículo”. Contudo as interações celulares entre a linhagem germinativa e os componentes somáticos do testículo são cruciais para a espermatogênese normal (AMORY; BREMNER, 2001. Assim, uma alteração em qualquer fase da espermatogênese pode levar à interrupção do funcionamento correto do tecido e culminar na infertilidade (AMORY; BREMNER, 2001).

Figura 3 Fases do processo espermatogênico. **a**. Representação de um túbulo seminífero e o espaço intersticial demonstrando a localização das células somáticas (células de Leydig, Sertoli e células mioides) e células de linhagem germinativa (espermatogônia, espermatócito e espermátide) presentes no túbulo seminífero. Diferenciação das células germinativas até a formação do espermatozoide. (adaptado de HOGARTH; GRISWOLD, 2010; JUNQUEIRA; CARNEIRO, 2013).



A espermatogênese pode ser dividida em três fases: mitótica, meiótica e espermiogênese (Figura 3b). Em camundongos na fase mitótica as espermatogônias se proliferam por mitose se diferenciando em espermatogônias do tipo B, posteriormente as espermatogônia do tipo B dão origem a espermatócitos pré-leptóteno, seguido por leptóteno, zigóteno, paquíteno e espermatócitos diplóteno, ao fim esses espermatócitos sofrem diacinese, completando a meiose I, formando espermatócitos II. Os espermatócitos II passam pela meiose II, desenvolvendo espermátides arredondas ao qual irão sofrer a espermiogênese, em um processo de 16 passos no camundongo. Esses passos incluem: formação de acrossoma, alongamento e maturação da cauda e alterações nucleares para formação de espermátides alongadas. A espermatogênese termina com a espermiação, que é a liberação de espermátides maduras presentes aderidas no epitélio seminífero para o lúmen, formando os espermatozoides. Durante a espermiação além das células de Sertoli, as células mioides peritubulares e as células contráteis, presentes no espaço intersticial, ajudam na contração do

túbulo seminífero para que os espermatozoides saiam dos túbulos (HESS; FRANCA, 2008; HOGARTH; GRISWOLD, 2010; KENT; GRISWOLD, 2014; MRUK; CHENG, 2015).

Em adultos, um testículo normal possui aproximadamente 300 lóbulos do qual cada lóbulo em média contém 400 a 600 túbulo seminíferos medindo entre 30-80cm de comprimento. Os túbulos seminíferos estão densamente enovelados e se juntam para formar os túbulos retos que irão compor a rede testicular na região do mediastino. A rede testicular converge posteriormente em 15-20 ductos eferentes que penetram através de uma área espessa da túnica albugínea para formar a cabeça do epidídimo. Uma vez no epidídimo os ductos eferentes convergem em um único túbulo para formar o corpo e cauda do epidídimo que sairá do epidídimo como ducto deferente (Figura 1b) (WOODWARD et al., 2002).

Os espermatozoides que saem do testículo, apesar de apresentarem morfologia completam, não apresentam motilidade nem capacidade para fertilizar o ovócito II. Para que possa adquirir a capacidade de fertilização, é necessário que os espermatozoides passem por diferentes fases de diferenciação pós-gonadal. Essas fases ocorrem durante a passagem do espermatozoide ao longo do túbulo epididimário (CORNWALL, 2009; DACHEUX; DACHEUX, 2014).

O epidídimo adulto é composto pelas regiões de cabeça, corpo e cauda, sendo em roedores a cabeça subdividida em segmento inicial (KRAWETZ, 2005). Os ductos eferentes sofrem mudanças no seu epitélio pseudoestratificado no decorrer de toda extensão epididimária, variando a altura do epitélio e a presença ou ausência de seus vários tipos celulares (células basais, células claras, células estreitas, células principais, células apicais e células halo) (CORNWALL, 2009).

No epidídimo o espermatozoide irá sofrer maturação, o que garante a produção de espermatozoides saudáveis. Durante a passagem pela cabeça até a cauda do epidídimo, os espermatozoides são banhados em um gradiente de RNases, glicosidases e proteases para a remoção de qualquer componente celular que possa ser libertado como parte da gotícula citoplasmática (KRAWETZ, 2005).

É na cabeça do epidídimo que as primeiras modificações espermáticas, como a migração da gota citoplasmática, o início da flagelação e a capacidade de ligação à zona pelúcida ocorrem (DACHEUX; DACHEUX, 2014). Na cabeça do epidídimo de roedores, as células basais reabsorvem cerca de 90% do fluido liberado pela rede testicular para a formação de um ambiente eletroneutro, e assim aumentar a concentração de espermatozoides. Essa absorção é regulada por estrógenos que se ligam principalmente ao receptor de estrógeno α (ERα) (HESS, 1997; HESS, 2003). O aumento na concentração de esperma melhora a

sobrevivência e maturação espermática e assegura que um grande número de espermatozoides sejam libertadas no momento da ejaculação, aumentando a aleatoriedade da fertilização e proporcionar a variação genética (HESS, 1997; CORNWALL, 2009; DACHEUX; DACHEUX, 2014).

A maturação dos espermatozoides no epidídimo é essencial para aquisição da fertilidade pelo gameta masculino, que envolve sucessivas alterações sequenciais sutis em vários subdomínios do gameta (BERNE; KOEPPEN; STANTON, 2009; DACHEUX; DACHEUX, 2014). Cada etapa de maturação é essencial para a qualidade espermática, porém vias da ativação da motilidade, iniciação de capacitação, ou outras mudanças no metabolismo espermático não foram totalmente identificados (DACHEUX; DACHEUX, 2014). Contudo é conhecido que a motilidade e aumento de ATP é adquirido pelos espermatozoides durante a passagem destes pelo corpo e cauda do epidídimo. Os espermatozoides também passam pelo processo de descapacitação, o qual envolve mudanças na membrana celular para evitar que a reação acrossômica ocorra antes do contato com o óvulo (BERNE; KOEPPEN; STANTON, 2009; DACHEUX; DACHEUX, 2014). Por fim, os espermatozoides armazenados na cauda são rapidamente transportados do epidídimo para a uretra por contrações peristálticas da espessa cobertura muscular do ducto deferente (MOORE; PERSAUD, 2012).

O ducto deferente é composto por um lúmen estreito e uma espessa camada de músculo liso que sai da cauda do epidídimo e termina na uretra onde libera seu conteúdo (BERNE; KOEPPEN; STANTON, 2009). O ducto deferente, antes de chegar na próstata se dilata formando a ampola, onde as vesículas seminais liberam seu conteúdo para dentro do ducto deferente. Na continuação, o ducto deferente penetra na próstata e se abre na uretra prostática onde liberará os espermatozoides e receberá o liquido produzido pela próstata (JUNQUEIRA; CARNEIRO, 2012).

O liquido seminal é formado pela liberação de fluido contido na vesícula seminal, próstata e glândula bulbouretral e é responsável pela capacitação espermática, influência na competição dos espermatozoides, transporte de feromônios que atuam como atrativo feminino, modificação da velocidade dos espermatozoides, facilitação da movimentação espermática, controle do pH além de possuir propriedades alospermicidas e ajudar no processo de nutrição espermática, reação acrossômica, e defesa espermática contra sistema imunológico feminino (POIANI, 2006).

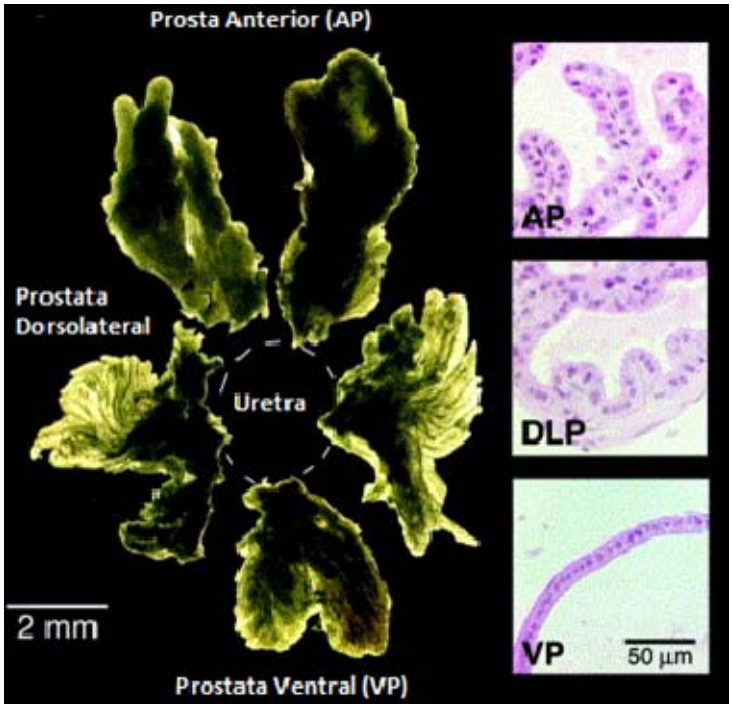
As vesículas seminais são glândulas compostas de dois tubos formada por uma mucosa pregueada forrada com epitélio cuboide e pseudoestratificado colunar, sendo sua principal função a secreção de substâncias importantes para os espermatozoides

(GONZALES, 2001; BERNE; KOEPPEN; STANTON, 2009). As vesículas seminais liberam cerca de 60% - 70% do volume presente no sêmen, sendo a principal fonte de frutose, citrato, inositol e prostaglandinas no esperma que são essenciais para a qualidade espermática (JUNQUEIRA; CARNEIRO, 2012). As vesículas seminais também secretam semenogelina, responsável por induzir a coagulação do sêmen imediatamente após a ejaculação pela formação de um tampão vaginal que impede o retrocesso do sêmen na vagina (MOORE; PERSAUD, 2012).

A próstata, juntamente com a glândula bulbouretral, compõe cerca de 40-30% do restante do fluido seminal, sendo a próstata responsável pela produção de grandes quantidades de citrato, zinco, espermina e fosfatase, além de antígeno prostático especifico (PSA), que liquefaz o sêmen coagulado após minutos, enquanto a glândula bulbouretral é responsável pela liberação de muco que lubrifica, limpa e tampona a uretra (LIMA, 2003; LEITÃO 2013).

A próstata humana é composta por epitélio basal e camada celular constituída por dois compartimentos distintos, o estromal e o epitelial. O compartimento epitelial é composto por células basais e células secretoras luminais, além de uma subpopulação celular com função neuroendócrina (CAMPOS et al., 2008). A próstata possui por um conjunto de 30 a 50 túbulos ramificados compostos por glândulas alveolares que envolvem uma porção denominada uretra prostática, além disso a próstata humana é dividida em zona central, no qual é composta por 25% do volume da glândula, a zona de transição e zona periférica que possui o restante da glândula (SHAPPELL, et al., 2004; JUNQUEIRA; CARNEIRO, 2012). Em roedores, a próstata composta de pares de lobos ventrais (VP), lobos dorsolaterais (DLP) e lobos anteriores (AP) (Figura 4). Histologicamente os lobos da próstata podem ser distinguidos de acordo com as dobras de seu epitélio: a porção AP apresenta epitélio com inúmeras dobras, já a DLP exibe menos dobras que a região AP e a região VP apresenta epitélio com mínimas dobras (MARKER, et al., 2003) (Figura 4).

Figura 4 Anatomia prostática de roedores adultos. Disposição dos lóbulos da próstata em torno da uretra juntamente fotomicroscopia de secções coradas com hematoxalina e eosina mostrando as diferenças dos canais prostáticos de cada lobo (adaptado de MARKER, et al., 2003). AP: Lobo anterior; DLP: Lobo dorolateral; VP: Lobo ventral.



A próstata é uma glândula dependente de andrógenos que possuem um papel central na manutenção do tecido da próstata, podendo regular o crescimento e diferenciação dos diferentes tipos de células epiteliais, direta ou indiretamente, via estroma (AUMÜLLER; SEITZ, 1990). Os efeitos dos andrógenos são mediados pela ativação do receptor de andrógeno (AR). Na próstata normal, o AR é expresso em altos níveis nas células do epitélio secretor e em um subconjunto de células musculares lisas no estroma (ROY-BURMAN, 2004; SUTHAGAR, 2008). Além do papel dos andrógenos na próstata, ela também responde a estrógenos através de receptores de estrogênio (ERα e ERβ), que são expressos no epitélio e no estroma prostático. Contudo o ERβ parece ser um regulador fisiológico do crescimento e diferenciação epitelial da próstata, estando presente no epitélio, onde é coexpresso com AR (WEIHUA, et al., 2001; CUNHA et al, 2002).

Apesar das grandes diferenças entre a próstata humana e de roedores ambas as espécies possuem órgãos sensíveis ao andrógeno e formam glândulas lobulares que têm uma tríade similar de células epiteliais distintamente diferenciadas e também funções semelhantes (SHAPPELL, 2004). A região DLP de rato tem sido por vezes indicada como a mais homóloga à zona periférica em humanos, apresentando maior quantidade de carcinomas,

enquanto a região AP apresenta semelhanças com a zona central, que é raramente um local de transformação neoplásica nos seres humanos (SHAPPELL, et al., 2004). Contudo, apesar do lobo ventral não possuir homologia direta com a próstata humana é o mais responsivo aos andrógenos e, portanto, é o lobo mais utilizado em estudos que focam lesões prostáticas (NETO, 2014).

O pênis humano é composto por três corpos eréteis: dois corpos cavernosos e um corpo esponjoso que envolve a uretra, enquanto o corpo do pênis do rato é formado por dois corpos cavernosos penianos e um corpo cavernoso uretral (CHIASSON, 1969). Histologicamente, o corpo cavernoso é constituído por tecido erétil com auréolas calibrosas, revestidas por uma espessa camada de tecido conjuntivo denso, denominada de túnica albugínea (MURAKAMI; MIZUNO, 1986). Já o pênis do rato apresenta um osso peniano localizado centralmente na glande (HEBEL; STROMBERG, 1976). Quando chega à uretra, os espermatozoides já estão envoltos pelo liquido seminal, denominado de sêmen. Em mamíferos o sêmen é ejaculado dentro do lúmen vaginal pelo pênis através de uma resposta a um arco reflexo que leva à estimulação sensorial do pênis, seguida de estimulação motora simpática do músculo liso do sistema reprodutor masculino e estimulação motora somática da musculatura associada à base do pênis (BERNE; KOEPPEN; STANTON, 2009; JUNQUEIRA; CARNEIRO, 2012).

1.2 Função das citocinas IL-10, IL-6 e TNF-α e das enzimas MPO e NAG nos tecidos prostáticos e testicular

Segundo Domiciano et al., (2017) "A inflamação é uma resposta celular fundamental de múltiplos passos a estímulos nocivos, tais como agentes patogênicos, toxinas, traumatismos ou lesões por calor. Assim, a inflamação tem um papel importante no sistema imunitário e na manutenção da função homeostática do tecido". As citocinas e as células do sistema imune possuem um papel fisiológico importante no sistema genital masculino mantendo a homeostase dos tecidos que compõe este sistema (FRACZE; KURPISZ, 2015).

TNF-α é secretado por monócitos e macrófagos e desempenha um papel central no início da resposta inflamatória estimulando a liberação de IL-6 por macrófagos e monócitos (HALES; DIEMER; HALES, 1999). No testículo TNF-α é expresso em espermatócitos e espermátides arredondadas, apresentando funções no controle do processo espermatogênico e inibindo a apoptose das células germinativas pelo bloqueio da ação do FasL em células de Sertoli (HALES; DIEMER; HALES, 1999; HEDGER; MEINHARD, 2003; BIALAS et al., 2009). Em contraste no ambiente inflamatório testicular o aumento de TNF-α está relacionado

ao aumento da apoptose devido sua ligação ao receptor TNFR1 em células germinativas (PÉREZ et a., 2013). Além disso, o TNF-α também pode levar a alterações na barreira hemato testicular e consequentemente induzir a perda de células germinativas (ZHANG et al., 2014). Esta citocina ainda pode ter uma função autócrina sobre as células de Leydig uma vez que estas células também liberam TNF-α, interferindo na esteroidogêsese (HEDGER; MEINHARD, 2003; BIALAS et al., 2009). Na próstata TNF-α está ligada a indução da apoptose em células prostáticas em condições fisiológicas e patológicas (CHOPRA et al., 2004). Estudos têm demonstrado que o TNF-α pode desempenhar ação pró-carcinogênica na próstata devido à sua capacidade de indução da ciclo-oxigenase-2 (COX-2) e outros fatores angiogênicos tais como metaloproteinases de matriz e quimiocinas (GALHEIGO et al., 2016).

Em testículos, a interleucina IL-6 é liberada por macrófagos intersticiais, células de Leydig, células de Sertoli, células germinativas e espermatozoides (POTASHNIK et al., 2005). Esta interleucina parece ter um papel importante no desenvolvimento, crescimento, proliferação e diferenciação do testículo além de ter uma ação autócrina/parácrina na regulação da espermatogênese e esteroidogênese (POTASHNIK et al., 2005; RIVAL et al., 2006). Na próstata a IL-6 é produzida por macrófagos residentes e células do epitélio prostático para compor o liquido seminal, cooperando com a defesa contra o sistema imune feminino dentro do ambiente vaginal (MATALLIOTAKIS et al., 1998). A IL-6 também é necessária para regular as funções linfocitárias locais importantes para a proteção imunológica dos tecidos testicular e prostático (HULEIHEL; LUNENFELD, 2004). Durante a inflamação a produção de IL-6 é aumentada devido à infiltração de células inflamatórias no testículo, e como TNF- α, este aumento está associado a perdas de células germinativas no epitélio seminífero devido ao rompimento da barreira hemato testicular (PÉREZ et a., 2013), enquanto na próstata o aumento da IL-6 tem sido correlacionado a patogênese no câncer de próstata, por estimular a iniciação e promoção do câncer (SFANOS; DE MARZO, 2012).

A IL-10 é uma das mais importantes citocinas anti-inflamatórias do sistema imunitário dos mamíferos (BIALAS et al., 2009). No sistema genital masculino, a IL-10 é liberada por macrófagos M2 e é responsável pode inibir o reconhecimento de antígenos por linfócitos T (BIALAS et al., 2009; BHUSHAN, et al., 2016). A IL-10 também demonstrou ser uma importante citocina contra os danos causados por torção testicular (OZTURK, 2014). Na próstata, esta citocina suprime a resposta Th-1, e regula a diferenciação de células B, natural *killer* e células T reguladoras e citotóxicas (MICHAUD et al., 2006). A IL-10 também mostrou inibir a angiogênese no câncer prostático agindo diretamente sobre as células de

câncer pela redução da produção de metaloproteinase 2 (TIMP-2) e metaloproteinase de matriz (MMP) -2 e -9 (ASADULLAH; STERRY; VOLK, 2003; MICHAUD et al., 2006).

Em condições fisiológicas normais células do sistema imune como monócitos, macrófagos, células dendríticas, células T e T reguladoras (Treg), células natural *killer* e mastócitos são encontradas no interstício do tecido testicular e prostático e juntamente com as células de Leydig, células de Sertoli e células da linhagem germinativa ajudam na manutenção da espermatogênese (MRUK; CHENG, 2015).

A infiltração de neutrófilos em tecidos é comumente avaliada por alterações na atividade da mieloperoxidase (MPO) que é uma enzima responsável por facilitar a conversão de peróxido de hidrogénio em ácido hipocloroso (ARNHOLD, 2004). Esta enzima é encontrada principalmente em neutrófilos, porém em determinados casos também são encontrados em monócitos e em macrófagos teciduais. (ARNHOLD, 2004; KLEBANOFF, 2005; CHOI et al., 2008). No caso de estímulos nocivos no testículo, ocorre a ativação de leucócitos na vasculatura testicular induzindo a ativação de neutrófilos que produzem grânulos compostos por peróxido de hidrogênio e MPO que pode levar a danos teciduais quando liberados (UZ et al., 2002). Ozturk et al. (2014), relataram aumento de MPO em tecido testicular após reperfusão, porém os danos foram amenizados pela liberação de IL-10 neste tecido. Roumeguère et al., (2011) demonstraram a presença de MPO em células do epitélio secretor prostático de próstata humana, entretanto a presença de MPO pode promover mutagênese através da modificação do DNA e induzir a disfunção da célula prostática. O aumento de MPO no tecido prostático, devido a exposição a compostos estrógenos, está relacionado ao aumento de infiltrado inflamatório com diminuição do tamanho deste tecido (STOKERAB; ROBINETTE; COOPER, 1999).

A análise de N-acetyl-β-D-glucosaminidase (NAG) é utilizada para quantificação de macrófagos ativos uma vez que esta enzima está presente em grandes quantidades em seus lisossomos presentes nestas células (REINER et al., 1981; BUENO et al., 2017). No testículo, os macrófagos apresentam importante papel na regulação do desenvolvimento e da esteroidoigênese das células Leydig (ZHAO et al., 2014; BHUSHAN, et al., 2016). O aumento de NAG em testículos está relacionado à alterações testiculares causadas por tóxicos levando a danos testiculares (AHMED et al., 2015; BUENO et al., 2017). Na próstata, os macrófagos e linfócitos são encontrados tanto no estroma quanto no epitélio prostático (DE MARZO et al., 2007). Adachi et al. (1989) observaram que NAG é encontrada no líquido seminal de homens saudáveis em altos níveis devido à produção desta enzima no tecido prostático, provavelmente pelos macrófagos residentes no epitélio prostático.

1.3 Linhagem celular de células de Leydig (TM3)

Como descrito anteriormente as células de Leydig em mamíferos estão localizadas no espaço intersticial testicular e são responsáveis pela produção de testosterona, um hormônio importante para a espermatogênese e para formação das características sexuais masculinas (TEERDS; HUHTANIEMI, 2015).

As células de Leydig da linhagem TM3 não são tumorigênicas é são derivadas de testículos de camundongo BALB/c apresentando como característica a retenção da maioria das funções *in vivo* incluindo a presença de receptores para LH. Na presença de LH estas células aumentam o monofosfato cíclico de adenosina (cAMP) e metabolizam o colesterol para a produção de testosterona (MATHER, 1980). As células TM3 também possuem receptores para fatores de crescimento epidérmico (EGF), receptor de andrógeno (AR) e receptor de estrogênio (ER) além de expressar o gene prostaglandina F2a (MATHER,1980; MATHER et. al, 1982). Harrison, et al. (2001) relataram a presença de receptores de inibina A nestas células o que permite a compreensão da ação desta proteína no controle da produção de testosterona.

Devido a estas características as células TM3 representam um modelo experimental amplamente utilizado para a avaliação *in vitro* da perturbação endócrina e vem sendo utilizada para a análise da ação de diversas substâncias como álcool (JANG et al 2002), genisteína (KUMI□DIAKA, RODRIGUEZ, GOUDAZE, 1998), *Morinda officinalis* (CHANG et al, 2008) e xenobióticos (CHEN et al., 2015).

1.4 Bisfenol A

O Bisfenol A (BPA, 2,2-bis (4-hidroxifenil) propano, CAS # 80-05-7) é uma molécula de hidrocarboneto, que ao se ligar a outras moléculas de hidrocarboneto forma um material altamente resistente e durável. Portanto desde 1940 o BPA é utilizado em grandes quantidades em todo o mundo para a produção de plástico e para a fabricação de revestimentos de resina epóxi para latas de alimentos e bebidas, além de também ser utilizada como componente de selantes dentários (VANDENBERG et al.,2009; GROFF, 2010; ERLER; NOVAK, 2010). A exposição ao BPA pode ocorrer de diversas formas, incluindo a alimentação uma vez que esta substância é liberada quando é exposta ao calor. Assim alimentos em embalagens produzidas com BPA podem conter quantidades significativas da substância (ERLER; NOVAK, 2010; ROGERS; METZ; YONG, 2013). A exposição ao BPA também inclui água e solos contaminados por descargas residuais provenientes de fabricas

que utilizam o BPA (GAROMA; MATSUMOTO, 2009; ERLER; NOVAK, 2010; ROGERS; METZ; YONG, 2013).

O BPA é considerado um desregulador endócrino uma vez que atua como um hormônio exógeno, sendo assim considerado um estrógeno não esteroidal. Esta molécula é capaz interferir na síntese, reserva, liberação, transporte, metabolismo, ligação, ação ou eliminação de hormônios naturais do organismo responsáveis pela regulação da homeostase e dos processos de desenvolvimento (KAVLOCK et al. 1996; WETHERILL et al., 2007; ERLER; NOVAK, 2010).

O principal mecanismo de ação do BPA como estrogênio, é sua interferência nos estrogênios endógenos (por exemplo, 17β-estradiol) perturbando a atividade adequada dos receptores hormonais nucleares de estrogênio (ER), além de atuar como antagonista do receptor de andrógeno (AR) num conjunto diversificado de tecidos-alvo tanto em humanos quanto em roedores (WETHERILL et al., 2007; PREETHI et al., 2014). O BPA se liga tanto ao ERα quanto ao ERβ, apresentando uma ligação mais forte ao ERβ (WETHERILL et al., 2007). A ligação desta molécula nestes receptores está relacionada a alterações na resposta fisiológica do tecido alvo e induz a alterações na expressão de genes responsáveis pela produção de hormônios além de alterar a estrutura do receptor resultando em uma diversidade de respostas transcricionais (WETHERILL et al., 2007). O BPA pode afetar múltiplos passos da ativação e função de AR incluindo a modulação da interação de AR com o seu co-regulador (ASC1) e a ligação de andrógenos endógenos (LEE et al., 2003; WETHERILL et al., 2007).

Em toxicologia a escolha da dose que será testada é importante para melhor compreensão dos danos causadas pelo tóxico. No caso do BPA foi estabelecido como o menor nível de efeitos adversos observados (LOAEL) a concentração de ≤ 50 mg/kg/dia de BPA, enquanto a dose onde não se observa efeito adverso (NOAEL) é de ≤ 5 mg/kg/dia de BPA (BRANIST et al., 2009; VANDENBERG et a al., 2013). Diversos estudos têm demonstrado alterações em tecidos como cérebro (ZALKO et al., 2016), tireoide (KOBAYASHI et al., 2005), mamas (FISCHER et al., 2016), além de alterações no desenvolvimento sexual de machos e fêmeas (CHRISTIANSEN et al., 2014) em doses abaixo da LOAEL e NOAEL. Com bases em estudos em animais a *United States Environmental Protection Agency (EPA)* definiu como dose oral de referencia (RfD), ou seja, a concentração aceitável de dose oral do BPA para humanos o valor de 50 µg/kg /dia, contudo assim como LOAEL diversos estudos têm demonstrado alterações em tecidos de animais em doses inferiores a RfD proposta pela EPA (BERONIUS et al., 2010; VANDENBERG et al., 2013). Atualmente a dose de ingestão

diária tolerável humana (IDT) proposta pela European Food Safety Authority (EFSA) é de 10 μg/kg/dia (ESFA, 2006). Em estudos in vitro tem sido considerado como a menor concentração que causa efeitos como $1x10^{-7}$ M. Entretanto, essa concentração pode ser variável de acordo com a célula testada, assim como as concentrações da média da concentração máxima inibitória (IC50) (VANDENBERG et al., 2013).

Estima-se que a ingestão humana diária, incluindo crianças e lactantes, seja de < 1μg/Kg de peso corpóreo/dia (KANG et al., 2007), contudo este dado pode variar de acordo com cada região. Lang et al. (2008) coletaram a urina de 694 homens e 761 mulheres da população americana com idades entre 18 a 74 anos e relacionaram as concentrações urinárias mais altas de BPA com o aumento da prevalência de doenças cardiovasculares, diabetes e anormalidades da enzima hepática nesta população. Ning et al. (2011), analisaram a urina de 3.423 chineses adultos com mais de 40 anos e observaram a presença de BPA na urina com a diabetes tipo-2 nesta população. Um estudo envolvendo 2.838 crianças e adolescentes da população americana associou o aumento de BPA na urina com a obesidade (TRASANDE; ATTINA; BLUSTEIN, 2012). Galloway et al. (2010) relataram um aumento na concentração sérica de testosterona com o aumento da presença de BPA na urina em 715 homens italianos entre 20 a 74 anos. Em relação a reprodução diversos estudos têm associado as altas concentrações de BPA em mulheres com problemas na implantação por fertilização *in vitro* (EHRLICH et al., 2012), assim como redução dos níveis de estradiol (BLOOM et al., 2011). Em homens o BPA está relacionado a altos níveis de testosterona, LH, e estradiol e de baixas concentrações de espermatozoides móveis (CALAFAT et al., 2008).

Em roedores, a presença de baixas doses de BPA pode levar à disfunção cerebral, alterações de comportamento durante o desenvolvimento e alterações metabólicas (VANDENBERG et al., 2013). No sistema reprodutor de fêmeas o BPA induziu a alterações na morfologia mamária relacionadas ao câncer e também a perturbações da meiose do oócito durante a formação dos folículos primário (RICHTER et al., 2007). Em machos o BPA leva a alterações hormonais como redução de testosterona e alterações na espermatogênese (RICHTER et al., 2007). *In vitro* diversos trabalhos têm demonstrado alterações em culturas de células devido ao BPA como em células neuronais (NEGRI-CESI, 2015), adipócitos, células do pâncreas (PROVVISIERO et al., 2016), monócitos (NERI, et al., 2015) e células mamárias (AGHAJANPOUR-MIR et al., 2016).

Por fim, o BPA também está relacionado a alterações na resposta imune devido a sua ligação aos ERα e ERβ ativando a vias de cascata da proteína quinases reguladas extracelularmente (ERK) /fator nuclear κB (NF-κB) que regula citocinas pró-inflamatórias,

como TNF-α e IL-6 e citocinas anti-inflamatórias, como TGF-β e IL-10 (KHAN; AHMED, 2014).

1.5 Bisfenol A e o Tecido Testicular e Prostático

Diversos estudos têm mostrado alterações testiculares e prostáticas devido a desreguladores endócrinos (COLBORN; VON SAAL; SOTO, 1993; PASQUALOTTO et al., 2004; PATRICK et al., 2016), porém dentre eles o BPA parece ter diversos efeitos no sistema reprodutor masculino (NANJAPPA et al., 2014; HO et al., 2017).

O BPA leva à alterações no eixo hipotalâmico-hipofisário-testicular. Este eixo é responsável pela produção da testosterona em células de Leydig assim como o funcionamento correto da espermatogênese (MANFO, et al., 2014). Trabalhos têm demonstrado alterações hormonais como o aumento dos níveis de LH e prolactina e redução das concentrações plasmáticas de testosterona testicular, devido a administração de BPA (1mg/kg) na água de bebedouros, ou por via subcutânea, durante 14 dias, em ratos adultos Wistar (TAKAO, et al., 1999; TOHEI, et al., 2001). Toyama et al. (2004) observaram que a administração subcutânea de BPA durante seis dias nas doses de 20 e 200 μg/kg em camundongos adultos (3 meses de idade) e ratos adultos (4 meses de idade) tem efeito estrogênico sobre os testículos induzindo a anomalias de cabeças em espermátides maduras no epitélio germinativo. A administração de BPA nas doses 0,02 a 50mg/kg durante 5 dias em ratos Sprague-Dawley prejudica a integridade e a função da barreira hematotesticular, consequentemente alterando o processo espermatogênico o que pode levar a prejuízos na fertilidade (LI, et al., 2009).

Em um estudo com humanos, Lassen et al. (2014) avaliaram amostras de urina, sêmen e plasma de 303 homens jovens em idade reprodutiva da população americana e demonstraram que 98% das amostras de urina apresentavam níveis detectáveis de BPA (0,12 ng/mL), o que estava associado a maiores níveis de testosterona circulante, LH e estradiol e uma menor porcentagem de espermatozoides progressivos móveis.

Já em próstata o BPA tem sido relacionado ao desenvolvimento de câncer neste tecido (MANFO et al., 2014; HO et al.,2017). Em humanos, um estudo envolvendo 60 pacientes de urologia encontrou uma associação com os níveis aumentados de BPA na urina com homens que apresentavam câncer de próstata. O mesmo não foi observado em homens sem câncer (TARAPORE et al., 2014). Em ratos expostos desde o nascimento até a fase adulta a baixa dose de 10 μg/kg de BPA foi o suficiente para aumentar a susceptibilidade a lesões neoplásicas na próstata. Os autores também evidenciaram alteração epigenéticas no tecido prostático (HO et al., 2006). Em ratos machos adultos descendentes de fêmeas expostas

via gavagem à dose de 25 μg/kg/dia, foi evidenciado aumento do peso da próstata ventral juntamente com o aumento da proliferação de células do epitélio e aumento da expressão de AR, resultando em uma suscetibilidade a inflamação crônica multifocal e hiperplasias reativas e atípicas na idade adulta (BERNARDO et al., 2015).

1.6 Bisfenol A e Células de Leydig

As células testiculares expressam um nível elevado de receptores de estrógeno (ERα, ERβ) juntamente com AR (N'TUMBA-BYN et al., 2012, ZHOU et al., 2002) e respondem ao LH que estimulam a esteroidogênese em células de Leydig (CHEN et al., 2016, DANKERS et al., 2013). Portanto alterações na função das células de Leydig pode afetar adversamente as funções testiculares levando à infertilidade (YANG et al., 2015).

O BPA funciona como um antagonista de AR e pode interagir com os subtipos de ER (ERα e ERβ) (LASSEN et al., 2014). BPA exerce um efeito inibitório sobre a esteroidogênese das células de Leydig que é provavelmente mediada através da ER (AKINGBEMI et al., 2004). Em cultura primária de células de Leydig humana fetal, concentrações superiores a 10^{-8} M de BPA durante 3 dias de exposição afetaram a função esteroidogênica levando à diminuição da produção de testosterona (N'TUMBA-BYN et al., 2012). Os descendentes de fêmeas de ratos que foram expostas na dose de 25 μg/kg por dia via gavagem durante os dias gestacionais 12 ao 21, apresentaram uma menor produção de testosterona pelas células de Leydig de machos adultos (ABDEL-MAKSOUD et al., 2015). Em ratos machos expostos 4 vezes por semana durante 6 semanas em doses de 20, 100 e 200 mg BPA/kg/dia induziu uma diminuição nos níveis de testosterona plasmática e testicular assim como diminuição de LH e consequentemente uma diminuição da expressões de enzimas esteroidogênicas e da proteína transportadora de colesterol em células de Leydig em todas as doses testadas. Além disso, a dose de 200 mg / kg de BPA foi associada a uma diminuição significativa no número de células de Leydig no testículo, assim como a expressão do ERα (NAKAMURA et al., 2010).

Roelofs et al. (2015), relataram alterações na esteroidogênese, assim como diminuição nos níveis de testosterona na linhagem celular de Leydig MA-10, expostas a concentrações de BPA de 10^{-5} a 10^{-8} M, neste estudo os autores também observaram modificações no AR devido a ação antagonista do BPA. Em cultura primária de células de Leydig imaturas de camundongos expostas à concentração de 10 μM de BPA foi observado um aumento na produção de andrógenos devido a atenuação da conversão de testosterona no metabólito α-androstane-3α,17β-diol. Esta conversão pode perturbar a diferenciação da célula de Leydig e criar ambientes parácrinos intra-testiculares anormais que podem alterar o

desenvolvimento adequado das células germinativas (SAVCHUK; SÖDER; SVECHNIKOV, 2013). Um estudo recente demonstrou que o BPA nas concentrações de 10^{-9} a 10^{-3} M durante 24, 48 e 72 h pode induzir células de Leydig de camundongos a modular perfis proteicos, inibir a proliferação celular e promover a migração e invasão celular *in vitro* (CHEN et al., 2016).

Portanto o Bisfenol A em altas doses e concentração pode induzir diversos distúrbios no sistema genital masculino. Porém ainda há poucos dados sobre os danos causados pelo BPA em baixas doses nesse sistema, ao qual é responsivo por receptores de andrógenos e estrógenos.

2 JUSTIFICATIVA

O Bisfenol A (BPA) é utilizado para a fabricação de produtos plásticos, portanto a população ainda está em contato com essa molécula principalmente pela exposição via oral. O principal mecanismo danoso desta molécula é sua ação sobre receptores de estrógeno e andrógeno, sendo assim considerado um desregulador endócrino.

A presença do BPA na urina em diversas populações do mundo está relacionada à doenças cardiovasculares, à síndrome metabólica e à alterações no tecido cerebral e na tireoide. Apesar do conhecimento dos efeitos do BPA em diversos tecidos humanos e de roedores, em alguns países como Estados Unidos, Canadá e Brasil, a utilização do BPA foi proibida somente em produtos como mamadeira e embalagens de alimento infantil. Atualmente doses de LOAEL, NOAEL e de RfD para BPA parecem estar equivocadas, uma vez que estudos têm demonstrado alterações em múltiplos órgãos em doses inferiores a estas, além disso há pouca informação referente aos danos causados ao sistema genital masculino no que diz respeito a ingestão humana diária (≈1μg/kg/dia) em adultos.

Estudos *in vitro* e *in vivo* têm demonstrado a ação do BPA diretamente sobre a linhagens celulares e tecidos responsivos a andrógenos e estrógenos. Contudo há a necessidade de mais informações sobre a ação do BPA em células de Leydig, uma vez que estas células são responsáveis pela produção de testosterona nos testículos, além de a literatura já apresentar descrições sobre que alterações neste hormônio, por meio de interação com tóxicos responsáveis por alterações no testículo e na próstata de roedores.

Portanto o atual trabalho tem alta aplicabilidade, uma vez que propomos o estudo com base em doses e concentrações de exposição humana. Assim, pretendemos com os resultados obtidos neste estudo, contribuir para a compreensão da atuação de baixas doses e concentrações de BPA tanto *in vivo* quanto *in vitro* no sistema genital masculino.

3 OBJETIVOS

3.1 Geral

O presente estudo tem como objetivo avaliar as alterações causadas pelo BPA em baixas doses nos tecidos testicular e prostático de camundongos machos adultos e sobre a linhagem de células de Leydig TM3.

3.2 Específicos

Avaliar os efeitos do BPA sobre células de Leydig.

Avaliar a morfologia testicular e prostática;

Avaliar a qualidade espermática;

Analisar alterações na produção total de testosterona;

Realizar análises de perfil inflamatório no testículo e próstata;

Contribuir com dados sobre os efeitos da ação do BPA em baixas doses no testículo e próstata de camundongos;

Contribuir com dados sobre os efeitos da ação do BPA em células de Leydig.

4 ARTIGO I

Baixas Doses de Bisfenol A Alteram a Qualidade Espermática e a Morfologia Testicular e Prostática com Redução da Testosterona em Camundongos Adultos

Low Doses of Bisphenol A alter Sperm Quality and Testicular and Prostatic Morphology with Testosterone Reduction In Adult Mice

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Low Doses of Bisphenol A alter Sperm Quality and Testicular and Prostatic Morphology with Testosterone Reduction In Adult Mice

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Abstract

The male genital system depends on the androgens and estrogens such as testosterone for its correct function. The endocrine disruptor Bisphenol A (BPA) in high doses cause modifications in various tissues principally by binding in the estrogen and androgen receptors. Therefore, the objective of this study was to evaluate changes in the testes, sperm and ventral prostate due to oral exposure to low doses of BPA. 60-day-old animals were divided into 3 groups in which they received via gavage: 1% DMSO (C), 1μg / kg BPA (BPA 1μg) and 10μg / kg BPA (BPA 10μg) daily for 50 days. At 110 days of age, the animals were weights and euthanized, blood was used for dosage of plasmatic testosterone levels. Testes, ventral prostate, vas deferens and seminal vesicle full and empty were weight. Testes and ventral prostate were used for analysis of the histological, inflammatory profile, stereological analysis of the prostate, morphometric and sperm count analysis of the testis. Sperm from the vas deferens was submitted to morphological and motility analysis. We results showed that the low doses of BPA alters the testicular and prostatic morphology, increased the prostatic and sperm morphology damages and decrease the motility sperm, as well as decreased the testosterone levels. We conclude the alterations caused in testes, prostate, sperm parameter by low doses of BPA is probably due to an action in estrogen and androgen receptors, since there was a decrease in total testosterone production without altering the number and kariometric in Leydig cells of mice exposed to BPA.

Key words: Bisphenol A, Testes, Ventral Prostate, Sperm parameters, Testosterone, Mouse.

1. Introduction

Bisphenol A (BPA) is a molecule of the hydrocarbon, used mainly in the production of plastic and epoxy resin coatings for food cans and beverage (Groff, 2010; Erler and Novak, 2010). Therefore, the main source of human contamination is through the consumption of food and water containing BPA, being the estimated value of daily human intake 1μg/kg/day of BPA (Kang et al., 2007; Erler and Novak, 2010). Several governmental entities have been concerned about establishing doses of BPA tolerable for human consumption, the US Environmental Protection Agency has established a tolerable daily dose of 50 μg/kg/day (EPA, 1998). However, the European Food Safety Authority placed a human tolerable daily intake (TDI) at a dose of 10 μg/kg/day (ESFA, 2006). Though, some studies have demonstrated that lower doses are associated with liver and pancreatic changes (García-Arévalo et al., 2016; He et al., 2016)

The BPA is considered an endocrine disrupter, since it acts as an exogenous hormone in the organism (Kavlock et al., 1996; Wetherill et al., 2007). The principal mechanism of action of BPA is its interference in the endogenous estrogens (e.g., 17β-estradiol) by binding estrogen receptors (ER) altering its function (Wetherill et al., 2007). This molecule also can act as an antagonist for the androgen receptor (AR) in a diverse set of target tissues in both humans and rodents (Preethi et al., 2014).

Testicular cells express a high level of estrogen receptors (ERα, ERβ) and AR (Zhou et al., 2002; N'Tumba-Byn et al., 2012) and respond to LH (Dankers et al., 2013; Chen et al., 2016). The function of testes is the production of gametes (spermatozoa) and the production of testosterone, to which are regulated by androgen and estrogens receptors (Amory et al., 2001). Similarly, the prostate is an androgen-dependent gland and the androgens having a central role in the maintenance of prostatic tissue regulating the growth and differentiation of different types of epithelial cells (Aumüller and Seitz, 1990). In addition the prostate tissue also responds to estrogens through estrogen receptors (ERα and ERβ) that are expressed in the prostatic epithelium and stromal (Cunha et al, 2002). ERβ appears to be a physiological regulator of prostate epithelial growth and differentiation, being present in the epithelium, where it is coexpressed with AR (Weihua et al., 2001). The prostate is divided into three lobes, however the ventral lobe being the most responsive to androgens, and therefore the most used in studies focusing on prostatic lesions (Scarano, 2009).

Cytokines and cells of the immune system they have an important physiological role in the male genital system maintaining the homeostasis of the tissues that compose this system (Fracze; Kurpisz, 2015). Cytokines such as IL-6, TNF-α and IL-10 they have a key role in the control of the spermatogenic process and the inhibition of recognition of self-antigens (Hales; Diemer; Hales, 1999; Rival et al., 2006; Bhushan, et al., 2016). However the binding of BPA in the estrogen receptors mediate the extracellular-regulated protein kinases (ERK)/nuclear factor κB (NF-κB) signal cascade increasing TNFα and IL-6 inducing an inflammatory process (Khan; Ahmed, 2014).

Although, there are studies about the BPA action on various tissues in concentrations above what is considered safe, there is still few data on the damage caused by low dose BPA on sperm, testosterone levels, testes and prostate tissues. The objective of present study was to investigate the toxic effects of BPA on the sperm parameters, testes and prostate ventral tissue at dosages doses similar to human ingestion in adult mice.

2. Materials and Methods

2.1 Ethical approval

All experimental procedures in this paper were in accordance with the Ethical Principles in Animal Research approved by the Ethics Committee on Animal Use of State University of Londrina, UEL, Londrina-PR, Brazil (CEUA/UEL number 161/2015) in accordance by the Brazilian College of Animal Experimentation.

2.2 Drugs

The bisphenol A (BPA, CAS No. 80-05-7) purchased from Sigma-Aldrich (St. Louis, MO) was diluted in dimethyl sulfoxide (DMSO, Mallinckrodt Chemicals, St. Louis, MO, USA) and solubilized in water and two solutions were prepared with the final concentrations of 1 μg/mL and 10 μg/mL of BPA, respectively. All solutions were prepared on day and kept without contact with light in glass containers

2.3 Experimental protocol

The Biotery Central, Biological Sciences Center, State University of Londrina (CCB-UEL), supplied 53-day-old male mice with approximately 40 g of body weight. The animals were randomly assigned and allocated into polypropylene cages (43 × 30 × 15 cm) with a shavings substrate under a 12-hour controlled lighting condition with controlled temperature (22.5 ± 2.5°C) and were given drinking water and food (Nuvital®) *ad libitum* during the acclimation time (7 days) and throughout the experimental period. After the acclimation period these animals with 60-day-old were separated into 3 groups: control (C), Bisphenol A 1 μg/kg/day (BPA 1μg), Bisphenol A 10 μg/kg/day (BPA 10μg). All animals were treated daily for 50 days via gavage, and the control group received 0.1mL of 1% DMSO in 99% water, the BPA 1μg group received solution containing 1 μg/kg BPA and the BPA 10μg group received solution containing 10 μg/kg of BPA. The doses of BPA used in the present study were lower than the dose recommended (RfD) of 50 μg/kg/day (EPA, 1998). The body weight were measured throughout the experiments.

2.4 Body and prostate weights

Mice were weighed with 110-day-old, anesthetized (0.1 ml ketamine (Quetamin®, Louveira, Brazil) and 0.1 mL xylazine (Anasedan®, Paulinia, Brazil)), euthanized by cardiac puncture, and collected blood. The testes, ventral prostate and vas deferens were removed and weights and the seminal vesicle was weight with and without fluid seminal. The blood were collected (between 8:00 a.m. and 12:00 p.m.) into heparinized tubes and was centrifuged (12000g, 15min, 4ºC) and the plasma was used for total testosterone dosage (n= 10/group).

Ventral prostate was used for histological analysis (n = 5/group) and inflammatory profile: Myeloperoxidase (MPO) activity (n= 5/group), N-acetyl-β-D-glucosaminide (NAG) activity (n = 5/group) and cytokine measurement (n = 5/group). The left testes was used for histological processes (n= 5/group) and sperm count (n=10/group) and the right testes was used for MPO activity (n= 5/group), NAG activity (n= 5/group) and cytokine measurement (n= 5/group). The sperm obtained by vas deferens and used for spermatic morphology (n=10/group) and motility (n=10/group).

2.5 Histological processing

The ventral prostate segment and left testes (5 per group) was removed, fixed in Metacarn (60% methanol, 30% chloroform, and 10% acetic acid), embedded in Paraplast® (Sigma-Aldrich) and sectioned into semi-serial sections at 5 µm. The slides with 4 sections were stained with hematoxylin and eosin (HE) and examined general histopathological and morphometric analysis using a photomicroscope (Opton) and BELView Software version 6.2.3.0 (BEL Engineering) for Windows at a magnification of 100x and 400x.

2.5.1 Histopathological analysis in testis and prostate

One hundred random testicular cross-sections per animal were observed into photomicroscope at a magnification 100x and the seminiferous tubules were divided into normal or abnormal, according to the cells present in the seminiferous tubules as describe by Favareto et al. (2011). The ventral prostate sections were qualitatively evaluated using the photomicroscope (100x and 400x) and photomicrography performed using BELView Software. The prostate lesions presented in the experimental animals was performed according to the Bar Harbor Classification System for the mouse prostate, developed by National Cancer Institute's Mouse Models of the Human Cancers Consortium, Prostate Steering Committee (Shappell et al., 2004).

2.5.2 Leydig Cells analysis

Nuclei of the adults Leydig cells (ALC) were counted in 10 random fields in each testis section stained with HE. Kariometric analyzes were performed in 50 random circular or elliptical nuclei of ALC per animal (Guerra et al., 2016). Major (D) and minor (d) diameter of the cell nuclei were obtained using using a photomicroscope and BELView Software at a magnification of 400x. Thereafter, the medium diameter (M) vas calculated using the formula $M= (D + d)/2$ and nuclear area (A) and volume (V) were obtained with the following formulas: $A= \pi \times 1/4 \times M^2$ and $V= \pi \times 1/6 \times M^3$, respectively (Cury et al., 2006).

2.5.3 Morphometric and Stereological analysis

The seminiferous tubule diameter and epithelium height were measured using BELView Software. Therefore, 10 random testicular cross-sections (stage IX of the seminiferous epithelium cycle) per animal were photomicrographs using a photomicroscope and BELView Software at a magnification of 100x. For each seminiferous tubule, the mean of values was calculated and used in the statistical analysis (Gonçalves et al., 2016). In the stereological analysis, 10 random cross-sections per animal of ventral prostate (Scarano et al., 2009) were captured using a photomicroscope and BELView Software a magnification of 400x. This analysis was performed using Weibel's multipurpose graticule with 168 points (Weibel, 1963) to compare relative proportions among the epididymal components (epithelium, stroma and lumen) in the experimental groups (50 sections per group for each epididymal region)

2.6 Spermatic parameters

2.6.1 Daily sperm production per testis

The left testes were weighed and homogenized as described previously by Robb et al. (1978), with the adaptations described by Fernandes et al. (2007). After dilution of the homogenate, a small sample was transferred to Neubauer chamber (4 fields per animal) for counting homogenization resistant spermatids (stage 16 of the spermatogenesis) using a light microscope (Leica Microsystems, Wetzlar, Germany). To calculate the daily production of sperm (DSP), 4.84 divide the concentration of spermatids per testis, which is the number of days in which mature spermatids are present in the seminiferous epithelium.

2.6.2 Sperm Motility

The left vas deferens was rinsed with 0.5 mL modified human tubal fluid (HTF) medium with gentamicin (Irvine Scientific) at 34ºC–36ºC to obtain spermatozoa. At the same temperature, a Makler counting chamber (Sefi-Medical) was loaded with a 10µL aliquot of the sperm solution prepared previously. Sperm motility was assessed by visual estimation (100 spermatozoa per animal) under a light microscope light microscope (Leica) at 100x magnification, and was performed by the same person

(FA.V.D.L.) throughout the study. Spermatozoa were classified as mobile or immobile (Siervo et al., 2015).

2.6.3 Sperm morphology

Contents of the right vas deferens were removed by internal rinsing with 1.0 mL of saline formol 10%. Smears into histological slides were prepared from this solution and observed in a light microscope (Leica) at 400× magnification. One hundred spermatozoa were analyzed per animal. Morphological analysis was classified into three general categories: normal morphology, head abnormalities (without characteristic curvature or isolated form, i.e., no tail attached) and tail abnormalities (broken, rolled into a spiral and isolated, i.e., no head attached). This analysis was performed as described by Siervo et al. (2015).

2.7 Testosterone levels

Blood plasma was used for determination of testosterone. The total testosterone present in plasma was measured by chemiluminescence (2nd Generation Testosterone, Architect System, Abbott, Wiesbaden, Germany), according to the manufacturer's recommendations.

2.8 Inflammatory profile

2.8.1 Myeloperoxidase (MPO) activity

MPO colorimetric assay was used to assess neutrophil migration into the right testes and ventral prostate. Frozen samples of the right testes and ventral prostate were homogenized and centrifuged (16.100 g, 12 min, 4 ºC) to obtain supernatant. 15 µL of the supernatant was placed in a 96-well plate in mixed with 200 µL, 50 mM phosphate buffer (pH 6.0), containing 0.167 mg/mL o-dianisidine dihydrochloride and 0.015% hydrogen peroxide (Bradley et al*.,* 1982; Valério et al., 2009) and the enzymatic activity was determined by spectrophotometer at 450 nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific®, Vantaa, Finland). The results of MPO activity

are expressed as the number of neutrophils per mg of tissue by using a standard curve of neutrophils (196 – 200,000 cells).

2.8.2 N-acetyl-β-D-glucosaminide (NAG) activity

NAG activity was determined by an adapted colorimetric method previously described by Horinouchi et al. (2013). 10 µL of supernatant (right testes and ventral prostate), previously described in MPO activity, was placed in a 96-well plate, followed by the addition of 80 µL of 50 mM phosphate buffer, pH 6.0. The reaction was initiated by the addition of 2.24 mM 4-nitrophenyl N-acetyl-β-D-glucosaminide. The plate was incubated at 37 ºC for 15 min, and the reaction was stopped by the addition of 100 µL of 0.2 M glycine buffer, pH 10.6. The enzymatic activity was determined by spectrophotometer at 400 nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific®, Vantaa, Finland). The results of NAG activity are expressed as the number of macrophages per mg of tissue by using a standard curve of macrophages (97 – 100,000 cells).

2.8.3 Cytokine Measurement

The samples of ventral prostate were homogenized in 400 µl of buffer containing protease inhibitors, and IL-6, IL-10 and TNF-α levels were determined by an enzyme-linked immunosorbent assay (ELISA) using eBioscience kits (Verri et al., 2006; Verri et al., 2007) according to the manufacturer's recommendations. The results are expressed as pitograms (pg) of cytokine/mg of ventral prostate.

2.9 Statistics Analysis

The data normality was evaluated using the Bartelett's test. The data were compared using ANOVA, followed by Tukey test or using the non-parametric Kruskal-Wallis test, followed by Dunn test, according to the data distribution. Differences were considered significant when $p < 0.05$. The statistical analyses were performed by GraphPad Prism (version 6.0). Values expressed as media ± SEM (standard error of the mean) our median [Q1(25% Percentile) – Q3 (75% Percentile)].

3. Results

3.1 Body and reproductive organs weight

The initial and final body weights, as well as the weight gain index were similar between the groups. The testes, ventral prostate, vas deferens and seminal vesicle full and empty weights also did not show significant differences between the tested groups, showed in Table 1.

3.2 Histopathological analysis in testis and prostate

In the testes the histopathological analysis do not were evidenced alterations in the tubules seminiferous (C: 88, 80 ± 3,262; BPA 1µg: 77, 60 ± 2,542; BPA 10µg: 84, 00 ± 3,286). The morphological evaluation of the ventral prostate showed regularity in the glandular architecture in the untreated group (C). However, the BPA 10µg group presented mucinous metaplasia and hyperplasia in prostate epithelium and presence cells in the glandular lumen, and in the BPA 1µg group there was alteration in the acinus as a presence of swelling. These alterations is showing in the figure 1.

3.3 Leydig Cells analysis

The interstitial testis analysis showed that the number of ALC do not differences between the C (11, 63 ± 0, 90), BPA 1µg (11, 47 ± 0, 69) and BPA 10µg (11, 04 ± 0, 5131) groups. The area nuclear of the ALC do not were altered for treatment with BPA (C: 38,03 ± 1,39 μm^2 ; BPA 1µg: 37,29 ± 0,50 μm^2; BPA 10µg: 38,41 ± 0,70 μm^2) as well as the volume (C: 176,80 ± 9,72 μm^3 ; BPA 1µg: 171,40 ± 3,47 μm^3; BPA 10µg: 179,10 ± 4,90 μm^3). Therefore, the BPA do not induced morphologic alterations in the Leydig cells in low doses.

3.4 Morphometric and Stereological analysis

The values are shown in Table 2. The BPA 1µg and BPA 10µg groups showed increased values in the seminiferous epithelium height compared to the C group ($p<0.01$

and $p<0.001$ respectively). The BPA10μg group presented a significant decrease in the seminiferous tubular diameter ($p<0.01$) in relation the C groups. In the prostate, the BPA groups (BPA 1μg and BPA 10μg) presented a significant increase in stroma compartment followed by a decrease in luminal compartment from ventral prostate compared to control group. The epithelium was not modified by the treatments tested. Demonstrated that the BPA induces alterations in the morphological both in testes and in the prostate.

3.5 Spermatic parameters

There was no difference between groups analyzed in relation the sperm number and the daily production of sperm in testis. In relation, the spermatic motility and spermatic morphology the BPA10μg group presented difference significate in all parameters analyzed, with increase in sperm immobile and increase of abnormal head and tail. All data are show in table 3.

3.6 Testosterone levels

In both groups exposed to BPA, serum concentrations of testosterone were reduced by 65% and 68% in dosages of 1 μg/kg and 10 μg/kg, respectively (Fig. 4.). Data are expressed as mean ± SEM in nanograms per deciliter.

3.7 Inflammatory profile in testes and prostate

The neutrophil recruitment to testicular tissue demonstrated increased in BPA 10μg group in relation to the other groups (Fig. 2. A), and cytokine IL-10 decrease in the BPA 10μg group (Fig. 2. C), other parameters did not present significance between the groups in the testis (Fig. 2. B, D and E). In the prostate the MPO activity was elevated only in the BPA 1μg group when compared to the other groups ($p <0.05$) (Fig. 3. A), however there were no significant differences with respect to the NAG activity (Fig. 3. B), and the IL-10, IL-6 and TNF-α cytokines (Fig. 3. C-D) among the groups tested.

4. Discussion

The circulating hormones, such the testosterone, during the reproductive years for developmental morphogenesis and functionality of testis and prostate gland in men are of extreme importance (Ellem and Risbridger, 2010; Salian-Mehta et al., 2014). In the testicles and prostate gland the endocrine regulation occurs through steroid hormones by interaction with their specific nuclear receptors, such as androgen receptors (AR) and estrogen receptors alpha and beta (ERα and ERβ) (Salian-Mehta et al., 2014). Compounds with estrogenic properties, such as bisphenol A, pose a major threat to organs that are sensitive to estrogens, as is the case of testis and prostate (D'Cruz et al., 2012).

In our study, there were no differences in body, testes, prostate, vas deferens and seminal vesicle weights. Similar with we results, adult Wistar rats treated daily by gavage with acute doses of BPA (0.02 mg/kg, 2 mg/kg, 10 mg/kg or 50 mg/kg) for 5-6 days did not present significant differences at 1–11 weeks after the treatment, with relation to body weight and testis weigh between treatment and control groups (Li et al., 2009). Male rats expose at high concentrations of BPA 40 mg/kg and 200 mg/kg orally for 28 days did not observe changes in body weight and prostate weight at the two doses tested (Yamasaki et al., 2002). However, Akingbemi et al. (2004) related the decreased of the seminal vesicle weight in males exposed from 21 days to 90 days of age the dose of 2.4 μg/kg BPA. In this same study, animals exposed from gestational day 12 to postnatal 21 to 2.4 μg/kg BPA had reduced testicular weight at 90 days of age. 21 and 180 day-old Sprague-Dawley rats exposed to intraperitoneal BPA at doses of 25 or 250 μg/kg during gestational days (GD) 10-21, decreased the body weight in animals treated with 25 μg/kg BPA, while the highest dose induced an increase in prostate weight only in 21 day-old animals (Brandt et al., 2014).

"In vitro" evidences shows that low doses of BPA increased cell proliferation of spermatogonia (Sheng and Zhu, 2011) and human seminoma by binding of BPA to ERα and ERβ, respectively (Bouskine et al., 2009). The presence of Bisphenol A in utero at the dose high of BPA 50 μg/kg, 1000 μg/kg BPA did not change the diameter of the seminiferous tubules (LaRocca et al., 2011). Nonetheless, low doses of BPA at 5 and 25μg/kg resulted in a decrease the seminiferous tubule diameter and the height seminiferous epithelium in adult rats exposed for 35 days, altering the morphology of testis which corroboted with we findings (Kazemi et al., 2016). Low doses of BPA led a reduced of the seminiferous tubule diameter but in contrast, the seminiferous epithelium presented an increase compared to control in this study.

In the prostate, the stereology analysis revealed that BPA remodeled of the luminal and interstitial compartments. Comparable with us results, Bernardo et al. (2015), reported increased interstitial compartment with decreased ventral luminal compartment at doses of 25 μg/kg/day and 250 μg/kg/day of BPA in 21-day-old rats exposed intrauterine on GD 12-20 days. In the study of Brandt et al. (2014), doses of 25 μg/kg/day and 250 μg/kg/day did not cause changes in prostate stereology in 21 and 180 day-old animals. Other endocrine disrupter, such as ethinylestradiol, have also been correlated with changes in prostatic stereology with increased epithelial and stromal compartment and decreased prostatic lumen (Perez et al., 2012; Falleiros-Júnior et al., 2016).

The histopathological analysis of this study showed that the BPA in these lower doses did not cause damage in the testes. Tyl et al. (2002) reported that intrauterine exposure of male rats at doses of 0.001, 0.02, 0.3, 5, 50, and 500 mg/kg/day of BPA did not were observe histopathological changes in the testis, epididymis and seminal vesicles at all doses tested. Li et al. (2009) also did not observe significant changes in testis of adult rats exposed to doses of 0.02, 2, 10 or 50 mg/kg/day of BPA. However, low doses of BPA (2.4 and 10 μg/kg) induced the loss of germ cells in the seminiferous epithelium with presence of the cell in the lumen of the seminiferous tubules in animals exposed during the neonatal phase (Salian et al., 2009).

Contrasting to the results the histopathologic of the testis, BPA leads to prostatic changes, increasing the susceptibility to the development of prostatic lesions. A number of studies have demonstrated the association of BPA to the development of prostate cancer, demonstrating the presence of inflammatory infiltrates (Stoker et al., 1999; Brandt et al., 2014), hyperplastic focus (Falleiros-Júnior et al., 2016), atrophic prostatic (Yamasaki et al, 2002) and squamous metaplasia (Ogura et al., 2007). Rats exposed from birth to adulthood at a low dose of 10 μg/kg of BPA increased the susceptibility to neoplastic lesions of the prostate, the authors also evidenced epigenetic changes in prostate tissue (Ho et al., 2006). In adult male rats descended from females exposed via gavage at a dose of 25 μg/kg/day, presented an increased in the ventral prostate weight, epithelial cell proliferation and AR expression, resulting in a susceptibility of the chronic multifocal inflammation and atypical hyperplasia in prostate (Bernardo et al., 2015). Simanainen et al., 2011 related the presence of prostatic changes as squamous metaplasia due to the inactivation of AR favoring a prostatic response to estrogen that can induce tumorogenesis. Therefore, BPA leads to prostatic changes acting primarily

on estrogen receptors increase the susceptibility to the development of prostatic lesions even at low doses.

Doses equal or greater than tolerable daily dose proposed for EPA (50 μg/kg and 1000 μg/kg) did not alter the daily sperm production in adult rodents exposed in the uterus to BPA (LaRocca et al., 2011). Wisniewski et al. (2015), observed a reduction of the total and diary production of into the doses 5 mg/kg and 25 mg/kg of BPA. Parallel to our results, adult male mice treated with low doses of BPA (0.2, 2 and 20 μg / kg) did not present changes in daily sperm production (Ashby et al., 2009). The adults rats exposure at doses of the 5 mg/kg e 25 mg/kg of BPA during 40 days, presented alterations in the integrity of the acrosome and plasma membrane with reduced of mitochondrial activity, in these animals also were observed an increase of abnormalities in the spermatic cells (Wisniewski et al., 2015). Studies have related changes in sperm morphology as well as the increase in the presence of immobile spermatozoa in animals and men exposed to BPA, correlating the decrease in testosterone concentration (Aikawa et al., 2004; Salian et al., 2009; Vitku et al., 2016). The present data demonstrated a reduction in testosterone production in animals after oral expousure to doses of 1 and 10 μg/kg BPA for 50 days. Aikawa et al. (2004) also related sperm alterations due the action of Bisphenol A on estrogen receptors present in germ cells. Therefore, the low doses of BPA can be able to induce the alterations in the sperm but do not reduce sperm production.

Testosterone binds to androgen receptors presents in the prostate gland and testes having an important role in prostate morphogenesis and prostate function, still the testosterone is responsible for spermatogenesis in the testes and changes in testosterone concentration can induce sperm damage (Datta and Tindall, 2013; Alukal and Lepor, 2016; O'Hara et al., 2016). In the prostate, this hormone is converted to dihydrotestosterone, which is the main androgen and act on prostatic morphogenesis, thereby decrease in this androgen leads to increased levels of estrogen related to the development of benign prostatic hyperplasia (Wu et al., 2015). Doses of the 5 mg/kg and 25 mg/kg of BPA during 40 days induced the reduction testosterone concomitant with alterations in the hypothalamic–pituitary–testicular axis and increased of the AR mRNA demonstrating a activing of feedback negative (Wisniewski et al., 2015). Wu et al. (2015), using oral doses of BPA 10, 30 or 90 μg/kg for 4 weeks in male rats, reported a decrease in testosterone production. In this study the testosterone reduction occurred in the two doses tested of BPA, however this reduction do not it is correlated with loss

in the Leydig cells, since we results do not demonstrated reduction in the number of Leydig cells, as well as the kariometric also do not was alters. Akingbemi et al. (2004) related that doses of the 2,4 μg/kg the BPA induced the reduction testosterone production in rats acting directly on the Leydig cells principally by binding to estrogen receptor.

In the testis, macrophages play an important role in the regulation of development and steroidogenesis of Leydig cells in rats, represent approximately 20% of all interstitial cells and present the phenotype of M2 macrophages with IL-10 appearing to play an important role in anti- inflammatory characteristics in the testicle of rodents (Zhao et al., 2014; Bhushan et al., 2016). Nevertheless, is possible find the macrophages with phenotype M1 represented 20% of all macrophages in the testis and the balance about the M1 and M2 macrophages is responsible for maintenance of homeostasis in this organ (Li et al., 2012). Still the increase of the M1 macrophages was related with chronic orchitis (Li et al., 2012). The MPO also can be produced by macrophages in other diseases, how in the atherosclerosis, and the macrophage myeloperoxidase have demonstrated that is responsible for the develop of this disease (Sugiyama et al., 2001). The presence of BPA in human and rodents has been related to the increase of the inflammatory response (Yan et al., 2008; Yang et al., 2009; Midoro-Horiuti et al., 2010). In our study, an increase in MPO activity could be correlated with a decrease in IL-10, probably by increase of macrophages myeloperoxidase that induced decrease of IL-10, since histopathological analysis do not was observed neutrophils infiltrated in the testicular tissue. Therefore, low doses of BPA may lead to changes in testicular immunoregulation.

In we study low dose of BPA (1μg/kg/day) showed increased MPO activity, but all pro-inflammatory cytokines tested showed no alterations, as well as NAG activity. Stoker et al., (1999) observed an increase of myeloperoxidase activity indicating an increase of neutrophils in the prostate using in male rats submitted to single dose of Bisphenol A (50 mg/kg) at 22 days of age. Pro-inflammatory cytokines such as IL-6 are associated with pathogenesis in prostate cancer, stimulating the initiation and promotion of cancer while, TNF-α released by mast cells is related to death of tumor cells (Sfanos; Tewari, 2014). Although unlike other tissues, the increase of IL-10 in humans may be related to progression of prostatic cancer since this cytokine suppresses the anti-tumor immune response (Dwivedi et al., 2015). As only the dose of 1μg/kg/day of BPA related

an increase of the MPO the damages find in the prostatic tissue is due the decrease of testosterone and not because the inflammatory process.

In conclusion, even the BPA in lower doses can modifies the prostate and testis tissue probably due its biding in the androgens and estrogens receptors leading to alterations in Leydig cells with reduced testosterone production without induced loss of these cells. The BPA also induced alterations in sperm quality and immune response in the testis. In resume, these data suggest that even at a dosage considered safe for humans, BPA exposure compromises prostatic and testis morphologic, sperm quality, testosterone production and immune response.

Conflict of interest

The authors declare no conflict of interest.

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Table 1

Body and reproductive organs weight (n=20/group)

	C	BPA 1µg	BPA 10µg
Initial body weight (g)	36.96 ± 0.71	36.87 ± 0.66	38.30 ± 0.62
Final body weight (g)	43.61 ± 1.05	44.79 ± 0.73	44.31 ± 0.71
Weight gain index (g)	7.81 ± 0.90	7.92 ± 0.66	6.36 ± 0.73
Testis (g)	0.13 ± 0.013	0.12 ± 0.01	0.12 ± 0.01
Ventral prostate (g)	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Vas deferens (g)	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
Seminal vesicle (full) (g)	0.37 ± 0.03	0.32 ± 0.01	0.30 ± 0.02
Seminal vesicle (empty)	0.21 ± 0.01	0.22 ± 0.02	0.21 ± 0.01

Values expressed as Mean ± SEM. ANOVA test with the post hoc Tukey's.

Table 2

Morphometric and Stereological analysis

	C	BPA 1µg	BPA 10µg
Morphometric analysis			
Seminiferous tubular diameter (µm)	205.3 [175.0 - 245.0] [a]	208.0 [169.8 - 247.4] [a]	194.4 [167.4 - 235.1] [b]
Seminiferous epithelium height (µm)	66.1 [58.8 - 77.0] [a]	74.54 [67.3 - 83.8] [b]	72.7 [62.7 - 81.2] [b]
Stereological analysis			
Lumen	50.0 [31.0 - 68.2] [a]	36.0 [20.0 - 51.0] [b]	33.0 [18.2 - 51.5] [b]
Epithelium	43.0 [26.0 - 50.2]	35.0 [28.0 - 56.0]	33.0 [23.7 - 44.7]
Stroma	76 .0[62.0 - 92.5] [a]	83.0 [74.0 - 107.0] [b]	92.0 [78.0 - 112.0] [b]

Values expressed as median [Q1 – Q3]. [a,b] Different letters indicate groups that differ statistically. Kruskal-Wallis test with the post hoc Dunn's.

Table 3
Spermatic parameters

	C	BPA 1µg	BPA 10µg
Sperm count (n=10/group)			
Sperm number in testis ($\times 10^6$)	25.89 ± 2.70	23.02 ± 0.43	23.02 ± 2.45
Daily production of sperm ($\times 10^6$)	112.50 ± 18.49	116.70 ± 16.40	86.35 ± 11.93
Spermatic Motility (n=10/group)			
% Mobile spermatozoa	71.30 ± 4.54 [a]	68.60 ± 2.74 [a,b]	44.60 ± 8.14 [b]
% Immobile spermatozoa	28.70 ± 4.54 [a]	31.60 ± 2.75 [a,b]	55.40 ± 8.14 [b]
Spermatic morphology (n=10/group)			
% Normal morphology	70.60 ± 0.80 [a]	66.80 ± 1.27 [a]	60.90 ± 1.63 [b]
% Abnormal morphology	29.40 ± 0.80 [a]	33.20 ± 1.27 [a]	39.10 ± 1.63 [b]
% Abnormal head	20.90 ± 0.76 [a]	19.33 ± 1.31 [a]	26.50 ± 2.06 [b]
% Abnormal tail	9.200 ± 1.26 [a]	13.33 ± 1.54 [a,b]	14.22 ± 1.39 [b]

Values expressed as Mean ± SEM. [a.b] Different letters indicate groups that differ statistically. ANOVA test with the post hoc Tukey's.

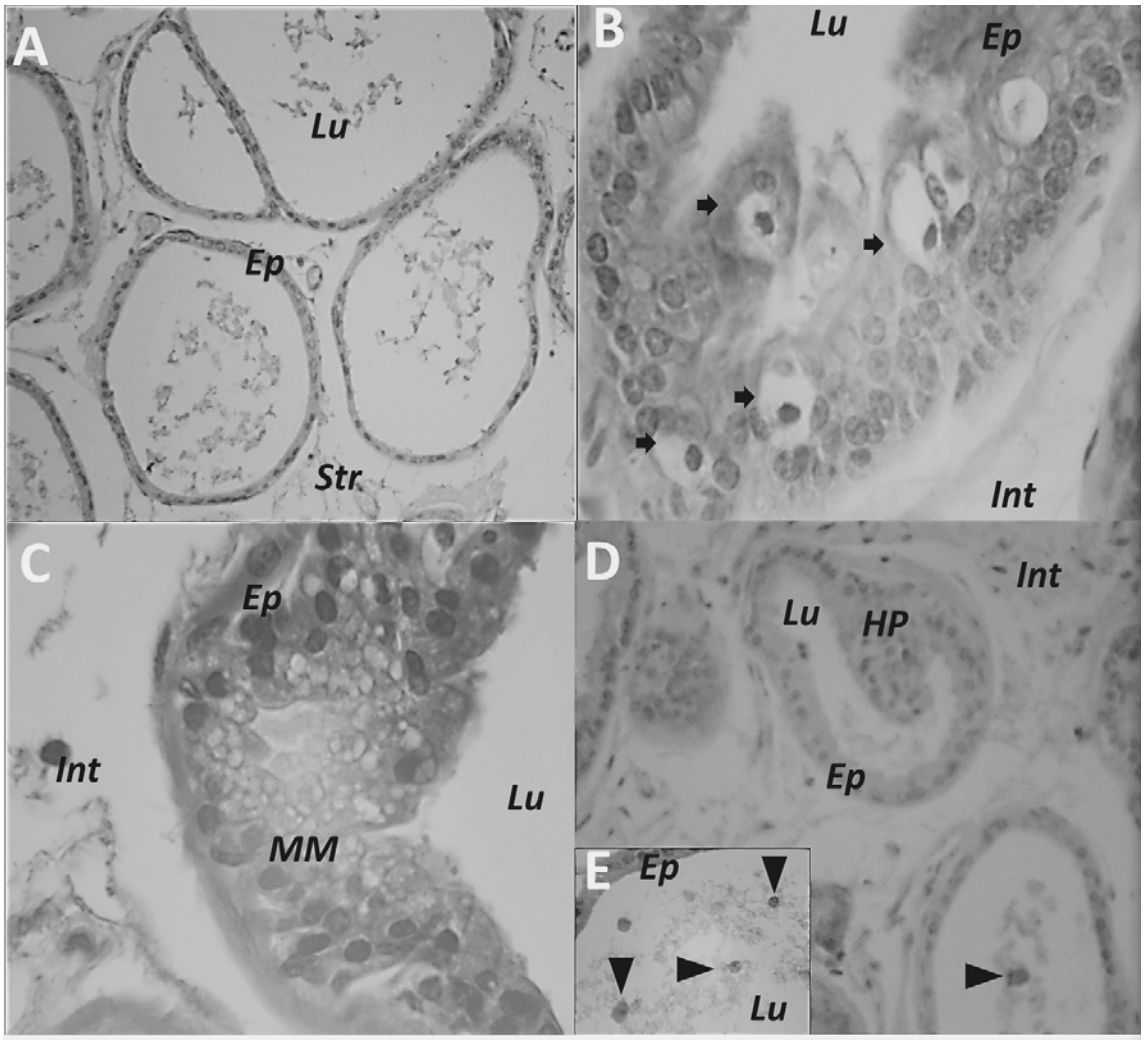

Fig. 1. Histological sections of prostate of the mice. **A**. Untreated Group: Acini predominantly composed of simple and low columnar epithelium supported by a delicate fibromuscular stroma in the ventral prostate. **B**. BPA 1µg. Presence of cellular swelling in the prostatic epithelium (arrow). **C-E**. BPA 10µg. **C**. Presence of mucinous metaplasia (MM). **D**. Presence of hyperplasia (AH) and cells in the lumen (arrowhead). **E**. Presence of cells in the lumen. Str= stroma, Ep= epithelium, Lu= Lumen. Magnification 100x (A and D), 400x (B,C and E).

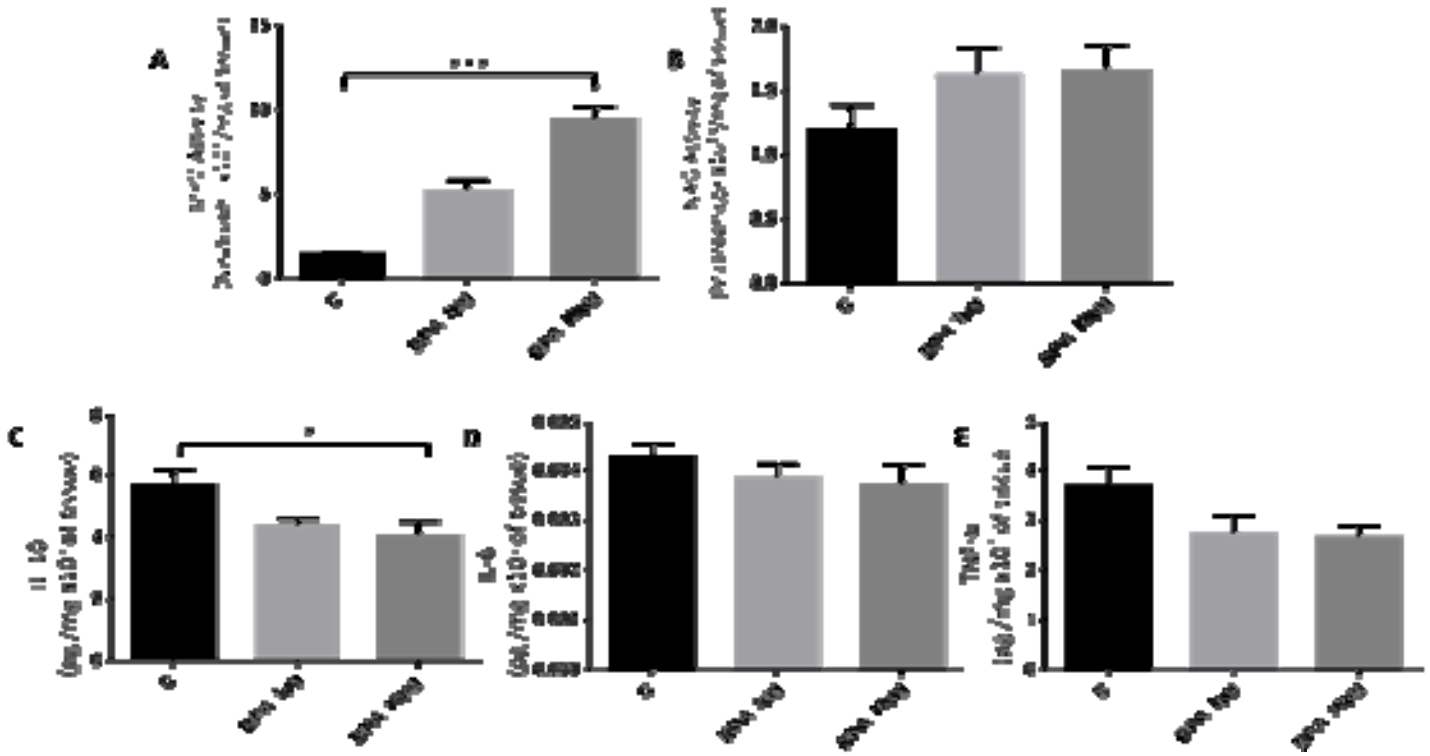

Fig. 2. Inflammation profile in testis of mice (n=5/group). Mice were treated with BPA 1μg/kg/day or 10μg/kg/day (BPA 1μg and BPA 10μg respectively) or 100μL of 1%DMSO in water (C). For the analysis of the inflammatory profile the dosages of (A) myeloperoxidase (MPO) activity and (B) N-acetyl-β-D-glucosaminidase (NAG) activity were performed and was measured by ELISA cytokines (C) IL-10 ,(D) IL-6 and (E) TNF-α. Results are expressed as mean ± SEM. * P <0.05. ANOVA followed by Tukey's.

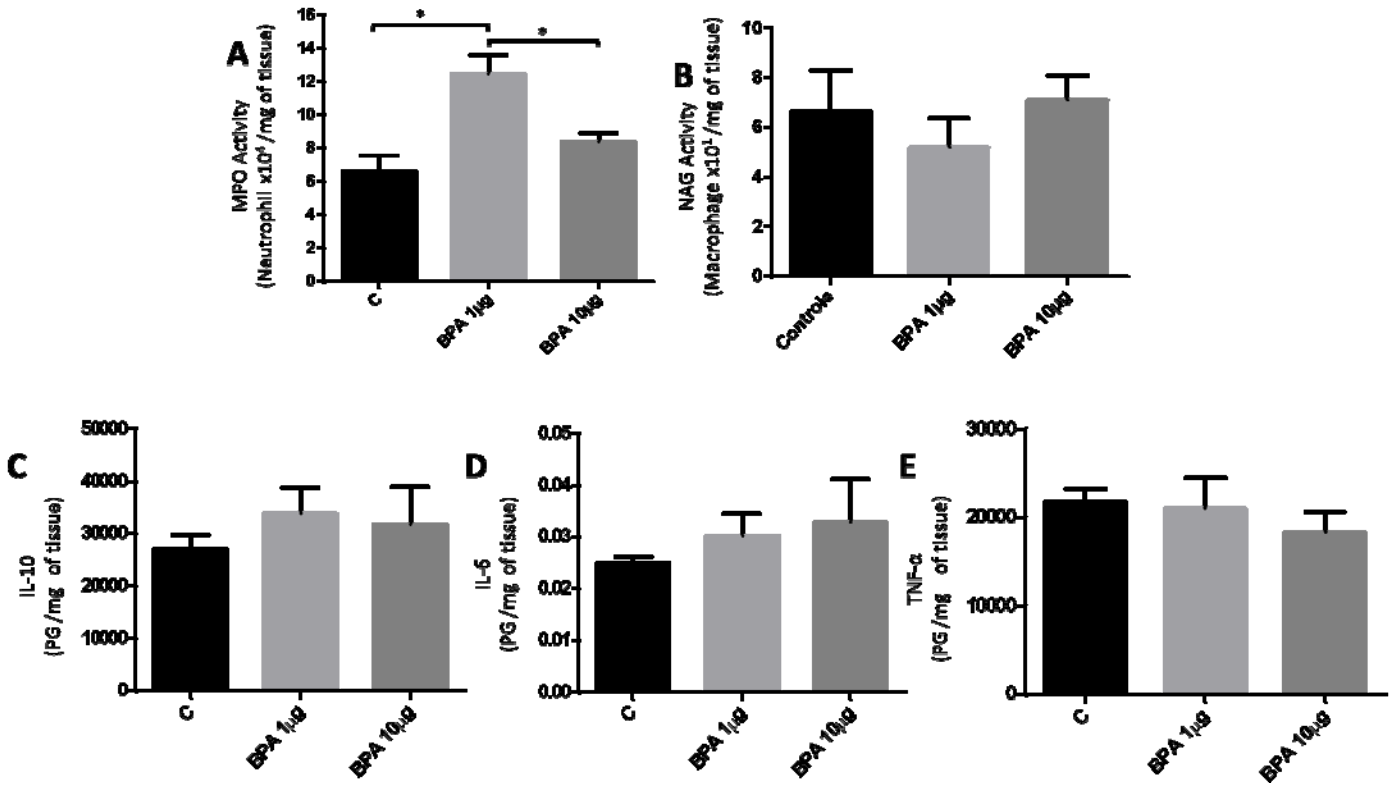

Fig. 3. Inflammation profile in ventral prostate of mice (n=5/group). Mice were treated with BPA 1µg/Kg/day or 10µg/Kg/day (BPA 1µg and BPA 10µg respectively) or 100µL of 1%DMSO in water (C). For the analysis of the inflammatory profile the dosages of (A) myeloperoxidase (MPO) activity and (B) N-acetyl-β-D-glucosaminidase (NAG) activity were performed and was measured by ELISA cytokines (C) IL-10, (D) IL-6 and (E) TNF-α. Results are expressed as mean ± SEM. * P <0.05. ANOVA followed by Tukey's.

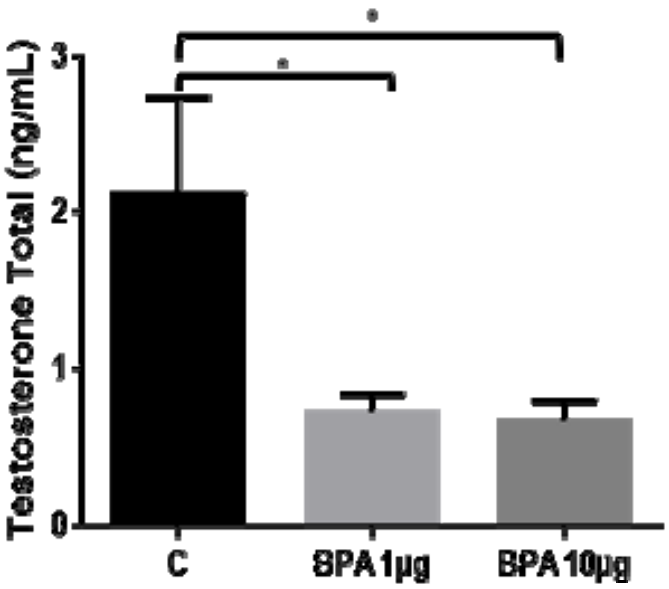

Fig. 4. Testosterone total measure for chemiluminescence in plasma. Data are presented as the mean ± SEM.* $p < 0.05$. ANOVA followed by Tukey's.

5 ARTIGO II

Bisfenol A Reduz a Produção de Testosterona em Células de Leydig TM3 Independentemente dos seus Efeitos sobre a Morte Celular e o Potencial de Membrana Mitocondrial

Bisphenol A Reduces Testosterone Production in TM3 Leydig Cells Independent of its Effects on Cell Death and Mitochondrial Membrane Potential

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Bisphenol A Reduces Testosterone Production in TM3 Leydig Cells Independent of its Effects on Cell Death and Mitochondrial Membrane Potential

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Abstract

Leydig cells are the major testosterone producing cells of the male reproductive system and damage in this cells can affect the spermatogenesis and consequently lead to infertility in men. Bisphenol A (BPA), an endocrine disrupter, impairs the male reproductive system by affecting sperm function. The present study aimed evaluated the effect on cell growth, viability and testosterone production in the TM3 Leydig cells of mice after the exposure at micromolar concentration of BPA. BPA (100 μM) reduced cell viability, as measured by the viability assay, and this correlated with increased cell death indicated by an increased sub-G1 phase population and higher number of cells labeled with Hoechst 3342. Staining with rhodamine 123 showed a decrease in metabolically active mitochondria at the highest tested dose of BPA (100 μM). However, BPA caused a decrease in testosterone production at all doses tested. Therefore, our results suggest that micromolar concentration of BPA is sufficient for inhibit the function of the TM3 cell, since these cells have an important role in the male system, the BPA can induced male infertility.

Keywords: Leydig Cell, Bisphenol A, Cell Death, Testosterone, Metabolically Active Mitochondria

Abbreviations: AR, Androgen Receptors; BPA, Bisphenol A; CI, Cellular Index; CYP11A1, Cytochrome P450 Family 11 Subfamily A Member 1; DMSO, Dimethyl Sulfoxide; ER, Estrogen Receptor; ERα, Estrogen Receptor Alpha; ERβ, Estrogen Receptor Beta; IC50, Half-Maximal Inhibitory Concentration; LH, Luteinizing Hormone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RTCA, Real-Time Cellular Analysis; StAR, Steroidogenic Acute Regulatory Protein.

1. Introduction

Infertility is a major problem for couples of reproductive age and currently affects ~9% of couples worldwide (Quah and Cockerham, 2008). In approximately 50% of these couples, infertility is due to male reproductive dysfunction (Anawalt, 2013). Among the factors responsible for the increase in male infertility, endocrine disrupters have been extensively studied (Maffini et al., 2006; Manikkam et al., 2013; Sweeney et al., 2015). According to the International Program on Chemical Safety (2002), an endocrine disruptor is a "chemical with a potential to interfere with the endocrine system and consequently cause adverse health effects." The endocrine disruptors may contribute to male reproductive dysfunction by interfering with spermatogenesis, a process highly dependent on hormones (Dohle et al., 2003; Sweeney et al., 2015; WHO, 2013).

Testosterone, the major hormone produced by the testes, is responsible for the embryonic development of the male reproductive system, and for the maintenance of spermatogenesis and inhibition of germ cell apoptosis in adulthood (Dohle et al., 2003). Testicular cells express a high level of estrogen receptors (ERα, ERβ) together with androgen receptors (AR) (N'tumba-byn et al., 2012; Zhou et al., 2002), and are responsive to luteinizing hormone (LH) that stimulates steroidogenesis in Leydig cells (Chen et al., 2016; Dankers et al., 2013). Thus, alteration of Leydig cell function can adversely affect testicular functions, leading to infertility (Yang et al., 2015).

TM3 cells have receptors for LH, epidermal growth factor, AR, and ER, and are able to metabolize cholesterol, the precursor for testosterone biosynthesis, in the presence of LH (Mather, 1980). Moreover, BPA binds to ER (Richter et al., 2007) and affects AR function (Teng et al., 2013). Therefore, the TM3 cell line exhibits important characteristics for the experimental analysis of the physiological effects of BPA on Leydig cells.

Bisphenol A (BPA, 2, 2-bis (4-hydroxyphenyl) propane) is an endocrine disrupter that is widely produced; about 3.5 million tons was produced worldwide in 2008. BPA is used to manufacture polymers such as polycarbonates, and is found in various products such as reusable plastic bottles, feeding bottles, cups, microwave ovenware, storage containers, and dental sealants (Kang et al., 2006; N'Tumba-Byn et al., 2012). Although BPA is banned from use in certain products such as bottles and disposable cups (Erler and Novak, 2010), many countries use this product without any prohibition or regulation. In a study by Calafat et al. (2008), 92% of the participants, representative of the U.S. population 6 years old and over, were found to have detectable levels of BPA (0.4-149 μg/L) in their urine. In the general population, the average serum concentration of BPA ranges from 0.2-20 ng/mL (0.1-10 μM) (Qian et al., 2014).

Exposure to xenoestrogens such as BPA may be the underlying cause of increased genital tract abnormalities and increased incidence of infertility observed in Europe and the U.S. over the last 50 years (Calafat et al., 2008; Vandenberg et al., 2007). Lassen et al. (2014) evaluated urine, semen, and plasma samples from 303 young men of reproductive age and demonstrated that 98% of the urine samples had detectable levels of BPA (0.12 ng/mL), and this was indeed associated with higher levels of circulating testosterone, LH, and estradiol, and a lower percentage of progressive mobile sperm.

In the male reproductive system of rodents, BPA causes several adverse effects such as decrease in sperm quality (Wisniewski et al., 2015), impairment of spermatogenesis (Tarapore et al., 2016), disruption of the hypothalamic-pituitary-testicular axis (Wisniewski et al., 2015) and reduction of testosterone production by the Leydig cells (Hong et al., 2016; N'tumba-byn et al., 2012). Several studies have demonstrated that BPA functions as an AR antagonist and can interact with both the ER subtypes, ERα and ERβ (Lassen et al., 2014).

BPA exerts an inhibitory effect on Leydig cell steroidogenesis, which is likely mediated through the ER (Akingbemi et al., 2004).

In this study we used techniques to demonstrate the behavior of TM3 Leydig cells, no tumorigenic, in the presence of BPA. We observed that micromolar concentrations of BPA were able to induce cellular dysfunction in these Leydig cells. Since these cells are responsible for production in testosterone, we demonstrate that the BPA can lead to infertility problems in male system.

2. Materials and Methods

2.1. Chemicals

BPA, CAS number 80-05-7, was purchased from Sigma-Aldrich (St. Louis, MO, USA). BPA was solubilized in dimethyl sulfoxide (DMSO, Mallinckrodt Chemicals, and St. Louis, MO, USA) to a concentration of 1 M, and diluted to experimental concentrations using culture medium. The final DMSO concentration in cell cultures was 0.025% v/v. The control group received an amount of DMSO equivalent to the treated group. The chemotherapeutic agent, doxorubicin (Zodiac, Brazil), was used at concentration of 0.5 μM as a positive control.

2.2. Cell culture

The mouse Leydig cell line, TM3 (ATCC® CRL-1714™), was kindly provided by Prof. Dr. Wamberto Antonio Varanda (Department of Physiology, Medical School of RibeirãoPreto/USP). Cells (passage number 3-5) were cultured in Dulbecco's modified Eagle medium/nutrient mixture F-12 Ham (Gibco®, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco®, Life Technologies, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Gibco®, Life Technologies, Carlsbad, CA, USA), and maintained at 37°C in a 5% CO_2 humidified incubator. Under these conditions, the cell viability remained high (>90%).

2.3. Cell viability assay

Cell viability was measured based on the MTT assay. This assay is based on the conversion of MTT (3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazoliumbromide) (Invitrogen, Life Technologies, USA) to formazan crystals by mitochondrial dehydrogenase in living cells, as described by Mosmann (1983). Exponentially growing cells ($3.125x10^3$ cell/well in 100 µL of culture medium) were seeded in 96-well culture and incubated at 5% CO_2 and 37°C for 24 h. After 24 h of incubation, concentration of BPA (0.5, 1, 5, 10, 50, 100, 250 e 500 µM) were added and remained in culture for 24 h and 48 h. After exposure, the treatments were removed, and 100 µL of MTT reagent (0.5 mg/ml) was added and kept at 37 °C for 4 h to form formazan crystals. The cell culture medium was aspirated, and 100 µL of DMSO was added to dissolve the crystals. The absorbance was measured at 570 nm using a spectrophotometer (Thermo^{TM} Plate). Three independent experiments were performed in quadruplicate. The percent of cell viability was calculated according to Huang et al. (2005).

2.4. Real-time cell growth kinetics xCELLigence

Real-time cellular index profiling was performed using the xCELLigence system (Real-Time Cell Analysis, RTCA) (Roche Diagnostic, Mannheim, Germany), according to the manufacturer's recommendations. The xCELLigence equipment monitors the cell biological status through impedance measurements. Alterations in cellular biological status, including the number of cells, cell viability, morphology and adhesion, change the impedance values, which are expressed through the Cell Index (CI). This is possible because of gold microelectrodes coupled to the base of a 96-well culture plate called an E-Plate. Those electrodes are connected to a computer that measures differences in impedance within an electrical circuit (Martinez-Serra et al. 2014). Thus, larger cell numbers are reflected in greater impedance values (Xing et al., 2006). The RTCA station remained in a humidified incubator containing 5% CO_2 at 37 °C. Briefly, 50 µL of medium was added to the E-plate,

and background was measured. After, the cells were seeded at a density of 3.125x 10^3 cells/well and incubated for 24h. After 24 h, when the cells were in logarithmic growth phase the BPA treatments (0.5, 1, 5, 10, 50, 100, 250 and 500 μM) were added and the impedance was monitored every 30 min for 96 h. The experiment was performed in triplicate. The cell growth curves were normalized to the Cell Index (CI) at the last time point before treatment addition using the RTCA software (Roche Diagnostic, Germany).

2.5. Cell cycle analysis

Cells were seeded in 6-well plates at a density of 90x10^3 cells/ well. After 24 h of incubation, BPA (1, 10 and 100 μM) were added for 24 and 48 h. Following the treatment period, cells were trypsinized, resuspended in DMEM and centrifuged for 5 min. The samples were treated with RNase (0.1 mg/ml; QIAGEN) for 30 min at 37 °C. After, the cell suspension was resuspended in 100 μL HFS (50 mg/mL propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100) (Sigma Aldrich, USA) and incubated for 30 min on ice in the dark for 30 min (Savio et al. 2014). Next, the fluorescence of propidium iodide was estimated by flow cytometry (Guava easy Cyte™, Millipore, USA). The data were analyzed using a Guava software (Millipore, USA). The percentages of cells in different phases of the cycle (G1, S and G2/M) as well as the percentage of cells with fragmented DNA (sub-G1 region) were estimated. For each sample examined, 5000 events were analyzed in three experiments.

2.6. Single-cell gel electrophoresis assay (comet assay)

To investigate the genotoxic potential of BPA was performed the comet assay according to the protocol of Tice et al. (2000) with some modification. For this purpose, cells were seeded in 6-well plates at a density of 90x10^3 cells/ well. After 24 h of incubation, BPA (1, 10 and 100 μM) were added. Doxorubicin 0.5 μM was used as a positive control for DNA damage induction, and the vehicle control contained culture medium with 0.025 % DMSO. After 3 h of exposure, the cells were trypsinized, resuspended in DMEM and centrifuged for 5

min. Subsequently, 20 μL of the cell suspension was mixed with 120 μL of low-melting point agarose (0.5%) and deposited onto a slide pre gelatinized with agarose (normal melting point, 1.5%), and then covered with a coverslip and left to solidify. The slides were subject to 1 h lysis solution (2.5M sodium chloride, 100mM EDTA, 10mM tris (hydroxymethyl)aminomethane (Tris), pH 10; 1% Triton X-100, and 10% DMSO) and subjected to alkaline denaturation for DNA unwinding (20 min). Electrophoresis (300 mA, 25 V, 20 min) were performed in a high pH buffer solution (200 mM EDTA, 10 N sodium hydroxide, and pH 13). After electrophoresis, the slides were neutralized (0.4 M Tris and pH 7.5), dehydrated with ethanol, stained with 100 μL of 0.002 mg/mL ethidium bromide solution and scored using a fluorescence microscope using EVOS® FL Auto Imaging System (Life Technologies, Waltham, MA, USA) equipped with a 420–490-nm UV excitation filter and a 520-nm barrier filter . The comets were classified by visual scoring into four categories based on the length of DNA migration (Collins et al., 2008): class 0, undamaged cells and nucleoids without tails; class 1, cells with damage and a tail smaller than the diameter of the nucleoid; class 2, cells with damage and a tail twice the diameter of the nucleoid; and class 3, cells with damage and a tail greater than twice the diameter of the nucleoid. The damage index (DI) was calculated as follows: $DI = N1 + (2 \times N2) + (3 \times N3)$, where N1, N2, and N3 represent the numbers of cells of damage levels 1, 2, and 3, respectively. For each sample examined, 100 nucleoids per slide were analyzed in three experiments. The cell viability analysis was conducted using the trypan blue stain exclusion method, and only treatments with an index greater than 80% were considered.

2.7. Chromatin staining by Hoechst-33342 dye

For visualizing the morphologic characteristics of cell death, we utilizing Hoechst 33342 a DNA-specific dye which enables the visualization of cell nucleus under fluorescence microscopy. Cells were seeded in 6-well plates at a density of 90x103 cells/ well. After 24 h

of incubation, BPA (1, 10 and 100 μM) were added for 24 and 48 h. After 24 h and 48 h of exposure, the cells were stained with Hoechst 33342 (2 μg/mL) for 15 min at room temperature and observed immediately using FLoid® Cell Imaging Station (magnification of 200x, blue filter, 390/ 40 nm excitation and 446/33 nm emission — Life Technologies, USA). 500 cells per well were analyzed in three experimental replicates and images were captured using FLoid® Cell Imaging Station. Cells with presence of fluorescence and nuclear condensation were considered cell death.

2.8. Mitochondrial membrane potential by Rhodamine-123 staining

The metabolically active mitochondria can be evaluated by staining Rhodamine 123, in which the presence of green fluorescence evidence mitochondrial membrane potential (Pajaniradje et al., 2014). TM3 cells were seeded in 6-well plates at a density of 90x103 cells/ well. After 24 h of incubation, BPA (1, 10 and 100 μM) was added for 24 and 48 h. The cells were washed two times with PBS (pH 7.4) and incubated with 1 mg/mL rhodamine 123 at 37°C for 30 min. The cells were then washed with PBS and were analyzed in three experimental replicates and images were captured using EVOS® FL Auto Imaging System, (magnification of 400x, green filter, 482/ 18 nm excitation and 532/59 nm emission — Life Technologies, Waltham, MA, USA).

2.9. Testosterone levels

Cells were seeded in 6-well plates at a density of $90x10^3$ cells/ well. After 24 h of incubation, BPA (1, 10 and 100 μM) were added and remained in culture for 48 h. Next 48 h of exposure, the culture medium were collected and frozen at -20 °C, until the day of the dosage. The total testosterone present in the culture medium were measured by chemiluminescence (2nd Generation Testosterone, Architect System, Abbott, Wiesbaden, Germany), according to the manufacturer's recommendations.

2.10. Statistical analysis

Data are presented as averages ± mean standard error of the three independent experiments. Differences were considered significant for P values < 0.05. The cell viability, cell cycle, morphologic analysis of cell death, comet assay and testosterone data were analyzed by analysis of variance (ANOVA) followed by Dunnett's test by Graph Prism (version 6.0). The IC50 values were calculated by RTCA software, with data measured by xCELLigence equipment.

3. Results

3.1. High concentrations of BPA decrease cell viability and alter cellular biological status

BPA was found to reduce the viability of TM3 cells in a concentration- and time-dependent manner. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay showed that at concentrations above 0.5 μM, 24 h of exposure to BPA inhibited cell viability (Fig. 1A) and reduced absorbance (Fig. 1B) compared to the untreated control group. However, the viability of TM3 cells did not decrease significantly even after 48 h of exposure to BPA at concentrations above 50 μM, demonstrating the resilience of these cells. The half-maximal inhibitory concentration (IC50) of BPA for TM3 cells after 48 h of exposure was measured to be 129.7 μM ($R^2 > 0.94$) using the MTT assay. Therefore, in subsequent experiments, BPA was used at concentrations of 1, 10, and 100 μM for the investigation of possible cellular changes at low concentrations.

The real-time cellular analysis (RTCA) was employed to assess the cellular biological status of TM3 cells treated with BPA. The TM3 cells exhibited a decrease in their cellular index (CI) after 34 h of exposure to BPA at concentrations above 10 μM (Fig. 2A, vertical red line). However, high concentrations of BPA (250 and 500 μM) exhibited cytotoxic effects as demonstrated by a decrease in the CI within a few hours of exposure (Fig. 2A). Fig. 2B shows that within the first few hours of exposure to BPA at concentrations of 50

and 100 μM, there was an initial increase in the CI of the TM3 Leydig cells, followed by a decrease thereafter. In contrast, BPA at 250 and 500 μM led to a decrease in the CI from the start of exposure (Fig. 2A and B), presumably due to morphological alterations induced by the high concentrations of BPA. A significant decrease in cell growth was also observed upon exposure to 100 μM BPA (Fig. 2B).

3.2. BPA induces an increase in the sub-G1 phase cell population but does not cause any genotoxic effects

There were no differences in the distribution of the TM3 cells in the G0/G1, S and G2/M phases of the cell cycle after treatment with BPA (1, 10, and 100 μM) for both 24 and 48 h of exposure (Fig. 3A, B, D, and F). Further, no genotoxicity was observed in the cells after 3 h of BPA exposure at any concentration tested (Fig. 4).

There was an increase in the sub-G1 phase cell population only upon exposure to 100 μM BPA for 24 h (control: 0.4%, 100 μM BPA: 3.46%, $p<0.05$) and 48 h (control: 0.46%, 100 μM BPA: 4.1%, $p<0.05$) (Fig. 3C and E). These results suggest that treatment with 100 μM BPA induced cell death in TM3 cells.

3.3. BPA induces cell death and alteration in mitochondrial membrane potential

The cell TM3 control, i.e., with no exposure to BPA, exhibited normal chromatin staining and nuclear morphology. On the other hand, treatment with 100 μM BPA led to an increase in the number of cells exhibiting strong fluorescence and chromatin condensation (Fig. 5A, head arrows), indicative of these cells becoming apoptotic. Fig. 5B shows an increase in the number of cells labeled with Hoechst 33342 upon exposure to BPA for 24 and 48 h.

A decrease in the metabolically active mitochondria was observed in BPA-treated cells after staining with a green fluorescent dye (Fig. 6). Compared to the untreated control cells, the mitochondrial membrane potential was decreased in the TM3 cells exposed to 100

μM BPA (Fig. 6). Doxorubicin is known to decrease the mitochondrial membrane potential, and was used as a positive control.

3.4. Exposure to BPA decreases the production of testosterone

The figure 7 show the reduce the testosterone production (1 μM: 1.013 ± 0.029; 10 μM: 0.963 ± 0.095; 100 μM: 0.803 ± 0.123) for BPA in the Leydig cells, compared the control (1.357 ± 0.029) ($p<0.05$), suggesting that BPA leads to impairment of steroidogenesis in these cells even at low concentrations.

4. Discussion

In the present study, we demonstrated a decrease in the viability of TM3 Leydig cells upon 48 h of exposure to BPA at concentrations above 50 μM. Further, we showed changes in the biological status of these cells within 34 h of exposure to BPA at concentrations above 10 μM. While BPA induced cell death at a concentration of 100 μM, it caused a decline in the production of testosterone at a low concentration (1 μM), indicating that BPA inhibits the steroidogenic function of these cells.

Studies have reported a decrease in cell viability upon exposure to BPA in several cell types (Hwang et al., 2013; Lin et al., 2013; Liu et al., 2013a). BPA decreased viability of human spermatozoa after 20 h of exposure at a concentration of 300 μM (Barbonetti et al., 2016). De Freitas et al. (2016) observed a 50% reduction in the viability of human Sertoli cells upon treatment with 10 μM BPA in 48 hours exposure. In our study, BPA decreased the viability of TM3 Leydig cells only at concentrations greater than 50 μM after 48 h of exposure.

The xCELLigence® RTCA system evaluates cellular changes and allows the dynamic monitoring of the effects of a compound on cell growth at various time points, without the need to disturb the cell culture or use of dyes that may negatively influence the results (Abassi

et al., 2009; Garcia et al., 2013). Inhibition of cell growth upon exposure to high concentrations of BPA (250 and 500 μM) began shortly after the addition of BPA, demonstrating a cellular response to the cytotoxic effects of these high concentrations. In both the MTT assay and the RTCA, it was possible to observe the cytotoxic effects of BPA on the TM3 cell line, but concentrations considered low doses (<1 μM) (Vandenberg et al., 2013) did not show cytotoxic effects even after 48 h of exposure.

The CI, measured by the RTCA, varies according to changes in the cellular biological state such as cell number, cell viability, morphology, and adhesion (Garcia et al., 2013; Semprebon et al., 2016). A toxic agent may increase the CI by inducing an increase in cell number or size, which may increase cell contact with the microelectrode sensors present in the E-plate of the RTCA system (Urcan et al., 2010). In contrast, the toxic compound may decrease the CI by inducing a decrease in cell size, increased cell detachment, or cell death (Urcan et al., 2010). In BALB/c mouse 3T3 fibroblasts, exposure to antipyrine led to a decrease in the CI in the first few hours, and an increase in the CI thereafter (Xing et al., 2006). Similarly, Garcia et al. (2013) observed a peak in CI in mouse L-929 fibroblasts soon after exposure to cadmium chloride. In the current study, we observed differences in the CI of TM3 cells in the first few hours after exposure to BPA, concentrations of 50 and 100 μM BPA caused an increase in the CI, while concentrations of 250 and 500 μM decreased the CI. These results indicate that exposure to BPA for a short time can lead to changes in the morphology of Leydig cells.

Genotoxic insults to DNA activate checkpoints in the cell cycle, causing a temporary stop in the cell cycle at the G1/S or G2/M phase transitions (Semprebon et al., 2015). Thus, cell cycle analysis of individual cells permits the assessment of changes in the cell cycle in response to intracellular and extracellular signals (Liang et al., 2016). In vivo studies have shown that BPA disrupts the progression of meiosis during spermatogenesis, leading to

changes in genes essential for cell division (Ali et al., 2014; Grassi et al., 2016; Wu et al., 2015). In mouse spermatogonial cell line (C18-4), a concentration of 10 μM BPA was sufficient to halt the mitotic progression at the G2/M phase transition (Liang et al., 2016). In our work, although exposure to 100 μM BPA for 48 h decreased the CI and viability of TM3 cells, no changes were observed throughout the cell cycle, indicating that BPA exposure did not induce DNA damage at any of the doses tested. Therefore, we can conclude that BPA did not cause any genotoxic effects in the TM3 cells.

Cells may undergo cell death if their DNA is damaged beyond repair (Cheung-Ong et al., 2013). In the cell cycle, cells with fragmented DNA are defined as "sub-G1," and chromatin condensation and disintegration in this population in addition to DNA fragmentation indicates the presence of apoptotic cells (Darzynkiewicz et al., 1992; Kajstura et al., 2007). Liang et al. (2016) reported an increase in apoptotic cells in the sub-G1 phase upon exposure to 50 μM BPA for 72 h in mouse C18-4 spermatogonial cell line. In our study, exposure to BPA (100 μM) caused an increase in the sub-G1 population, consistent with a decrease in cell viability, and this corroborated with the results obtained through Hoechst 33342 and rhodamine 123 staining.

Mitochondrial dysfunction is involved in apoptosis; at the onset of the execution phase or "non-return point" in apoptosis, the mitochondria undergo remodeling to release apoptogenic proteins into the cytoplasm, thus initiating the apoptotic cascade ultimately leading to cell death (Liu et al., 2013b; Perkins et al., 2016). Wang et al. (2010) reported that in vivo exposure of mice to BPA (480 mg/kg) for 14 days increased the number of apoptotic germ cells in their testes, simultaneously, there was an increase in the levels of Bax, a proapoptotic protein, and active caspase-9, suggesting that BPA-induced apoptosis occurs via the mitochondrial apoptotic pathway. Exposure of human spermatozoa to 300 μM BPA for 4 h resulted in a decrease in their mitochondrial membrane potential, leading to cell death

(Barbonetti et al., 2015). BPA (1000 μM) induced apoptosis in primary cell cultures of mouse embryonic midbrain cells, and this was associated with an increased expression of Bax and p53 mRNA, indicating that BPA induced apoptosis in these cells via the p53-mitochondrial apoptosis pathway (Liu et al., 2013b). In rat insulinoma (INS -1) cells, BPA (0.02, 0.2 and 2 μM) induced fragmentation of mitochondria, reduction of mitochondrial mass and membrane potential, depletion of ATP, release of cytochrome c from the mitochondria into the cytosol, and the activation of caspases, while doses of 0.2 and 2 μM led to an increase in apoptotic cells (Lin et al., 2013).

Leydig cells in the testes are the primary source of testosterone, which acts to regulate spermatogenesis (Walker, 2010). Moreover, decrease in the levels of testosterone drives metabolic changes in men and increases their chances of developing cardiovascular disease and obesity (Jørgensen et al., 2016). Mitochondria contain proteins and enzymes essential for testosterone biosynthesis such as the steroidogenic acute regulatory protein (StAR) that mediates the rate-limiting step in steroidogenesis, and the enzyme cytochrome P450 family 11 subfamily A member 1 (CYP11A1) that converts cholesterol to pregnenolone. Mitochondrial dysfunction may thus adversely affect testosterone biosynthesis (Akingbemi et al., 2004; Chen et al., 2006; Ye et al., 2011). In antral follicle cells in primary culture, Peretz and Flaws (2013) observed a decrease in the expression of the StAR and CYP11A1 genes upon exposure to BPA (10 and 100 μg/mL) for 72 and 24 h, respectively, concomitant with a decline in testosterone production after 72 h of exposure. Similar to the results obtained in our experiment, Feng et al. (2016) reported a decrease in testosterone production in H295R human adrenocortical carcinoma cell line after 48 h of exposure to BPA (1, 10, and 10 μM).

Upon exposure to low concentrations of BPA (0.01 nM), adult mice Leydig cells in primary culture presented inhibition of testosterone production, associated with the suppression of the gene encoding the steroidogenic enzyme cytochrome P450 17α-

hydroxylase/17,20 lyase (Akingbemi et al., 2004). Similarly, in our experiment, low doses of BPA were able to induce a decrease in testosterone production, culminating in changes in steriodogenesis by TM3 Leydig cells.

In conclusion, we observed diminution of testosterone levels in the TM3 Leydig cell line in response to BPA, independent of cell death or changes mitochondrial membrane potential induced by BPA, showing that this molecule is capable of inducing the cellular dysfunction in these cells. This is a new approach, which could be associated with the problem of infertility. Our results may provide an explanation for fertility dysfunction in males exposed chronically to low concentrations of BPA, since the Leydig cells is responsible for production of testosterone in men.

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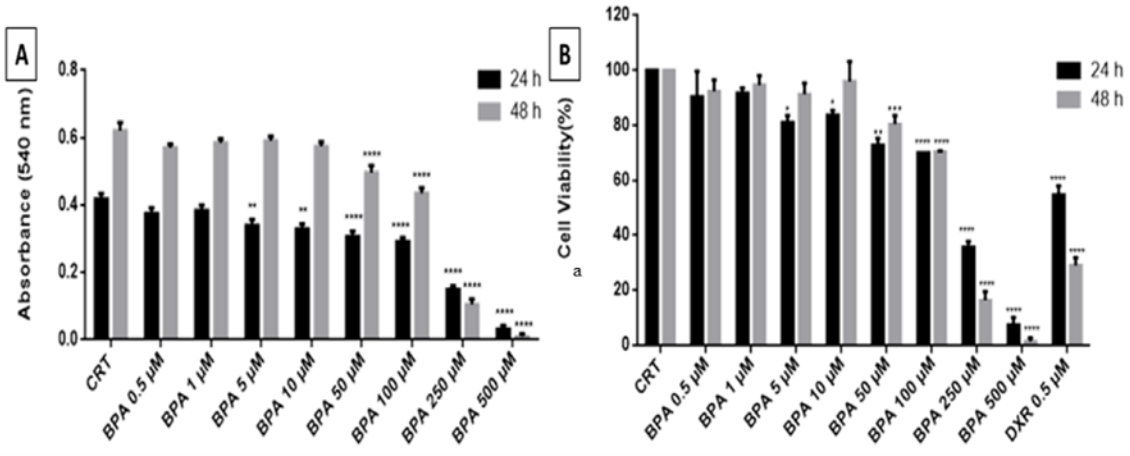

Fig.1. Absorbance values and percentage of cell viability (%) of Leydig cell (TM3) exposed to BPA with 0.5–500 µM during 24 and 48 h measured using to the MTT assay. (A) Absorbance value and (B) cell viability obtained using the MTT cytotoxicity assay in TM3 after 24 and 48 exposition of BPA. Control (DMSO 0.025%); DXR 0.5 µM. Results are presented as the mean ± SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ****$p < 0.0001$ relative to control using ANOVA followed by Dunnett.

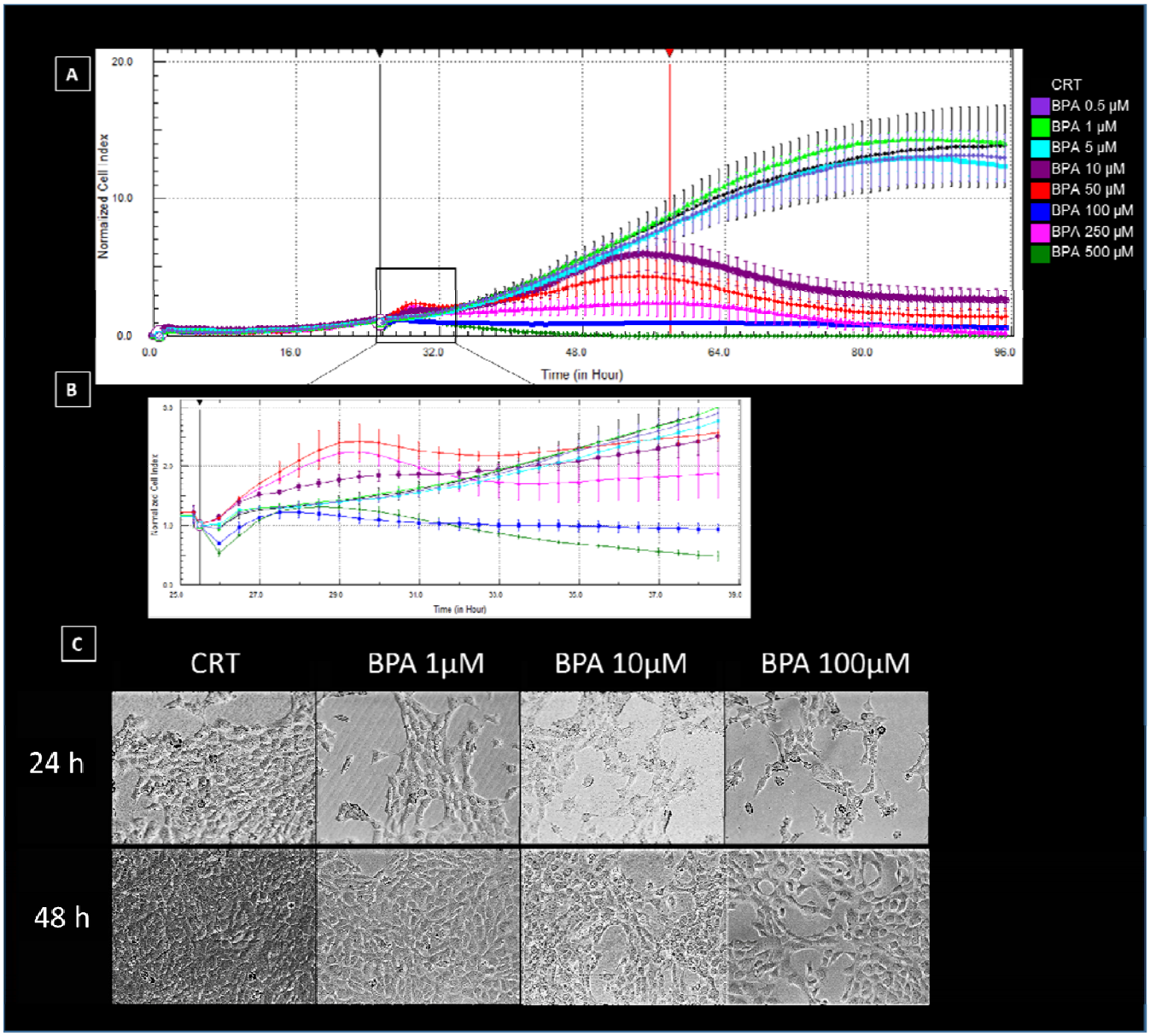

Fig. 2. The kinetics of cell growth in real-time monitoring of TM3 cells obtained using the xCELLigence system after exposure to BPA (0.5–500 µM) for 24 and 48 h. (A) Growth curves of TM3 cells exposed to BPA, vertical line black represents the addition of BPA, vertical line red represents the start of decrease of cell growth in minor concentration of BPA. (B) Greater magnification of image A showing alterations of cellular biological status soon after the addition of BPA. (C) Photomicrograph of TM3 cells after treatment with BPA µM (1, 10 µM and 100 µM) in 400x magnification. The results are presented as the means of three replicates with standard error bars.

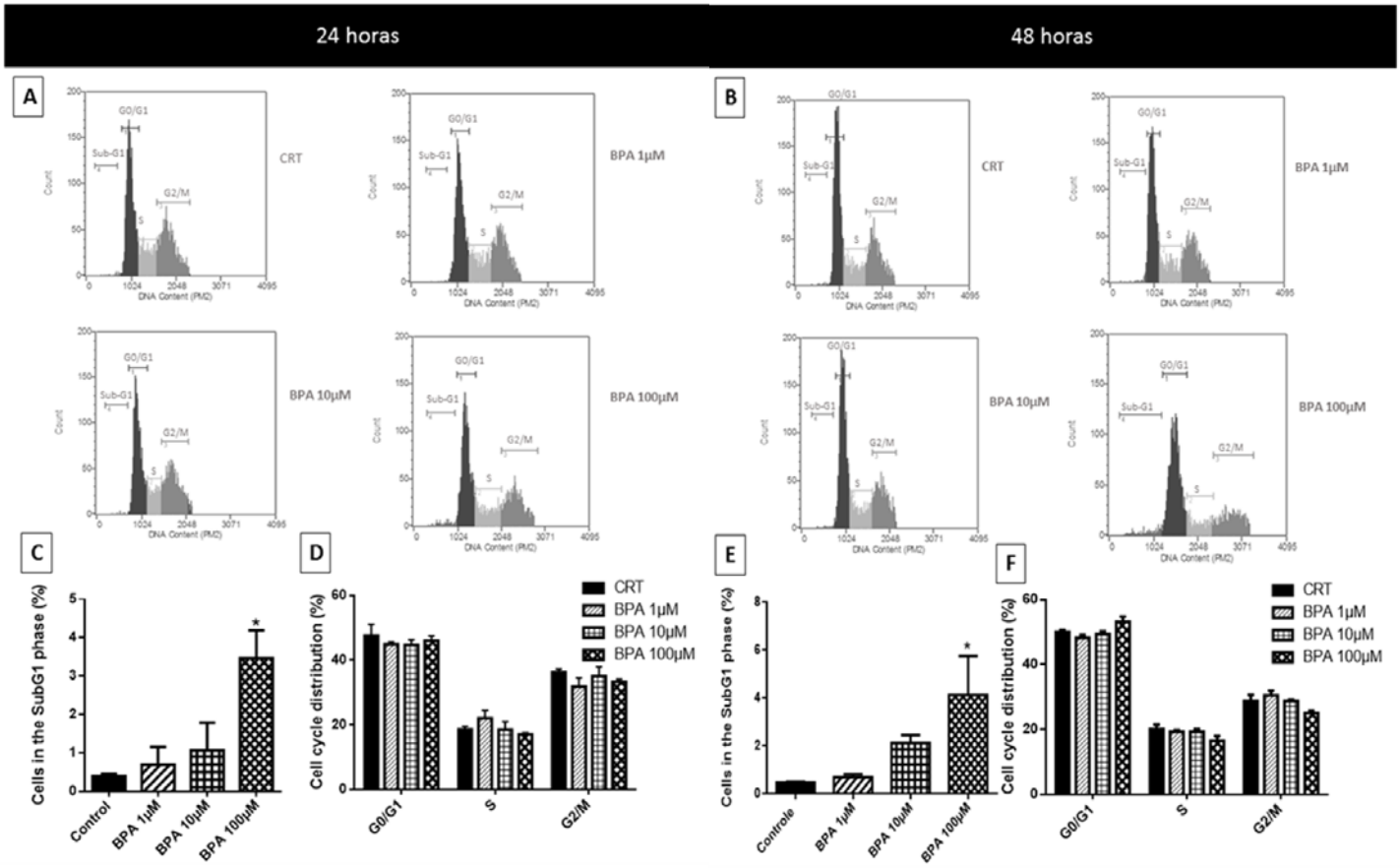

Fig. 3. TM3 cells in cell cycle analysis (G0/G1, S, G2/M and subG1) after exposure to BPA (1 μM, 10 μM and 100 μM) by flow cytometry using propidium iodide labeling. (A) Representative histograms indicate the number of cells (vertical axis) vs. DNA content (horizontal axis) after exposure to BPA during 24 (B) and 48 h. (C and E) Percenter of the cell in the SubG1 phase after exposure to BPA during 24 and 48 h respective. (D and F) TM3 cell cycle distribution after exposure to BPA during 24 and 48 h respective. Results are presented as the mean ± SEM of three independent experiments. * $p < 0.05$, relative to control using ANOVA followed by Dunnett.

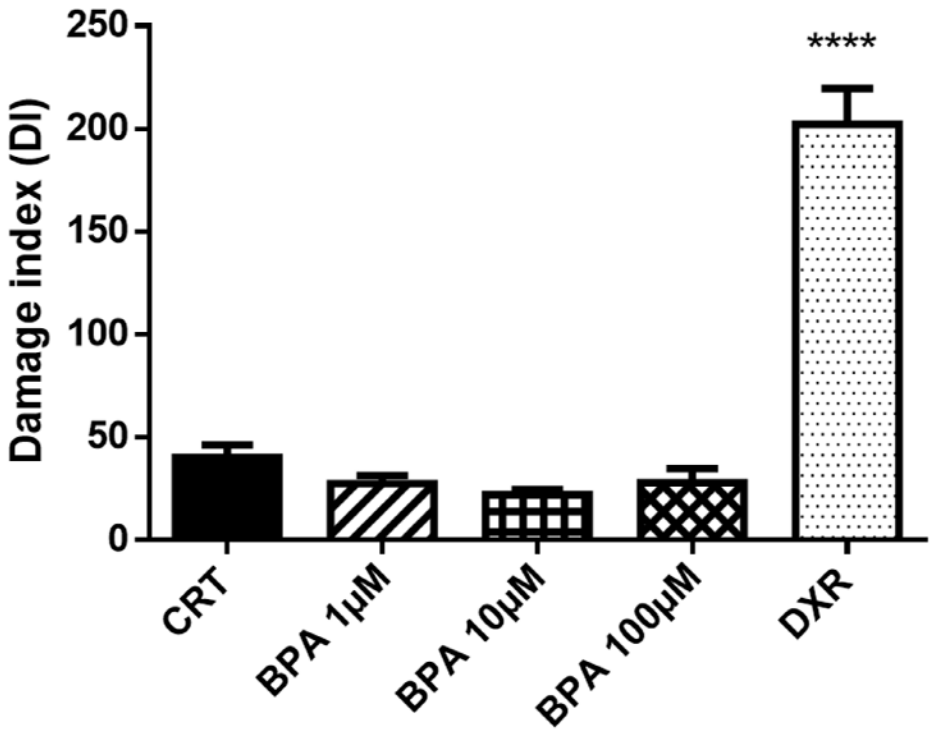

Fig. 4. Damage index (DI) after 3 h of exposure of TM3 cells to BPA measured using the Comet Assay. Data are presented as the mean ± SEM of three independent experiments. **** $p < 0.0001$ relative to the control group using ANOVA followed by Dunnett.

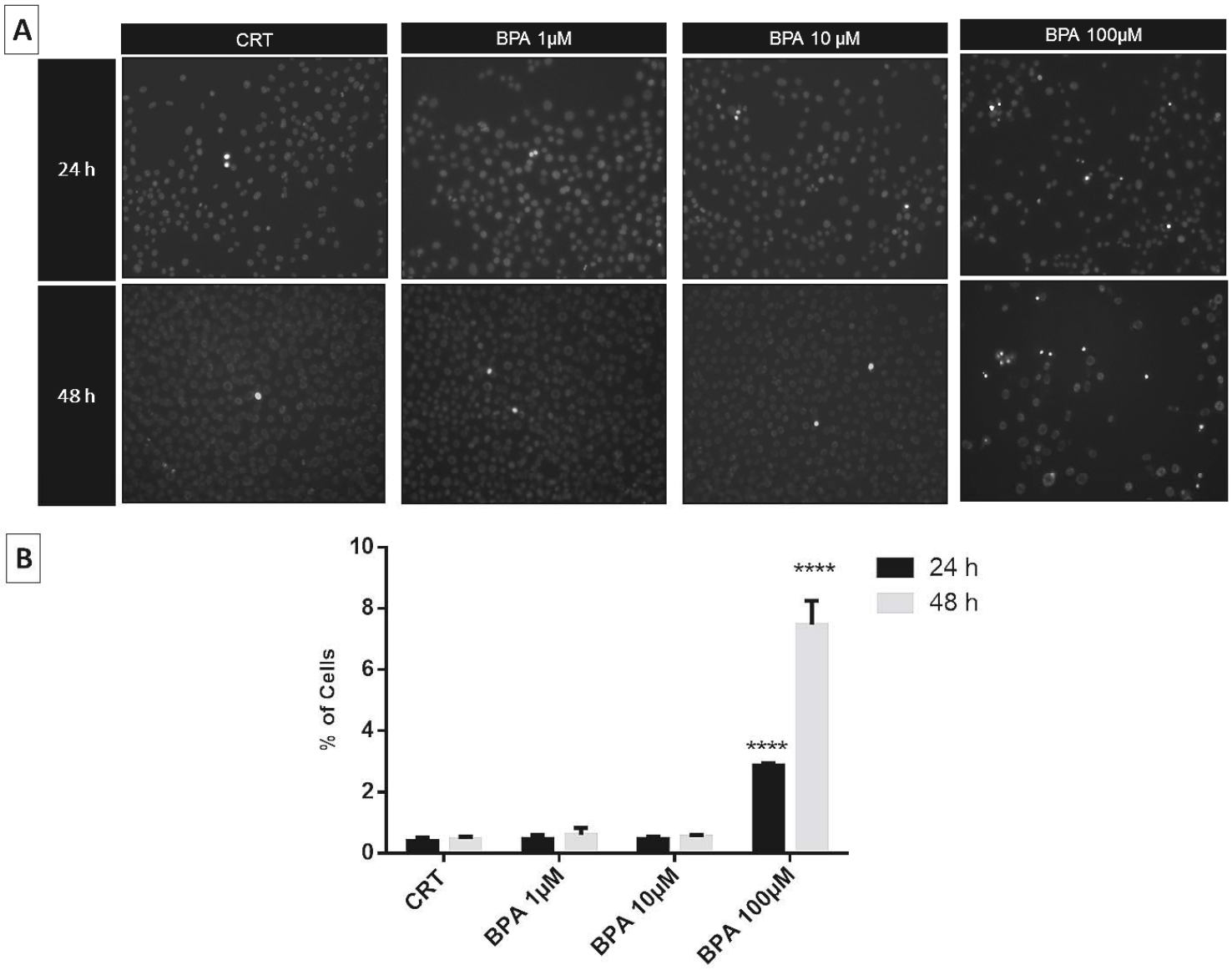

Fig. 5. Cell death induction for BPA (1 µM, 10 µM and 100 µM) in TM3 cell. (A) photomicrographs showing morphology and the staining with Hoechst 33342 of TM3 cells (magnification = 200x).(B) Percentage of TM3 cells in cell death after 24 h and 48 h of treatment. Results are mean ± SEM of three independent experiments. **** $p < 0.0001$ compared to control using ANOVA followed by Dunnett.

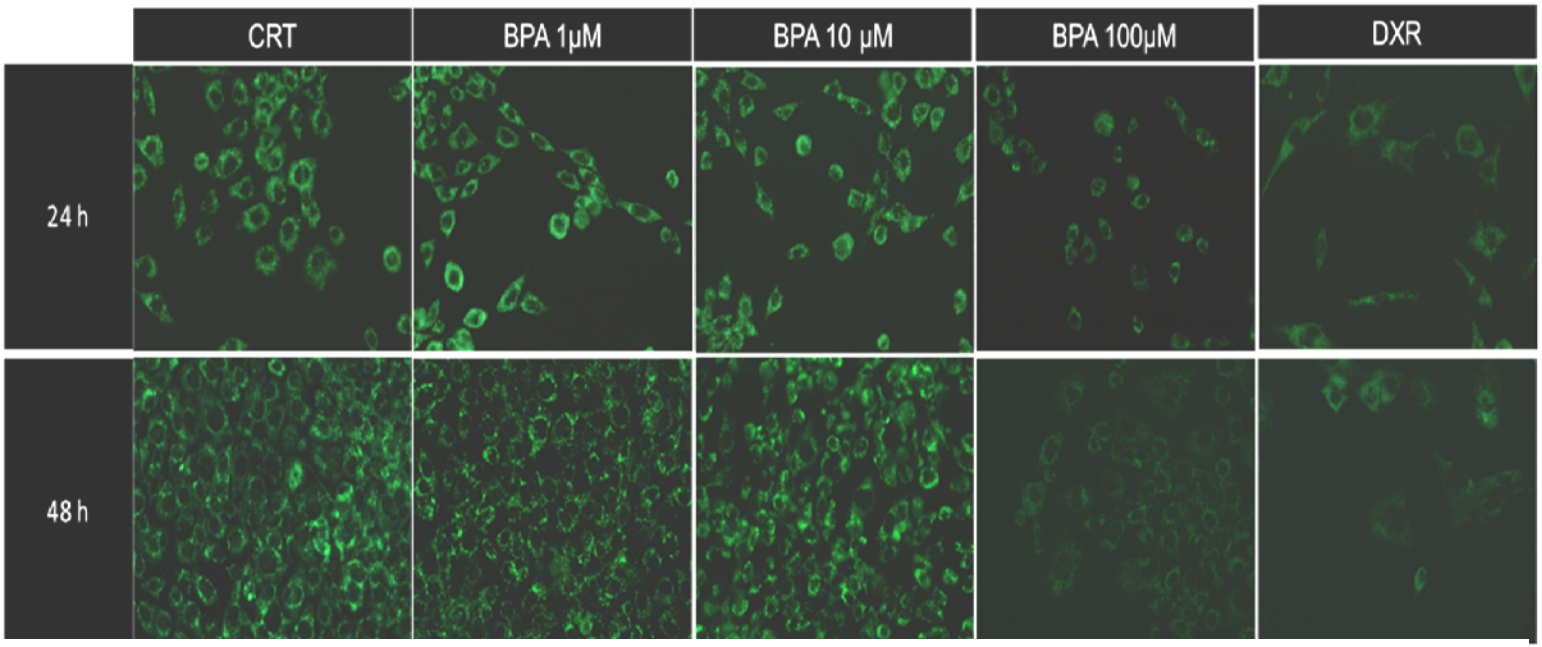

Fig. 6. Mitochondrial fluorescence staining using rhodamine 123 of Leydig cell (TM3) after BPA exposure during 24 and 48 h. After exposure to BPA (100 μM) and doxorubicin (DXR) the mitochondrial membrane potential was decreased in TM3 cells as evidenced by decreased fluorescence as compared to untreated cells (CRT). 400x magnification

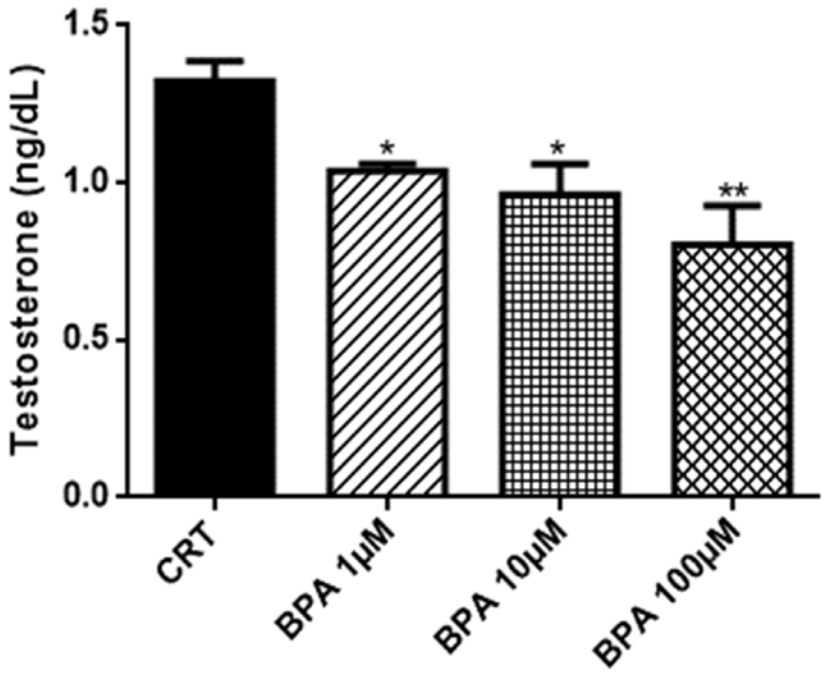

Fig. 7. Testosterone dosage measure for chemiluminescence in the culture medium of TM3 cell after exposition of BPA. Data are presented as the mean ± SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$ relative to the control group using ANOVA followed by Dunnett.

6 CONSIDERAÇÕES FINAIS

In vivo baixas doses de BPA foram capazes de induzir a alterações na morfometria testicular ao qual o diâmetro dos túbulos seminíferos reduziu apenas no grupo BPA 10μg, no entanto a altura do epitélio seminífero aumentou nos dois grupos de BPA (BPA 1μg e BPA 10μg). A maior dose de BPA causou aumento de MPO com diminuição da IL-10 nos testículos. Na próstata os grupos BPA 1μg e BPA 10μg apresentaram alterações histopatológicas e morfométricas e aumento de MPO. O BPA também levou a perda da qualidade espermática na maior dose testada. Já todas as doses testadas foram capazes de reduzir a produção de testosterona em mais de 60% quando comparada ao controle, porém sem alterar o número de células de Leydig.

In vitro na concentração de 100 μM o BPA induziu alterações na viabilidade celular, na cinética de crescimento celular em tempo real, no potencial de membrana e aumentou o número de células em morte celular. Porém todas as concentrações testadas (1, 10 e 100 μM) de BPA induziram a uma perda da função destas células com a redução de testosterona.

Nós concluímos que em doses semelhantes a exposição via oral humana o BPA causou alterações morfológicas nos testículos e próstata com diminuição da qualidade espermática e redução da testosterona. O BPA também levou a disfunção celular das células de Leydig com diminuição da produção de testosterona. Em conjunto, nossos resultados demonstram que o BPA é capaz de atuar diretamente nas células de Leydig provavelmente por ação dos receptores de estrógeno e andrógeno levando a disfunção destas células e reduzindo a produção de testosterona, que pode estar relacionado a todas alterações observadas nos testículos e próstatas e nos espermatozoides.

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ANEXOS

ANEXO A

Universidade
Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 161/2015 **Londrina, 06 de Agosto de 2015.**

Prezada Pesquisadora,

Certificamos que o projeto intitulado **"Avaliação dos efeitos do bisphenol A sobre a linhagem celular TM3 e órgãos reprodutores masculino de camundongos adultos"**, protocolo CEUA nº **11335.2015.77**, sob a responsabilidade de **Glaura Scantamburlo Alves Fernandes**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL), em reunião realizada em **04/08/2015**.

O projeto tem como objetivo avaliar a citotoxicidade causada pelo BPA sobre a linhagem de células de Leyding TM3 e avaliar a exposição de baixas doses de BPA nos testículos e epidídimos de camundongos Swiss machos adultos. Para isso os animais serão distribuídos em 3 grupos. Dois grupos serão tratados com Bisphenol A nas doses de 1 ou 10 μg/Kg de peso corpóreo por gavagem. O terceiro grupo (grupo controle) receberá apenas o veículo (óleo de milho e DMSO) em igual volume. No 50º dia experimental, será coletado 0,3mL de sangue da veia caudal de cada animal para a análise de corticosterona. Após isso os animais serão anestesiados com a associação de xilazina e quetamina e mortos por punção cardíaca, sendo colhido 1mL de sangue em tubos heparinizados para as dosagens dos hormônios FSH, LH e testosterona. Após a eutanásia, 10 animais de cada grupo terão os testículos direitos retirados e congelados para posterior determinação de número de espermátides maduras. Os testículos esquerdos serão congelados para avaliação de parâmetros inflamatórios. Do ducto deferente direito serão obtidos os espermatozóides para avaliação da morfologia espermática e do esquerdo serão utilizados para avaliação da motilidade espermática. Os testículos e epidídimos direitos serão retirados para análises histopatológicas, morfológicas e estereológicas.

Vigência do Projeto	30/09/2015 a 29/09/2018
Espécie/linhagem	Camundongo heterogênico / Swiss
Nº de animais	60
Peso/Idade	50 dias
Sexo	Macho
Origem	Biotério Central da Universidade Estadual de Londrina
Amostras a serem coletadas	Sangue, testículo, vesícula seminal, próstata, ducto deferente, epidídimo

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações.

Coloco-me à disposição para quaisquer esclarecimentos que se fizerem necessária. Sem mais para o momento, subscrevo, cordialmente,

Waldiceu Ap. Verri Junior
Prof. Dr. Waldiceu Aparecido Verri Junior
Coordenador da CEUA/UEL

Ilma. Sra.
Profa. Dra. Glaura Scantamburlo Alves Fernandes
Coordenadora do Projeto
Departamento de Biologia Geral / Centro de Ciências Biológicas
Com cópia para André Junior da Conceição (Chefe da DP-IC/PROPPG), Luiz Carlos Juliani (Diretor do Biotério Central da UEL), Chefe do Departamento de Biologia Geral e Diretor(a) do Centro de Ciências Biológicas.