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Desreguladores endócrinos versus Ginsenosídeos: modulação da via não genômica ativada por GPR30 e estresse oxidativo em células de Sertoli humanas (HSeC)

ANDRÉ TEVES AQUINO GONÇALVES DE FREITAS

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração Biologia Celular e Estrutural, Instituto de Biociências, Câmpus de Botucatu, UNESP, para obtenção do título de Doutor.

Orientador: *Prof. Dr. Wellerson Rodrigo Scarano*

BOTUCATU – SP
2019

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2019

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSANGELA APARECIDA LOBO-CRB 8/7500

Freitas, André Teves Aquino Gonçalves de.
Desreguladores endócrinos versus Ginsenosídeos : modulação da via não genômica ativada por GPR30 e estresse oxidativo em células de Sertoli humanas (HSec) / André Teves Aquino Gonçalves de Freitas. - Botucatu, 2019

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências de Botucatu
Orientador: Wellerson Rodrigo Scarano
Capes: 20601000

1. Barreira hematotesticular. 2. Sertoli, Células de. 3. Desreguladores endócrinos. 4. Ginseng. 5. Produtos químicos.

Palavras-chave: Barreira hematotesticular; Células de Sertoli; Desreguladores endócrinos; Monobutil ftalato; Panax ginseng.

Dedicatória

Como tem sido desde a concepção, em cada passo, em cada gesto, por tanto carinho, por todo o apoio, pelo amor incondicional, agradeço e dedico mais uma conquista aos meus pais e exemplos, Rita e Durval.

Agradecimentos

Seguindo a dedicatória, estendo o agradecimento aos meus pais à minha família, de sangue e coração. Á todas as tias, madrinhas e primos e especialmente às minhas irmãs, avó, prima e sobrinhos. Sem a base de vocês, sem o colo, o calor, o amor e a segurança, não chegaria nem na metade do caminho.

Ao Lucas, pela vida compartilhada, pelo abrigo, refúgio, pela compreensão, pela paz e por tanto amor. Obrigado por estar ao meu lado sempre.

À Suki, por todos os dias de amor incondicional, por alegrar a minha casa, a minha rotina e minha vida. Por dividir todos os momentos, por me fazer largar o computador pra fazer carinho e levar a vida com mais leveza.

Aos amigos, espalhados pelo mundo, que, independente de distância ou compromissos, sempre se fizeram presentes em todos os momentos. Os abraços, os sorrisos, as lágrimas, a escolha de serem parte da minha história e de quem eu sou foram, são e sempre serão motivo de gratidão eterna.

Ao meu orientador, Wellerson, pela oportunidade de estar no LabDECA, de adentrar essa equipe e aprender em conjunto. Por todo o crescimento científico e pessoal, pelo incentivo e confiança de sempre. Aos que dividiram comigo a bancada, os estágios, reuniões e desafios, tornando o trabalho mais leve, prazeroso e possível, meu obrigado cheio de saudades.

À UNESP, por toda a minha formação. À cada docente, servidor e colega que fez parte dessa trajetória. Aos alunos que me permitiram ensinar e me mostraram o prazer de estar em sala de aula. Compartilhar esses anos com vocês fez de mim alguém muito melhor.

À Botucatu, cidade que me recebeu, me encantou e me deu um novo lar. Muito obrigado!

“Sempre tem gente pra chamar de nós
Sejam milhares, centenas ou dois
Ficam no tempo os torneios da voz
Não foi só ontem, é hoje e depois
São momentos lá dentro de nós
São outros ventos que vêm do pulmão
Ganham cores na altura da voz
E os que viverem verão”

Luiz Tatit/Marcelo Jeneci

Sumário

Resumo

Desreguladores endócrinos versus Ginsenosídeos: modulação da via não genômica ativada por GPR30 e estresse oxidativo em células de Sertoli humanas (HSec)

Citocinas e proteínas quinases são fundamentais para o controle do processo espermatogênico, estando diretamente envolvidas na dinâmica da barreira hematotesticular. Diferentes mecanismos de controle são modulados por receptores como o GPR30, que ativa rapidamente diferentes vias de sinalização, responsáveis pelos processos de proliferação, sobrevivência e morte celular. Os desreguladores endócrinos (DEs) possuem grande afinidade pelo GPR30, além de potencial para ativar vias de estresse oxidativo e a abertura da barreira. Antagonistas funcionais dos DEs, como o *Panax ginseng*, podem ser protetores contra seus efeitos. Considerando a importância das vias de sinalização que regulam a espermatogênese e a constante exposição ambiental aos DEs a que estamos submetidos, este trabalho objetiva estudar a possível modulação da via não genômica ativada por GPR30 e do estresse oxidativo em células de Sertoli expostas a baixas doses do DE Monobutil Ftalato (MBP) bem como o potencial papel citoprotetor do GIM-1 (metabólito do *P. ginseng)* sobre essas células. Para tal, as células de Sertoli humanas (HSec) foram mantidas sobre matriz artificial, simulando o ambiente *in vivo.* A exposição ao MBP e ao GIM-1 foi realizada nos tempos de 30min, 1, 12, e 48 horas, em doses pré-estabelecidas pelo ensaio do MTT (teste de toxicidade) em 4 grupos: controle, MBP, GIM-1 e MBP + GIM-1. A morfologia celular foi avaliada pela coloração com Hematoxilina e Eosina, evidenciando efeitos deletérios do MBP sobre a distribuição celular e adesão na membrana basal; O grupo GIM-1 foi semelhante ao controle e o MBP+GIM-1 apresentou um aspecto intermediário. Para avaliar o estresse oxidativo, os marcadores enzimáticos glutationa peroxidase, superóxido dismutase e catalase foram analisados por método colorimétrico, sendo o MBP capaz de reduzir a atividade das três enzimas e o GIM-1 de aumentá-las e atenuar os efeitos do MBP no grupo MBP+GIM-1. A quantificação de proteínas por Western Blot mostrou que o MBP inibiu a expressão de NRF2 e aumentou a de caspase 3 clivada, ativou o receptor GPR30, PKA, Src, EGFR e a via da ERK1/2, enquanto o GIM-1 aumentou a expressão de SIRT1 e NRF2 e inibiu PKA, Src e as vias da ERK1/2 e AKT. O MBP também aumentou a expressão da Cofilina, diminuindo a intensidade de actina-F na superfície celular. A exposição combinada evidenciou o antagonismo entre os compostos. Nossos resultados mostram efeitos deletérios do MBP na linhagem HSec, através do estresse oxidativo e da via do GPR30, evidenciando o importante papel citoprotetor da GIM-1 sobre eles.

Palavras-chave: células de Sertoli, barreira hematotesticular, desreguladores endócrinos, monobutil ftalato, *Panax ginseng*, GIM-1, HSec, GPR30, estresse oxidativo.

Abstract

Endocrine disruptors versus Ginsenosides: modulation of GPR30/GPER1 activated pathway and oxidative stress in human Sertoli cells (HSec)

Cytokines and kinases protein are essential to control the spermatogenic process, being directly involved in the blood-testis barrier control. Activation of these mechanisms is modulated by receptors such as GPR30, which rapidly activates different signaling pathways responsible for proliferation and cell death processes. Endocrine Disruptors (EDs) have high affinity for GPR30, causing oxidative stress and possible barrier rupture. Functional antagonists of EDs, such as *Panax ginseng*, may be protective against their effects. Considering the importance of the signaling pathways that regulate spermatogenesis and the constant environmental exposure to the EDs to which we are subject, this work aims to study the possible modulation of the non-genomic pathway activated by GPR30 and oxidative stress in Sertoli cells exposed to low doses of the ED Monobutyl Phthalate (MBP) and the possible cytoprotective role of GIM-1 (*P. ginseng* metabolite) on these pathways. To this end, HSec human lineage cells were maintained on artificial matrix, simulating in vivo environment. Exposure to MBP and GIM-1 was performed at 30 min, 1, 12 and 48 hours at pre-set MTT (toxicity assay) levels in 4 groups: control, MBP, GIM-1 and MBP + GIM-1. Morphology and cell adhesion were evaluated by staining with Hematoxylin and Eosin, evidencing deleterious effects of MBP above cell distribution and adhesion in basement membrane; GIM-1 group was similar to Control and MBP+GIM-1 showed an intermediate aspect. In order to evaluate oxidative stress, the enzymatic markers glutathione peroxidase, superoxide dismutase and catalase were analyzed by colorimetric method, being MBP able to reduce all enzymatic activity and GIM-1 to promote it and attenuate MBP effects in MBP+GIM1. Quantification of proteins by Western Blot showed that MBP inhibited NRF2 expression and increased Cleaved-caspase3, activated the GPR30 receptor, PKA, Src, EGFR and the ERK1/2 pathway, while GIM-1 enhanced SIRT1 and NRF2 expression and inhibited PKA, Src, ERK1/2 and AKT pathways. MBP also enhances Cofilin expression, decreasing F-actin intensity in cell surface. The combined exposure evidenced the antagonism between the compounds. Our results show deleterious effects of MBP on the HSec line, through oxidative stress and GPR30 pathway, evidencing the important cytoprotective role of GIM-1 on them.

Key words: Sertoli cells, blood-testis barrier, endocrine disruptors, monobutyl phthalate, Panax ginseng, GIM-1, HSec, GPR30, oxidative stress.

Capítulo 1

Introdução e Revisão da Literatura

1. Introdução

1.1 A célula de Sertoli e a barreira hematotesticular

Os testículos, as gônadas masculinas, são protegidos por uma túnica fibrosa denominada túnica albugínea. Essa túnica prolonga-se para o interior do testículo formando septos que dividem a gônada em lóbulos, compostos por um emaranhado de túbulos seminíferos, que convergem para a zona posterior do testículo, formando a rede testicular. Desta, saem cerca de quinze canais, os dúctulos eferentes, que confluem para o epidídimo. O túbulo seminífero não é penetrado por vasos sanguíneos, vasos linfáticos ou nervos. Estes se encontram no interstício entre os túbulos, onde são encontradas as células de Leydig, responsáveis pela produção dos hormônios sexuais masculinos, sobretudo testosterona, que coordena e mantém o desenvolvimento dos órgãos genitais masculinos e dos caracteres sexuais secundários (Waites & Setchell, 1975; Cheng & Mruk, 2010a; McAninch, 2010).

É nos túbulos seminíferos que ocorre a espermatogênese, processo de produção dos espermatozoides, que se divide em quatro fases importantes: Multiplicação, Crescimento, Maturação e Diferenciação ou Espermiogênese, iniciando na puberdade e perdurando por toda a vida do homem (Skinner, 2005).

A fertilidade masculina e o processo de espermatogênese estão diretamente relacionados à capacidade de produção de fatores determinantes para o desenvolvimento das células germinativas por células específicas, as células de Sertoli (Griswold, 1998). Encontradas no epitélio dos túbulos seminíferos, denominado epitélio germinativo ou seminífero, essas células apresentam grande dimensão, envolvendo todo o epitélio germinativo (aproximadamente 17-19% do volume do epitélio seminífero em ratos) e possibilitando o desenvolvimento de um grande número de células germinativas, na porcentagem estimada em 1:50 no testículo de ratos adultos (Figura 1) (McAninch, 2010; Weber et al., 1983).

O número dessas células no epitélio germinativo pode determinar o tamanho testicular, o número de células germinativas por testículo e a produção de espermatozoides propriamente dita. Além disso, as células de Sertoli são responsáveis por proporcionar um ambiente protegido e altamente especializado, no interior dos túbulos seminíferos, para o desenvolvimento das células germinativas (Skinner, 2005).

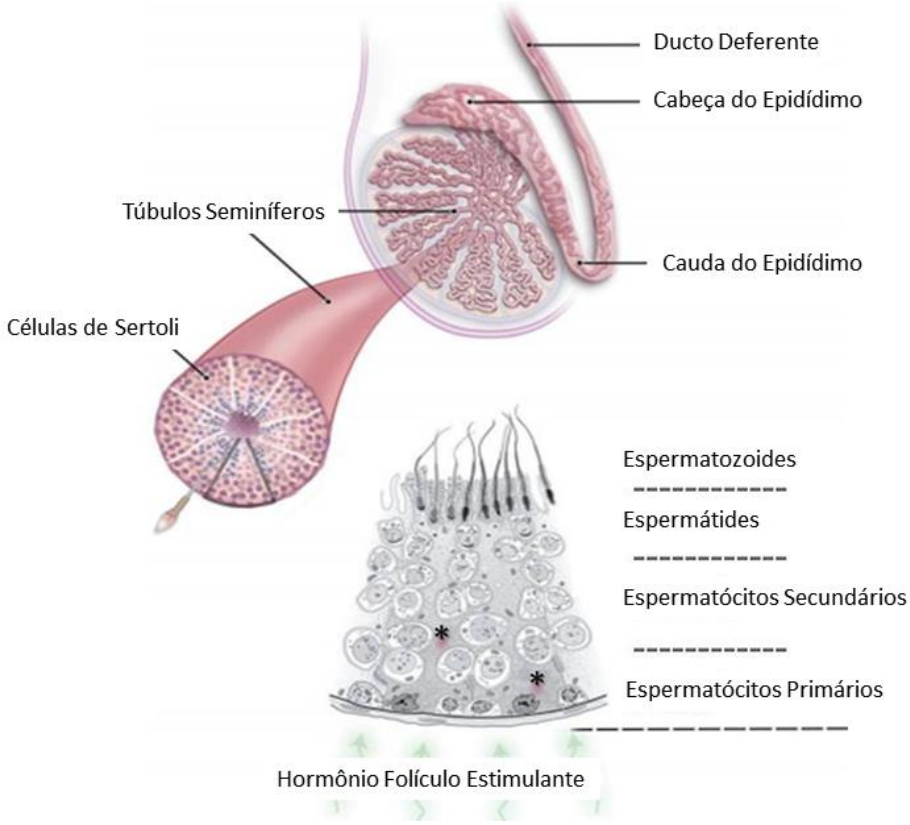


Figura 1. Imagem representativa de um corte sagital de testículo e de um corte transversal do túbulo seminífero, evidenciando o epitélio germinativo sustentado pelas células de Sertoli* (adaptada de Samplaski et al., 2010).

Na espécie humana, as células de Sertoli têm algumas peculiaridades, sendo uma delas a presença dos corpos nucleares, que são organelas esféricas compostas por uma cápsula microfibrilar e com grânulos em sua região central. Além disso, as células humanas, diferentemente de outras espécies, apresentam alinhamento nuclear em uma posição intermediária no interior da célula. Inclusões e organelas típicas são encontradas na base celular (Skinner, 2005).

As células de Sertoli são responsáveis pelo fornecimento de substâncias necessárias para o metabolismo das células germinativas (lactato, transferrina, proteínas andrógeno-dependentes); fatores de crescimento como o fator de célula-tronco, fator transformador de crescimento alfa e beta (TGF-α e TGF-β), fator de crescimento homólogo à insulina (IGF-I), fator de crescimento fibroblástico (FGF), fator de crescimento epidermal (EGF) e hormônios que regulam o desenvolvimento das

estruturas reprodutivas masculinas, como a substância inibidora-mulleriana (MIS) e a inibina (Skinner, 2005).

Estruturalmente, as células de Sertoli são unidas por junções de oclusão e conectadas por junções comunicantes, denominadas *junções gap*, que possibilitam a troca iônica e química entre as células e a interação com a matriz extracelular, o que é importante para a coordenação do ciclo do epitélio seminífero. O forte complexo de adesão entre as células de Sertoli forma a barreira hematotesticular (Mruk & Cheng, 2004).

A barreira hematotesticular é um conceito originalmente baseado em observações no início do século 20, quando corantes administrados em animais de laboratório falhavam em corar os testículos e o cérebro. Esses achados levaram aos conceitos de barreira hematotesticular e barreira hematoencefálica (Cheng & Mruk, 2011). O termo barreira hematotesticular, também conhecido como Sertoli cell seminiferous epithelium barrier, contudo, foi primeiramente utilizado por Chiquoine (1964) em um estudo que avaliou os efeitos da toxicidade do cádmio relacionados à necrose testicular.

A barreira hematotesticular nos mamíferos, diferente de outras barreiras hematoteciduais, é constituída predominantemente por junções especializadas entre células de Sertoli adjacentes próximas a membrana basal no epitélio seminífero, dividindo o epitélio em compartimento basal e luminal, também conhecidos por especialização basal e luminal ou apical (Cheng &Mruk, 2010).

No rato, a barreira hematotesticular começa a se formar aproximadamente entre 15-16 dias pós-natais, se completando entre 18-21 dias, coincidindo com o tempo em que as células de Sertoli acabam de se dividir (Toyama et al., 2001).

Uma das funções da barreira hematotesticular é a restrição do fluxo paracelular de biomoléculas (por exemplo, água, eletrólitos, íons, nutrientes, hormônios, fatores parácrinos e moléculas biológicas) através do epitélio das células de Sertoli no compartimento luminal, regulando assim a entrada de substâncias nutricionais, moléculas vitais e tóxicos nocivos para o compartimento apical em que o desenvolvimento pós-meiótico das células germinativas ocorre (Cheng & Mruk, 2012).

Outra função associada é a criação de uma barreira imunológica, conferindo o estado de imunoprivilégio dos testículos, que permite que a resposta imunológica a autoantígenos que residem dentro das células germinativas em desenvolvimento durante a espermatogênese, muitos dos quais são expressos transitoriamente, seja suprimida (Fijak et al., 2011; Meinhardt & Hedger, 2010). Isto é necessário para evitar a produção de anticorpos antiespermatozóides e doença autoimune, o que leva à infertilidade masculina (Francavilla et al., 2007).

Quando as espermatogônias, células germinativas quiescentes desde o período fetal, iniciam o a espermatogênese através do processo de meiose, ainda estão na região basal dos túbulos seminíferos, fora do espaço delimitado pela barreira hematotesticular. Os espermatócitos primários, formados nessa fase, quando em pré-leptóteno, sinalizam para a abertura da barreira, entrando na região de imunoprivilégio para então completar seu desenvolvimento até a formação dos espermatozoides (Moore & Persaud, 2013). A partir desse momento, se tornam dependentes das células de Sertoli para o fornecimento de nutrientes e fatores de crescimento para o seu desenvolvimento (Mruk & Cheng, 2004).

Embora o epitélio seminífero tenha seus próprios mecanismos de defesa imunossupressora, antibacteriana e antiviral, as células de Sertoli, por si só, podem desempenhar um papel crítico na manutenção dos testículos como um órgão imunologicamente privilegiado, secretando moléculas imunossupressoras para bloquear a resposta imune para autoantígenos expressos transitoriamente em células germinativas em desenvolvimento, durante a espermatogênese (Selawry & Cameron, 1993; Mital et al., 2011). No entanto, as identidades das biomoléculas imunossupressoras permanecem desconhecidas, sendo supostamente compostas por um conjunto de moléculas, incluindo citocinas (por exemplo, interleucinas, interferons) e prostaglandinas (Yang, 2010; Mruk & Cheng, 2004; Meinhardt & Hedger, 2010).

Outra função importante da barreira hematotesticular é a de conferir polaridade celular no epitélio seminífero, evidenciada pela localização dos núcleos das células de Sertoli, que são restritos ao compartimento basal, encontrando-se adjacentes à túnica própria e as organelas citoplasmáticas (por exemplo, aparelho de Golgi, lisossomos) que nas células de Sertoli não são uniformemente distribuídas no citoplasma (Mruk & Cheng, 2010).

Mruk & Cheng (2010) afirmam também que especialização apical confere polaridade as espermátides durante a espermiogênese, usando módulos de polaridade semelhantes, de forma que o desenvolvimento das espermátides pode ser devidamente orientado para ocupar o mínimo de espaço no epitélio com a cabeça do espermatozoide apontando para a membrana basal e as caudas para o lúmen do túbulo seminífero.

Alguns dos fatores ultraestruturais mais distintos e proeminentes da barreira hematotesticular são os pacotes de filamentos de actina, que se distribuem na membrana plasmática e conferem resistência adesiva para a barreira (Mok et al., 2012). A actina existe tanto como actina globular (actina-G) quanto como actina filamentosa (actina-F) (Figura 2). Para facilitar o movimento das espermátides através do epitélio, é criada uma rede ramificada de actina, que elimina a "rigidez" associada aos feixes, desestabilizando as especializações. Além disso, a actina-F pode ser despolimerizada por proteínas encontradas nas especializações e assim convertida em actina-G para facilitar a movimentação das espermátides (Cheng & Mruk, 2011).

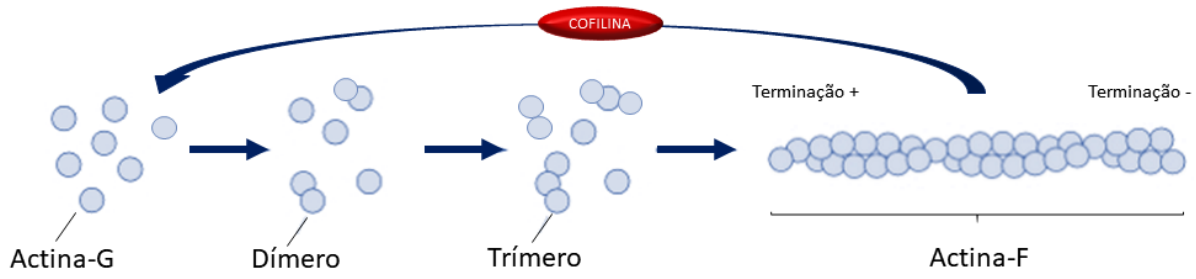


Figura 2. Polimerização da Actina-G em Actina-F, através do processo de nucleação e ação da Cofilina no sequestro de monômeros, despolimerizando os filamentos de actina, aumentando a concentração de actina-G e possibilitando a abertura da barreira hematotesticular.

Essas proteínas são chamadas de ABPs (Proteínas que se ligam a actina), uma família com a vasta função de controlar os mais diversos processos celulares que envolvem actina, com formação de estruturas complexas e tridimensionais, como a barreira hematotesticular (Lappalainen, 2016). Entre elas destacamos a Cofilina, proteína que exerce funções como o sequestro de monômeros de actina (Figura 2). Para que haja formação dos filamentos, é necessário que ocorra o processo de nucleação, onde monômeros são estabilizados e ocorre a formação de dímeros e trímeros. A

Cofilina sequestra os monômeros, impedindo essa formação e levando, consequentemente, ao aumento da concentração de actina-G (Sibley, 2010).

As Cofilinas podem ainda participar da quebra de filamentos de actina, por alterações mecânicas e de flexibilidade (McCullough et al., 2011) e causar a despolimerização da actina-F, induzindo a dissociação dos monômeros (Carlier et al., 1997).

Esses pacotes de filamentos de actina ficam entre as cisternas no reticulo endoplasmático e a especialização basal, sendo encontrados nos dois lados das células de Sertoli (Lie et al., 2010). As junções de oclusão (tight junctions), por outro lado, aparecem como uma ligação entre membranas plasmáticas de células de Sertoli justapostas, sendo encontradas entre as especializações basais das células de Sertoli, perto da membrana basal. Notamos que quando a especialização foi primeiramente identificada como um componente estrutural crucial da barreira hematotesticular, era chamada de “especialização juncional” (Flickinger &Fawcett, 1967), mas renomeada 10 anos depois (Russell, 1977).

Essas junções são os únicos tipos de junção de oclusão no epitélio e endotélio de mamíferos, contribuindo para a barreira de permeabilidade através de um epitélio ou endotélio. Nos testículos, diferente do observado em outros epitélios, elas são encontradas adjacentes à membrana basal e encostadas às junções de aderência e aos desmossomos, separando os compartimentos basal e adluminal e controlando o fluxo transepitelial de moléculas e íons (Lui et al., 2003).

Pela coexistência das junções com a especialização basal no epitélio seminífero próximo a membrana basal ainda é difícil identificar proteínas que são únicas à especialização basal, pois as supostas proteínas das junções de oclusão (ocludina, claudina, JAM-A, JAM-B) estão localizadas no mesmo sítio que as da especialização basal (N-caderina, β-catenina) (Yan & Cheng, 2005).

Não obstante, Yan & Cheng (2005) mostram que muitas proteínas são comuns a especialização apical e basal (N-caderina, E-caderina, β-catenina, α-catenina, nectinas...). Por exemplo, a N-caderina (proteína da especialização basal) e a ocludina (proteína da tight junction) estão localizadas no mesmo sítio da barreira hematotesticular, mas não tem interação direta proteína-proteína, estando

estruturalmente ligadas pelos seus adaptadores periféricos correspondentes, α-catenina e a ZO-1, respectivamente. Assim, ultraestruturas, como os pacotes de filamentos de actina podem ser utilizados para "reforçar" a função adesiva das células na tight junction da barreira hematotesticular. No entanto, quando o testículo é exposto a um tóxico ou quando o epitélio seminífero está no estágio VIII do ciclo epitelial, onde a barreira hematotesticular sofre reestruturação para acomodar o transito dos espermatócitos em pré-leptóteno, α-catenina e ZO-1 se tornam "livres" e deixam de ter interação física entre elas, de forma que uma quebra da interação proteína-proteína dentro do complexo ocludina-ZO-1 ou N-caderina-α-catenina na tight junction ou na especialização basal não leva a quebra imediata do outro, e vice-versa.

Já nas junções de aderência, encontramos proteínas sinalizadoras que conferem *cross-talk* entre os diferentes tipos de junção para coordenar a migração de espermatócitos em pré-leptóteno. Como observado na figura 3, caderinas e cateninas formam um forte complexo de ligação, principalmente entre N-caderinas e β-cateninas, sendo interrompido momentaneamente, por endocitose mediada por TGF-β2, para permitir a migração dos espermatócitos primários e rapidamente retomada, via receptor de androgênios (Li et al., 2011).

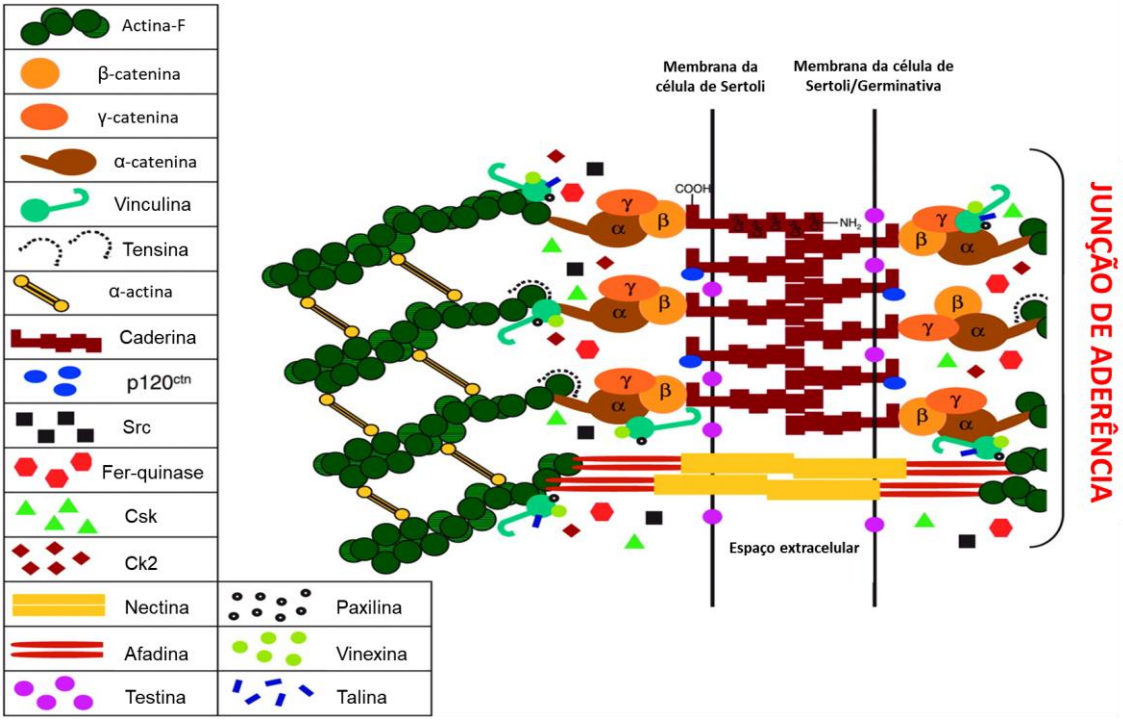


Figura 3. Representação esquemática dos componentes proteicos das junções de aderência da barreira hematotesticular (Extraído e adaptado de Cheng & Mruk, 2002).

1.2 Controle da barreira

Processos fundamentais para a espermatogênese, como a regulação de proliferação e diferenciação das espermatogônias, a progressão do ciclo celular, espermiogênese, espermiação, adesão celular e a manutenção do estado de imunoprivilégio dos testículos são mediados por receptores celulares, por vias genômicas altamente descritas, reguladas por Receptores de Androgênios (AR), e por vias não genômicas recentemente descobertas recentemente (Walker et. al., 2009; Wang et al., 2009; Lamont & Tindall, 2010; Ruwanpura et al., 2010; Verhoeven et al., 2010; Willens et al., 2010; O'Donnell et al., 2011; Meng et al., 2011; Shupe et al., 2011).

O estado de fosforilação de proteínas integrais de membrana, adaptadores periféricos, quinases, fosfatases e proteínas periféricas em junções de barreira, desempenha um papel fundamental na determinação da função adesiva na interface célula-célula (Figura 4). A fosforilação é controlada por proteínas quinases *nonreceptor*, em resposta às alterações no ambiente e ao estresse oxidativo (Cheng & Mruk, 2002;

Hawkins & Davis, 2005; Xia et al., 2005a; Suzuki & Hara, 2011). Citocinas e proteínas quinases, como o proto-oncogene Src e as proteínas da via da MAPK (proteínas quinases ativadas por mitógenos), são também fundamentais para o controle da barreira por estarem diretamente envolvidas na fosforilação e endocitose das proteínas juncionais, alterando assim a intensidade de adesão da barreira (Cheng & Mruk, 2012).

Parte de uma família de proteínas quinases *non-receptor*, a Src atua nas junções gap, desmossomos, especialização basal e nas junções de oclusão, sendo expressa em quase todas as células de mamíferos. Ela também é um componente integral dos complexos de proteína baseados em ocludina, N-caderina e ZO-1 (*Zonula Ocludens 1*) na barreira, e possivelmente regula a adesão celular através da sua capacidade de manter e/ou alterar o estado de fosforilação dessas proteínas, regulando assim a sua cinética de endocitose, reciclagem e transcitose durante a espermatogênese (Mruk & Cheng, 2010).

Cheng & Mruk (2011) demonstraram que alguns complexos proteicos de adesão (ex., ocludina-ZO-1, N-caderina-β-catenina, claudina-5-ZO-1), esteroides (ex., testosterona, estradiol-17β), proteínas quinases nonreceptor (ex., focal adhesion kinase, c-Src, c-Yes), proteínas de polaridade (ex., PAR6, Cdc42, 14-3-3), proteínas de vesícula endocítica (ex., clatrina, caveolina, dinamina 2), e proteínas reguladoras de actina (ex., Eps8, complexo Arp2/3), estão trabalhando juntos, aparentemente sob influência de citocinas (ex., fator transformador de crescimento-β3, fator de necrose tumoral-α, interleucina-1α) na criação de uma "nova" barreira hematotesticular atrás dos espermatócitos em trânsito, enquanto a "antiga" vai se degenerando aos poucos, de forma que a barreira imunológica é mantida durante a travessia dos espermatócitos pela barreira.

Além disso, Src tem participação importante em vários eventos celulares, como o controle de migração, proliferação, diferenciação, citoesqueleto, sobrevivência, apoptose, sinalização de cálcio e a própria espermatogênese, como apontado por Xiao e colaboradores (2017). O mesmo grupo mostrou que ratos *knockout* para Src são inférteis, além de evidenciar seu papel na degradação de restos citoplasmáticos da espermiogênese e na ativação de outros complexos proteicos moduladores das funções celulares.

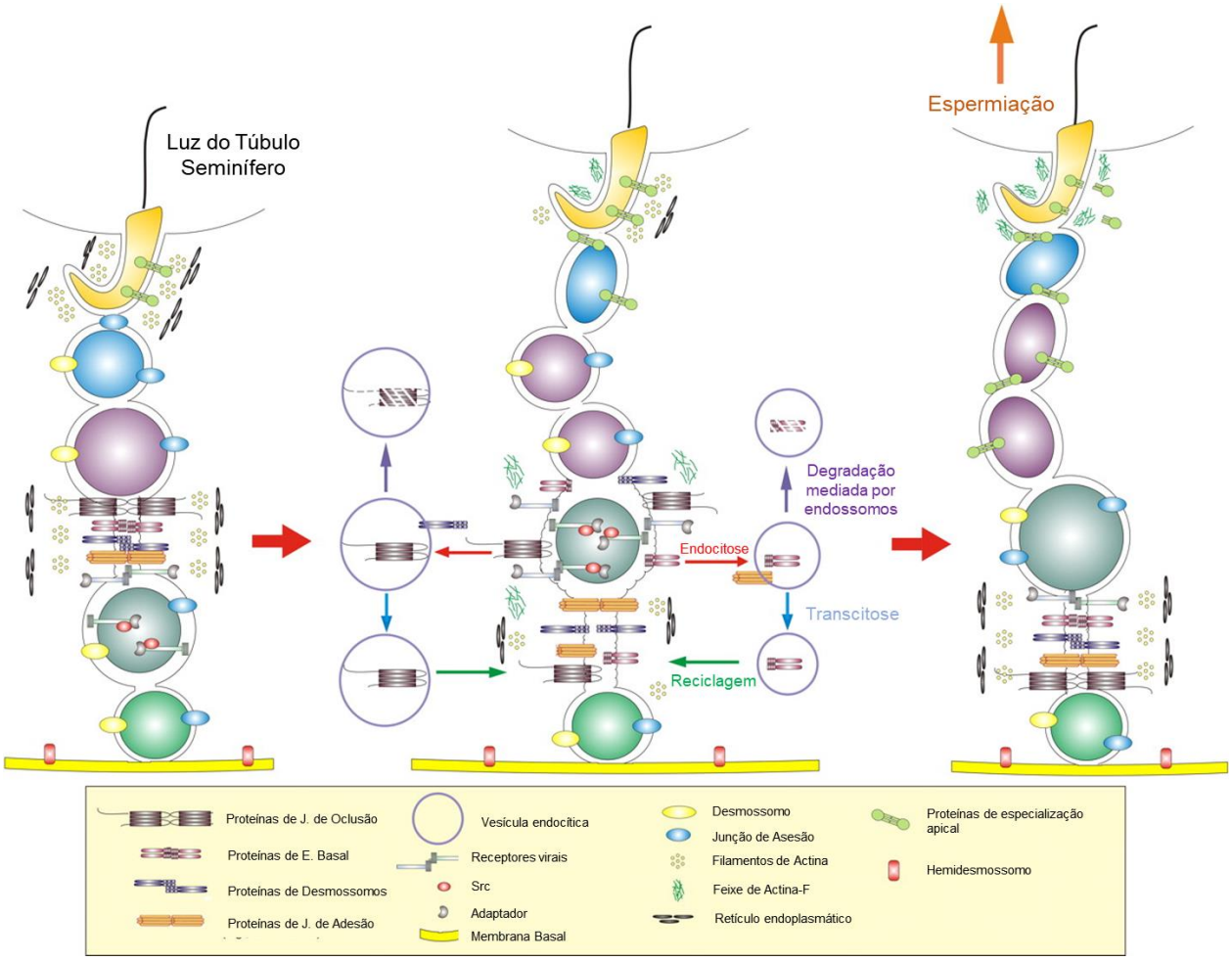


Figura 4. Representação esquemática dos mecanismos de abertura e fechamento da barreira hematotesticular através dos processos de endocitose, transcitose e reciclagem de proteínas. Podemos evidenciar o papel da Src na coordenação do estado de fosforilação das proteínas de junção e, consequentemente, na dinâmica da barreira (Extraído e adaptado de Cheng & Mruk, 2012).

A ativação desses complexos de proteínas, por sua vez, recruta outros adaptadores, mediando diferentes vias de sinalização, envolvendo GTPases e MAPKs, e regula uma série de eventos e funções celulares em condições normais e patológicas, incluindo a espermatogênese (Lui et al., 2003; Xia et al., 2005; Loveland et al., 2007; Massague, 2008; Worthington et al., 2011). Por exemplo, sinalização envolvendo proteínas da família das MAPKs como a p38-MAPK (Lui et al., 2003; Wong et al., 2004) ou a ERK1/2 (Xia & Cheng, 2005) pode perturbar a adesão da barreira, das espermatogônias e a função da barreira. ERK1/2, AKT e fosfatidilinositol-3-quinase (PI3K) também exercem modulação sobre as junções de oclusão (Cheng & Mruk,

2012). Li e colaboradores (2001) demonstraram que os inibidores e/ou ativadores de proteína-quinases e fosfatases poderiam "manipular" a função dessas junções (Xia & Cheng, 2005; Zhang et al., 2005).

Independentemente da via de ativação de Src, estudos na última década têm mostrado que a ativação de PI3K também é crucial para regular o crescimento celular, proliferação, diferenciação, apoptose e tráfego intracelular de proteínas em vários epitélios (Kwiatkowska, 2010; Cockcroft & Garner, 2011). Em testículos de ratos adultos, PI3K é localizada intensamente nas especializações apicais e é ativada durante a perda de espermátides, induzida por ajudina, que imita o processo de espermiação (Siu et al., 2005).

Os estudos de Xia e colaboradores (2006) e Li e colaboradores (2006) têm mostrado que as citocinas tais como TGF-βs, interferon-γ e fator de necrose tumoral (TNF-α), também podem perturbar, de maneira reversível, a barreira hematotesticular in vivo, por *down-regulation* do nível de proteína das junções de oclusão e da especialização basal através de um mecanismo ainda a ser definido. Yan e colaboradores (2011) demonstraram que o TGF-β2 perturba a barreira hematotesticular ao acelerar a cinética da internalização das proteínas integrais de membrana ocludina e N-caderina, mas não da JAM-A, seguido pela degradação de proteínas endocíticas.

Além do controle hormonal e da interação direta na interface entre células de Sertoli, é importante ressaltar que em testículos de mamíferos adultos, essas células estão em estreito contato com a membrana basal, uma forma modificada da matriz extracelular (MEC). A MEC desempenha um papel significativo na regulação da espermatogênese, particularmente das células de Sertoli e da dinâmica da barreira hematotesticular. Proteínas quinases, como a c-Src, medeiam a sinalização na interface célula-matriz extracelular, regulando a adesão, a progressão do ciclo celular, sobrevivência, proliferação e diferenciação. Estudos recentes relacionam casos de infertilidade masculina, com comprometimento da espermatogênese, à estruturas anormais da MEC. Evidências apontam ainda para o papel essencial de componentes da MEC, como colágenos e lamininas, no controle dos níveis de proteínas integrais de membrana na interface das células de Sertoli (Siu & Cheng, 2008; Xiao et al., 2012).

1.3 Receptores envolvidos

Por serem as únicas células, nos testículos, com receptores para testosterona e Hormônio folículo-estimulante (FSH), as células de Sertoli são as principais mediadoras dos sinais endócrinos fundamentais para o controle da espermatogênese (Walker et al. 2005). O hormônio mais importante nesse processo é o Hormônio Liberador de Gonadotrofina (GnRH), produzido pelo hipotálamo. O GnRH induz a produção de FSH e Hormônio luteinizante (LH), pela hipófise anterior, que agem no testículo permitindo o controle do potencial espermatogênico. O LH se liga aos receptores na superfície das células de Leydig, nos testículos, e estimula a produção de testosterona, um hormônio esteroide que se difunde nos túbulos seminíferos (Walker et al. 2005).

Evidências mostram que o controle do processo espermatogênico é mediado pelas células de Sertoli pela ação dos andrógenos testiculares via receptor de Androgênio, tendo em vista que em animais *knockouts* para o AR ocorre interrupção da espermatogênese, interferindo em alvos moleculares específicos (Verhoeven et al., 2010) – Figura 5. Alterações estruturais e na síntese desse receptor ocasionam a deficiência no transporte de testosterona e consequentemente infertilidade masculina ou insensibilidade parcial ou completa de androgênios (Heinlein et al., 2001).

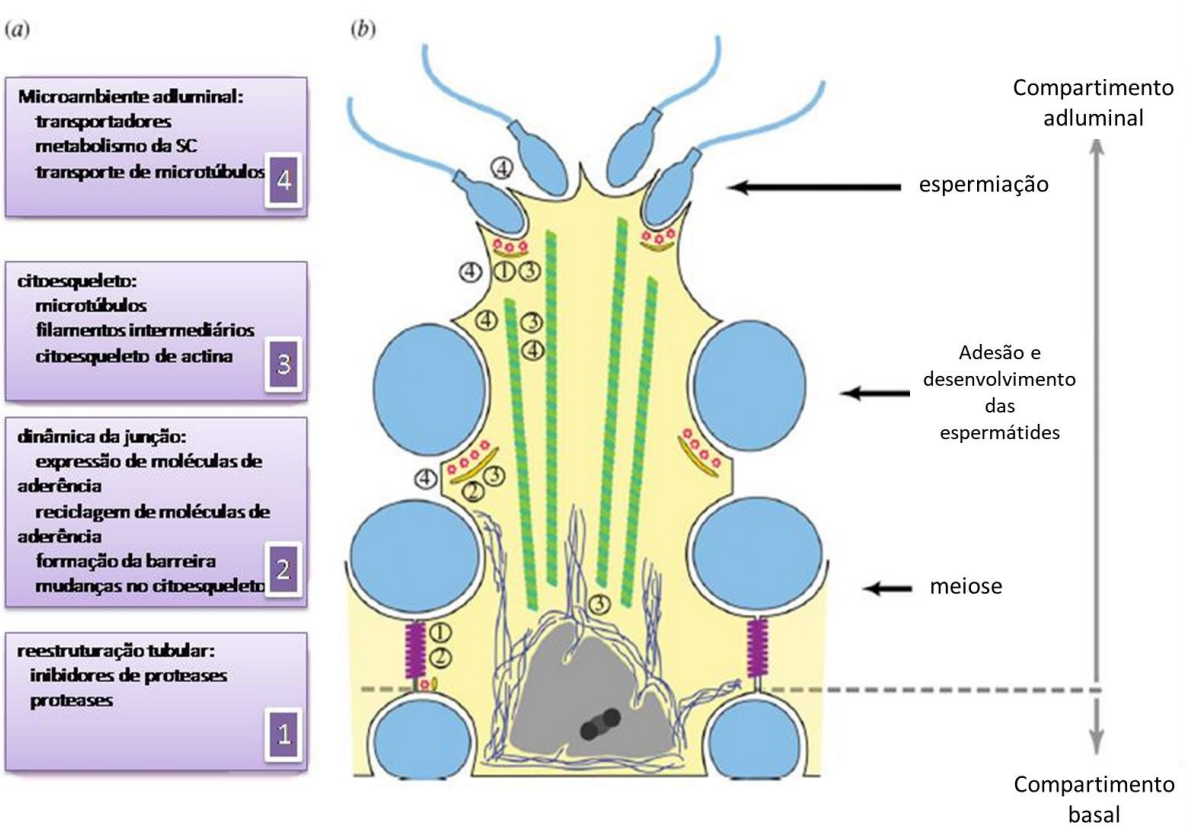


Figura 5. Representação esquemática dos mediadores moleculares da ação androgênica sobre a espermatogênese. *(b)* indica as principais fases da espermatogênese afetadas pelos androgênios. Em *(a)*, um número de processos e alvos moleculares que são identificados como potenciais alvos de ação androgênica. Em amarelo (célula de Sertoli); em azul (células espermatogênicas). Microtúbulos e filamentos intermediários são representados em verde e azul escuro. Junções de oclusão são representadas em púrpura (Extraído e adaptado de Verhoeven et al., 2010).

Willems e colaboradores (2010) mostraram que os andrógenos desempenham papel fundamental na espermatogênese e, sob várias condições, eles são capazes de manter a fertilidade na ausência virtual de hormônio folículo-estimulante (FSH). É interessante que a maioria dos dados indica que o receptor de andrógeno (AR) não é expresso nas células germinativas e que estas se desenvolvem normalmente na ausência de expressão autônoma de AR na célula. Isso indica que os andrógenos afetam o desenvolvimento das células germinativas indiretamente, agindo nas células somáticas do testículo. Considerando que, em outras células somáticas do testículo a expressão de AR é estabelecida durante a vida embrionária, a expressão de AR nas células de Sertoli começa tarde (4-5 dias pós-natais em ratos e camundongos, 4-8 anos em homens).

A regulação de quase todos os aspectos da espermatogênese pelos andrógenos inclui proliferação e diferenciação das espermatogônias, a progressão do ciclo celular de células germinativas pela meiose, espermiogênese, espermiação e adesão celular na interface Sertoli-Sertoli e Sertoli-Célula Germinativa no epitélio seminífero e para conferir privilégio imunológico no testículo (Wang et al., 2009).

Esses efeitos são mediados por caminhos genômicos e/ou não genômicos. Por exemplo, foi mostrado que o knockout de AR (AR-KO) específico para as células de Sertoli em camundongos levou a infertilidade, manifestada por impedimento meiótico (De Gendt et al., 2004). Além disso, camundongos *Sertoli cell-especific AR-KO* mostraram uma barreira hematotesticular deficiente, que estava associada a uma expressão reduzida de claudina-11, ZO-1, ocludina e gelsolina, mas com um aumento significante da expressão de vimentina. Também foi observado que a maturação e polarização das células de Sertoli também foram perturbadas nos camundongos *Sertoli cell-especific AR-KO*, e que a JAM-C (marcador da especialização apical, restrito a ela) mostrou redução significativa, ilustrando que os andrógenos são cruciais tanto para as funções da especialização apical quanto para a basal (Willems et al., 2010).

Além disso, a retirada de testosterona resultou no distanciamento de espermátides em desenvolvimento (estágio VIII a XIX das espermátides) a partir de células de Sertoli do epitélio seminífero, demonstrando que a testosterona é importante na adesão celular (Yan e colaboradores, 2008).

Yan e colaboradores (2008) mostraram que a testosterona promove a função da barreira hematotesticular por "desaceleração" da cinética da endocitose de proteínas. A testosterona melhorou significativamente a cinética de internalização de proteínas de membrana integrais das tight junctions (JAM-A e ocludina) e proteína de membrana integral da especialização basal (N-caderina) na barreira hematotesticular das células de Sertoli, de forma específica.

Os andrógenos, de acordo com Yan e colaboradores (2011), facilitam o tráfico de proteínas integrais da membrana entre a superfície da célula e o citosol, bem como a sua reciclagem do citosol de volta para a superfície da célula em vez de aumentar a expressão de proteínas da barreira hematotesticular, que poderia reforçar sua integridade. Os andrógenos provavelmente exercem os seus efeitos através do deslocamento de proteínas integrais de membrana dentro do microambiente da barreira,

como por remontagem (ou "selagem") da barreira sob a migração dos espermatócitos em pré-leptóteno. Considerando que citocinas promovem a endocitose de proteínas de forma semelhante à testosterona, observou-se que a dinâmica da barreira hematotesticular é regulada pela coordenação de ambas as classes de biomoléculas, as quais afetam diferencialmente o destino da endocitose de proteínas de membrana integrais na barreira.

Além do receptor de androgênio, responsável pelo controle dos mecanismos desencadeados pelos androgênios acima descritos, estudos apontam a importância do receptor de estrogênio (ER) para o controle das funções testiculares (Fietz et al., 2014).

O estrógeno exerce as suas funções biológicas através da sua interação com o receptor de estrogênio, em suas formas α e β. Em testículos de mamíferos adultos, ERα está praticamente restrito a células de Leydig e células mioides peritubulares, enquanto o ERβ está praticamente restrito às células de Sertoli e espermatócitos (Carreau & Hess, 2010).

Estudos recentes identificaram um terceiro receptor de estrogênio: o GPR30 (receptor acoplado à proteína-G, um receptor transmembrana, também chamado de GPER1). Identificado como uma nova proteína ligante ao 17β-estradiol (E2), é estruturalmente distinto dos receptores estrogênicos clássicos α e β. Além de diferenças estruturais, essa proteína desempenha funções diferentes dos receptores anteriormente descritos. Quando ligado ao E2, é capaz de mediar rápidos eventos não genômicos de sinalização – com efeitos em questão de segundos ou minutos (Prossnitz et al., 2008).

Yang e colaboradores (2017) inferiram que o GPR30 participa do controle das funções da Célula de Sertoli, uma vez que é encontrado em células humanas, de roedores, células de Leydig e células diploides da linhagem germinativa.

A ligação ao GPR30 rapidamente ativa diferentes vias de sinalização (Figura 6), incluindo a transativação do receptor de EGF (Fator de crescimento epidermal ligado à heparina), levando a rápida fosforilação das MAPKs ERK1/2, estimulação da adenilato ciclase, com consequente aumento de cAMP e expressão de PKA, mobilização de cálcio intracelular e ativação da via da PI3K/AKT, responsáveis ainda pelos processos de proliferação e sobrevivência celular (Maggiolini & Picard, 2009).

O GPR30 é também um importante sítio de ligação para FSH, atuando diretamente na evolução do processo de espermatogênese. A ligação do FSH ao receptor, ativando a proteína G acoplada, aumenta a expressão de cAMP, que induz o aumento de PKA,

responsável por intermediar as respostas da célula de Sertoli ao FSH, além de participar diretamente do processo de alongamento das espermátides e formação de espermatozoides férteis (Gupta, 2005; Chauvigné et al., 2014) – Figura 6.

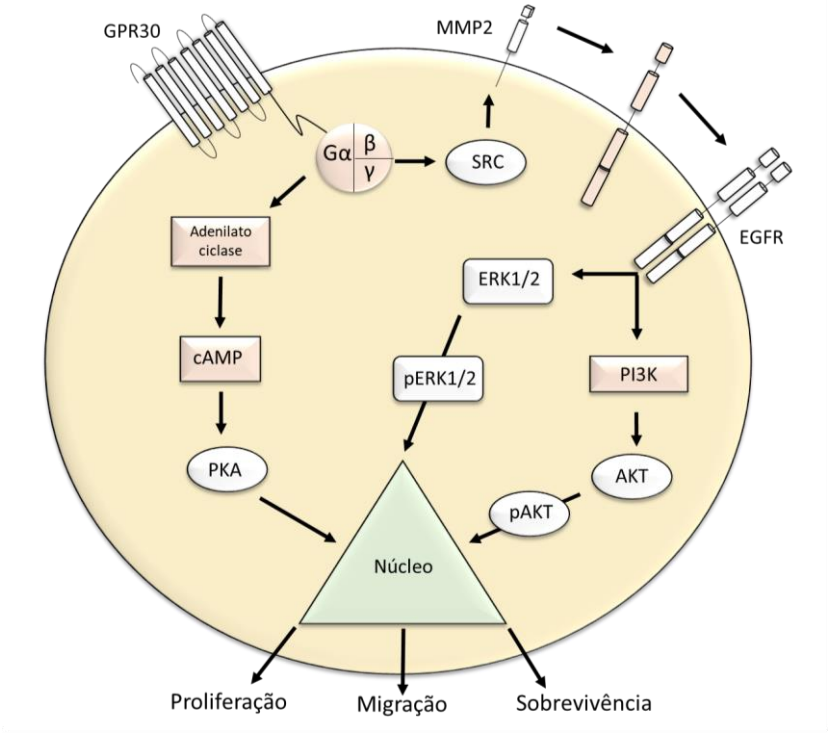


Figura 6. Vias de sinalização ativadas pelo GPR30. Em branco estão os alvos do presente estudo.

Assim como a Src, a PKA tem a função de ativar a via ERK1/2 que, juntamente com a via da PI3K/AKT, é uma importante via de sinalização mediadora da ação hormonal sobre as células de Sertoli (Crepieux et al., 2001; 2001Meroni et al., 2002). A via das MAPKs ERK1/2 retransmite, amplifica e integra os sinais provenientes de uma grande variedade de estímulos e induz uma resposta fisiológica adequada, incluindo a proliferação celular, a diferenciação, o desenvolvimento, respostas inflamatórias e apoptose em células de mamíferos (Zhang & Liu, 2002). Usualmente, essa via é mais associada a proliferação e sobrevivência celulares, mas sabe-se que ela pode também estar envolvida na regulação do metabolismo celular. Por outro lado, a sinalização pela via PI3K/AKT contribui para diversos processos, incluindo a progressão do ciclo celular, crescimento celular, sobrevivência e migração, e transporte vesicular intracelular (Vanhaesebroeck et al., 2010).

1.4 Desreguladores Endócrinos e Estresse Oxidativo

Desreguladores endócrinos são definidos pela *American Environmental Protection Agency* (EPA) como "agentes exógenos que interferem na produção, liberação, transporte, metabolismo, ligação, ação ou eliminação de hormônios naturais presentes no organismo, responsáveis pela manutenção da homeostase e regulação do processo de desenvolvimento" (Kavlock et al., 1996).

De acordo com dados do *American Center for Disease Control* (*Third National Report on Human Exposure to Environmental Chemicals*, 2005) os humanos estão expostos a, no mínimo, centenas de químicos ambientais, dos quais, dezenas deles são conhecidamente DEs e essa exposição pode ocorrer através do ar, alimentação, água ou contato com uma grande variedade de produtos de consumo.

A detecção consistente de resíduos de desreguladores endócrinos (DEs) no soro humano, plasma seminal e fluido folicular aumentou a preocupação de que a exposição ambiental aos DEs está afetando a fertilidade humana (Younglai et al., 2002). Embora não sejam considerados grandes teratógenos, a função reprodutiva é particularmente suscetível aos DEs, desencadeando alterações morfológicas e funcionais (Sikka & Wang, 2008; Diamanti-Kandarakis et al., 2010), sendo fundamental o estudo de seus efeitos sobre os componentes do sistema genital masculino.

Atualmente sabe-se que os DEs agem via receptores nucleares esteroides, receptores hormonais esteroides não nucleares, receptores não esteroides, vias enzimáticas envolvidas na biossíntese e/ou metabolismo esteroide e através de vários outros mecanismos que estão associados aos sistemas endócrino e genital, com particular afinidade pelo receptor GPR30 (Diamanti-Kandarakis et al., 2009; Hu et al., 2013).

De acordo com Sheehan e colaboradores (1999), a exposição a qualquer nível de DEs pode causar anormalidades reprodutivas ou endócrinas. Contrariamente ao esperado, a exposição a baixas doses pode exercer efeitos mais significativos que altas doses (Welshons et al., 2003; vom Saal & Hughes, 2005; vom Saal et al., 2007).

Já é bem estabelecido na literatura que os hormônios exercem ação em concentrações extremamente baixas no soro, tipicamente na faixa entre picomolar e nanomolar. Muitos estudos mostram que os DEs podem agir na faixa entre nanomolar e micromolar, com alguns agindo em níveis de picomolar. Por definição uma dose baixa é aquela que se encontra na faixa de exposição humana ou uma dose menor do que a tipicamente utilizada ou definida como a menor dose com efeitos adversos observados (LOAEL, do inglês "lowest observed adverse effect level") (Vandenberg et al., 2012).

Ainda quanto à exposição aos DEs, Diamanti-Kandarakis e colaboradores (2009) ressaltam a existência de um intervalo de tempo entre o período de exposição e a manifestação fisiopatológica, ou seja, as consequências da exposição durante as fases iniciais de desenvolvimento, por exemplo, podem não ser imediatamente aparentes, mas podem se manifestar na idade adulta e/ou durante o envelhecimento.

O grupo de moléculas identificado como DEs é altamente heterogêneo e inclui químicos sintéticos utilizados como solventes/lubrificantes industriais e seus subprodutos (bifenis policlorados – PCBs, bifenis polibromados - PBBs, dioxinas), plásticos (bisfenol A – BPA), plastificantes (ftalatos), pesticidas (metoxiclor, diclorodifeniltricloroetano – DDT), fungicidas (vinclozolin) e compostos farmacêuticos (dietilestilbestrol - DES) (Diamanti-Kandarakis et al., 2009).

O monobutil ftalato (MBP) é o metabólito ativo do di-n-butil-ftalato (DBP), um desregulador endócrino utilizado em muitos produtos de uso cotidiano, como plásticos, tubos de PVC, tintas, colas, repelentes, perfumes, spray de cabelo e esmalte de unhas. Por não ser ligado ao produto final, o DBP pode se mover do produto para o meio ambiente durante longos períodos de tempo. Estudos encontraram 400-700 mg/kg de ftalatos na poeira de residências domésticas e cerca de 111ng/ml na urina de crianças (Becker et al., 2004; Frederiksen et al., 2011).

A estrutura molecular do MBP apresenta uma cadeia hidrofóbica (Figura 7), que permite sua inserção na membrana celular, justificando sua presença em amostras de sangue e urina e intensificando o alerta de sua exposição (Hu et al., 2014).

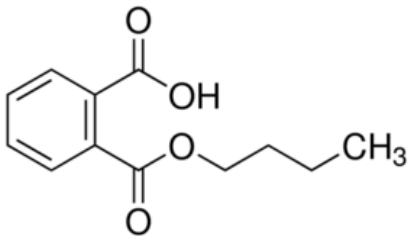


Figura 7. Estrutura química do MBP

Os ftalatos causam uma larga série de problemas adversos à saúde, incluindo danos ao fígado, aos rins e aos pulmões bem como anormalidades no sistema genital e no desenvolvimento sexual (Wei et al., 2001). Auharek e colaboradores (2010) mostraram que a exposição fetal de ratos ao DBP reduz a produção fetal de testosterona e o número de células de Sertoli ao nascimento. Yao e colaboradores (2010) relataram que a lesão da célula de Sertoli em roedores após exposição ao mono-2-etilhexil-ftalato (MEHP) resultou na ativação da matriz de metaloproteinase 2 e aumentou a apoptose de células germinativas. A exposição de camundongos ao MEHP diminuiu a expressão de ocludina nas *tight junctions* e provocou o aparecimento de lacunas entre as células de Sertoli adjacentes.

Além da afinidade e potencial de ativação dos ftalatos sobre o receptor GPR30, seus efeitos levam ao aumento de estresse oxidativo, atuando diretamente sobre enzimas (por exemplo, superóxido dismutase, catalase e glutationa peroxidase) que modulam as espécies reativas de oxigênio (ROS) que, por sua vez, estimulam a via das MAPKs (Wong & Cheng, 2011; Hu et al., 2013). Evidências crescentes demonstram uma relação entre o aumento do estresse oxidativo e a ruptura de junções celulares na barreira hematotesticular, sendo observado em cerca de 80% dos homens inférteis que tiveram exposição prolongada a tóxicos ambientais (Kiziler et al., 2007; Tremellen, 2008; Venkatesh et al., 2011).

Muitas condições ou eventos associados à infertilidade masculina estão associados ao estresse oxidativo, dado que o processo de espermatogênese e os espermatozoides são muito suscetíveis ao ataque por ROS (Sikka, 2001). A proteção da peroxidação lipídica é principalmente devida à atividade da glutationa-peroxidase (GPx), uma enzima importante para manter a espermatogênese, a motilidade e a viabilidade dos espermatozoides (Griveau et al., 1995).

A ativação anormal de PI3K é a marca registrada do rompimento de junção induzido por estresse oxidativo (Wong & Cheng, 2011). Rasool e colaboradores (2014)

mostraram que a peroxidação lipídica, fruto da redução de Superóxido Dismutase e Catalase, é uma das principais causadoras de danos celulares, destruindo células germinativas (das espermatogônias aos espermatozoides) e células de Sertoli.

Em alguns casos de alterações de fertilidade, além da redução da atividade enzimática, estudos observaram um aumento na expressão de 8-hidroxi-2'-desoxiguanosina (8-OHdG), um importante indicador de lesão oxidativa no DNA (Fardilha et al., 2015). O aumento da 8-OHdG é resultante do acúmulo das espécies reativas de oxigênio, que condicionam danos sucessivos ao DNA. A molécula tem o potencial de emparelhar com resíduos de adenina, aumentando a taxa de translocações espontâneas. Por ser constantemente excisada pelos mecanismos de reparação do DNA, a 8-OHdG é facilmente detectada no plasma, sendo um dos marcadores de danos por ROS mais utilizados na atualidade (Teixeira, 2013).

Além disso, o sistema antioxidante mediado por NRF2 foi reconhecido por Ohta e colaboradores (2008) como um alvo molecular primário contra estresse oxidativo, por acúmulo nuclear e consequente indução de expressão de enzimas citoprotetoras e proteínas relacionadas. Estudos anteriores reportaram que ratos *knockout* para NRF2 apresentaram aumento de peroxidação lipídica no testículo e epidídimo, com baixos níveis de antioxidantes, resultando em redução de motilidade espermática (Nakamura et al., 2010).

Outro alvo importante no estresse oxidativo são as Sirtuínas (SIRTs), uma família de desacetilases que requerem NAD+ como cofactor para a reação de desacetilação. Por esse motivo pensa-se que essas desacetilases respondem a mudanças no ambiente, estresse oxidativo e metabolismo (Houtkooper et al., 2012). Existem sete sirtuínas em mamíferos, sendo a SIRT1 a mais estudada, principalmente, por sua regulação de diversos alvos e funções celulares, bem como seu potencial terapêutico. A SIRT1 tem sido cada vez mais reconhecida por seu papel no silenciamento de genes, resistência ao estresse, apoptose, senescência, senilidade e inflamação (Hwang et al., 2013).

Como consequência do estresse oxidativo e dos danos ao DNA, quando não há reparo, temos a apoptose, um mecanismo ativo de regulação gênica, desencadeado por sinalizações intercelulares que resultam na morte celular programada (Willingham, 1999). Entre as proteases responsáveis pelo processo apoptótico, através de clivagens proteolíticas, podemos destacar as Caspases e, entre elas, a Caspase-3. Quando ativa,

em sua forma clivada, o processo apoptótico é irreversível, de modo que a detecção de Caspase-3-clivada é um importante marcador de morte celular (Grutter, 2000).

1.5 Panax Ginseng

A raiz do *Panax ginseng* (*P. ginseng*) é um dos medicamentos tradicionais usados com mais frequência nos países asiáticos (Won et al., 2014). Estudos demonstraram que os extratos de *P. ginseng* têm muitas atividades biológicas, incluindo efeitos anti-inflamatórios (Yayeh et al., 2012), antidiabéticos (Xiong et al., 2010), antitumorais (Du et al., 2011), neuroprotetores (Zheng et al., 2011), cardioprotetores (Wang et al., 2010), e hepatoprotetores (Lee et al., 2005). Além disso, *P. ginseng* tem efeitos potentes sobre a função sexual, e pode combater a disfunção erétil (Choi et al., 1995), disfunção testicular senil (Hwang et al., 2010), e dano testicular induzido por dioxina (Hwang et al., 2004).

Saponinas do *P. ginseng*, comumente referidas como ginsenosídeos, são considerados os principais componentes ativos responsáveis pelas atividades farmacológicas do *P. ginseng*. As ações farmacológicas dos ginsenosídeos são o resultado da sua biotransformação no intestino humano (Hasegawa et al., 1997), sendo hidrolisados por bactérias intestinais antes da absorção. O metabólito-I intestinal do ginseng (GIM-I; [20-*O*-β-(D-glucopyranosyl)-20(S)-protopanaxadiol ou composto K) é o metabólito bacteriano intestinal final em seres humanos (Figura 8) (Kang et al., 2002), transformado a partir dos ginsenosídeos Rb1, Rb2 e Rc (Chen et al., 2008).

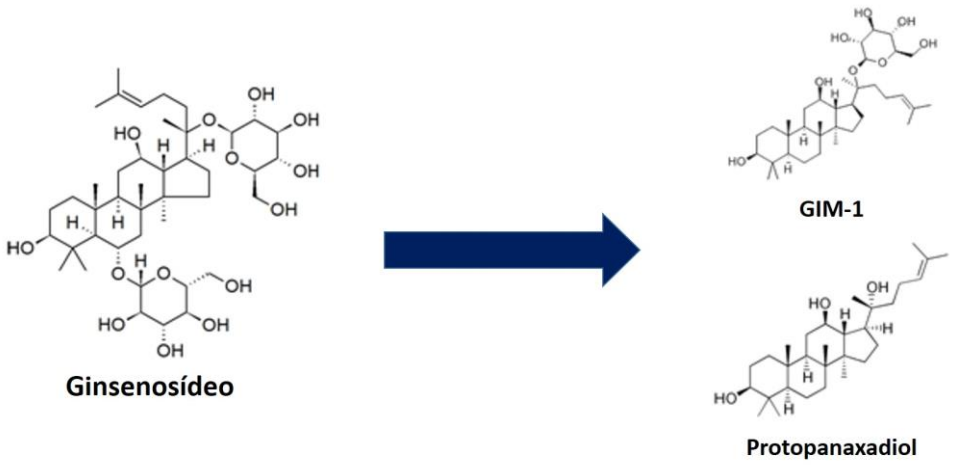


Figura 8. Metabolização e estrutura molecular do GIM-1.

O GIM-I exerce vários efeitos farmacológicos, tais como: antienvelhecimento, antiestresse, antimetastáticos (Sun et al., 1995; Hasegawa et al., 1997; Lee et al., 1998) e antioxidantes (Zang et al., 1996).

Potenciais antagonistas funcionais dos DEs, como o *Panax ginseng,* apresentam capacidade de inibição da via da MAPK. Por exemplo, Lui e colaboradores (2003) mostram que a ruptura da barreira, induzida por cádmio, pode ser parcialmente bloqueada e retardada com o uso de um inibidor de p38-MAPK.

1.6 Justificativa

Tendo em vista a grande importância das células de Sertoli para a reprodução masculina, estabelecer relações de causa e efeito entre desreguladores endócrinos amplamente dispersos no ambiente, como o MBP e seus precursores metabólicos, e essas células, são fundamentais para a compreensão dos possíveis riscos a que estamos expostos.

Trabalho anterior do nosso grupo (Freitas et al., 2016) mostrou que a exposição ao MBP leva à degradação de proteínas específicas estruturais e reguladoras da barreira hematotesticular, sendo necessária a compreensão dos mecanismos celulares envolvidos nesse processo. Nesse sentido, agentes citoprotetores, como o *Panax ginseng*, poderiam amenizar os efeitos nocivos do MBP, interferindo na ativação ou inibição de vias celulares específicas ou reduzindo o estresse oxidativo, melhorando as condições de sobrevivência celular.

1.7 Objetivos

Considerando a importância das vias de sinalização para as funções regulares da célula de Sertoli, esse trabalho objetiva avaliar se o MBP é capaz de ativar a via do GPR30 e mobilizar as proteínas das vias envolvidas na resposta a esse receptor e sua habilidade em alterar a resposta antioxidante em células de Sertoli humanas (HSec) expostas a uma dose de baixa toxicidade; e ainda se, nessas condições experimentais, o *Panax ginseng* atuaria como um citoprotetor modulando as vias ativadas pelo GPR30 ou aumentando a resposta antioxidante em um possível ambiente de estresse oxidativo.

1.7.1 Objetivos específicos

- Avaliar a morfologia e comportamento de adesão das células de Sertoli em sistema de cultivo sobre gel de membrana basal artificial após os diferentes sistemas de tratamento;

- Caracterizar a ação do Monobutil Ftalato sobre o receptor GPR30, bem como sobre as vias ativadas por ele e seus reflexos no comportamento celular;

- Avaliar os efeitos do GIM-1 sobre as células de Sertoli, avaliando seu potencial de proteção contra os possíveis efeitos nocivos do MBP, tanto sobre a via ativada pelo GPR30 como sobre o estado REDOX das células;

- Analisar os parâmetros de estresse oxidativo e correlacionar à ação das enzimas responsáveis pela defesa antioxidante frente aos diferentes estímulos de tratamento.

Capítulo 2

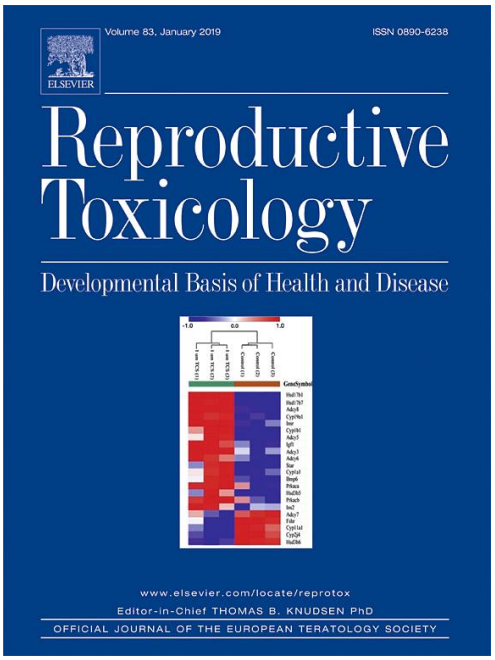
2.1 Panax ginseng methabolit (GIM-1) prevents oxidative stress and apoptosis in human Sertoli cells exposed to Monobutyl-phthalate (MBP)

Submetido para Reproductive Toxicology em 06/11/2018

Devolvido para revisão em 26/11/2018

Devolvido para a revista em 21/01/2019

Aceito para publicação em 22/02/2019



Manuscript Details

Manuscript number	RTX_2018_385_R1
Title	Panax ginseng methabolit (GIM-1) prevents oxidative stress and apoptosis in human Sertoli cells exposed to Monobutyl-phthalate (MBP)
Article type	Full Length Article

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Keywords	Sertoli cells; oxidative stress; monobutyl phthalate; Panax ginseng; GIM-1
Taxonomy	Reproductive System Development, Systems Biology, Experimental Models in Systems Biology
Manuscript region of origin	South America
Corresponding Author	Wellerson Scarano
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Panax ginseng methabolit (GIM-1) prevents oxidative stress and apoptosis in human Sertoli cells exposed to Monobutyl-phthalate (MBP)

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This study evaluated oxidative stress markers in Human Sertoli cells cultivated on Geltrex® and exposed to Monobutyl Phthalate (MBP), and the potential cytoprotective role of GIM-1 on the antioxidant response. Exposure was performed at 30min, 1, 12 and 48 hours into 4 groups: control, MBP (10µM), GIM-1 (0,05µM) and MBP+GIM-1. Morphology was evaluated. Antioxidant enzymes were analyzed by colorimetric method; NRF-2, SIRT-1, 8- OHdG and Cleaved Caspase-3 by Western Blot. Larger spaces between cells were shown in MBP treatment; GIM-1 was similar to Control and MBP+GIM-1 showed an intermediate aspect. MBP reduced enzymatic activity of all enzymes and NRF-2 expression, increasing cleaved Caspase-3 expression; while GIM-1 increased antioxidants markers alone and attenuated MPB effects in MBP+GIM-1. MBP induced deleterious effects on Sertoli cells, increasing the oxidative stress, apoptosis and modifying their distribution in culture; however, GIM-1 acted as an important cytoprotective agent reversing our attenuating MBP effects.

Key words: Sertoli cells, oxidative stress, monobutyl phthalate, Panax ginseng, GIM-1.

Highlights

- MBP increases apoptosis in HSec Sertoli cells.
- MBP induces oxidative stress by suppressing antioxidant enzymes.
- GIM-1 improves antioxidant mechanisms in HSec Sertoli cells.
- GIM-1 is an important antagonist for MBP effects.

1. Introduction

Male fertility and the spermatogenesis process are directly related to the capacity of Sertoli cells to produce determinant factors for germ cell development and sperm protection [1]. Joined by tight junctions and connected by gap junctions, these cells form a blood-testis barrier [2], which creates a protective status on the seminiferous tubules against immune response and reactive oxygen species (ROS) attack [3,4], leading to germ cell death and consequently male infertility [5].

Endocrine disruptors (EDs) are exogenous agents that interfere with the maintenance of homeostasis and the regulation of the developmental process [6]. The reproductive function is particularly susceptible to EDs, triggering morphological and functional changes [7,8]. Consistent detection of ED residues in human serum and seminal plasma has increased concern that its exposure to the environment is affecting human fertility [9], and a low dose may significantly affect it further [10,11, 12], being fundamental to the study and correlation of its effects on the male genital system.

Monobutyl phthalate (MBP) is the active metabolite of di-n-butyl phthalate (DBP), used in many everyday products, such as plastics, cosmetics, paints and clothes. Studies have found 400-700 mg/kg of phthalates in dust from household waste and about 111ng/ml in children's [13, 14]. Phthalates cause a wide range of adverse health problems, such as abnormalities in the genital system and sexual development [15]. Auharek et al. [16] showed a decrease in fetal testosterone and the number of Sertoli cells at birth after fetal exposure to DBP.

The effects of Phthalates lead to increased oxidative stress, acting directly on enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase, which modulate reactive oxygen species (ROS) [17, 18]. Evidence has shown a

relationship between increased oxidative stress and male infertility, with high ROS levels found in about 68% of infertile man [19, 20, 21]. Rasool et al. [22] showed that lipid peroxidation, a result of a reduction in SOD and Catalase, is one of the main causes of cellular damage, destroying germ and Sertoli cells. Similarly, Griveau et al. [23] showed that lipid peroxidation is important for spermatogenesis maintenance as well as for sperm viability and motility.

Other important ROS targets are the antioxidant system mediated by NRF-2 (nuclear factor erythroid 2-related factor 2), a primary molecular target [24], and the Sirtuins (SIRTs), is SIRT-1, and the most studied target for its role in gene silencing and resistance to stress, apoptosis and senescence [25]. Another molecular biomarker to evaluate endogenous oxidative DNA damage is 8-hydroxy-deoxyguanosine (8-OHdG), an ROS product to attack guanine, which is involved in mutagenicity [26].

The root of Panax ginseng (P. ginseng) is one of the traditional medicines most frequently used in Asian countries [27]. The intestinal metabolite-I of ginseng, GIM-I, is the final intestinal bacterial metabolite in humans [28], transformed from the ginsenosides Rb1, Rb2 and Rc [29]. GIM-I exerts several pharmacological effects, such as antiaging, anti-stress, anti-metastatic [30, 31] and antioxidants [32].

Overall, Sertoli cells are fundamental for spermatogenesis and the reproductive success in humans and animals. Our previous findings have showed that MBP was able to reduce the expression of important molecules for hematotesticular barrier integrity in HSeC cells [33]. Some reports have shown that phthalates were able to increase oxidative stress in different types of cells [17, 18], and it is known that hematotesticular barrier integrity can be affected by oxidative stress in Sertoli cells. Thus, we tested the hypothesis that MBP increases oxidative stress and cell death rates in human Sertoli

Cells (HSec), and in addition to this, that Panax ginseng can act as a cytoprotectant against MBP effects reestablishing the oxidative balance.

2. Material and methods

2.1 Sertoli Cells and Cell Culture

The HSec line of the Sertoli cells was purchased from Lonza (Walkersville-MD, USA), thawed and grown in DMEM/Han's F-12 medium (1:1) (LGC Biotechnology, SP, Brazil) as described by the manufacturer, at 37°C, 5% CO2 and 95% humidity. The culture medium was supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 500µL of antibiotic-antimycotic solution containing 100 units/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml fungizone (Gibco, Invitrogen-USA).

For cell expansion the artificial basement membrane (GeltrexTM, Gibco by Life Technologies, Grand Island, NY, USA) was previously placed in 25cm³ bottles, covering the entire bottom. The bottles were heated at 37°C for 1 hour for solidification and then $1x10^6$ cells were plated on that membrane with 5mL culture medium, changed every 4 days, or until they reached 80% confluency. When the growth limit was reached, the cells were resuspended with trypsin/0.25% 1x EDTA (Gibco) and divided into three 25cm flasks prepared with the same protocol.

2.2 MTT Assay

For the assessment of cytotoxicity of GIM-1, the MTT assay [34] was used. After reaching 75% confluency state, $1x10^6$ cells were exposed to 11 increasing concentrations of 0.05µM to 500µM GIM-1 (Ginseniside CK, 20mg, ALB Technology Limited, Henderson, NV, USA) at pre-established times of 12, 48 and 72 hours.

After treatment, medium was aspirated and cells were incubated with MTT solution (0.5 mg/ml culture medium) for 4 hours at 37°C. At the end of this period the reaction mixture was carefully aspirated and 200ul of Dimethyl Sulfoxide (DMSO), Sigma-Aldrich, was added to each well. After 10 minutes, the analysis was done on a spectrophotometer (Biotek, ELX 50) at 550nm. Each parameter was evaluated in triplicates. Positive and negative controls were conducted under identical conditions. Data are presented as percentage (%) of mitochondrial activity ± standard deviation of independent experiments on the same plaque.

In order to determine the dosage of Monobutyl Phthalate, the data of Freitas et al. [33] were used, which determined by the MTT test and literature analysis the non-cytotoxic dose of 10 μM MBP exposure for the Sertoli cells.

2.3 Cell treatment

Based on MTT test results and literature for the determination of 10 μM MBP and 0.05 μM doses of GIM-1, cells were treated for 30 minutes, 1 hour, 12 hours and 48 hours at respective doses in 4 groups: Control, MBP, GIM-1 and MBP+GIM-1. Exposure to each dose of the toxic agents was done in triplicates, using 0.05% DMSO as vehicle. Control group was exposed to 0.05% DMSO in culture medium and was evaluate in all treatment periods.

After the exposure period, the cells were resuspended with 0.25% trypsin/EDTA and centrifuged 2x in PBS. Cell pellets obtained were subsequently subjected to protein extraction.

2.4 Morphological evaluation and Stereology

Cell culture for morphological evaluation assays was performed in 6-well culture plates on coverslips previously acidified with HCl for greater cell adhesion and sterilized in UV light for 2 hours, until the cells had cell junctions when observed under the optical microscope, when they received the respective treatments in triplicates [33].

To evaluate the morphology, cells were stained by hematoxylin and eosin. The cells were fixed in cold absolute methanol for 10 minutes [33, 35], passed rapidly in 95%, 80%, 70% ethanol, washed in water and exposed to hematoxylin for 15 seconds. The cells were then washed in water, exposed to eosin for 40 seconds and rinsed again. Then, exposed for 1 minute in 70%, 80%, 90% ethanol, 3 times in 100% ethanol, ethanol + xylene and 2 times in xylene. One drop of Permount was used to fix the cover slip and the analyzes were performed using an image analyzer system (Axio Vision 4.8) coupled to the AxioLab.A1, Zeiss photomicroscope.

To evaluate cell morphology and dispersion, 10 microscopic fields (x400) from each triplicate (30 fields/treatment) were digitalized. The proportion of intercellular spaces were measured according to the Weibel grating points system, which consists of a test system of lines and dots in a grid containing 168 points [36]. Results were expressed in percentage (intercellular spaces proportion) as the median ± interquartile range.

2.5 Western Blotting

Protein extraction and Wester Blotting protocols were performed according to Freitas et al. [33]. Nitrocellulose membranes were subsequently incubated with the primary antibodies as specified in Table 1, for NRF-2 (Abcan Inc., Cambridge, MA, USA), SIRT-1 and β-actin (Santa Cruz Biotechnology Inc., Dallas, TX, USA), 8-

hydroxydeoxyguanosine (8-OHdG) (Millipore, Temecula, CA, USA) and Cleaved Caspase-3 (Cell Signaling,Danver, MA, USA); in TBST overnight. After washing, the membranes were incubated in specific secondary antibody diluted in 3% milk for 1.5 hours. The immunoreactive components were revealed by the GE® luminescent kit (luminol).

Antibody	Clone	Concentration	Dilution
Anti-SIRT-1	sc15404	1µg/mL	1:200
Anti-β-Actina	sc47778	1µg/mL	1:200
Anti-NRF-2	Ab62352	3,63 µg/mL	1:200
Anti-8-OHdG	Ab5830	N/A	1:1000
Anti-Cleaved Caspase-3	9661	0,57µg/mL	1:350

Table 1. Primary antibodies, specification, concentration and dilution.

2.6 Evaluation of Antioxidant Enzymes

2.6.1 Determination of the activity of glutathione peroxidase (GSH-Px)

The activity of glutathione peroxidase was determined according to the method of Nakamura et al. [37] in the presence of hydrogen peroxide. The reaction mixture was prepared with sodium phosphate buffer (50 mM, pH 7.0), NADPH2, sodium azide, EDTA, reduced glutathione (GSH) and glutathione reductase. Through the oxidation of NADPH2 to 340nm in the presence of glutathione reductase, which catalyzes the reduction of oxidized glutathione (GSSG), GSH-Px activity was determined.

2.6.2 Determination of superoxide dismutase activity (SOD)

The activity of superoxide dismutase was determined by the technique of Ewing & Janero [38], in sodium phosphate buffer (50 mM pH 7.4), based on the ability of the

enzyme to inhibit the reduction of nitroblue-tetrazolic (NBT) by radicals generated by electrons yielded by NADH2, in the presence of phenazine metasulfate. When the sample is added, the rate of NBT reduction is inhibited, depending on the SOD activity present in the sample.

2.6.3 Determination of catalase activity

Catalase activity was determined in 50 mM potassium sodium phosphate buffer, pH 7.0, using 0.5 mL of sample and hydrogen peroxide (30%). The readings were performed at 240 nm [39].

2.7. Statistical Analysis

The comparison between the experimental groups was performed using the ANOVA test, followed by the non-parametric Mann-Whitney test for each time, being considered differences where $p \leq 0.05$.

3. Results

3.1. MTT Assay

For the exposure of cells to different doses of GIM-1, MTT showed that none of the doses used had a cytotoxic effect on the HSec line in any of the observed periods (Figure 1). The 0.05µM GIM-1 dose presented a slight increase in viability when compared to the control group in all three periods, therefore this dose was chosen to treat cells.

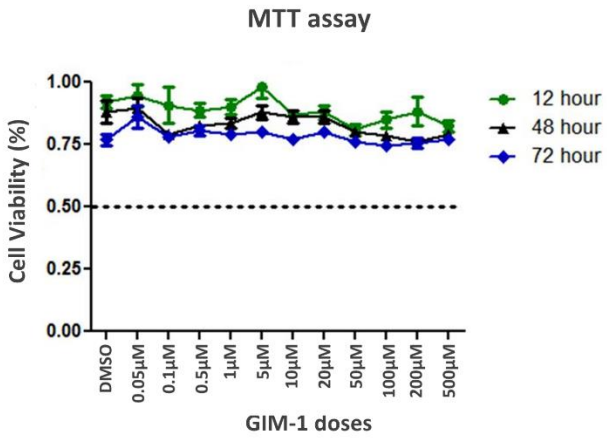


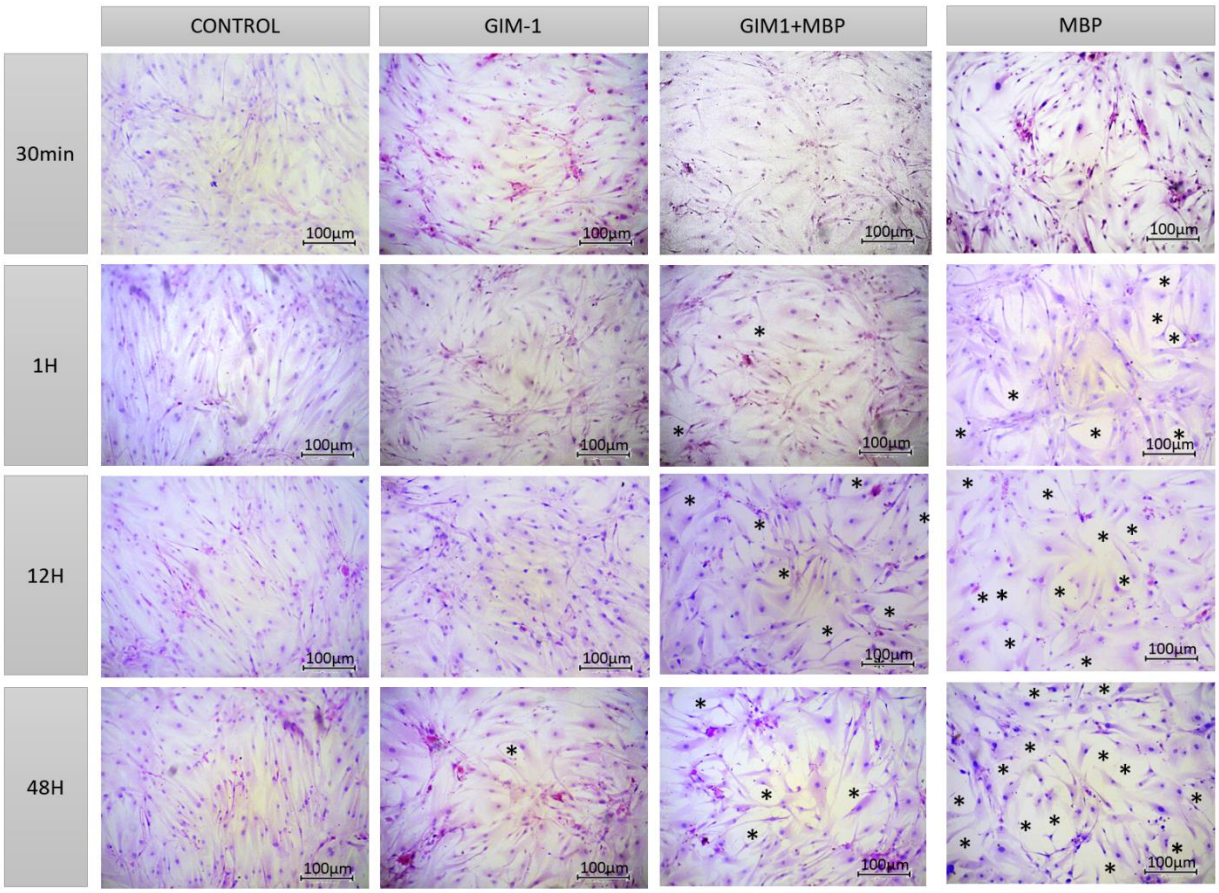
Figure 1. MTT assay for GIM-1. HSec Cells were exposed to eleven GIM-1 doses, concentration-dependent toxicity is shown by the dose effect curve. Graph represents relative cell viability after 12, 48 and 72 hours of exposure, expressed as mean ± SEM of seven plate's measurements. Results are representative of two independent experiments.

3.2. Morphological and stereological evaluation

After an optical microscopy analysis and stereology, it was observed that exposure to GIM-1 did not alter HSec lineage morphology or behavior, whereas MBP led to a loss of density and cell distribution, with an increase in intercellular spaces after an exposure time of 1 hour (Figures 2a and b).

MBP+GIM-1 exposure showed a reduction in deleterious effects when compared to the MBP exposure group but also showed that normal characteristics are lost compared to control (Figures 2a and b).

a.


CONTROL
GIM-1
GIM1+MBP
MBP
30min
1H
12H
48H
100µm

b.

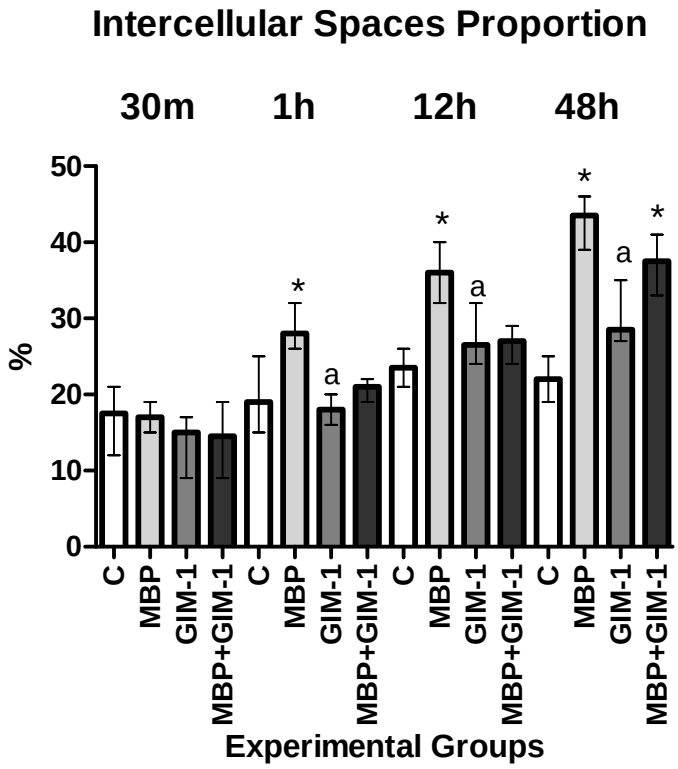

Intercellular Spaces Proportion
30m
1h
12h
48h
%
50
40
30
20
10
0
C
MBP
GIM-1
MBP+GIM-1
*
a
Experimental Groups

Figure 2. a: HSec Sertoli Cells stained by Hematoxylin and Eosin (Optical microscope) from different treatments. GIM-1 exposure doens´t affect HSec cells morphology or behavior, while MBP exposure lead to loss of cell density. GIM-1+MBP exposure shows less damage then in MBP group. Asterisks represent significant loss of cell density. Magnification: x200. **b:** Proportion of intercellular spaces (%) measured by stereological analysis in HSeC Sertoli cells stained by H&E. The asterisk represents statistical difference in relation to Control at different times of exposure; the letter "a" represents statistical difference in relation to MBP exposure at different times of exposure. Data were expressed by median ± interquartile range ($p<0.05$).

3.3. Western Blotting

3.3.1. Protein Expression Overview

NRF-2 expression decreased after being exposed to MBP for 30 minutes and increased after being exposed to GIM-1 for the same period relating to the control (Figure 3; Table 2). SIRT1 expression was not modified after MPB and MBP+GIM-1 exposure, but increased after 30 min and 1 hour of being exposed to GIM-1 compared to the control (Fig. 3; Table 2). 8-OHdG expression reduced after 1 hour of being exposed to MBP, after 30 min, 1 and 12 hours of GIM-1 exposure and after 1, 12 and 48 hours of being exposed to MBP+GIM-1 (figure 3; Table 2). In relation to cleaved caspase 3, there was an increase after 30 min, 12 and 48 hours of being exposed to MBP and after 30 min of being exposed to MBP+GIM-1 compared to control (figure 3; Table 2).

Comparing the treated groups among them, NRF2 expression decreased after 30 min and 1 hour of being exposed to MBP+GIM-1 compared to GIM-1 (Figure 3). There was an increase of SIRT1 expression in the MBP+GIM-1 group in relation to MBP (30 min) and a decrease in the MBP+GIM-1 group related to GIM-1 (1 hour) (Figure 3). After 48 hours of being exposed to MBP+GIM-1, there was a decrease in the 8OHdG

expression in relation to MBP and GIM-1 exposure (Figure 3). Finally, cleaved caspase 3 decreased in MBP+GIM-1 exposure relating to the MBP group (Figure 3).

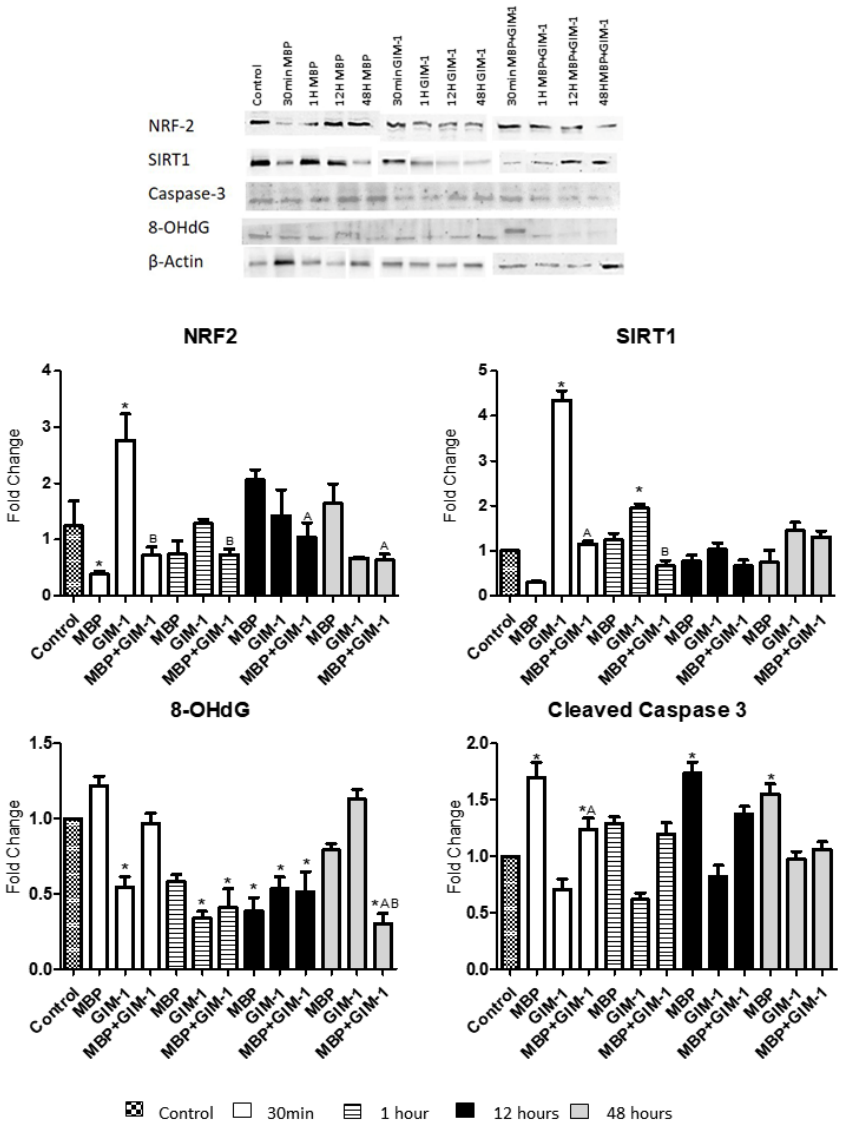


Figure 3: Western blot for proteins NRF-2, SIRT-1, 8-OHdG, Cleaved Caspase-3, and β-actin in the experimental groups: MBP, GIM-1 and MBP+GIM-1 for 30 minutes, 1, 12 and 48 hours; each band representing a pool with samples of three cell cultures from the protein extracts of cell cultures. Fold change charts comparing experimental groups to control and among themselves. Each bar represents the average followed by the Standard Error of three different bands referring to the experimental triplicates. Statistical difference $p \leq 0.05$ is represented in relation to the groups (*) Control; (A) MBP; (B) GIM-1. Controls did not differ from each other, they are represented as a single bar on the left.

3.5. Evaluation of Antioxidant Enzymes

An evaluation of Catalase revealed that MBP was able to reduce its activity significantly after 48 hours of exposure, while GIM-1 increases its activity after 1 hour. MBP+GIM-1 did not alter enzymatic activity compared to the control group (Figure 4 and Table 2).

The activity of Superoxide Dismutase was also reduced by MBP after being exposed for 30 min and 48 hours and was not altered by GIM-1. MBP+GIM-1 exposure resulted in a reduction after being exposed for 30min and 1 hour (Figure 4 and Table 2).

Peroxidase activity was significantly reduced only after being exposed to MBP for 48 hours, and no alterations were observed among groups or in other exposure times (Figure 4 and Table 2).

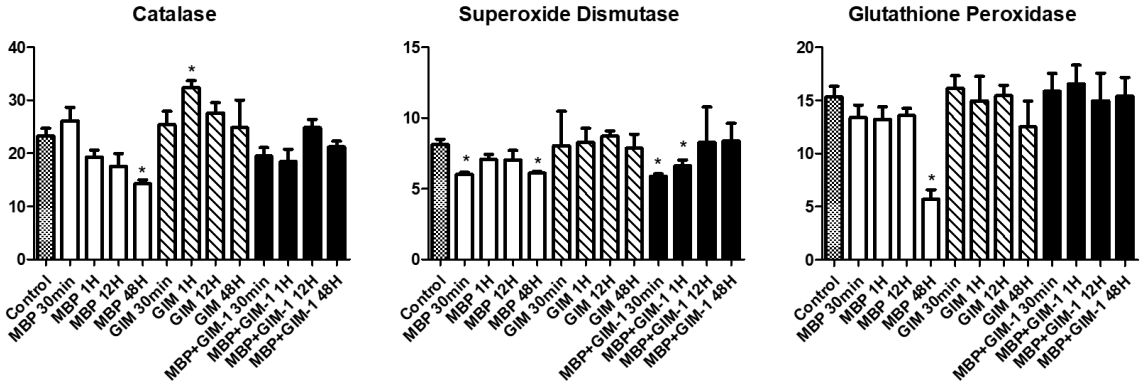


Figure 4. Relative activity of the enzymes Catalase, Superoxide Dismutase and Glutathione Peroxidase after exposure to MBP, GIM-1 and MBP + GIM-1 for 30 min, 1, 12 and 48 hours. Each bar represents the average followed by the Standard Error for the experimental triplicates. Statistical difference: * $p \leq 0.05$. Controls did not differ from each other, they are represented as a single bar on the left.

		SIRT-1	NRF-2	Casp3	8-OHdG	SOD	GPx	Cat
MBP	30min	=	-	+	=	-	=	=
	1H	=	=	=	-	=	=	=
	12H	=	=	+	=	=	=	=
	48H	=	=	+	=	-	-	-
GIM-1	30min	+	+	=	-	=	=	=
	1H	+	=	=	-	=	=	+
	12H	=	=	=	-	=	=	=
	48H	=	=	=	=	=	=	=
MBP + GIM-1	30min	=	=	+	=	-	=	=
	1H	=	=	=	-	-	=	=
	12H	=	=	=	-	=	=	=
	48H	=	=	=	-	=	=	=

Table 2. Summary of results from protein expression and enzyme activity of experimental groups in relation to the control in all the times of exposure. Simbols meaning: (+) increase; (-) decrease and (=) no difference in relation to the control.

4. Discussion

Sertoli cells *in vitro* culture have been established, with native cell characteristics such as morphology, cell junctions and functional aspects well reported [40, 41]. The 3D culture system, as used in our experiment, also improves the knowledge about Sertoli cell biology of and its junctions [42], mimicking condition observed *in vivo*.

Previous reports demonstrated that Monobutyl Phthalate at 10µM of ,as used in this study, has been described as environmentally relevant, with a direct correlation to human exposure [14, 18, 33, 41, 43, 44]. Hu et al. [45] also determined that an EC50 of

MBP in Sertoli cells from rats is 16.21mM and that the highest dose without adverse effects (NOAEL) is 1mM [46].

In order to estimate the lowest non-cytotoxic dose of GIM-1 with potential protective effect on HSeC cells, MTT results showed that 0.05Mm of GIM-1 did not differ from higher doses tested. Thus, we selected a lower dose that, theoretically, could be easily applied for human consumption.

Morphology and cell distribution in the monolayer were similar between GIM-1 and control groups, showing that GIM-1 preserves the characteristics of the HSeC line. On the other hand, the cells exposed to MBP showed an increase in intercellular spaces after being exposed for 30 min. MBP+GIM-1 exposure showed that GIM-1 was able to attenuate MBP effects on cell death, decreasing the intercellular spaces in the monolayer. In agreement with these findings, previous study showed that MBP exposure was able to reduce adhesion molecule expression and the number of HSeC cells in the monolayer [33]. Considering that Sertoli cells take place in spermatogenesis control, the reduction in the stable number of these cells against an insult can impair considerably the spermatogenesis [47,48], highlighting the potential protective role of GIM-1 in our experiment.

In order to evaluate if the morphological changes observed in this experiment has association with oxidative stress and apoptosis in the HSec line, we used NRF-2 and SIRT-1 proteins, with nuclear action, cleaved caspase-3, an apoptosis marker, 8-OHdG, a DNA damage marker, and enzymes with known antioxidant activity: catalase, superoxide dismutase and peroxidase [17].

MBP exposure was able to reduce NRF-2 expression after being exposed for 30 min; and no alterations in expression were observed for NRF-2 for other exposure

periods, and for SIRT-1 expression. Additionally, MBP led to a reduction in the activity of three antioxidant enzymes after being exposed for 48 hours, besides a rapid reduction of Superoxide Dismutase after 30 minutes.

GIM-1 exposure led to increased expression of NRF-2 and SIRT-1 after being exposed for 30 minutes, maintaining this increase in SIRT-1 expression after being exposed for 1 hour. Additionally, there was an increase in catalase activity after being exposed for only 1 hour, while there was no change in the activity of the other enzymes at any time of exposure to GIM-1.

In the other hand, combined treatment (MBP+GIM-1) did not lead to any change in SIRT-1 or NRF-2 expression over the observed periods, showing that the compounds potentially cancel out their effects. Furthermore, MBP+GIM-1 exposure did not show change in Catalase or Peroxidase activity. Superoxide Dismutase activity was reduced up to 1 hour of exposure, but its activity recovered to normal levels by combined treatment after being exposed for 12 and 48 hours, demonstrating that GIM-1 was able to maintain an oxidative balance in the long term.

As observed in figure 7A, NRF-2 and SIRT-1 exert important role in oxidative stress prevention [24, 25]. In this way, Li et al. [43] observed an initial increase in nuclear NRF-2 after two days of heat stress in rats, followed by a reduction after 12 days. Since NRF-2 acts as an important factor in antioxidant enzymes activation, the behavior of NRF-2 expression, can represent an initial response of HSeC to the insult followed by an adaptation to this condition.

Superoxide Dismutase, Catalase and Glutathione Peroxidase convert superoxide radicals into water and oxygen, eliminating the toxicity of the compounds in the cell, as seen in figure 7 B [22, 23]. Our results showed that MBP led to a reduction in the

activity of three antioxidant enzymes after being exposed for 48 hours, besides a rapid reduction of Superoxide Dismutase after 30 minutes. Huang et al. [49], in an *in vivo* and *in vitro* study with nonylphenol, an endocrine disruptor, observed the same reduction effects on SOD and Glutathione Peroxidase expression in isolated Sertoli cells and epididymal sperm from Sprague-Dawley rats.

Bak et al. [50] showed that ginseng increased the expression of NRF-2 in a dose-dependent manner, which facilitates the activation of the antioxidant response [51]. Our results also showed that GIM-1 was able to increase NRF-2 expression, and MBP+GIM-1 exposure did not alter NRF-2 expression, which leads us to hypothesize that GIM-1 was able to neutralize the influence of MBP on NRF-2 protein expression, and consequently, the antioxidant enzymatic response. In this sense, He et al. [52] showed that ginseng was able to inhibit the reduction of SOD and Glutathione Peroxidase activity induced by irradiation. Also corroborating our data, Wang et al. [53] not only reported an increase in Catalase, but also SOD and Peroxidase, with a significant decrease in lipid peroxidation after administration of ginsenosides in Sertoli cells. Although several lines of evidence indicate that SIRT-1 is a possible candidate for redox modulation [25, 54], in this study, we did not observe reduction in its expression after exposure to MBP. According to Furukawa et al. [55], SIRT-1 reduction precedes its transcriptional alteration, suggesting a post-translational regulation of SIRT-1 by oxidative stress. In this sense, probably in our model, exposure periods to MBP may not have been sufficient to modulate SIRT-1 expression. On the other hand, studies showed that ginsenosides were able to increase SIRT-1 expression in rats' livers [56] and also described the action of 14 different ginseng compounds, with antiproliferative and SIRT-1 promoter activity in liver cells [57]. Luo et al. [58] showed that prolonged consumption of ginseng reduced cardiac injury in rats mediated by an

increased expression of SIRT-1. Thus, our results agree with those previously described regarding the increase of SIRT-1 after exposure to GIM-1. However, it seems that the stimulus only holds in the short term with the absence of oxidative stress. In one sense, MBP appears to interfere with the activation of SIRT-1 by GIM-1, when cells are exposed to a combined treatment.

MBP exposure increased Cleaved Caspase-3 after being exposed for 30 minutes, 12 and 48 hours, showing an MBP role in improving apoptosis and leading to loss of cell density as shown here in the morphology evaluation. GIM-1 exposure did not change cleaved Caspase-3 expression after being exposed for 30 minutes, 1 and 12 hours. In its turn, MBP+GIM1 exposure, after 30 minutes it was possible to observe an increase in Cleaved Caspase-3 expression as found in the MBP group, however, there was no difference in caspase 3 expression at other exposure times.

Pourhassanali et al. [59] found increased cleaved caspase-3 mRNA expression levels in TM4 Sertoli cells after ethanol exposure, decreasing cell survival, evidencing Sertoli cells sensitivity in responding to some toxicological insults. Recent studies have reported a connection between SIRT-1 upregulation and cleaved caspase-3 suppression (as presented in figure 7A) in rat Sertoli cells [60] and described ginsenosides apoptosis inhibition by cleaved caspase-3 suppression [61]. GIM-1 increased SIRT-1 expression in our study and, although cleaved caspase-3 expression was maintained in relation to the control group, it was significantly decreased in comparison to the MBP group, pointing again for a protective role of GIM-1 in cell survival.

8-OHdG is one of the major products of DNA oxidation [62]. Curiously, all treatments led to a reduction in the expression of 8-OHdG at different levels and times of exposure. Although MBP exposure is expected to lead to DNA damage, this event is

associated with the loss of repair system efficiency, which is commonly linked with the aging process [63] or to high toxicity exposure, as observed by Fenga et al. [26] after benzene exposure. In our experiment, MBP was probably not able to damage DNA since MBP levels in our in vitro assay were not cytotoxic and because the HSeC cells were relatively young, which increased their efficiency for repair. Additionally, and agreeing with our results, Li et al. [64] and Huang et al. [46] found that ginsenosides reduced 8-OHdG in oxidative stress models, indicating its protective effect on lipid peroxidation and DNA oxidative damage.

In conclusion, our data indicates that human Sertoli cells (HSeC line) were sensitive to an environmental relevant dose of MBP by increasing oxidative stress markers and cell death; on the other hand, GIM-1 played an important protective role, inciting the antioxidative response, maintaining an oxidative balance in long term and reducing apoptosis caused by MBP exposure.

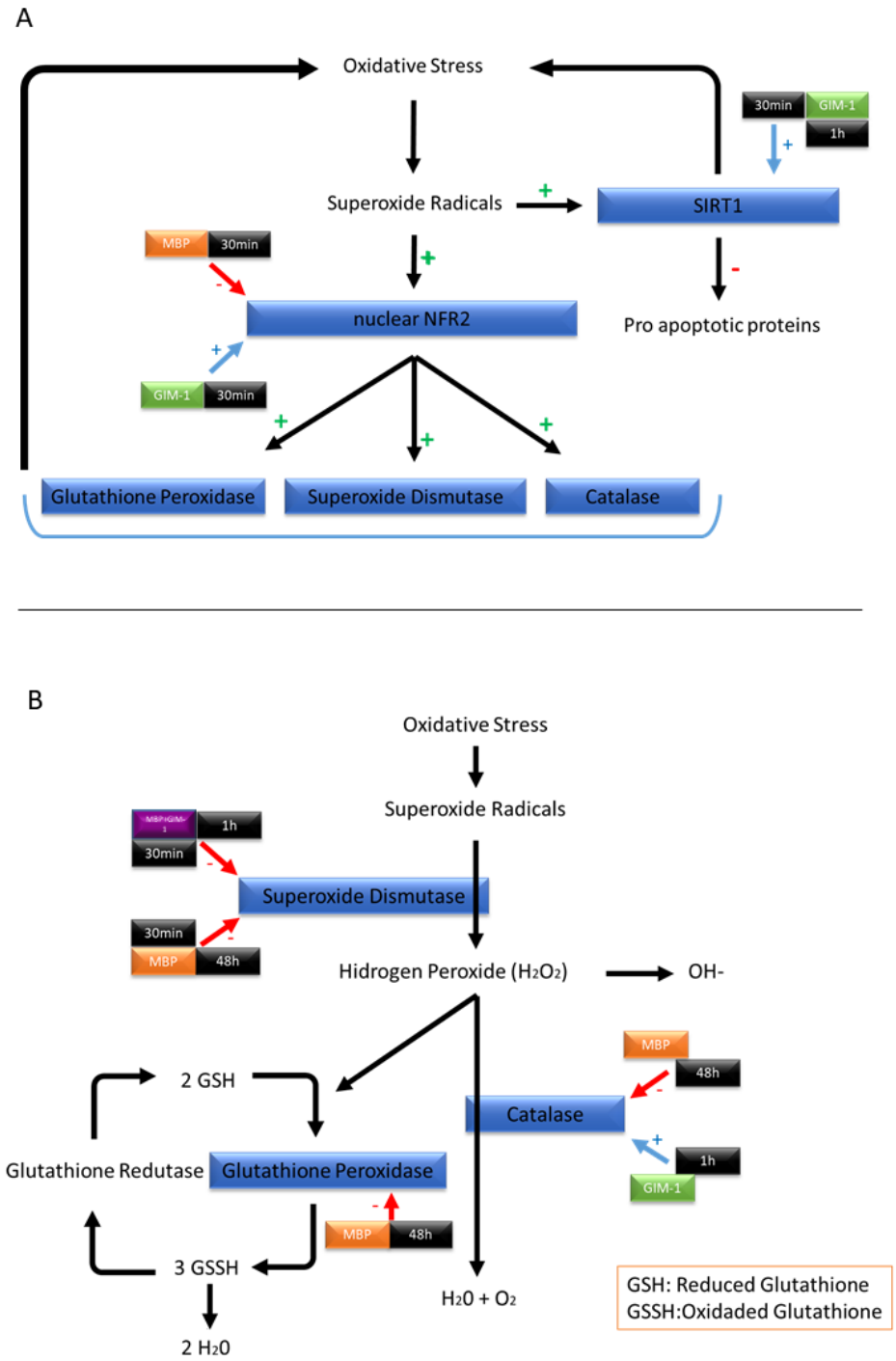


Figure 7: Representative scheme of the effect of oxidative stress on Sertoli cells and effect of MBP, GIM-1 and MBP + GIM-1 treatments under the HSec lineage. (A) The presence of superoxide radicals activates cellular defense pathways, increasing the expression of nuclear NRF-2 and SIRT-1. NRF--2 protein stimulates the activity of antioxidant enzymes, such as Glutathione Peroxidase, Superoxide Dismutase and Catalase, while SIRT-1 plays an important role in inhibiting proapoptotic proteins. (B) Antioxidant enzymes convert reactive oxygen species, producing water and oxygen and combating oxidative stress. The red and blue arrows represent inhibition and stimulation, respectively, by the treatments applied.

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001", by the São Paulo State Research Foundation (FAPESP; Process: 2012/00253-3) and by the National Council for Scientific and Technological Development (CNPq, Process: 306900/2016-5).

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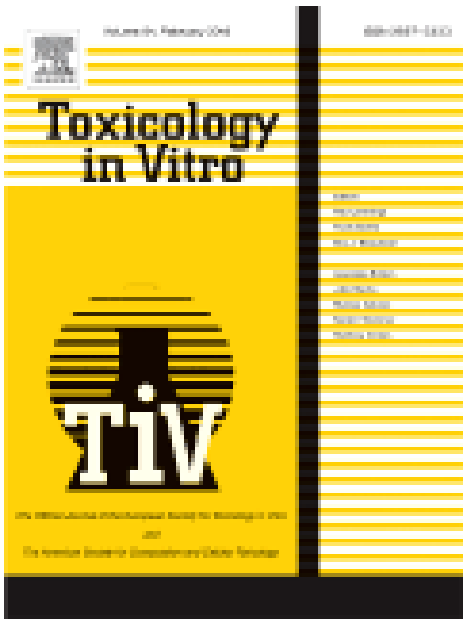
Capítulo 3

3.1 Monobutyl-phthalate (MBP) action by GPR30/GPER1 receptor in human Sertoli cells can be modulated for P. ginseng metabolite (GIM-1)

Será submetido para Toxicology in Vitro em fevereiro/2019



Monobutyl-phthalate (MBP) action by GPR30/GPER1 receptors in human Sertoli cells can be modulated for P. ginseng metabolite (GIM-1)

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Abbreviations: AR: Androgen Receptor; BPA: Bisphenol A; DMEM: Dulbecco's Modified Eagle Medium; EDs: endocrine disruptors; EGFR: epidermal growth factor receptor; ER: Estorgen Receptor; GIM-1: Ginseng intestinal metabolite-I; GPR30: G-protein coupled receptor 30; HSec: Human Sertoli cells line; MBP: Monobutyl Phthalate.

Monobutyl-phthalate (MBP) action by GPR30/GPER1 receptors in human Sertoli cells can be modulated for P. ginseng metabolite (GIM-1)

Blood-testis barrier and spermatogenic process control using receptors such as GPR30 are fundamental for male fertility. Endocrine Disruptors (EDs) have a high affinity for GPR30, possibly causing barrier rupture. Functional antagonists of EDs, such as Panax ginseng, may protect against these effects. Given the importance of these pathways and the constant environmental exposure to EDs, this study aims to look at the possible modulation of the non-genomic pathway activated by GPR30 in Sertoli cells exposed to low doses of Monobutyl Phthalate (MBP) and the possible cytoprotective role of GIM-1 (P. ginseng metabolite) on these pathways. For this purpose, HSec human lineage cells were maintained on an artificial matrix, and exposed to MBP and GIM-1 for 30 min, 1, 12 and 48 hours in 4 groups: control, MBP, GIM-1 and MBP+GIM-1. Quantification of proteins using Western Blot showed that MBP activates GPR30 receptor, PKA, Src, EGFR and the ERK1/2 pathway, while GIM-1 inhibited PKA, Src, ERK1/2 and AKT pathways. MBP also enhances Cofilin expression, decreasing F-actin intensity on the cell surface. The combined exposure demonstrated the antagonism between compounds. Our results show the effects of MBP on the HSec line, through GPR30 pathway, demonstrating the important cytoprotective role of GIM-1.

Key words: Sertoli cells, blood-testis barrier, endocrine disruptors, monobutyl phthalate, Panax ginseng, GIM-1.

Highlights

- MBP activates GPR30 pathway in HSec Sertoli Cells.
- GIM-1 acts as a cytoprotective compound for HSec Sertoli Cell.
- MBP enhances Cofilin expression and affect F-actin on cell surface.
- MPB+GIM-1 treatment was not able to avoid MBP deleterious effects, but they were attenuated by GIM-1.

1. Introduction

Sperm formation and the consequent success of male fertility are directly related to the functions of nutrition and the protection of germ lineage by Sertoli cells (Griswold, 1998). The fundamental processes for spermatogenesis and the formation and maintenance of the blood-testis barrier (BTB) are regulated by cellular receptors, such as the Androgen Receptor (AR) and Estrogen Receptors (ER). In addition to the classical ERα and ERβ receptors, recent studies have identified a third estrogen receptor: GPR30 (a G-protein coupled receptor, a transmembrane receptor, also called GPER1). In addition to structural differences, this protein performs different functions, mediating rapid non-genomic signaling events - with effects in a matter of seconds or minutes (Prossnitz et al., 2008).

Binding to GPR30 rapidly activates different signaling pathways (Figure 1), including EGFR (epidermal growth factor receptor) transactivation, through metalloproteinase-2 (MMP2), leading to rapid phosphorylation of ERK1/2 MAPKs and the activation of the PI3K/AKT pathway, responsible for cell proliferation and survival (Maggiolini & Picard, 2009) and participating in the control of Sertoli cells functions (Yang et al., 2017).

Inhibitors and activators of protein kinases and phosphatases, such as cofilin and Src, can manipulate the blood-testis barrier, acting at cellular junctions through their ability to maintain and/or alter the phosphorylation state of their proteins (Mruk & Cheng, 2010).

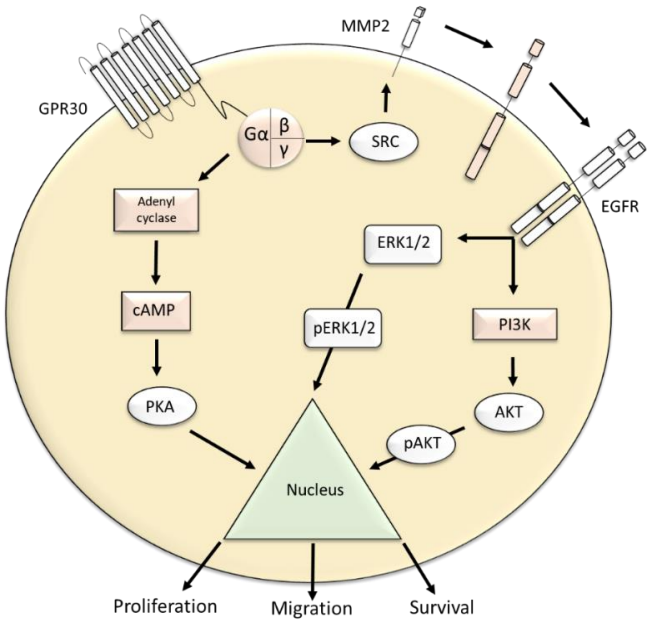

Figure 1. GPR30 pathway.

Potential activators of the GPR30 receptor are the Endocrine Disruptors (EDs), compounds that mimic the hormonal action in our bodies, with affinity for this receptor. In recent years, detection of significant levels of EDs in urine, blood, seminal plasma and house dust samples has been drawing attention to these compounds (Younglai et al., 2002; Becker et al., 2004; Sikka & Wang, 2008, Diamanti-Kandarakis et al., 2009; Frederiksen et al., 2011; Hu et al., 2013; Hu et al., 2014).

Among them, we highlight Monobutyl Phthalate (MBP), a metabolite that is present in daily products and capable of, at low doses, causing structural and functional abnormalities in the reproductive and endocrine system (Sheehan et al., 1999; Welshons et al., 2003; vom Saal & Hughes, 2005; vom Saal et al., 2007). Our previous results showed that MBP was able to decrease BTB adhesion protein expression in human Sertoli cells in culture (Freitas et al., 2016).

As a potential MBP functional antagonist, studies indicate the intestinal metabolite-I of Panax ginseng (GIM-I; [20-O-β-(D-glucopyranosyl)-20(S)-protopanaxadiol or compound K)], the final metabolite in the human organism, with the ability to inhibit the MAPK pathway (Kang et al., 2002; Lui et al., 2003).

Based on the environmental relevance of MBP as an important endocrine disruptor with a negative repercussion for male reproductive development and function, ally to our previous results that showed that MBP destabilize BTB adhesion molecules and increase oxidative stress in Sertoli cells, this study was performed to understand if the GPR30 receptor would be activated by MBP and which pathways could be modulated by it. In this context, and since our previous data showed that GIM-1 was able to attenuate oxidative stress in Sertoli cells caused by MBP (Freitas et al., 2019), GIM-1 was tested as a possible functional antagonist of MBP.

2. Material e methods

2.1 Sertoli Cells culture

The HSeC line of the Sertoli cells was purchased from Lonza (Walkersville-MD, USA), thawed and grown in DEMEM/Han's F-12 medium (1:1) (LGC Biotechnology, SP, Brazil) as described by the manufacturer, at 37°C, 5% CO2 and 95% humidity. The culture medium was supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 500µL of antibiotic-antimycotic solution containing 100units/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml fungizone (Gibco, Invitrogen-USA).

For cell expansion the artificial basement membrane (GeltrexTM, Gibco by Life Technologies, Grand Island, NY, USA) was previously placed in 25cm³ bottles, covering the entire bottom. The whole cell culture protocol was made according to Freitas et al., 2016.

2.2 Cell treatment

For the treatments, HSec cells were divided into 4 groups (Control, MBP, GIM-1 and MBP+GIM-1) and treated with doses pre-established in the literature (Freitas et al. 2016; Freitas et al., 2019), using as a vehicle, for both MBP (10μM) and GIM-1 (0.05μM) 0.05% DMSO. Control group received only the vehicle at the same dose. Each group was observed in 4 different periods: 30min, 1 hour, 12 hours and 48 hours, always in triplicates and with a specific control.

After the exposure period, cells were resuspended with 0.25% trypsin/EDTA and centrifuged 2x in PBS. Cell pellets obtained were subsequently subjected to protein extraction.

2.3 Western Blotting

Protein extraction and the Western Blotting protocol were performed according to Freitas et al., 2016. Nonspecific protein binding was blocked by incubating the membranes in 5% skim milk in Tris-NaCl buffer containing 0.2% Tween 20 (TBST) for 1 hour at room temperature. The membranes were subsequently incubated with the primary antibodies specific for GPR30, ERK1/2, pERK1/2, AKT, pAKT, Cofillin, pCofilin, EGFR (Abcan Inc., Cambridge, MA, USA), PKA, Src, pSrc (Cell Signaling, Danvers, MA, USA), MMP2 and β-actin (Santa Cruz Biotechnology Inc., Dallas, TX, USA); in TBST overnight. After washing, membranes were incubated in specific secondary antibody diluted 3% skim milk for 1.5 hours. The immunoreactive components were revealed by the GE® luminescent kit (luminol).

Antibody	Clone	Dilution	Concentration	Antibody	Clone	Dilution	Concentration
Anti-GPR30	Ab39742	1:250	4µg/mL	**Anti-Cofilin**	Ab42824	1:1000	1µg/mL
Anti-ERK1/2	Ab17942	1:500	1µg/mL	**Anti-pCofilin**	Ab12866	1:1000	N/A
Anti-pERK1/2	Ab4819	1:300	0,8µg/mL	**Anti-EGFR**	Ab2430	1:1000	0,2µg/mL
Anti-AKT	Ab18206	1:500	1,4µg/mL	**Anti-Src**	#2149	1:1000	N/A
Anti-pAKT	Ab38449	1:500	2µg/mL	**Anti-pSrc**	#2101	1:800	N/A
Anti-MMP2	Sc6838	1:800	2,5µg/mL	**Anti-β-Actin**	sc47778	1:200	1µg/mL
Anti-PKA	#4782	1:1000	N/A				

Table 1. Primary antibodies specification, clones, dilution and concentration.

2.4 Immunofluorescence

For F-actin filaments label, Phalloidin Tetramethyl rhodamine B isothiocyanate (TRITC) (Sigma-Aldrich, St Louis, MO, USA), was used. As described by Freitas et al. (2016), stock solution was made in methanol, at 500ug/ml, following producer guideline procedures. After cell culture and treatment, cells were washed with phosphate buffered saline (PBS) and fixed in 3.7% formaldehyde solution in PBS. 0.1% TRITON® X-100, diluted in PBS, was used for cell permeabilization. Staining with 500ug/ml fluorescent phalloidin conjugate solution in PBS was performed for 40 minutes and washed with PBS. DAPI was used for nuclear labeling. Only cells from MBP group in 1- and 12-hours treatments were used in order to evaluate the relation between cofilin expression and F-actin intensity. Confocal images were taken in a Leica TCS SP5 microscope.

3. Statistical analysis

The comparison between the experimental groups was performed using the ANOVA test, followed by the non-parametric Mann-Whitney test for each time, being considered differences where $p \leq 0.05$.

4. Results

4.1 Monobutyl Phthalate exposure

Exposure to a 10μM dose of MBP (Figure 2) showed rapid activation of the GPR30 receptor, with a significant increase after 30min of exposure and consequent reduction in expression after 12 and 48 hours. PKA expression also increased rapidly after 30 min of exposure, with no change in the other periods. Expression of Src, in its total form, increased after 30 minutes and was then reduced over periods of 1 and 12 hours, while its phosphorylated form was reduced after being exposed for 30 minutes, 1, 12 and 48 hours. MMP2 expression did not change in any period. The EGFR receptor had increased expression after being exposed for 30 minutes. Expression of ERK1/2 had a significant increase after being exposed for 12 hours, a result also presented for its phosphorylated form. The AKT expression was not altered in any period, both in its common and phosphorylated form.

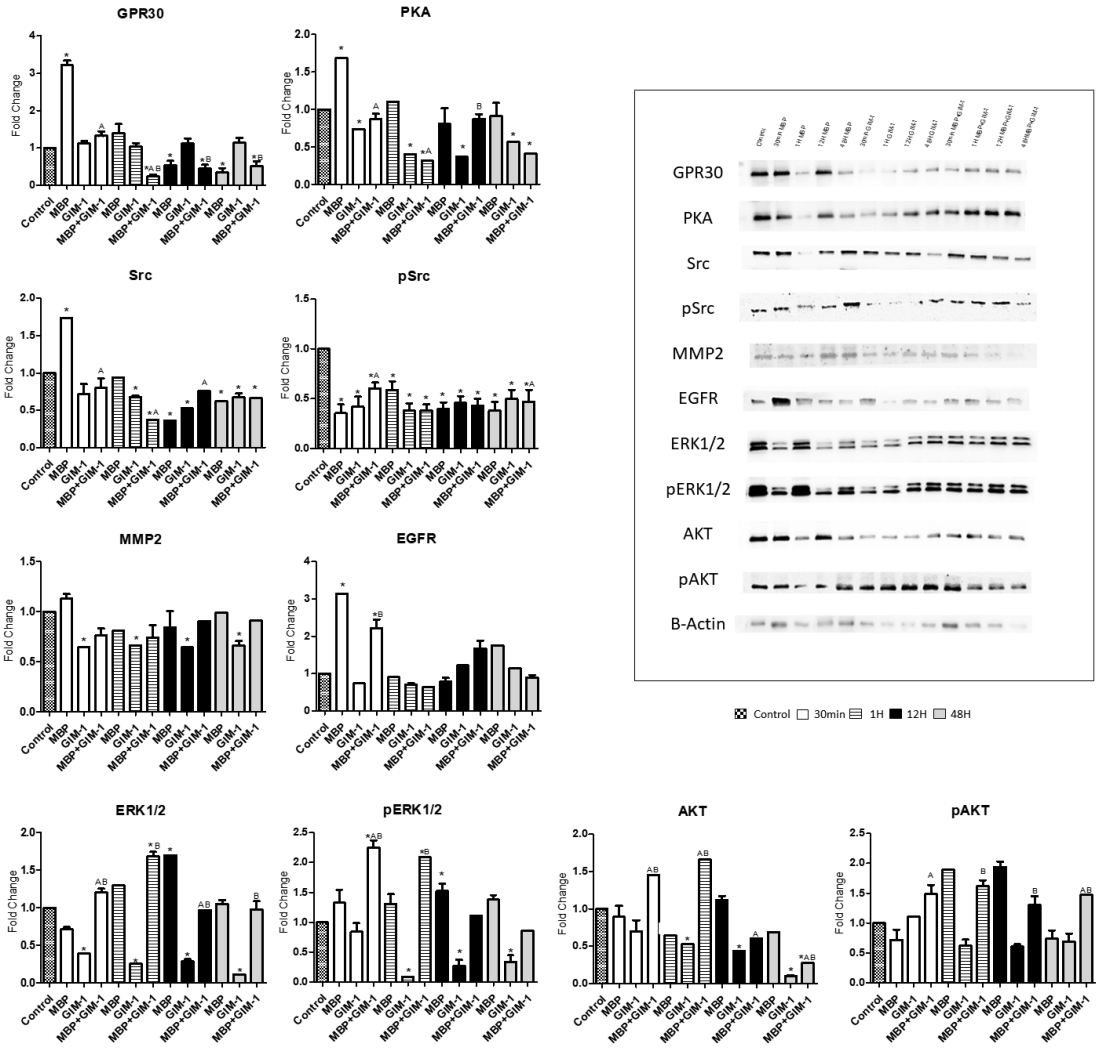

Figure 2. Western blot for proteins GPR30, PKA, Src, pSrc, MMP2, EGFR, ERK1/2, pERK1/2, AKT, pAKT and β-actin in the experimental groups: MBP, GIM-1 and MBP+GIM-1 for 30 minutes, 1, 12 and 48 hours; each band representing a pool with samples of three cell cultures from the protein extracts of cell cultures. Fold change charts comparing experimental groups to control and among themselves. Each bar represents the average followed by the Standard Error of three different bands referring to the experimental triplicates. Statistical difference $p \leq 0.05$ is represented in relation to the groups (*) Control; (A) MBP; (B) GIM-1. Controls did not differ from each other, they are represented as a single bar on the left.

4.2 GIM-1 exposure

Exposure to GIM-1 (Figure 2) did not change GPR30 expression, but reduced PKA, MMP2 and pSrc expression in all observed periods, as well as the total Src after 1, 12, and 48 hours. EGFR expression was also not altered.

On the other hand, we observed a significant reduction in ERK1/2 expression at all exposure times, as well as a reduction of its phosphorylated form after being exposed for 1, 12 and 48 hours. AKT expression also decreased after 1, 12 and 48 hours, with no changes in its phosphorylated form.

4.3 MBP+GIM-1 exposure

Compared to the control group, the combined exposure of MBP with GIM-1 (Figure 2) reduced GPR30 expression after 1, 12 and 48 hours, the same effect observed for PKA expression. The total Src expression was not altered, while its phosphorylated form was reduced after being exposed for 30 minutes, 1 and 12 hours. MMP2 Expression remained unchanged and EGFR expression was increased after 30 minutes.

ERK1/2 expression was increased after 1 hour in its total form and after 30min and 1 hour in its phosphorylated form. Total AKT expression was reduced after being exposed for 48 hours, with no changes in its phosphorylated form.

When compared to the MBP group, we observed that the combined exposure decreased GPR30 expression in relation to the MBP group at 30 minutes and 1 hour, and was also observed in relation to PKA and Src expression. The phosphorylated form of Src was increased over 30 minutes and 48 hours. EGFR Expression was reduced after being exposed for 30 minutes, while total ERK1/2 expression was increased over the same period and after 12 hours and pERK1/2 expression after 30 minutes. AKT expression was also increased after 30 minutes and 1 hour, with a reduction after being exposed for 12 and 48 hours. An increase of pAKT was also observed after 30 minutes and 48 hours.

In relation to the GIM-1 group, GPR30 expression was reduced after 1, 12 and 48 hours, with increased PKA and Src expression in a 12-hour period. ERK1/2 expression was increased at all periods in its total form and after 30 minutes and 1 hour in its phosphorylated form. AKT expression was increased after 30 minutes, 1 hour and 48 hours, also increasing in its phosphorylated form after 1, 12 and 48 hours.

		GPR30	PKA	Src	PSrc	MMP2	EGFR	ERK1/2	pERK1/2	AKT	pAKT	Cofilin	pCofilin
MBP	30min	↑	↑	↑	↓	=	↑	=	=	=	=	↑	=
	1H	=	=	↓	↓	=	=	=	=	=	=	=	↓
	12H	↓	=	↓	↓	=	=	↑	↑	=	=	↓	↓
	48H	↓	=	=	↓	=	=	=	=	=	=	=	↓
GIM-1	30min	=	↓	=	↓	↓	=	↓	=	=	=	=	↓
	1H	=	↓	↓	↓	↓	=	↓	↓	↓	=	=	↓
	12H	=	↓	↓	↓	↓	=	↓	↓	↓	=	=	↓
	48H	=	↓	↓	↓	↓	=	↓	↓	↓	=	↓	↓
MBP + GIM-1	30min	=	=	=	↓	=	↑	=	↑	=	=	=	↓
	1H	↓	↓	=	↓	=	=	↑	↑	=	=	↓	↓
	12H	↓	=	=	↓	=	=	=	=	=	=	=	↓
	48H	↓	↓	=	=	=	=	=	=	↓	=	↓	↓

Table 2. Western Blotting summary results of experimental groups in relation to the control in all the times of exposure. Symbols meaning: (↑) increase; (↓) decrease and (=) no difference in relation to the control.

4.4 Cofilin Western Blot and F-actin Immunofluorescence

Western Blotting assay for Cofilin revealed that MBP, after being exposed for 30 minutes, lead to an increase of its expression, followed by a decrease after 12 hours, while its phosphorylated form was reduced after being exposed for 1, 12 and 48 hours.

GIM-1 exposure reduced total Cofilin expression in 48 hours and its phosphorylated form in all the periods. MBP+GIM-1 exposure reduced Cofilin expression in 30 minutes, 1 and 48 hours in its total form and after 30 minutes and 1 hour in its phosphorylated form (Figure 3).

Since an increase in cofilin can lead to F-actin depolymerization, we performed an immunofluorescence assay in order to evaluate F-actin intensity and the distribution on the cell surface in the MBP group, in which an increase in Cofilin expression was observed after

30 minutes of MBP exposure. For that, we evaluated HSeC cells at 1 hour and 12 hours, after Cofilin expression recovery.

After being exposed for 1 hour, we observed the loss of F-actin intensity, without changes in its distribution through the cells. After being exposed for 12 hours, F-actin intensity shows a similar recovery to that of the control group.

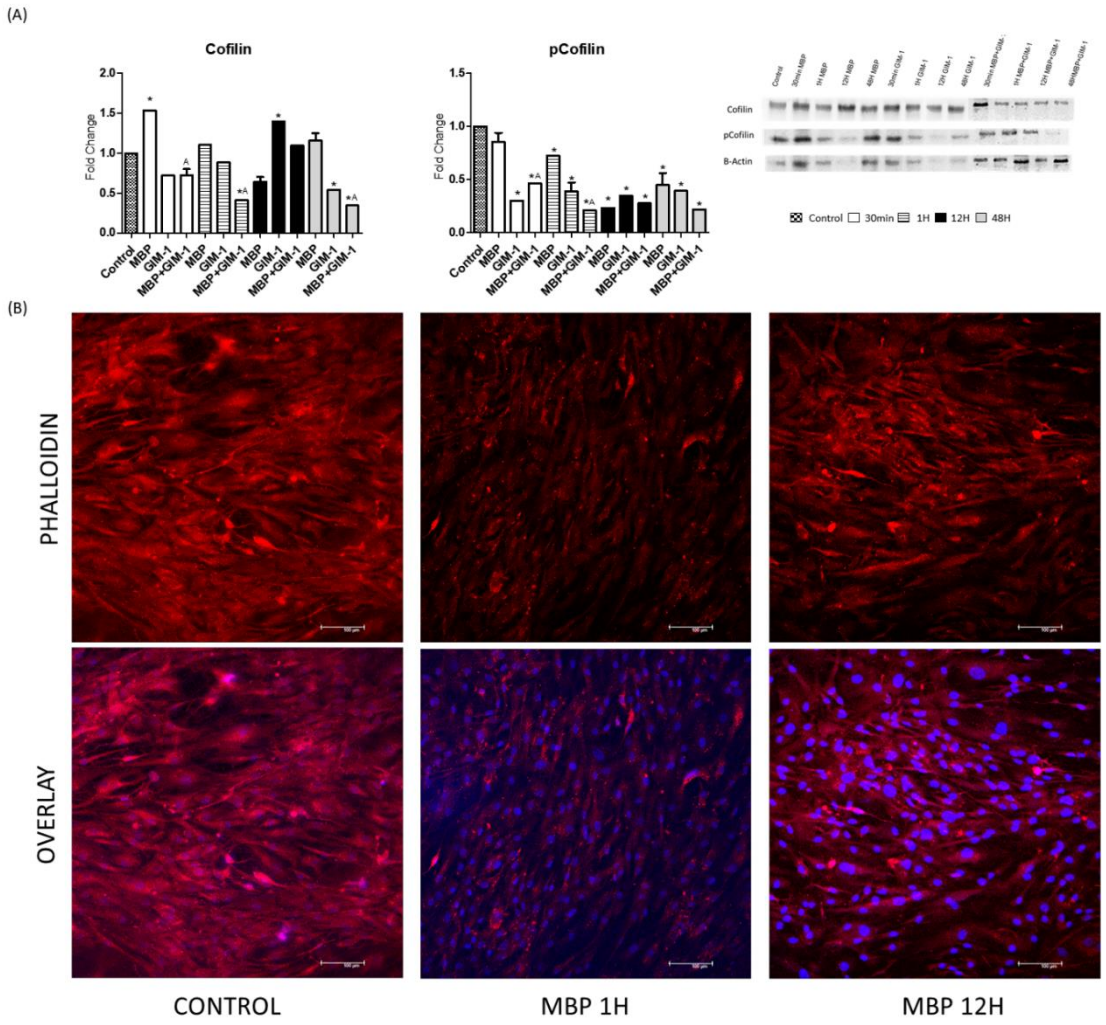

Figure 3. (A) Western blot for proteins Cofilin, pCofilin and β-actin in the experimental groups: MBP, GIM-1 and MBP+GIM-1 for 30 minutes, 1, 12 and 48 hours; each band representing a pool with samples of three cell cultures from the protein extracts of cell cultures. Fold change charts comparing experimental groups to control and among themselves. Each bar represents the average followed by the Standard Error of three different bands referring to the experimental triplicates. Statistical difference $p \leq 0.05$ is represented in relation to the groups (*) Control; (A) MBP; (B) GIM-1. Controls did not differ from each other, they are represented as a single bar on the left. (B) HSec Sertoli

Cells stained by Phallodin-TRITC conjugated (Fluorescence microscope) and DAPI from different treatments. Magnification: x200.

5. Discussion

To evaluate the effects of the compounds on the GPR30 pathway proteins, a Western blotting assay was performed. Exposure of the HSec line to the MBP showed a rapid activation of the GPR30 receptor after being exposed for 30 minutes, showing that GPR30 is a binding site for MBP in cells.

Confirming our data, a rapid increase in GPR30 expression was observed by Ge et al. (2014) after exposure of mice Sertoli cell (TM4 strain) to the endocrine disruptor Bisphenol A (BPA). Hu et al. (2013) observed that MBP increases GPR30 gene expression in a short period of exposure (5 to 15 minutes), which justifies the consequent rapid increase in protein expression of the receptor, with a reduction of the expression after a period of more than 24 hours, thus considering GPR30 a biochemical marker to observe the rapid response of Sertoli cells to MBP.

The increase in PKA, observed after being exposed for 30 minutes, can directly influence the activation of ERK1/2, in addition to other effects mediated by FSH, such as maturation of spermatids (Crépieux et al., 2001) and control of tight junctions between Sertoli cells, participating in the blood-testis barrier (Li et al., 2001). MBP, as an endocrine disruptor, mimics the effects of FSH when binding to GPR30.

The expression of Src is fundamental for the maintenance of spermatogenesis (Chojnacka & Mruk, 2015), as stated by the study with Src knockout mice, with consequent infertility (Xiao et al., 2017). However, the initial increase of Src can be alarming, since it is directly involved in the process of opening the barrier, which requires a specific control and, if excessive, can also lead to infertility (Hamaguchi et al., 1993).

Although the literature points to phthalates enhancing MMP2 expression (Yao et al., 2009; Zhang et al., 2016), MBP did not lead to changes related to control group. In relation to EGFR expression, increased after being exposed for 30 minutes, our findings agree with that of Grindler et al. (2018), who identified EGFR as a critical mediator of phthalates effects, promoting cell proliferation and apoptosis.

An increase in ERK1/2 and pERK1/2 expression, observed here after being exposed for 12 hours to MBP, following the activation of GPR30 and PKA, was also observed by Ge et al. (2014). Those authors showed that Bisphenol A acts via GPR30, activating the EGFR signaling cascade and phosphorylating ERK1/2, which led to an increase in Sertoli cells proliferation. Previous studies have also shown that endocrine disruptors can activate the EGFR-ERK pathway via GPR30 (Prossnitz & Barton, 2011), stimulating the MAPK pathway and cell proliferation (Alonso-Magdalena et al., 2012). This can lead to an increase in cell proliferation with consequent contact induced apoptosis.

Choi et al. (2014) showed that the endocrine disruptor nonylphenol induces apoptosis in Sertoli TM4 cells by the activation of the MAPK pathway and release of calcium. This observed increase in pERK may lead to a breakdown of cell junctions between Sertoli cells and between them and germ cells, as observed by Siu et al. (2005).

On the other hand, maintenance of AKT and pAKT expression levels after exposure to MBP, as compared to the control group, was not observed by Hu et al. (2014), who reported that MBP leads to rapid phosphorylation of key proteins in the MAPK and PI3K/AKT pathways and may induce problems in the occlusion junctions. An increase in PI3K/AKT pathway expression, by increased PI3K and consequent AKT phosphorylation, could affect Sertoli cell functions and blood-testis barrier integrity, as shown by Cao et al. (2015) in rat cells.

Conversely, Huang et al. (2016) observed a reduction in PI3K, AKT and pAKT expression after nonylphenol exposure to rat Sertoli cells, also reducing the pAKT/AKT phosphorylation ratio. The difference in the data observed may be due to the different models and species in the study.

Total Cofilin expression was increased by MBP, which is alarming for cell adhesion in blood test barriers. Cofilin is an actin-binding protein that increases the rate of actin turnover, leading to depolymerization of F-actin and loss in cell adhesion (Toshima et al., 2001). These changes, affecting boundary tissues, may lead to desquamation of the germinal epithelium and consequent infertility (Marettová et al., 2010).

Immunofluorescence assay for F-actin revealed that MBP reduced F-actin intensity after 1 hour, without changes in its distribution. These findings agree with Nishida (1985), who observed that cofilin causes a decrease in F-actin concentration by increasing the rate of G-actin loss. Zhang et al. (2008) reported no significant effect on fluorescent intensity and the distribution between control and MBP-treated cells after 24 hours, showing a possible fast cell recovery and the importance of the quick effects of GPR30, mainly in an environment of constant MBP exposure.

The exposure of cells to GIM-1, unlike that seen in the MBP-exposed group, did not alter GPR30 expression, showing that the pathway is not activated by the compound. PKA expression was reduced in all periods, along with Src expression, reduced after 1, 12 and 48 hours. Huang et al. (2018) also showed that ginseng inhibited PKA expression, attenuating cardiotoxicity, but the effects of this reduction is not clear on the Sertoli cell function.

As for MMP2 and EGFR expression, recent studies have shown that ginsenosides, including GIM-1, downregulated MMP2 gene and protein expression (Kim et al, 2016; Song et al., 2018) and EGFR expression, being an important target in abnormal cell proliferation and apoptosis mediated by EGFR (Sathishkumar et al., 2013; Li et al., 2018). Our findings show that GIM-1 reduced MMP2 expression, but no changes in EGFR were observed.

PKA and Src reduction are in concordance with the reduction in total ERK1/2 expression at all observational periods and pERK1/2 after being exposed for 1, 12 and 48 hours. Irfan et al. (2018) also showed that ginsenosides inhibits Src expression and Wang et al. (2012) showed that Panax ginseng inhibits MAPKs. Also confirming our data, Kee et al. (2017) showed that ginseng, in three different cell types, in vitro, suppressed ERK phosphorylation, thereby inhibiting the production of proinflammatory cytokines.

Wang et al (2012) also showed that the treatment of Sertoli cells with ginsenosides reduced ERK1/2 phosphorylation and is an important therapeutic target in reducing cytotoxicity induced by environmental toxicants. Deleterious effects mediated by MAPKs pathway, including changes in spermatogenesis, cell functions and the opening of blood-testis barriers by TGF-β2 can be prevented and reversed by the administration of ginsenosides (Lui et al., 2007, Li et al., 2009).

We also observed a reduction in AKT expression levels after being exposed to GIM-1 for 1, 12 and 48 hours, with no changes in its phosphorylated form. Contrary to what we observed here, Wu et al. (2016) showed high phosphorylation of ERK1/2 and AKT after administering ginsenosides in neural cells. In a liver cell study, Bak et al. (2016) and Zang et al. (2017) also observed that ginseng stimulated the phosphorylation of AKT and ERK1/2 and PI3K and AKT, respectively. This difference can be mainly due to the different cell types, since cellular proliferation, stimulated by these pathways, does not occur naturally in adult Sertoli cells.

Cofilin expression was reduced after 48 hour and pCofilin in all periods, showing that GIM-1 has a role in reinforcing cell adhesion, maintaining overall actin polymer length (Bamburg et al., 1999).

When the combination of MBP and GIM-1 was administered to the cell culture, GPR30 receptor activation didn't occurred, with a significant reduction observed after 1, 12 and 48 hours in relation to Control group, showing that GIM-1 could inhibit MBP from binding into the receptor. As well, PKA and Src expression were significantly reduced, showing GIM-1 effects against MBP, but the increase in EGFR expression, similar to MBP group, shows that this receptor may be activated by a different pathway and GIM-1 was not able to avoid it.

The interference of the compounds on ERK1/2 expression is clear, since the combined exposure did not lead to any of the changes observed when the compounds were given individually. The combined exposure also did not alter AKT and pAKT levels, as observed in the MBP-exposed group and contrary to the reduction of AKT observed after exposure to GIM-1, showing possible interference of MBP in this process.

When compared to the MBP group alone, the combined exposure showed a reduction in the expression of GPR30 in 30min and 1 hour, with a subsequent reduction in PKA, Src and EGFR, which demonstrates again the influence of GIM-1 on the effects observed with the MBP. Kang et al. (2002) also reported the potential of GIM-1 against testicular toxicity but Wang et al. (2012) demonstrated the inhibition of ERK1/2 phosphorylation by GIM-1 in a combined exposure with BPA in Sertoli cells.

Differently, ERK1/2 phosphorylation was higher than the MBP group after 30 minutes, as its total expression, showing that other pathways may be involved in this process. A reduction in the Cofilin expression, in both total and phosphorylated forms, shows a possible maintenance of cell adhesion, as F-actin would keep its structure in cell junctions.

In relation to the GIM-1 group, GPR30 expression was reduced, showing that it might influence the MBP connection with the receptor. The increase observed in the expression of PKA, ERK1/2, pERK1/2, AKT and pAKT shows that, although attenuated by GIM-1, the GPR30 pathway is still activated by MBP.

Here, we have demonstrated MBP action trough GPR30 pathways, in a time dependent manner, with relevant increases in proliferation and apoptotic proteins, also changing important modulators and barrier structure. These changes may justify our previous studies (Freitas et al., 2019), which are MBP, trough GPR30, capable of causing cytotoxic effects in HSeC Sertoli cells, with the probable disruption of the blood-testis barrier and consequences to male fertility. GIM-1 acted as a functional antagonist of MBP, but we showed that the combined exposure was only able to attenuate MBP effects in these doses, not preventing all MBP interference on the GPR30 pathway.

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001", by the São Paulo State Research Foundation (FAPESP; Process: 2012/00253-3) and by the National Council for Scientific and Technological Development (CNPq, Process: 306900/2016-5). We would like to thank Dr. Daniela Carvalho dos Santos, Shelly Favorito de Carvalho and Electronic Microscopy Center/IBB-UNESP for technical support.

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Capítulo 4

Conclusão Geral e Referências Bibliográficas

4.1 Conclusão Geral

Utilizando a linhagem HSec de células de Sertoli humanas conclui-se que:

1. O Monobutil Ftalato é um importante agente estressor, atuando tanto na redução da atividade enzimática responsável pela eliminação das espécies reativas de oxigênio quanto na expressão de proteínas reguladoras da homeostase celular, induzindo ainda a expressão de proteínas pró apoptóticas. Além disso, o MBP ativa diretamente a via do GPR30, mobilizando proteínas das vias envolvidas em sua resposta, como PKA, Src e EGFR e, consequentemente, estimula a expressão e fosforilação da via da ERK1/2. A nível de superfície celular, o MBP se mostrou capaz de reduzir a intensidade de actina-F, através da expressão de Cofilina, potencialmente afetando a adesão celular entre as células de Sertoli e levando ao aumento de espaços intercelulares. Com a compreensão da importância da manutenção da integridade das células de Sertoli e de todo o ambiente imunoprotegido por elas, através da barreira hematotesticular, o MBP é um importante objeto de estudo por seus efeitos deletérios em doses de exposição humana.

2. O GIM-1 apresenta importante atividade protetora sob as células de Sertoli, combatendo o estresse oxidativo através do estímulo de todo o sistema antioxidante e inibindo ou amenizando os efeitos adversos causados pelo MBP, de forma que a continuação de seu estudo, principalmente em doses variadas, pode resultar em uma importante terapêutica para a integridade do processo de espermatogênese e consequente restauração da fertilidade masculina.

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