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

PG-BGA

POTENCIAL DE DIFERENCIAÇÃO DE CÉLULAS-TRONCO MESENQUIMAIS TECIDO ADIPOSO HUMANAS EXPOSTAS AO 2,3,7,8-TETRACLORODIBENZO-P-DIOXINA (TCDD) E BISFENOL A (BPA)

Helga Caputo Nunes

Tese apresentada ao Instituto de Biociências, Câmpus de Botucatu, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração Biologia Celular Estrutural e Funcional.

Profa. Dra. Flávia Karina Delella

BOTUCATU – SP
2018

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ELENICE DEFFUNE

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Epígrafe

"A ciência é a tentativa de fazer com que a diversidade caótica da nossa experiência sensível corresponda a um sistema lógico uniforme de pensamento"
Albert Einstein, 1950.

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SUMÁRIO

LISTA DE ABREVIATURAS

ABCG2: *ATP-binding cassette sub-family G member 2*

AhR: Receptor aril hidrocarboneto

ANOVA: Análise de variância

ARs: Receptores de andrógeno

ASCs: *Adipose-derived stem cells*

BADGE: *Bisphenol A diglycidyl ether*

βHCH: *β-hexachlorocyclohexane*

BAK: *Bcl-2 homologous antagonist/killer*

BMI: *Body mass index*

BM: *Bone marrow*

BPA: *Bisfenol A*

CD: *Cluster of differentiation*

CFUs: *Colony forming units*

CTRL: *Control*

CYP1A2: Citocromo P450 1A2

CTAs: Células-tronco adultas

CTEs: Células-tronco embrionárias

CTMs: Célula-tronco mesenquimais

CTM-TAs: Células-tronco mesenquimal derivada de tecido adiposo

CTs: Células-tronco

DEs: Disruptores endócrinos

DEHP: *Bis-2-ethylhexyl phthalate*

DMEM: *Dulbecco´s Modified Medium*

DMSO: Dimetilssulfóxido

DNA: *Deoxyribonucleic acid*

EDCs: *Endocrine disrupting chemicals*

EPA: *United States Environmental Protection Agency*

ERs: Receptores de estrógeno

FBS: *Fetal bovine serum*

FDA: *Food and Drug Administration*

FITC: *Fluorescein isothiocyanate*

GMPs: *Good Manufacturing Practices*

hASCs: *Human adipose stem cells*

HeLa cells: *Henrietta Lacks cell line*

HLA-DR: *Human leukocyte antigen–antigen D related*

ICI 182 780: ER antagonist

IFN-γ: *Interferon gamma*

IL-1: *Interleucin 1*

iPSCs: *Induced pluripotent stem cells*

ITD: Ingestão tolerável diária

LPL: *Lipoprotein lipase*

MTT: (*4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide*

PBS: *Phosphate buffered saline*

PBST: *Phosphate buffered saline with Tween 20*

PCBs: *Polychlorinated biphenyls*

PCDDs: *Polychlorinated dibendibenzodioxins*

PCDFs: *Polychlorinated dibenzofurans*

PI: *Propidium iodide*

PLA: *Processed lipoaspirate*

POPs: Poluentes persistentes orgânicos

PPAR: *Peroxisome proliferation agonist receptor*

pp-DDE: *Dichloro-diphenyl-dichloroethylene*

rACs: *Rat adipose stem cells*

RIPA: *RadioImmuno Precipitation Assay lysis buffer*

RSa cells: *Human embryonic clonal cell line*

SE: *Standard deviation*

SEM: *Standard error medium*

SOX2: *SRY (sex determining region Y)-box 2*

TBT: *Tributyltin*

TCDD: Tetracloro-dibenzo-p-dioxina

3T3-L1: *Preadipocytes*

TNF-α: *Tumor necrosis factor alpha*

TRs: Receptores para tireoide

UCLA: *University of California in Los Angeles*

WHO: *World Health Organization*

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RESUMO

Células tronco mesenquimais (CTMs) possuem papel relevante na manutenção da homeostase e reparação em casos de lesão tecidual através da renovação do estoque celular. Tais células são implicadas na medicina regenerativa e terapia celular como fontes promissoras, sendo o tecido adiposo uma rica e relevante fonte dessas células. Nesse sentido, as células tronco mesenquimais derivadas de tecido adiposo (CTMs-TA) surgem como alternativa segura em estudos pré-clínicos e clínicos. As chamadas "Boas Práticas em Cultura Celular" (do inglês *Good Manufacturing Practices* - GMPs) tem sido utilizadas como uma nova abordagem para obtenção de controle de qualidade para fins terapêuticos. Neste contexto, o controle toxicológico dessas células surgem como uma questão relevante. Considera-se que os disruptores endócrinos mimetizam a ação de certos hormônios endógenos. Dessa forma, o BPA é conhecido por mimetizar o receptor de estrógeno e também causar alterações no processo de adipogênese. Já o TCDD possui afinidade com o receptor aril hidrocarboneto (AhR), assim como interfere através das vias de sinalização de estrógenos e andrógenos. Desta maneira, o presente estudo teve como objetivo geral testar o potencial de diferenciação das CTMs-TA humanas e de rato na presença dos compostos BPA e TCDD. Os objetivos específicos foram: a quantificação de proteínas relacionadas ao fenótipo de células-tronco, bem como de receptores específicos de ligação desses compostos, testes para verificação do possível perfil apoptótico e genotóxico dessas substâncias e quantificação do potencial de diferenciação adipogênica e osteogênica dessas células quando expostas. Primeiramente, CTMs-TA advindas de cultura primária humana ou de rato, foram obtidas, expandidas e caracterizadas. Assim, essas células foram expostas à 1uM e 10uM de BPA e 10nM de TCDD durante 7 dias. Análises por citometria de fluxo, ensaios de MTT, Western Blot para as proteínas ABCG2, BAK, SOX2, AhRe ERβ, ensaio de Apoptose/necrose, ensaio de genotoxicidade e quantificação espectrofotométrica do potencial de diferenciação das CTMs-TA foram realizados e analisados. A análise do MTT revelou que o BPA (1uM) para CTMs humanas alterou a atividade metabólica no sentido anti-proliferativo, já para as células de rato, tanto o BPA (10uM) quanto o TCDD (10nM) apresentaram caráter indutor de proliferação. Nas células humanas, o TCDD aumentou a concentração das proteínas ABCG2 e AhR. Para BAK, SOX2 e ERβ, nenhuma diferença foi observada entre

exposições ou grupos analisados Ambos compostos testados induziram apoptose nas células de rato e mostraram-se genotóxicos após a análise do ensaio do cometa. Para a quantificação da diferenciação adipogênica, a concentração de 1uM de BPA foi capaz de induzir maior diferenciação, o que não ocorreu com nenhuma das concentrações dos compostos quanto à diferenciação osteogênica. No presente trabalho foram analisadas tanto CTMs humanas quanto de ratos, e, nesse sentido, foi possível verificar acentuada diferença entre os comportamentos celulares de cada espécie e verificar a existências de poucos trabalhos sobre a presente temática. Sendo assim, com os presentes achados, é possível concluir que o BPA na concentração de 1uM apresentou atividade tóxica para hASCs fortalecendo os achados de que doses baixas têm maior ação prejudicial que doses mais elevadas. Além de enfatizar a ação do TCDD via receptor AhR nas CTMs, os nossos resultados mostram que o TCDD elevou da expressão da proteína ABCG2 nessas células, o que indica que este composto pode alterar o fenótipo de células-tronco bem como a expressão de receptores do tipo bomba de influxo e efluxo de xenobióticos.

ABSTRACT

Mesenchymal stem cells (CTMs) plays a relevant role in the maintenance of homeostasis and repair in cases of tissue injury through cell stock renovation. Such cells are implicated in regenerative medicine and cell therapy as promising sources, been the adipose tissue a rich and relevant source. In this sense, mesenchymal stem cells derived from adipose tissue (ASCs) appears to be a safe alternative in preclinical and clinical studies. The so-called Good Manufacturing Practices (GMPs) have been used as a new approach to obtaining quality control for therapeutic purposes. In this context, the toxicological control approach becomes a relevant issue. Endocrine disrupting chemicals are thought to mimic the action of certain endogenous hormones. Thus, BPA is known to mimic the estrogen receptor and also cause changes in the process of adipogenesis. TCDD, on the other hand, has affinity with the aryl hydrocarbon receptor (AhR), as well as interfering with the estrogen and androgen signaling pathways. In this way, the present study had the rationale to test the differentiation potencial of human and rat MSCs in the presence of BPA and TCDD. The specific objectives were: quantification of proteins related to the stem cell phenotype, as well as specific binding receptors of these compounds, verification of apoptotic and genotoxic profile of these substances and also the quantification of the potential of adipogenic and osteogenic differentiation of these cells when exposed. First, ASCs from human or rat primary culture were obtained, expanded and characterized. Thus, these cells were exposed to 1uM and 10uM BPA and 10nM TCDD for 7 days. Flow cytometric assays, MTT assays, Western Blot for ABCG2, BAK, SOX2, AhR and ERβ assays, Apoptosis/ necrosis assay, genotoxicity assay, and spectrophotometric quantification of ASCs differentiation potential were performed and analyzed. MTT analysis revealed that BPA (1uM) for human ASCs altered the metabolic activity in an anti-proliferative sense, whereas for both rat cells, both BPA (10uM) and TCDD (10nM) had a proliferation-inducing behaviour. In human cells, TCDD increased the concentration of ABCG2 and AhR proteins. For BAK, SOX2 and ERβ, no difference was observed between exposures or groups analyzed. Both compounds tested induced apoptosis in rat cells and were genotoxic after comet assay analysis. For the quantification of adipogenic differentiation, the concentration of 1uM of BPA was able to induce greater differentiation, which did not occur with any of the concentrations of the compounds regarding the osteogenic differentiation. In the present study, was investigated both human and rat mesenchymal stem cells, accordingly, we observed marked difference

between the cellular behaviors from diferente species and few studies about the current moment. Thus, with these findings, we conclude that BPA in the concentration of 1uM showed toxic activity to hASCs strengthening the findings that low doses has more harmful action than higher doses. In addition to emphasizing the action of TCDD via the AhR receptor on the MSCs, our results show that TCDD increased expression of ABCG2 protein in these cells, suggesting that this compound may alter stem cells phenotype as well as expression of pump-like receptors of influx and efflux of xenobiotics.

I. INTRODUÇÃO

1. Células-tronco e suas aplicações na Medicina Regenerativa

As primeiras concepções sobre células-tronco (CTs) surgiram na literatura científica a partir dos achados de **Haeckel** em **1868**, estabelecendo assim o conceito de *stamzelle*, ou seja; "uma determinada célula comissionada ou indiferenciada responsável por produzir novas células com o intuito de reparar o corpo" **(Armstrong et al., 2012)**. Somente depois dos trabalhos de **James Till** e **Ernest McCulloch** no ano de **1961** sobre achados com CTs hematopoiéticas, é que se teve a comprovação da existência dessas populações. Nos vinte anos subsequentes, as pesquisas com CTs começaram a ampliar drasticamente. Achados como a descoberta de CTs estromais no sangue por **Friedenstein** e **colaboradores (1966)** e a derivação de CTs de carcinoma embrionário por **Martin** em **1981** eram os tipos celulares mais estudados até então **(Armstrong et al., 2012)**. Estudos experimentais e clínicos já eram realizados desde 1939 com essas células, mas somente em1969 é que se realizou o primeiro transplante de CTs hematopoiéticas alógenas com sucesso para o tratamento de leucemia humana **(Thomas & Storb, 1970; Henig & Zuckerman, 2014)**.

Quase dois séculos após os primeiros relatos e achados, é sabido atualmente que as CTs são populações de células somáticas específicas responsáveis pela gênese (embrionárias) ou renovação tecidual (adultas) **(Wankhade et al., 2016)**. As células-tronco embrionárias (CTEs) são as células geradoras de todos os tecidos de um embrião, são embriologicamente formadas a partir do estágio de mórula, quando o embrião possui de 5 à 7 dias de desenvolvimento. Após esse período, no estágio de blástula; a massa interna do blastocisto é retirada e esse tecido é então cultivado. Tais células são capazes de originar os três folhetos embrionários conhecidos possuindo, dessa maneira, características de totipotência. À partir de 6 semanas de desenvolvimento do embrião, tais células agora possuem características de pluripotência, ou seja, a capacidade de gerar células advindas dos 3 folhetos embrionários vai diminuindo à medida em que o embrião se desenvolve **(Kaufman et al., 1983;Martin, 1981)**.

As células-tronco adultas (CTAs) por outro lado, são populações residentes específicas responsáveis pela manutenção e o reparo de tecidos adultos. São definidas por sua habilidade em manterem-se em estado indiferenciado por longos períodos, ou seja,

possuem capacidade de se autorrenovar e gerar células dos tecidos específicos ao nicho* que pertencem em condições *in vivo*, além de manterem-se quiescentes **(Barker et al, 2010)**. *In vitro*, também são capazes de diferenciarem-se em tipos celulares distintos dos nichos aos quais pertencem. Didaticamente, possuem características de pluripotência, multipotência e unipotência dependendo de estágio de *stemness*** ou comissionamento **(Wankhade et al., 2016)**. Além disso, as CTAs têm sido identificadas praticamente em todos os tecidos humanos e muitos dos tecidos da maioria dos mamíferos utilizados como animais experimentais **(Wankhade et al., 2016)**.

Em 2007 outro tipo celular, as células-tronco pluripotentes induzidas (iPSCs), foram produzidas artificialmente por técnicas de engenharia genética através da reprogramação de fibroblastos dérmicos murinos para expressar genes característicos de CTEs **(Takahashi et al., 2007)**.

Em relação à viabilidade e aplicabilidade dessas células, de forma geral, as CTEs humanas por sua origem e natureza tornam difíceis de serem obtidas por possuírem implicações éticas. Em contraste, as CTAs, dependendo do sítio doador, são de fácil coleta e encontradas em concentrações razoáveis, mas por outro lado, podem ser limitadas em sua capacidade de diferenciação **(Wankhade et al., 2016)**. A **tabela 1** abaixo apresenta, de forma sucinta, os principais tipos de células-tronco descobertas e desenvolvidas até a atualidade.

*Nichos: são microambientes fisiológicos, constituindo-se por células especializadas, que sinalizam através de moléculas da superfície celular e controlam, por exemplo, a taxa de proliferação das CTs, determinando a diferenciação das células progenitoras e protegendo essas populações de processos apoptóticos **(Rizvi & Wong, 2005)**.

***Stemness:* propriedade de uma célula em apresentar um ou certos genes que torna possível a expressão do potencial de auto-renovação e diferenciação multilinhagem **(Leychkis; Munzer; Richardson, 2009)**.

Tabela 1. Classificação Células-Tronco (CTs). Fonte: Instituto de Pesquisas com Células-Tronco (IPCT) http://celulastroncors.org.br/, 2017.

TIPO DE CÉLULA-TRONCO	POTENCIAL DE DIFERENCIAÇÃO	FUNÇÕES	FONTES
CT EMBRIONÁRIA	**TOTIPOTENTE, PLURIPOTENTE**	**Teoricamente podem ser utilizadas para reparo e tratamento de todos os òrgãos**	**Massa interna do blastocisto e embrião de 4 à 5 dias**
CT ADULTA	**PLURIPOTENTE, MULTIPONTENTE**	**Teoricamente podem ser utilizadas para reparo e tratamento de muitos ógãos**	**Tecido fetal, cordão umbilical, tecidos adultos**
CT PLURIPOTENTE INDUZIDA	**PLURIPOTENTE**	**Geneticamente reprogramadas para comportarem-se como CT pluripotentes *"stem cell like"***	**Inicialmente reprogramadas à partir de fibroblastos dérmicos de camundongo**

Já as células-tronco mesenquimais (CTMs) primeiramente assim nomeadas por **(Caplan, 1991)** são células que não possuem um número fixo de divisões mitóticas. Sua progênie é afetada por um número de fatores, em várias vias específicas de desenvolvimento. Fatores intrínsecos como extrínsecos se combinam para controlar os padrões moleculares e celulares de expressão, que resultam em tecidos específicos desempenhando funções também específicas baseadas em seu repertório molecular **(Caplan, 1991)**. Tais células são encontradas virtualmente em todos os tecidos mesenquimais, apresentando relevante papel na manutenção da homeostase e reparo em casos de injúria tissular através da renovação do estoque celular, mantendo assim, tal nicho ativo **(Tuan et al., 2003)**.

Dependendo do sítio doador, as CTMs são de fácil coleta e relativamente abundantes **(Mizuno et al., 2012)**, quando cultivadas, possuem a capacidade de manterem-se geneticamente estáveis por diversas passagens mantendo o potencial de diferenciação. Possuem ainda, potencial imunomodulatório bem documentado, sendo capazes de inibir ou estimular uma gama de mecanismos para alvos do sistema imune humano já elucidados **(Follin et al., 2016)**.

Para datar, o primeiro estudo clínico realizado com CTMs hematopoiéticas ocorreu em 1995, tendo como resultados a ausência de efeitos colaterais negativos aos pacientes **(Bielski et al., 1998)**. CTMs foram também identificadas em tecidos esqueléticos, como ossos e cartilagens **(Bruder et al., 1994)** e um dos primeiros estudos clínicos com essas células nesses tecidos ocorreu em combinação com biomateriais para reparo de fratura de ossos longos, no qual demonstrou alta eficácia **(Bianchi et al., 2001)**. Sendo assim, o campo de estudos com CTMs têm crescido substancialmente desde o ano 2000, principalmente após a comprovação de que CTMs cultivadas *in vitro* possuem propriedades imunossupressivas após tratamento com citocinas inflamatórias como IFN-γ, IL-1, ou TNF-α **(Ménard &Tarte, 2013)**. Tais achados foram primeiramente utilizados para o transplante de CTs hematopoiéticas no tratamento da doença do enxerto versus hospedeiro **(Sundin et al., 2006)**, com frequente ocorrência de resistência às drogas normalmente utilizadas. Tal transplante mostrou-se eficiente em diminuir reações de rejeição **(Tan et al., 2012)**, possivelmente por essas células possuírem efeitos tróficos mediados por inúmeros fatores de crescimento e citocinas que secretam **(Phinney & Prockop, 2007)**.

Em **2001**, **Zuk e colaboradores** publicaram um estudo descrevendo uma nova população de CTMs isoladas à partir de tecido adipose (CTM-TA). Tal tecido, assim como a medula óssea, é derivado do mesênquima embrionário contendo uma fração estromal facilmente identificável. Inicialmente chamadas de *processed lipoaspirate (PLA) cells*, isoladas à partir de conteúdo de lipoaspirado, e, assim como as CTMs de outros tecidos, essas células também podem ser diferenciadas nas linhagens adipogênica, osteogênica, miogênica e condrogênica, bem como ser caracterizadas imunofenotipicamente com marcadores já existentes para CTMs de outras regiões. Desde então, as CTMs-TA apresentam-se como uma das populações mais promissoras dentre as CTs adultas identificadas, possuindo potencial para diferenciarem-se também em linhagens não mesodermais **(Zuk, 2010)**. Atualmente é sabido que tais células são capazes de diferenciarem-se em neurônios *(neurons like cells)* **(Choi et al., 2012)**. Como fonte de CTMs tanto para estudos experimentais quanto clínicos, o tecido adiposo apresenta-se como um farto e acessível reservatório, sendo essas células obtidas através de procedimento cirúrgico, tanto em cirurgias eletivas estéticas quanto reparadoras, sendo que, normalmente, tal tecido é descartado como lixo infectante e posteriormente incinerado. Hoje tem-se o tecido adiposo como uma rica e promissora fonte de CTMs tanto para transplante autólogo como alógeno apresentando-se viável, frequentemente eficaz e seguro em estudos pré-clínicos e clínicos para aplicações na medicina

regenerativa e terapia celular **(Gimble et al, 2010)**.

Desta maneira, o potencial uso de terapias baseadas em CTs para o reparo e regeneração de vários tecidos e órgãos oferece uma mudança de paradigma que pode gerar soluções terapêuticas alternativas a um grande número de doenças **(Keung et al, 2013)**. Portanto, como anteriormente citado, com a descoberta terapêutica dessas populações celulares, o campo da medicina regenerativa evoluiu tremendamente. As últimas décadas experimentaram grandes saltos na pesquisa com CTs voltadas para a área de engenharia de tecidos e tratamentos de diversas doenças, como por exemplo doenças degenerativas e crônicas e, apesar de avanços consideráveis, a disponibilidade dessas células em condições de uso imediato, permanece ainda um desafio tanto para pesquisadores como clínicos **(Wankhade et al., 2016)**. Ainda, com respeito à aplicação das CTs na medicina regenerativa, torna-se imprescindível que haja condições ideais para a adoção desse processo como tratamento propriamente dito, sendo assim, a adesão desta como ferramenta estratégica para uso na medicina personalizada tende a ser uma realidade dentro de alguns anos. Mas, no entanto, é necessário que se tenha pleno conhecimento e controle de todos os processos que cercam a manipulação celular do início ao fim. Para isso, a implantação de metodologias para controle de qualidade em produção são altamente necessárias, como por exemplo, a adoção das chamadas GMPs (G*ood Manufacturing Practices*), práticas que tem como finalidade seguir a inúmeros e rígidos critérios para a obtenção de elevado nível de qualidade em terapia celular. **(Gimble et al., 2010; Sensebé et al., 2013)**.

2. Poluentes Orgânicos Persistentes (POPs)

"A maré de produtos químicos nascidos da era industrial têm surgido para engolfar nosso meio ambiente, ocasionando dessa forma, uma drástica mudança na natureza dos mais sérios problemas de saúde pública". Rachel Carson, Silent Spring, 1962 **(Carson, 1962)**.

Poluentes Orgânicos Persistentes (POPs) são compostos altamente estáveis persistindo no ambiente após a sua liberação, resistindo à degradação química, fotolítica e biológica. Têm a capacidade de bioacumular em organismos vivos, sendo tóxicos para estes, incluindo o homem **(Jones & De Voogt, 1998)**. Atuam negativamente, sobretudo como desreguladores dos sistemas reprodutivo, imunológico e endócrino, sendo também apontados como carcinogênicos **(Jones & DeVoogt, 1998)**. Como característica relevante, os POPs podem ser transportados a longas distâncias pela água, vento ou pelos

próprios animais. Podem ser divididos em: pesticidas organoclorados, bifenilos policlorados (PCBs); dioxinasedibenzo furanos policlorados, sendo estes resultantes principalmente de incinerações industriais **(Ritter et al., 1995)**, como exemplificados na **Figura 1** e **Tabela 2**. No organismo, os POPs não são facilmente degradados por enzimas metabolizadoras de xenobióticos, possivelmente pelo alto grau de halogenação*. No entanto, alguns POPs ligam-se frequentemente com alta afinidade a certos receptores xenobióticos, assim como a algumas enzimas metabolizadoras xenobióticas como a CYP1A2 (citocromo P450 1A2), sem, no entanto, passar por transformação catalítica **(Slezak et al, 1999)**. Tal ligação desempenha importante papel na distribuição dos POPs. Devido à sua hidrofobicidade, POPs tendem a distribuir-se em compartimentos lipofílicos, como particularmente o tecido adiposo **(La Merrill et al., 2013)**. No que se refere à saúde humana e meio ambiente, os efeitos dos POPs foram publicamente discutidos pela comunidade internacional durante a Convenção de Estocolmo para Poluentes Persistentes Orgânicos em 2001 com a intenção de eliminar ou restringir severamente sua produção **(Europeian Union, 2004)**.

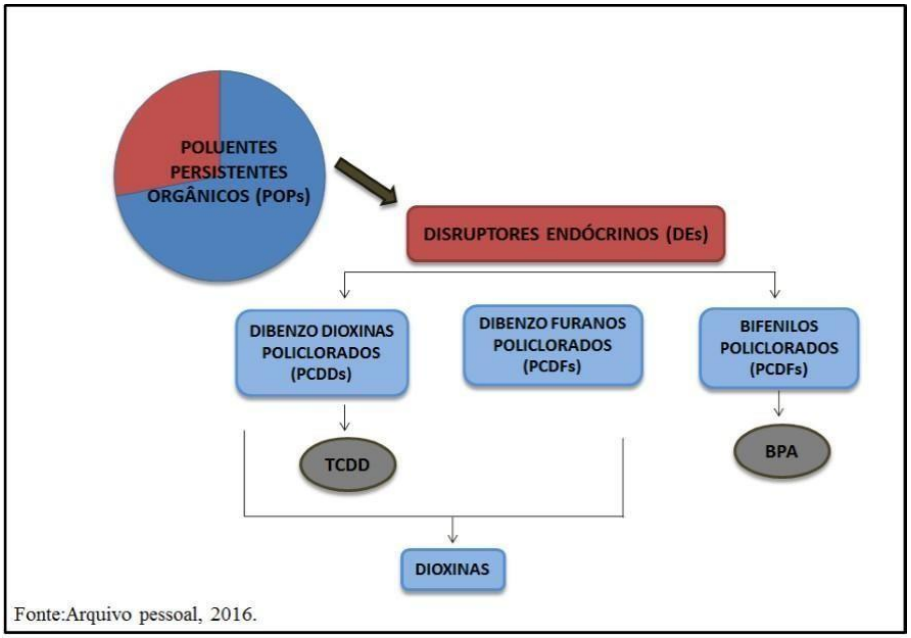


Figura1. Esquema classificação POPs. Arquivo Pessoal, 2016.

*Halogenação: reação química no qual um átomo de hidrogênio é substituído ou incorporado à um átomo de halogênio (flúor, cloro, bromo, iodo e astato).

Tabela 2. Classificação Poluentes Persistentes Orgânicos (POPs). Official Journal of the European Union, Regulation (EC) No 850/2004 of the European Parliament ando of the Council, 2004.

Poluentes Persistentes Orgânicos (POPs)	Pesticidas	Químicos Industriais	Resíduos Resultantes de Processos Industriais
	Aldrin	Bifenilos policlorados	Dioxinas
	Chlordane	hexachlorobenzenos (HCBs)	Furanos
	Dichlorodiphenyltrichloroethano (DDT)		HCB hexachlorobenzeno
	Dieldrin		Bifenilos policlorados
	Endrin		
	HCB hexachlorobenzeno		
	Hepatochlor		
	Mirex		
	Toxapheno		

3. Disruptores Endócrinos

Disruptores endócrinos (DE) são compostos naturais ou sintéticos que possuem a capacidade de alterar funções endócrinas geralmente mimetizando ou bloqueando hormônios endógenos **(Schug et al., 2011)**. São ainda definidos pela Sociedade Mundial de Endocrinologia como: "um composto químico exógeno (não natural), ou uma mistura deles, no qual interferem em quaisquer aspectos hormonais" **(IPCS, 2002)**. A ideia de que componentes químicos xenobióticos poderiam modular de forma inapropriada o sistema endócrino, causando assim efeitos prejudiciais à vida selvagem e de seres humanos, foi primeiramente proposta em uma reunião divisor de águas; a

conferência de *Wingspread* **(Colborn, 2004)**. Tais compostos têm tido ao longo dos anos, posições controversas em relação à comunidade científica e a indústria, produzindo uma grande e longa discussão.

Atualmente, inúmeros estudos têm sido realizados e a maioria deles mostra que tais substâncias são indiscutivelmente prejudiciais à saúde de animais e seres humanos, atuando através da alteração dos níveis hormonais basais, inibindo ou estimulando a produção e o metabolismo dos mesmos, ou ainda, modificando a forma pela qual os hormônios transitam pela circulação, afetando assim as funções que tais hormônios controlam **(Schug et al., 2011)**. Bioquimicamente, existem duas vias pelas quais um composto químico pode atuar como um DE: por ação direta no complexo proteico que compõe um receptor para determinado hormônio ou por ação direta numa proteína específica que controla algum aspecto da entrega desse hormônio ao sítio alvo **(WHO, 2010)**. Em relação à especificidade, acreditava-se originalmente que tais compostos exerciam sua atividade somente através de receptores para hormônios nucleares, incluindo os receptores de estrógeno (ERs), andrógeno (ARs), progesterona e tireoide (TRs) entre outros. No entanto, evidências recentes mostram que os mecanismos pelos quais os DEs atuam parecem ser muito mais extensos. De fato, foi demonstrado que além de alterarem os padrões de sinalização de receptores nucleares, eles também são capazes de agir através de receptores não esteroidais, co- ativadores transcricionais, vias enzimáticas envolvidas na biossíntese esteroidal e/ou metabólica, e numerosos outros mecanismos que convergem para sistemas endócrinos e reprodutivos **(Schug et al., 2011).**

Deste modo, os diferentes sistemas afetados pelos DEs incluem todos os sistemas hormonais que por sua vez controlam desde o desenvolvimento e função de órgãos reprodutivos até os tecidos e órgãos que regulam o metabolismo e saciedade **(WHO, 2012)**. Os efeitos colaterais nesses sistemas podem levar à obesidade, infertilidade ou fertilidade reduzida, diabetes durante idade adulta e doenças cardiovasculares, assim como uma variedade de outras doenças como mostrado na figura abaixo **(Barker; Bartfeld; Clevers, 2010)**.

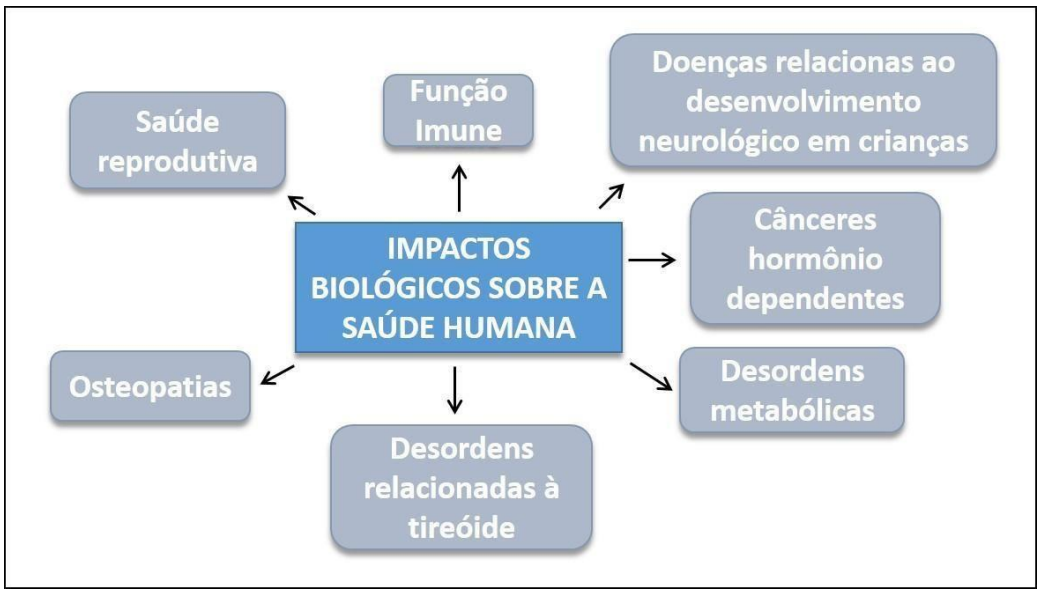


Figura 2. Diagrama "Impactos Biológicos dos Disruptores Endócrinos Sobre a Saúde Humana". Fonte: Adaptado de **Jones; De Voogt, 1998.**

4. BPA: 4, 4'-dihidroxi-2, 2-difenilpropano

Classificação Química

O Bisfenol A (BPA) ou 4, 4'-dihidroxi-2, 2-difenilpropano é um composto químico sintético, que apresenta estrutura química composta por 2 anéis de fenol conectados por uma ponte de metil, com 2 grupos de metil anexados à essa ponte **(Carlisle et al., 2009) (Figura 3)**. É utilizado na produção do policarbonato de Bisfenol A, o policarbonato mais comum, e de outros plásticos. A maioria dos produtos plásticos como por exemplo, utensílios de cozinha, mamadeiras, embalagens para alimentos cujo interior é envolvido com resinas do tipo epóxi, equipamentos esportivos, equipamentos e dispositivos médicos, certos polímeros utilizados como selantes dentários, CD´s, equipamentos eletrônicos, retardantes de chamas, tintas, adesivos, revestimentos protetores, vidros automotivos, proterores de vidro do tipo *insul films*, materiais de construção, discos compactos, lentes ópticas, papel térmico, revestimentos de papel como revelador em corantes e para encapsulação de peças elétricas e eletrônicas utilizam o BPA como parte de suas composições **(Carlisle et al., 2009)**. Tendo a sua produção mundial aproximada em 5 milhões de toneladas **(Vogel, 2009)**, o BPA é caracterizado como um disruptor endócrino químico, possuindo a capacidade de mimetizar o hormônio estrógeno no organismo **(Schug et al., 2011)**. Sintetizado pela primeira vez em 1891, o BPA foi utilizado na década de 1930 como medicamento pela descoberta de suas propriedades estrogênicas **(Vogel, 2009)**.

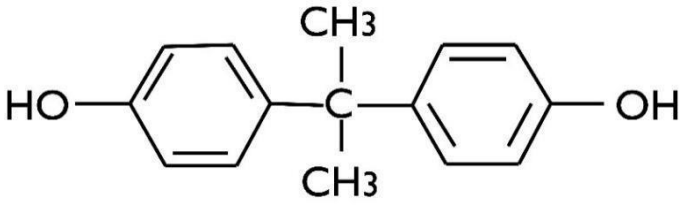


Figura 3. Molécula 4, 4'-dihidroxi-2, 2-difenilpropano. Fonte: University of North Carolina at Chapel Hill. http://bpatruth.web.unc.edu/about/.

Ação Metabólica

O BPA é capaz de se ligar a um agonista do receptor de estrógeno, embora com afinidade consideravelmente menor que o estradiol **(Kuiper et al., 1998)** e tem sido observado possuir atividade agonista e antagonista ao receptor de estrógeno em estudos realizados *in vitro* **(Sawai; Anderson; Walser-Kuntz, 2003)**. O BPA também possui a capacidade de se ligar ao receptor aril hidrocarboneto **(Bonefeld-Jørgensen et al., 2007)** e ao receptor para o hormônio tireoidiano **(Heindel et al., 2015)**, que possui papel adicional em inibir a atividade transcricional estimulada pela triiodotironina (T3) **(Moriyama et al., 2002)**. A exposição humana potencial ao BPA é 400 vezes menor do que o máximo aceitável ou dose referência que é de 0,05mg/kg de peso corporal. O BPA se liga à receptores de estrógeno, particularmente aos subtipos alfa, beta, gama e antagonistas aos receptores ativados por proliferador de peroxissomos (PPAR - *peroxisome proliferation agonist receptor*) **(Bonofiglio et al., 2005)**, como representado na **Figura 4**.

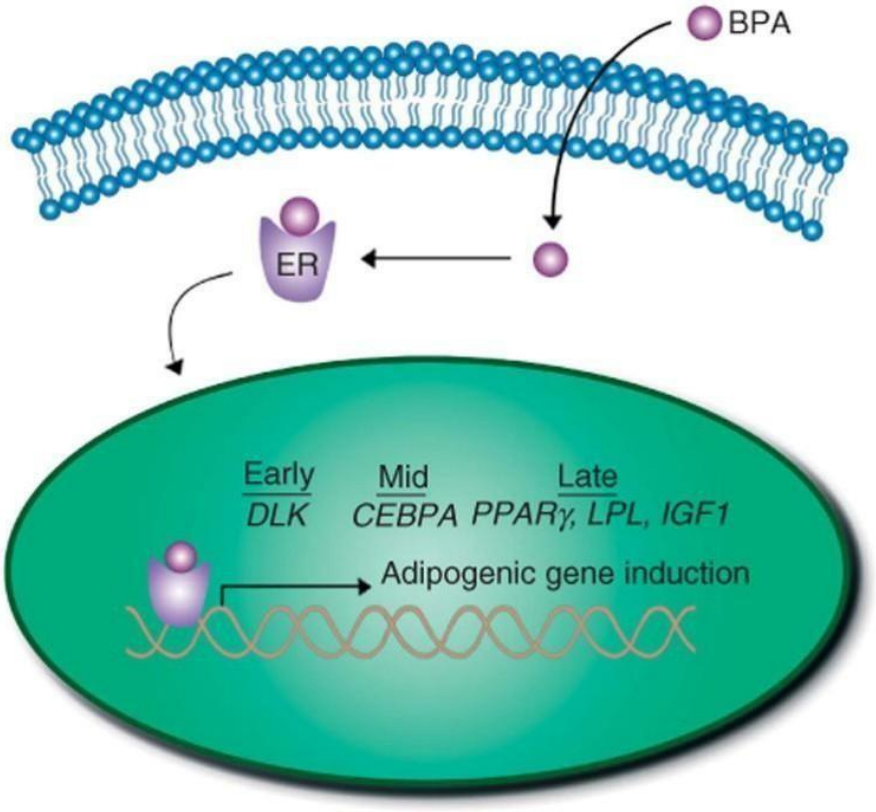


Figura 4. Representação dos Efeitos do BPA sobre a adipogênese em CTMs-TA. O diagrama ilustra o BPA adentrando a célula e então interagindo com o receptor ER, sendo translocado até o núcleo, onde ocorre a transcrição de genes chave no processo de adipogênese acelerando e aumentando dessa maneira o processo de diferenciação de CTMs-TA em adipócitos maduros. Fonte: **(Ohlstein et al., 2014).**

Aspectos Ambientais

O BPA foi usado primeiramente na fabricação de plásticos e produtos contendo tal composto sendo comercializados por mais de 50 anos. Hoje, o BPA é um monômero chave na produção de resinas epóxi **(Calafat et al., 2008)**. A contaminação ocorre primariamente através da dieta e ar, poeira e água também são possíveis fontes de exposição.

Desde a década de 1930 existe a suspeita de que o BPA seja prejudicial à saúde humana em relação à estrogenicidade **(Ashby et al., 2000)**. Em 2008, após vários estudos financiados pelo governo dos Estados Unidos questionarem a sua segurança, alguns varejistas retiraram das prateleiras produtos contendo BPA. Um estudo coordenado pelo FDA (*Food and Drug Administration*) de 2010 levantou questões preocupantes quanto à exposição de fetos, bebês e crianças pequenas a esse composto. Agências regulamentadoras dos Estados Unidos e Europa regurlarmente apresentam documentos

com normas instrutivas sobre o uso e manipulação de produtos contendo BPA **(EPA, 2010)**. A principal fonte de exposição humana ocorre através da ingestão de alimentos que foram armazenados ou reaquecidos em recipientes contendo BPA, mas dados recentes sugerem que há pelo menos alguma exposição da água potável, selantes dentários, exposição dérmica através de papel térmico e, em menor extensão, a inalação de partículas de poeira doméstica. Tal composto encontra-se amplamente difundido no meio meio ambiente, comprovado pelo fato de que mais de 90% dos indivíduos apresentem níveis detectáveis de BPA presentes na urina, fonte primária de excreção. **(Vandenberg et al., 2007;Calafat et al., 2008;Lee et al., 2008; Edginton; Ritter, 2009)**.

5. TCDD: 2,3,7,8-tetraclorodibenzo-para-dioxina

Classificação Química

O 2,3,7,8-tetracoloro dibenzo-p-dioxina (TCDD) é o composto de maior toxicidade pertencente à família dos hidrocarbonetos halogenados poliaromáticos que incluem as dioxinas ou compostos químicos organoclorados (policloradosdibenzo-dioxinas (PCDDs)), no qual o TCDD faz parte; os furanos (policloradosdibenzo-furanos (PCDFs)), bifenilos e naftalenos. Dos 210 congêneros de dioxinas, apenas 17 são tóxicos. O TCDD, é uma molécula que apresenta quatro átomos de cloro como ilustrado na **Figura 5 (Marinković et al., 2010)**. Trata-se de substância lipofílica que resiste à degradação biológica e ambiental, permanecendo no meio-ambiente.

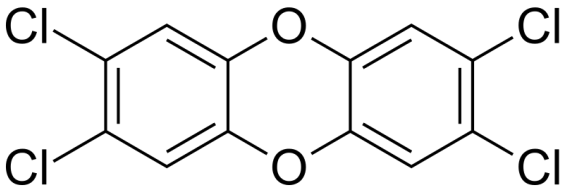


Figura 5. Molécula 2,3,7,8-tetraclorodibenzo-p-dioxina. Fonte: Wikipedia.org.

Ação Metabólica

Uma vez no organismo parte do TCDD é metabolizada e eliminada e parte é armazenada no tecido adiposo (bioaculumação). De modo geral, o armazenamento de POPs no organismo acredita-se ocorrer primariamente nos adipócitos **(Bourez et al., 2012)**, célula cujo citoplasma é praticamente composto por deposições de triglicerídeos **(Sbarbati et al., 2010)**. Tal composto é capaz de se ligar, com alta afinidade a um receptor citosólico conhecido como aril hidrocarboneto (AhR) **(Figura 7)**, responsável por mediar

respostas pleiotrópicas em vários tecidos e células alvo, levando a mudanças na expressão gênica, que por consequência podem alterar o metabolismo celular, diferenciação e proliferação **(Poland; Knutson, 1982; Hankinson, 1995)**. De forma geral, a transferência dos POPs através da corrente sanguínea a outros tecidos e órgãos, implica em fatores farmacocinéticos como volume tecidual, localização anatômica e fluxo sanguíneo como influenciadores na distribuição desses compostos até o tecido adiposo **(La Merrill et al., 2013)**. Desta forma, é corrente afirmar que os tecidos possuem fluxo sanguíneo total limitado (dependendo do tecido), o que significa que a distribuição dos químicos contidos na corrente sanguínea ocorre de forma mais eficiente nos compartimentos onde a vascularização é maior; portanto, não será homogênea no tecido total **(La merrill et al., 2013)**. A Organização Mundial de Saúde define como a ingestão tolerável diária (ITD) para o TCDD numa faixa que vai de 1 à 4 pg/kg/dia (picogramas por kilo de peso corporal por dia) para adultos. A Agência de Proteção Ambiental dos Estados Unidos (*EPA*) estima que a média da ITD nos Estados Unidos varia entre 0,5 à 1 pg/kg/pc/d.

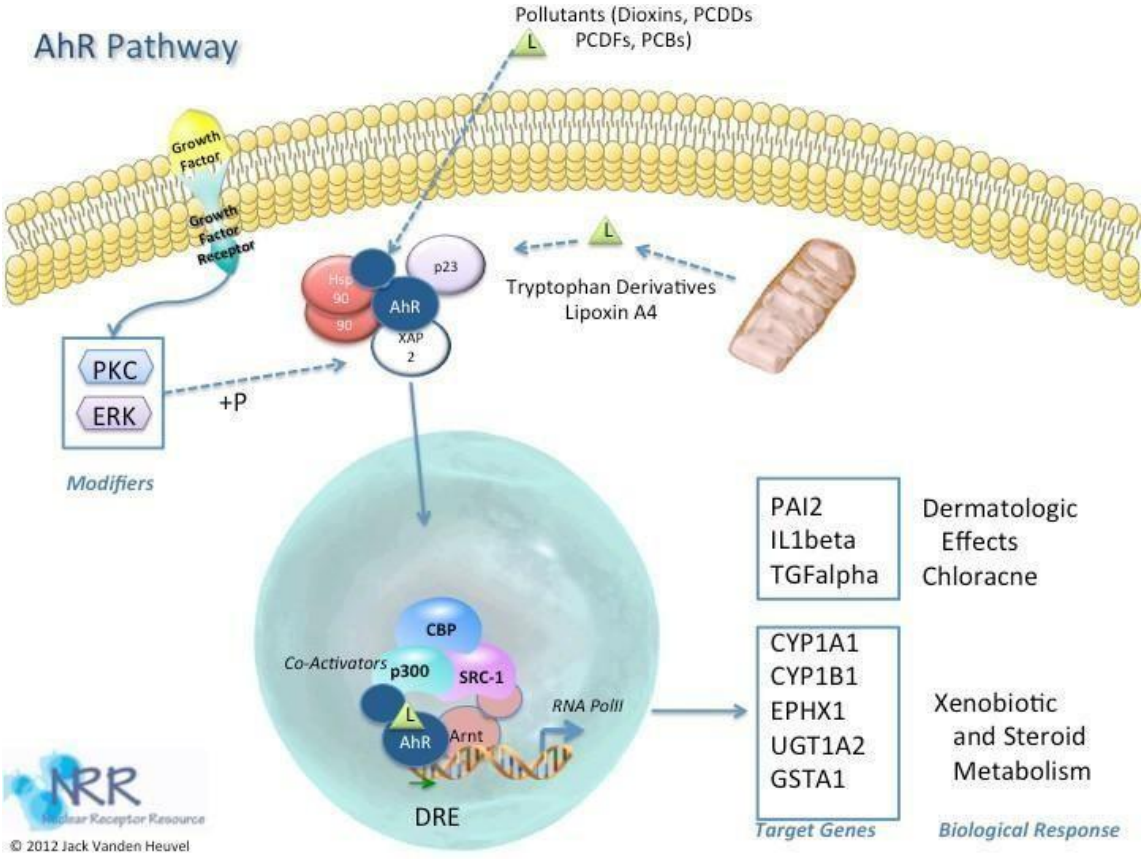


Figura 6. Esquema ilustrativo da via do AhR. Fonte: Nuclear Receptor Resource. http://nrresource.org/.

Aspectos Ambientais

O TCDD, assim como as demais dioxinas, são subprodutos de processos industriais como o branqueamento de papel e celulose, fabricação de certos pesticidas e da incineração de plástico e resíduos hospitalares **(Foster et al, 2010)**.Também é produzido como resíduos de processos de combustão naturais como na queima da madeira e atividade vulcânica **(WHO, 2010)**. A exposição humana ocorre através do consumo de alimentos contaminados, especialmente os ricos em gordura, como leite, queijo, carnes, produtos de *fast foods* e o leite materno **(Foster et al, 2010)**, bem como a inalação, absorção pela pele e água contaminada.

Quanto à toxicidade, o TCDD é classificado como carcinógeno humano, possuindo também efeitos não carcinógenos, como a indução de aterosclerose, hipertensão e diabetes **(Marinković et al., 2010)**. A exposição a longo prazo causa alterações nos sistemas nervoso, imune, reprodutivo e endócrino, enquanto que exposições agudas podem alterar a função hepática bem como causar cloracne (ácne severa por exposição ao cloro) **(Marinković et al., 2010)**.

O TCDD é o congênero pertencente ao grupo das dibenzo dioxinas policlorados de maior toxicidade. Tal composto pode ocorrer naturalmente na natureza, fruto de atividade vulcânica e combustão de madeira. Porém, a maior produção ocorre devido à atividade antrópica como por exemplo, em processos industriais, combustão incompleta de produtos contendo cloro resultantes de incineração, carvoarias, produção de pesticidas derivados de policlorofenois, produção de metais e principalmente no processo de clareamento da celulose para produção de papel **(Marinković et al., 2010, Fiedler, 2007)**.

Em 10 de julho de 1976, uma explosão numa fábrica de triclorofenol em Seveso na Itália, liberou aproximadamente 30kg de TCDD na área ao entorno, resultando na maior exposição conhecida em população humana **(Chevrier et al., 2014)**. Desde então, inúmeros estudos e programas de vigilância têm sido conduzidos na população remanescente concluindo inúmeros danos agudos, crônicos e doenças possivelmente relacionadas à exposição ao TCDD no que se refere à cloracne, desordens reprodutivas, desordens neurológicas e cânceres diversos **(Pesatori et al., 2003)**.

6. Poluentes Orgânicos Persistentes (POPs) e o Tecido Adiposo

Os POPs são compostos químicos tipicamente lipofílicos e hidrofóbicos, acumulando-se desta forma, preferencialmente no solo e em matéria orgânica e em menor

concentração em corpos aquáticos **(Jones; De Voogt, 1998)**. Como a maioria dos POPs não são facilmente biotransformados pelos organismos, quando em contato, esses poluentes tendem à bioacumularem-se em tecidos onde sua concentração aumenta de acordo com o nível da posição ocupada na cadeia alimentar, fazendo com que predadores de topo, como por exemplo os seres humanos, sejam os mais expostos **(Covaci et al., 2008; Verreault et al., 2008)**. Sendo assim, em organismos vivos, estes acumulam-se em altas concentrações no tecido adiposo, e em menores concentrações no fígado e pele **(Covaci et al., 2008)**. Vários grupos avaliaram de forma independente a ingestão diária total de alguns POPs em seres humanos e estimaram as taxas de ingestão de POPs variando de 400 à 1200 ng/dia, uma vez que os POPs são altamente lipofílicos (todos log *$Kow \geq 5$) **(Baars et al., 2004; Becker et al., 2006; Voorspoels; Covaci; Neels, 2008)**.

Kow*: coeficiente de partição octanol/água. É realizado tomando aproximadamente volumes iguais de octanol (um álcool "oleoso") e água, misturando-os juntos e adicionando o produto químico de interesse na mistura e homogeneizando. A proporção da quantidade no óleo para a quantidade que foi dissolvida na água é o coeficiente de partição octanol / água ou *Kow*. Este número indica se um produto químico é um lipofílico (que tem afinidade por gordura) ou hidrofílico (que tem afinidade por água) **(Jayjock et al., 2007).

Como é sabido, o tecido adiposo é altamente vascularizado, composto por vários tipos de células. Os adipócitos maduros diferenciados representam cerca de um terço de todas as células presentes no tecido adiposo, sendo o restante constituído pelas células estromal-vasculares incluindo células de músculo liso, células endoteliais, fibroblastos, macrófagos, monócitos, leucócitos e pré-adipócitos ou células-tronco ainda indiferenciadas **(Avram; Avram; James, 2005; Cinti, 2006; Vázquez-Vela; Torres; Tovar, 2008).**

Estudos recentes correlacionaram a presença de tais contaminantes em células do tecido adiposo causando possíveis alterações na regulação da homeostase energética em adipócitos **(Dirinck et al., 2011).** Tais estudos estão em acordo com outras pesquisas *in vitro* e em animais que sugerem que certos POPs podem alterar a via de sinalização da insulina e interferir no processo de adipogênese **(Piaggi et al., 2007; Kim et al., 2012)**. Dessa forma, têm sido recentemente demonstrado que alguns congêneros de POPs também são capazes de modificar determinadas vias de sinalização envolvidas na resposta imune/inflamatória, câncer e metabolismo, causando efeitos disruptores preferencialmente em precursores adipocitários do que em adipócitos maduros, sugerindo assim que células indiferenciadas talvez sejam mais sensíveis aos efeitos dos POPs do

que as já diferenciadas **(Kim et al., 2012; Bourez et al., 2012)**. Embora o tecido adiposo seja o reservatório de POPs de maior significância em organismos vivos, não existem ainda informações suficientes sobre os mecanismos de entrada desses compostos nas células do tecido adiposo **(Barouki, 2014; Müllerová & Kopecký, 2007).** De acordo com sua hidrofobicidade, acredita-se que os POPs atravessem a membrana celular por difusão passiva. No entanto, estudos toxicocinéticos considerando essas variáveis ainda são raros **(Bourez et al., 2012)**

II. JUSTIFICATIVA E RELEVÂNCIA DO TEMA

Os seres humanos estão expostos diariamente aos POPs presentes no meio ambiente. Considerando que ambos os compostos aqui apresentados são os disruptores endócrinos mais estudados e dois dos de maior relevância toxicológica, torna-se importante estudar os efeitos dessas substâncias lipofílicas sobre o potencial de diferenciação das células-tronco mesenquimais oriundas de tecido adiposo, células estas de grande aplicabilidade no campo da terapia celular e medicina regenerativa. Além disso, o presente estudo propõe-se a contribuir com o crescente interesse em estudos que visam as chamadas GMPs (*Good Manufacturing Practices*) em cultura celular para fins terapêuticos, práticas que tem como finalidade seguir a inúmeros e rígidos critérios para a obtenção de elevado nível de qualidade em terapia celular.

Desta maneira, este trabalho utilizou CTMs-TA humanas e de ratos, ambas advindas de culturas primárias. A utilização das células de ambas as espécies se deu pelo fato das CTMs humanas apresentarem taxa de amplificação lenta quando comparada às CTMs de rato, fato este comprovado pelo maior período necessário para a obtenção e montagem do banco de células humanas, quando comparado às células de rato.

Atualmente são escassos na literatura os dados científicos relativos à ação dos compostos BPA e TCDD sobre as CTMs (como mostra a tabela 3), o que justifica a importância de novos estudos e de revisões sobre essa temática.

Tabela 3. Resultado do levantamento bibliográfico realizado em Janeiro de 2018 no site de base de dados médicos e biomédicos PUBMED.

PALAVRAS-CHAVE	NÚMERO DE ARTIGOS	TIPO CELULAR	REFERÊNCIA BIBLIOGRÁFICA
Adipose Stem Cell AND Bisphenol A	7	1. Murine mesenchymal stem cell line C3H/10T1/2; 2. Human and mouse bone marrow primary stem cell culture and 3T3-L1 mouse cell line; 3. 3T3-L1 preadipocytes and human adipose stem cells (hASCs) cell line; 4. Human adipose stem cells (hASCs) primary culture; 5. Multipotent murine mesenchymal stem cells (C3H10T1/2) 6. Human Bone Mesenchymal Stem Cells primary culture (hBMSCs); 7. Human embryonic derived mesenchymal stem cells (hES-MSCs) and Human adipose stem cells (hASCs) primary culture;	1. Biemann et al, 2012 2. Chamorro-Garcia et al, 2012 3. Linehan et al, 2012 4. Ohlstein et al, 2014 5. Biemann et al, 2014 6. Leem et al, 2016 7. Wang et al, 2016
ASCs AND BPA	1	Human adipose stem cells (hASCs) primary culture;	Ohlstein et al, 2014
Adipose Stem Cell AND 2,3,7,8-tetrachlorodibenzo-p-dioxin	3	1. 3T3-L1 preadipocytes 2. adult neural stem/ precursor cells 3. Human adipose stem cells (hASCs) primary culture;	1. Liu et al, 1996 2. Fernández et al, 2010 3. Kim et al, 2012
ASCs AND TCDD	0		

III. HIPÓTESE

Considerando estudos prévios e recentes sobre o tema, a presente hipótese aqui apresentada é a de que o BPA e o TCDD possam alterar os processos de diferenciação adipogênica e osteogênica das CTMs-TA quando expostas à diferentes concentrações.

IV. OBJETIVO GERAL

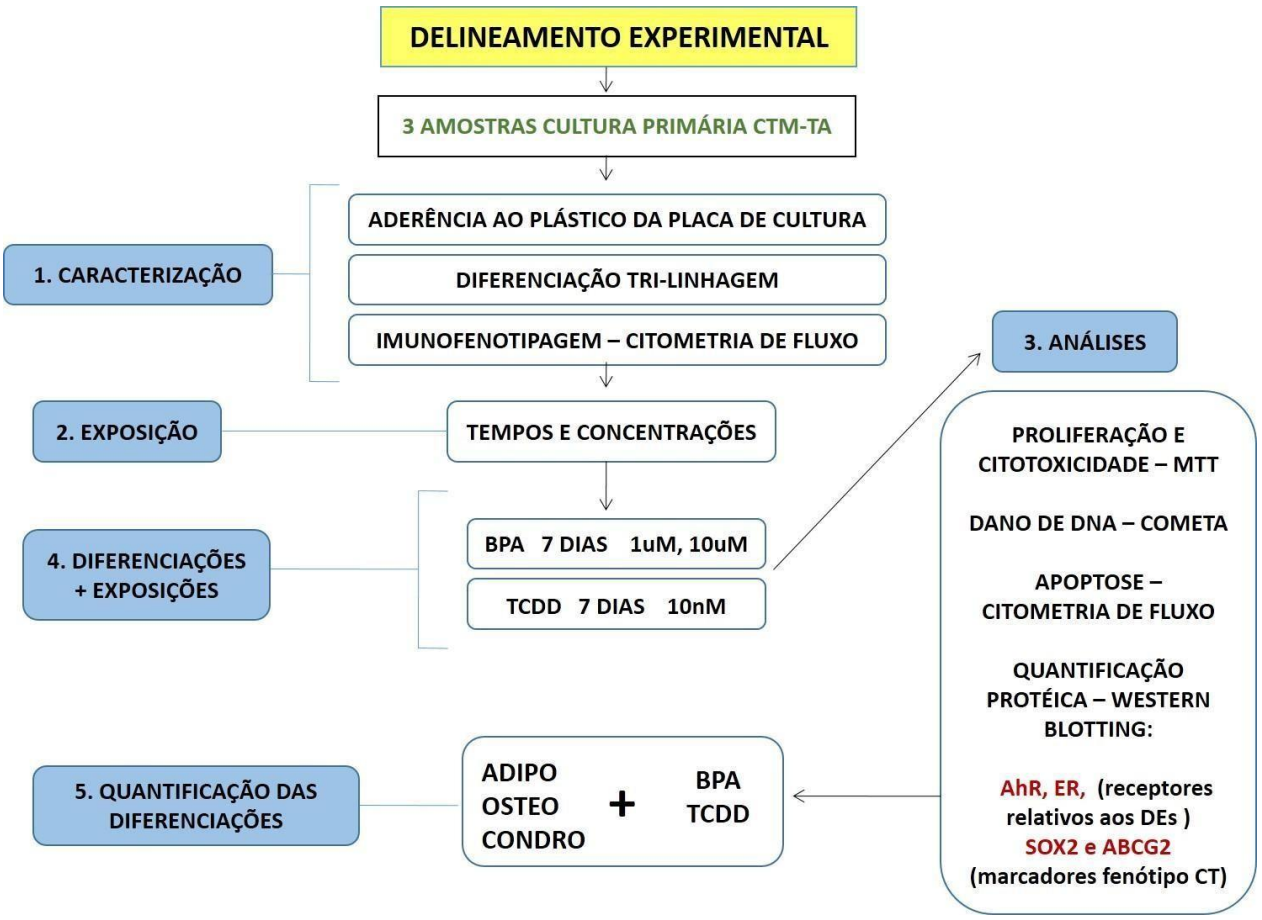
O presente projeto de doutorado teve como objetivo geral testar o potencial de diferenciação das CTMs-TA na presença dos disruptores endócrinos BPA e TCDD através de análises multiparamétricas.

V. OBJETIVOS ESPECÍFICOS

Os objetivos específicos foram:

- ✓ Coletar, isolar e amplificar CTMs-TA de amostras de tecido adiposo adulto humano e de ratos Wistar.
- ✓ Estabelecer um banco celular em condições de criopreservação;
- ✓ Caracterizar tais amostras utilizando kits de diferenciação tri-linhagem e imunofenotipagem para marcadores específicos de membrana;
- ✓ Monitorar a taxa de proliferação e de morte celular das amostras expostas ao BPA e ao TCDD e seus controles;
- ✓ Analisar as possíveis taxas de apoptose/necrose por citometria de fluxo quando as amostras expostas
- ✓ Analisar a possível genotoxicidade dos compostos através do Ensaio do Cometa
- ✓ Avaliar a expressão proteica do receptor para estrógeno (ERβ) e arilhidrocarboneto (AhR) e das proteínas ABCG-2, SOX-2 e BAK das CTMs-TA expostas aos disruptores endócrinos.

VI. FLUXOGRAMA DO DESENHO EXPERIMENTAL



VII. REFERÊNCIAS DA INTRODUÇÃO

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Resultados

Os resultados obtidos nesta tese estão divididos em 3 capítulos apresentados na forma de artigos científicos:

Capítulo I: *Artigo de Revisão*
"Bisphenol A and Mesenchymal Stem Cells: Recent Insights".

Capítulo II: *Artigo Original*
"Human Adipose-derived Mesenchymal Stem Cells Exposed to BPA and TCDD: A Protein Assessment study".

Capítulo III: *Artigo Original*
"BPA and TCDD Action on Rat Adipose-derived Mesenchymal Stem Cells (rASCs) Differentiation Potential".

Capítulo I: Review

BISPHENOL A AND MESENCHYMAL STEM CELLS: RECENT INSIGHTS

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ABSTRACT

Mesenchymal stem cells (MSCs) are found in all adult mesenchymal tissues, presenting a relevant role in the maintenance of homeostasis and repair in cases of tissue injury through the renewal of the cellular stock and can be isolated from human and animal sources. MSCs has been implicated in regenerative medicine and cell therapy and, in this sense, adipose tissue is a rich and promising source of these cells. The adipose-derived stem cells (ASCs) are actually often effective and safe, used in preclinical and clinical studies for both autologous and allogeneic transplantation, for example. In this way, the potential use of stem cells based therapies for the repair and regeneration of various tissues and organs provides important contribution to alternative therapeutic solutions to a large number of diseases. However, it is necessary to have control of all processes surrounding cell manipulation prior to its use. Exposure of humans to the endocrine disrupter bisphenol A (BPA) has been associated with increased weight and obesity, but the mechanisms by which BPA increases adipose tissue in humans remains to be determined. It is known that BPA is classified as a potent endocrine-disrupting chemical for interfering with adipogenesis. Currently, few studies report the action of BPA on the integrity and capacity of MSCs differentiation. Thus, this review aims to present a survey and discussion of the main results of BPA action on MSCs currently presented in the literature.

Keywords: mesenchymal stem cells, adipose derived stem cells, endocrine disrupting chemicals, bisphenol A and regenerative medicine.

INTRODUCTION

Mesenchymal Stem Cells

MSCs are a heterogeneous cell population that comprises different progenitors possessing the ability to repair tissues, support hematopoiesis, and regulate immune and inflammatory responses **(De Luca et al. 2017)**. These precursors can give rise to a variety of cell types, including adipocytes, osteoblasts, chondrocytes, myocytes, β-pancreatic islets cells and, potentially, neuronal cells **(Hashemian et al. 2015; Almalki & Agrawal, 2016; Gao et al. 2016)** and they can be found virtually in all tissues **(Tuan et al. 2003)**. This population were first identified and isolated from bone marrow more than 40 years ago **(Friedenstein et al. 1966)**. In 2006, when discussing the characteristics of MSCs, the International Society of Cellular Therapy established three minimum biological parameters to better identify these kind of cells, that is:(i) plastic adherence in *in vitro* standard culture conditions;(ii) expression of cluster of differentiation (CD) CD105, CD73, and CD90 and no expression of CD45, CD34, CD14 or CD11b, CD79a, or CD19 and HLA-DR (human leukocyte antigen–antigen D related) surface markers; (iii) *in vitro* differentiation to osteoblasts, adipocytes, and chondrocytes **(Dominici et al. 2006; Kim et al. 2013; Yu et al. 2014)**. MSCs can be expanded *in vitro* by consecutive passaging without significant alteration of their major properties **(Bernardo & Fibbe 2013)**. It also can release chemokines and cytokines exerting paracrine effects **(De Luca et al. 2017)**. For these reasons, this population have been extensively studied and analyzed to serve as relevant tools in treating many types of diseases.

Adipose derived stem cells

As previously stated, MSCs can be found in practically all tissues. However, until the year of 2000, adult stem cell lines seemed to be derived exclusively from hematopoietic tissue, mesenchymal tissues, neural stem cells and muscle satellite cells. In 2001, a group from the University of California in Los Angeles (UCLA) discovered a stem cell population derived from adipose tissue. Due to their isolation from human lipoaspirates, they were first termed "processed lipoaspirate cells", but they are now called "adipose-derived stem cells" (ASCs). The current term is more descriptive, as they proposed that ASCs was a multilineage stem cell population that could be isolated from the stromo-vascular fraction of adipose tissue **(Zuk et al. 2001)**.

In addition, to prove the ability of ASCs to be a multilineage cell population, Zuk

and co-authors (2001), utilizing additional approaches such as the expression of multiple lineage-specific genes and functional biochemical assays, confirmed that both differentiation capacity and clonogenicity are important requirements for ASCs characterization. When considering this issue, one of the main challenges with adult stem cell identification is the heterogeneity of their tissue of origin. The observed multilineage differentiation by ASCs may simply be due to the presence of multiple precursor populations, each completing their development **(Klimczak & Kozlowska, 2016)**. Therefore, Zuk group found a way to circumvent this problem by isolating a stem cell and combining this with proof of the cell multipotency. By doing this, they demonstrated differentiation capacity and clonogenicity, which proposed a new adult stem cell population **(Zuk et al. 2001)**. Since 2002, many groups have confirmed this proposal in both human and animal ASCs populations **(Klimczak & Kozlowska, 2016)**. Additionally, these cells also have the ability to differentiate into neuronal-like cells, which has been confirmed by numerous studies since it was first discovered **(Safford et al. 2002; Ashjian et al. 2003)**.

For this reason, the use of adipose tissue-derived precursors as a therapeutic tool has grown considerably in the past years, and has trigged the growth of a new research field and industry worldwide **(Bourin et al. 2014)**. In this way, regenerative medicine has evolved tremendously with recent advances in stem cell research. The last decades have shown flashes of the astonishing potential of these cells in tissue regeneration **(Law & Chaudhuri, 2013)**. Despite these advances, the availability of stem cells remains a challenge for both scientists and clinicians with a real interest in regenerative medicine **(Wankhade et al. 2016)**. ASCs main advantage over mesenchymal stem cells derived from other sources, e.g. from bone marrow, is that they can be accessible and repeatable harvested using minimally invasive techniques with low morbidity **(Frese et al. 2016)**. Still, the ideal stem cell population should be present and accessible in abundant numbers, harvestable by a relatively noninvasive procedure, able to differentiate into a variety of cell lineages, easy to transplant to an autologous or allogeneic host, and able to be manufactured in accordance with the currently accepted good manufacturing practice guide lines set by the FDA **(Gimble et al. 2010)**.

Furthermore, according to many studies it is known that our environment is contaminated with different chemical substances that can intrinsically alter the homeostasis and physiology of biological systems, leading to significant impact on human and animal health **(Muncke, 2009; Kirkley & Sargis, 2014)**. Following this line

of thinking, there are some organic compounds that are obesogenic, possessing the capacity to bind to hormone receptors and to accumulate in the fat tissue, besides cause weight alterations in all organisms **(Janesick & Blumberg, 2016)**. These molecules could as well impact/alter the stem cell biology, impair their differentiation efficiency and compromise their therapeutic use as recently reviewed for MSCs **(Bateman et al. 2017)**. In this work we will focus the attention on the effects of BPA on MSCs.

Obesogens

Obesogens are chemical compounds that can boost weight-gain by altering the number of adipocytes, increasing their ability to store fat, or by modifying several homeostatic processes **(Janesick & Blumberg, 2016)**. These processes include the amount of calories burned at rest, the energy balance to favor storage of calories and the mechanisms through which the body manages appetite and satiety **(Janesick & Blumberg, 2012)**.The obesogen hypothesis has gained visibility in recent years with the identification of obesogenic chemicals that promote adipogenesis and obesity in animals and humans **(Newbold et al. 2009; Merrill & Birnbaum, 2011; Tang-Péronard et al. 2011; Janesick & Blumberg, 2012)**. Some of these compounds act through the mimicking of endogenous hormones like estrogens, testosterone, and thyroid among others.

Endocrine Disruptors Chemicals

Endocrine disruptors chemicals (EDCs), are compounds present in our environment, food, and consumer products that interfere with the biosynthesis, metabolism, and action of hormones resulting in alterations of normal homeostatic control or reproduction **(Diamanti-kandarakis et al. 2009)**. EDCs are produced as pesticides, plasticizers, or solvents. It has been found that when they are absorbed into the body they can either mimic or block hormones and disrupt the normal functions of the organism **(Schug et al. 2011)**. Therefore, they were initially thought to exert their actions solely through nuclear hormone receptors, including estrogen receptors (ERs), androgen receptors (ARs), progesterone receptors, thyroid receptors (TRs), and retinoid receptors, among others **(Diamanti-kandarakis et al. 2009)**.

These substances are typically hydrophobic and lipophilic, which means that they work best in an environment that has a low concentration of water and a lot of fatty acids. In aquatic systems and soils, EDCs are easily degraded to solids, while in organisms, they

are partitioned into lipids. By doing this, the chemicals avert an unfavorable aqueous phase where they accumulate in cells and fatty tissue. This confers persistence on the chemical in biota since metabolism is slow and EDCs may therefore amass in food chains **(Jones & De Voogt 1998).**

Bisphenol A

Bisphenol A (BPA) is a synthetic chemical that, because of its structure, is multifaceted. The BPA (4, 4'-dihydroxy-2, 2-diphenylpropane) molecule constitutes two phenol rings connected by a methyl bridge, with two methyl groups bound to the bridge **(Carlisle et al. 2009)**. BPA is also an endocrine disruptor, resulting in the activation of estrogen receptors a(ERa) and b(ERb) **(Wozniak et al. 2005; Welshonsetal. 2006; Le et al. 2008; Kim et al. 2012; Chamorro-garcía et al. 2012)**. Furthermore, recent studies have demonstrated that the G protein-coupled receptor-30 (GPR-30), a transmembrane receptor structurally unrelated to the nuclear ERs, mediates rapid actions of estrogens and xenoestrogens, as BPA (Wang et al, 2017).

BPA is used in the fabrication of polycarbonate plastic and epoxy resins, which can be used in impact-resistant safety equipment and baby bottles, as protective coatings inside metal food containers, and as composites and sealants in dentistry **(Calafat et al. 2008)**.

This compound is one of the highest-volume chemicals produced globally with >8 billion pounds produced each year **(Vandenberg et al. 2010)**. Because of its mass production and widespread adoption, the probability of environmental contamination with BPA has increased. Environmental evictions are possible via industrial wastewater treatment systems or sewage treatment plants that receive this compound. Other possible sources of BPA are found in the environment, such as waste plastics in waste landfills and sewage sludge from wastewater treatment facilities. There is a considerable amount of monitoring data on BPA in Europe, United States, and Japan, and certain levels of BPA have been detected in many different biologic samples **(Yamamoto et al. 2001; Cousins et al. 2002)**. BPA can be detected in human blood, plasma, urine, saliva, amniotic fluid, placental tissue, follicular fluid, breast milk, and adipose tissue **(Vandenberg et al. 2010)**. More than 90% of people tests positive for BPA in urine and blood **(Heindel et al. 2015)**, and infants and children are the most affected **(Calafat et al, 2008)**. Some studies have demonstrated that BPA is detected in amniotic fluid **(Ikezuki et al. 2002; Engel et al. 2006; Corrales et al. 2015)**, and that BPA levels are eight times higher in early pregnancy than in later pregnancy **(Ikezuki et al. 2002)**, at concentrations ranging from 0.5 nM to 40

nM **(Ikezuki et al. 2002; Welshonsetal. 2006; Calafat et al. 2008)**.

Data from multiple sources show that the amount of BPA that humans are exposed to may cause adverse health effects, among others diabetes, obesity, abnormal neurobehavior, developmental effects, thyroid and reproductive dysfunctions, etc **(Chevrier et al. 2013, Porreca et al. 2017, Rochester et al. 2013).** This has raised concerns among regulatory agencies all over the world **(Vandenberg et al. 2010)**.

The effects of BPA on human health have also been a concern, and mutagenicity by BPA was found in human RSa cells (human embryonic clonal cell line established by double infection with Rous sarcoma virus and Simian virus 40, and HeLa cells) **(Suzuki & Fuse 1981)** within the range of 10uM to 100nM **(Takahashi & Oishi, 2001)**. Even though the direct genotoxic activity of low-dose BPA has not been reported, exposure to BPA at environmental relevant dose, primarily during the developmental stages, represents a risk for carcinogenesis **(Cuomo et al. 2017)**. Thus, BPA has become an environmental contaminant of considerable interest **(Cousins et al. 2002)**.

The mechanism of action of BPA is associated with its shared homology with estrogen and the up-regulation of downstream targets, including peroxisome proliferator-activated receptor gamma (PPARg (PPARG)) and lipoprotein lipase (LPL) genes, based on results from in vitro experiments on rodents **(Melzer et al.2011)**. BPA can still activate GPR30 receptor modulating, for example, ERK and AKT pathways, which are responsible for survival and cell death processes **(Wang et al. 2017)**.

Effects of BPA on MSCs

The endocrine-disrupting chemical bisphenol A has been shown to accelerate the rate of adipogenesis as seen in **Masuno et al.** study **(2005)** where BPA (80uM) induced adipocyte differentiation and adipogenic marker genes in 3T3-L1 preadipocytes (a cell line derived from mouse) during six days. BPA also increased the amount of triglyceride accumulation during 14 days of differentiation in 3T3-L1 preadipocytes. However,when tested the same conditions (80uM during 14 days) in human primary preadipocytes, such data were not similar for human cells **(Linehan et al. 2012)**. Based on these discrepant results we will here present the majority of findings related to MSCs exposed to the BPA endocrine disruptor during *in vitro* differentiation.

MSCs, especially adipose stem cells are a useful model for studying changes in the programming of adipogenesis because they are the cells that give rise to adipocyte progenitors *in vivo* **(Avram et al. 2005)**. Based on this assumption, few studies have

looked at the relationship between the differentiation potential of different MSCs when exposed to BPA. The concern about the possible contamination of these cells by endocrine disruptors began with the adoption of Good Manufacturing Practices (GMPs) in the process of producing MSCs for therapeutic purposes. Thus, recently, groups such as **Biemann** and colleagues **(2012)** were one of the first to study the endocrine-disrupting chemicals that can affect the adipogenic differentiation of MSCs. Therefore, they showed that BPA and other EDCs such as DEHP (bis (2-ethylhexyl) phthalate) and TBT (Tributyltin) affect the adipogenic differentiation of murine mesenchymal stem cells (MSC, C3H/19T1/2) in concentration, stage, and compound-specific manner. During 6 and 14 exposure days, BPA (10uM) decreased subsequent adipogenic differentiation of MSCs when cells were exposed during undifferentiated growth. **Chamorro-garcía** and colleagues **(2012)** also used bone marrow primary culture of MSCs and 3T3-L1 cells to study the adipogenic capacity of BADGE (Bisphenol A diglycidyl ether) and BPA and evaluated their effects on adipogenesis, osteogenesis, gene expression, and nuclear receptor activation. They found tha tBPA (1nM, 10nM, 100nM, 1uM) during 14 days of exposure failed to promote adipogenesis in MSCs, but induced adipogenesis in 3T3-L1 cells, which was the same outcome that was found in Linehan´s study. In 2014, Biemann and colleagues conducted another study with multipotent murine mesenchymal stem cells (C3H10T1/2) that were exposed to EDC mixtures in high concentrations, i.e. MIX-high (10 μmol/l BPA, 100 μmol/l DEHP, 100 nmol/l TBT), and in environmentally relevant concentrations, i.e. MIX-low (10 nmol/l BPA, 100 nmol/l DEHP, 1 nmol/l TBT). The exposure was performed either for the entire culture time (0–12days) orat distinct stages of adipogenic differentiation. Results showed that MIX-high increased the development of adipocytes and the expression of adipogenic marker genes independently of the exposure window. The total amount of glyceride content was not increased. The low-concentrated EDC mixture had no obvious impact on adipogenesis. They found that in EDC mixtures, the adipogenic effect of TBT and DEHP predominates single effects of BPA. Mixture effects of EDC are not deducible from single compound experiments.

When talking ASCs, **Ohlstein et al. 2014** determined the effects of BPA on adipogenesis of cultured human ASCs, which are precursors to mature adipocytes. Cells were exposed to increasing concentrations of BPA (100pM–10uM). BPA significantly enhanced adipogenesis at a concentration of 1uMafter 21 days of culture. Additionally, we found that BPA increased transcription of the estrogen receptor (ER (ESR1)) and that treatment with the ER antagonist ICI 182780 blocked the effects of BPA, indicating that

BPA may act via an ER-mediated pathway. In 2016, Leem and coleagues, conducted a study where they analysed if high BPA concentrations (250uM, 500uM during 18h) has cytotoxic action on human bone marrow MSCs; and they found that this compound has a disturbing action on cateninin b-catenin signaling via a superoxide anion overload. In the same year, **Wang et al**, developed a 3D model to study the BPA action on human embryonic MSCs and also to compare these results with ASCs. They found that BPA didn´t induce adipogenesis on ASCs and didn´t presented significant effect on embryonic MSCs. Table 1 presents a summary of all of these results.

Table I: Summary of the main results of the published scientific work involving the key words: Adopose Stem Cell and Bisphenol A.

Title/Author/Year	Cell Type	Objectives	Results
Endocrine disrupting chemicals affect the adipogenic differentiation of mesenchymal stem cells in distinct ontogenetic Windows. **Biemann et al, 2012**	C3H/10T1/2 mouse MSCs embryosarcoma cells CGR8 mouse embryonic stem cells.	To analyse of BPA,DEHP and TBT affect the adipogenic differentiation of C3H/10T1/2 and CGR8cells in a concentration-, stage- and compound-specific manner.	BPA (10 uM) decreased subsequent adipogenic differentiation of C3H/10T1/2, when cells were exposed during undifferentiated growth DEHP (100 uM) during the hormonal induction period,and TBT (100 nM) in all investigated stages, enhanced adipogenesis.
Bisphenol A Diglycidyl Ether Induces Adipogenic Differentiation of Multipotent Stromal Stem Cells through a Peroxisome Proliferator–Activated Receptor Gamma-Independent Mechanism. **Chamorro-Garcia et al, 2012**	Mouse bone marrow MSCs (Invitrogen Carlsbad, CA) Human bone marrow mononuclear cells (Lonza Walkersville, MD) 3T3-L1 mouse pre-adipocyte cells.	To study the adipogenic capacity of BADGE and BPA and evaluated their effects on adipogenesis, osteogenesis, gene expression, and nuclear receptor activation.	BADGE induced adipogenesis in human and mouse MSCs, as well as in mouse 3T3-L1 preadipocytes; BPA failed to promote adipogenesis in MSCs, but induced adipogenesis in 3T3-L1 cells; Neither BADGE nor BPA activated or antagonized retinoid “X” receptor (RXR) or PPARγ in transient transfection assays.

Bisphenol A-Mediated Suppression of LPL Gene Expression Inhibits Triglyceride Accumulation during Adipogenic Differentiation of Human Adult Stem Cells. **Linehan et al, 2012**	hASCs Human adult stem cells (Zen-Bio NC, USA).	To study if BPA can accelerate the rate of adipogenesis and increase the amount of triglyceride accumulation of hASCs.	BPA (0.08uM, 8uM, 80uM) during 14 days of differentiation dramatically reduced triglyceride accumulation and suppressed gene transcription of the lipogenic enzyme, lipoprotein lipase (LPL). BPA can reduce triglyceride accumulation During adipogenesis by attenuating the expression of LPL gene transcription.
Bisphenol A enhances adipogenic differentiation of human adipose stromal/stem cells. **Ohltein et al, 2014**	hASCs Human adipose stem cells.	Determine the effects of BPA on adipogenesis of cultured human adipose stromal/stem cells (ASCs), precursors to mature adipocytes.	BPA(1mM) significantly enhanced adipogenesis after 21 days of culture. BPA increased transcription of the estrogen receptor (ER) and the treatment with the ER antagonist ICI 182 780, blocked the effects of BPA, indicating that BPA may act via an ER-mediated pathway.

Adipogenic Effects of a Combination of the Endocrine-Disrupting Compounds Bisphenol A, Diethylhexylphthalate, and Tributyltin. **Biemann et al, 2014**	C3H/10T1/2 mouse MSCs embryo sarcomacells.	To investigate the effects of a simultaneous exposure of BPA, DEHP, and TBT on mesenchymal stem cell differentiation into adipocytes.	BPA(10 µmol/l), DEHP(100 µmol/l) and TBT (100 nmol/l) increased the development of adipocytes and the expression of adipogenic marker genes independently of the exposure window. BPA(10 nmol/l), DEHP(100 nmol/l) and TBT(1 nmol/l) had no obvious impact on adipogenesis during 12 days.
BPA-Toxicity via Superoxide Anion Overload and a Deficit in b-Catenin Signaling in Human Bone Mesenchymal Stem Cells. **Leem et al, 2016**	hBMSCs human bone marrow MSCs.	To verify if BPA (250uM, 500uM during 18h) has cytotoxic action.	BPA causes a disturbance in b-catenin signaling via a superoxide anion overload.
Development of a Three-Dimensional Adipose Tissue Model for Studying Embryonic Exposures to Obesogenic Chemicals. **Wang et al, 2016**	hASCs Human Adipose Stem Cells hES Human embryonic MSCs.	To develop a 3D human tissue system that is able to model the effects of obesogens (BPA-10uM, 20uM, 40uM) (TBT - 1nM,5nM, 10nM), (BPS - 10uM, 20uM, 40uM) in vitro in order to better understand the impact of obesogens on early development.	hASCs were not induced to adipogenesis and hES didn´t show any significant effect when exposed to these compounds.

DISCUSSION

MSCs show promise in the field of regenerative medicine because they can modulate numerous incurable diseases **(Pittenger et al. 2016)**. Currently, there are 333 published studies conducted with MSCs in clinical trials, such as cardiovascular diseases **(Faiella & Atoui, 2016)**, diabetic nephropathy **(Liu & Tang, 2016)**, diverse brain injuries (including stroke, neural trauma, heatstroke) **(Hsuan et al. 2016)** and many others. The importance of these cells in cell therapy is enormous. For this reason, they are widely tested because of their multiple biological functions, including multilineage differentiation, tissue-repair promotion, anti-inflammatory mediation and immunosuppression. Since 2000, the MSC field has moved rapidly with the demonstration that ex vivo-grown had immuno suppressive properties after treatment with inflammatory cytokines such as IFN-γ, IL-1, orTNF-α **(Krampera et al. 2006)** and acted on all effectors of innate and adaptive immunity **(Ménard & Tarte, 2013)**.

When talking about their clinical properties these cells afford several advantages, such as availability and easy of harvesting; safety with very low possibility of malignant transformation after infusion of allogeneic cells, which is common in the case of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs); and the lack of ethical issues that occur with the application of human ESCs **(Kim & Park, 2017)**.

However, it is well known that this new and exciting branch of stem cell biology presents hurdles of unexplained issues that have yet to be overcome. For example, in terms of the mechanisms underlying MSC biological functions, it was originally thought that cells originating from damaged tissue differentiate and replace damaged cells **(Yuet al., 2014)**. Nonetheless, subsequent research showed that MSC engraftment and differentiation at injury sites are very low and transient **(Katsha et al., 2011)**. Therefore, for successful cell-based therapies, a significant number of cells are needed, requiring extensive ex vivo cell expansion. Owing to prolonged ex vivo expansion needed in the clinic to obtain a sufficient number of cells for therapy, long-term culture will likely evoke continuous changes in MSCs, including cellular senescence **(Parketal.2005; Younget al. 2013)**. Besides this, other concerns impose limitations in MSCs clinical use such as the safety, efficacy, and reproducibility of MSC production, donor eligibility and screening, facilities, environmental controls, and storage **(Sensebé et al., 2013)**.

Taking into consideration the environmental control, one such important sub issue is the toxicological quality of cells. It is well known that EDCs exert a strong influence on exposed organisms, which can change both organs and systems. This subsequently leads to the development of short or long-term diseases. So, producing cells according to Good

Manufacturing Practices (GMPs) is aglobal challenge for the production of all cells for use in humans **(Sensebé et al., 2013)**.

As has been shown, there are very few studies conducted using MSCs from different sources and species exposed to BPA that have, to date, demonstrated that in all of them, cells were somehow affected mainly into their adipose differentiation process. Nonetheless, different authors demonstrated miscellaneous results leading to think that such findings may possibly be explained by the fact that different cells from different animals certainly will respond in a variable way. Concentrations and exposure times caused into these cells particular results, what is in fact very understandable. Another relevant concern is the concentrations used. Because most of the studies exposed the cells to extremely high concentrations that surely doesn´t mimic the real environment. Therefore, until we have more studies taking into account the same cell types and using the same concentrations and times of exposure, only then we can reach more solid conclusions regarding the biochemical and molecular effects of BPA on MSCs.

Considering the strengths and weaknesses of MSCs in ex vivo cultures would provide us with some novel approaches for overcoming limitations to their therapeutic efficacy and maximize their clinical value. In summary, it is possible to conclude that diverse populations within the heterogeneous group of MSCs **(Phinney & Prockop, 2007)** can exhibit divergent behaviors when exposed to BPA or others EDCs. Studies that are more systematic are needed to characterize these behaviors.

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Capítulo II: Original Article

Human Adipose-derived Mesenchymal Stem Cells Exposed to BPA and TCDD: A Protein Assessment study

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Abstract

Human adipose derived mesenchymal stem cells (hASCs) are a cell population with some properties of self-renewal and multi differentiation potential. Nowadays, these populations are been using in regenerative medicine as a tool to restore tissues and whole organs. In these context, the so-called Good Manufacturing Practices (GMPs) emerge as a new approach. This is used in cell culture for therapeutic purposes, following numerous and strict criteria for obtaining high level of qualityin cell therapy. A concern within quality control in cell therapy, is the chemical contamination. Endocrine disrupting chemicals (EDCs) are xenobiotics that mainly mimics the action of some endogenous compounds. BPA has been shown to mimic estrogen receptors and also can cause adipogenesis dysfunction, among others. TCDD has affinity with aryl hydrocarbon receptor (AhR) and has long been known to interferes through estrogen and androgen signaling pathways. So, this study aimed to better understand the effects of these EDCs on proteins related to stem cell phenotype and specific receptors binding sites. For this, hASCs were exposed to 1uM - 10uM of BPA and 10nM of TCDD. MTT assay were performed to access cell cytotoxicity and Western Blot assays to quantify ABCG2, BAK, SOX2, ERβ and AhR proteins. BPA (1uM) was responsible for the least mitochondrial dehydrogenase reducing activity compared to control [0.562 to 0.376] and TCDD had no significant effect on mitochondrial activity. ABCG2 quantification showed a 1.95 fold higher when cells were exposed to 10nM of TCDD. AhR quantification showed a 1.16 fold higher when cells were exposed to 10nM of TCDD. For BAK, SOX2 and ERb quantifications, no difference was seen between the exposures or groups analyzed. There are very few studies conducted using hASCs exposed to BPA and TCDD and we found that hASCs were affected somehow by these compounds. So, it is possible to conclude that 1uM of BPA has toxic activity to hASCs strengthening the findings that low doses has harmful action than higher doses. This is important to note, because BPA in low doses can possibly interefe in intrisic hASCs functions. Besides this, TCDD also exhibit ABCG2 increased expression in these cells, which indicates other hASCs alterations by such compound. In addition, TCDD was able to increase AhR expression, endorsing existing data.

Key words: hASCs, cell therapy, GMPs, BPA, TCDD.

Introduction

hASCs are a mesenchymal stem cell population with some properties of self- renewal and multi differentiation potential. These cells can develop into adipocytes **(Halvorsen et al., 2001),** osteoblasts **(Huang et al., 2017),** chondrocytes **(Estes et al., 2006)**, myocytes **(Choi et al., 2010)**, neurocytes **(Choi et al., 2012)**, and other cell types **(Lindroos et al, 2011)**. Compared to other stem cells populations, hASCs have two main advantages: it can be easily accessible from subcutaneous liposuction in large numbers **(Baer & Geiger, 2012)** and also have no ethical and political issues compared to embryonic stem cells because they can be derived from autologous fat **(Simonacci et al., 2017).** These two characteristics make these cells become a more acceptable solution for tissue and organ transplantation in regenerative medicine and clinical studies **(Gimble et al., 2010).**

Organ and tissue loss through disease and injury triggers the development of therapies that can regenerate tissues and decrease reliance on transplantations. Regenerative medicine, an interdisciplinary field that applies engineering and life science principles to promote regeneration, can possibly restore diseased or injured tissues and whole organs **(Mao & Mooney 2015).** Therefore, promising preclinical and clinical current evidence support the possibility for treating both chronic diseases and acute insults **(Jaklenec et al., 2012; Bailey et al., 2014).** For these reasons, hASCs hopes to treat and cure several diseases, triggering and fascinating researchers, and clinicians.

Endocrine disrupting chemicals (EDCs) are described as "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process." according to the U.S Environmental Protection Agency (EPA) **(Diamanti-Kandarakis et al., 2009).** They belong to the persistent organic pollutants (POPs) superfamily that is a vast set of substances that are claimed to cause severous deleterious alterations in biological systems **(Merrill & Birnbaum, 2011)**. So, the idea that xenobiotic chemicals could inappropriately modulate the endocrine system thereby causing detrimental effects in wildlife and humans was a relevant concern that has been arisen through the last past 20 years **(Blumberg et al., 2011).**

These compounds were originally thought to exert actions primarily through nuclear hormone receptors, including estrogen (ERs), androgen (ARs), progesterone, thyroid (TRs), and retinoid receptors, among others **(Needham et al., 2007).** Currently, basic scientific research shows that the mechanisms are extensive than firstly identified. Hence, EDCs act via nuclear receptors, nonnuclear steroid hormone receptors (*e.g.*, membrane ERs), nonsteroid receptors

(*e.g.*, neurotransmitter receptors such as the serotonin receptor, dopamine receptor, norepinephrine receptor), orphan receptors (*e.g.*, aryl hydrocarbon receptor (AhR) an orphan receptor), enzymatic pathways involved in steroid biosynthesis and/or metabolism, and numerous other mechanisms that converge upon endocrine and reproductive systems **(Patisaul & Adewale 2009; Gore, 2010)**.

BPA considered an EDC member, is a synthetic monomer used in the production of polycarbonate plastics, epoxy resin linings of canned foods and beverage containers, dental sealants, and thermal receipt paper **(Calafat et al., 2008; Seachrist et al., 2016).** EPA estimates that over 1 million pounds of BPA leaches into the environment each year and over 90% of tested humans have detectable BPA in their systems with the highest levels found in infants and children **(Kuroda et al., 2003; Lee et al., 2008).** The use of BPA in food and beverage containers accounts for the majority of daily human exposure; estimated human consumption of BPA from epoxy-lined food cans alone was 6.6 μg/person-day **(Howe & Borodinsky, 1998).** Warming the plastic, such as in a microwave, increases the leaching of BPA into liquids **(Carlisle et al., 2009).**

There is a regular number of *in vitro* and animal evidence supporting a role of BPA in the setting of several diseases like diabetes, cardiovascular disease, and obesity. BPA is structurally similar to 17β-estradiol and thus binds to estrogen-related receptors (ER) such as ERα, ERβ and ERγ, the G protein-coupled estrogen receptor GPR30, and the peroxisome proliferator-activated receptor gamma (PPAR-γ) **(Fenichel et al., 2013; Delfosse et al., 2015)**. While the mechanisms of action are not totally understood, binding of BPA to these receptors has been shown to cause insulin resistance, adipogenesis, pancreatic beta-cell dysfunction, inflammation, and oxidative stress **(Alonso-Magdalena et al., 2006; Akingbemi et al., 2010).** Thus, xenoestrogens can act directly on gene expression via nuclear ERα and ERβ, or indirectly via multiple other cellular signaling pathways, some of which can be rapidly activated and at very low concentrations **(Blumberg et al., 2011).** Taken together, all this data show how much BPA is potentially harmful to animal and human health that should be extensively studied.

Another well known as much toxic compound is TCDD that is a persistent lipophilic environmental contaminant produced as a residual by-product of various burn and chemical processes of chlorine containing components. **(Pohl et al., 1998).** This compound is classified as dioxins, which are omnipresent contaminants with decades of persistence in the environment or animal organisms. These substances are highly lipophilic, accumulating in the adipose fraction of organs and tissues. Human exposure is mostly determined by the consumption of

products of animal origin rich in fat (meat, milk, cheese, seafood) **(Schecter et al., 2006)**, and due to continuous accumulation, blood dioxin levels strongly increase with age **(Patterson et al., 2009; Papke, 2014).** The aryl hydrocarbon receptor (AhR) has affinity with TCDD, so, many of the effects are modulated by this receptor. Considerable related compounds share the capacity of binding to AhR (the reason for "dioxin-like" term) and consequently have similar effects **(Pesatori et al., 2003).** AhR has long been known to interfere with signaling through estrogen and androgen signaling pathways; although, the specific mechanisms were not understood **(Blumberg et al., 2011)**. Considering that both compounds are the two currently most studied EDCs having relevant toxicological importance, this study is mandatory to try to understand the effects of these substances on some proteins related to stem cell phenotype maintenance and specific receptors binding site.

Taken up that stem cells has a large applicability in the field of cell therapy and regenerative medicine, the present study looks to contribute to the growing interest in studies aimed at the so-called GMP (Good Manufacturing Practices) in cell culture for therapeutic purposes, following numerous and strict criteria for obtaining a high level of quality in cell therapy. Therefore, this study aimed to better understand the effects of these EDCs on proteins related to stem cell phenotype and specific receptors binding sites.

Methods

Human subjects

Human samples used in this study were harvested from three Caucasian females with a BMI below 25 (35 to 42 years old and an average BMI of 23.4), were obtained from subcutaneous abdominal adipose tissue of who underwent elective abdominoplasty procedures at the Department of Surgery and Orthopedics of Sao Paulo State University, School of Medicine, Botucatu-SP, Brazil. Patients agreed to donate tissue fragments by filling in a consent form, and all protocols were approved by the Governmental Ethics Committee on human biologic materials (Process number: CAAE: 32712514.4.0000.5411).

Cell Culture and Media

Basically, all chemicals were purchased from Life Techonologies (Grand Island, NY USA) Sigma-Aldrich (St. Louis, MO USA), Thermo Fisher (Waltham, MA USA) Abcam (Cambridge, UK), Santa Cruz Biotechnology Inc, (Dallas, TX USA), BioRad (Hercules, CA

USA). ASCs were maintained as subconfluent monolayers in basic medium DMEM-F12 containing 10% fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin.

Adipose tissue fragments were stored at 4°C (24hrs) in HEPES medium containing 2% penicillin/streptomycin and 2% amphotericin B. The fragments were washed PBS and the samples were submitted to the previously described enzymatic digestion protocol **(Rodbell, 1964)** to obtain ASCs which were then seeded into cell culture and cultivated until the fourth passage. The stemness of ASCs was assessed based on their ability to adhere to plastic, to aquire fibroblast-like morphology, ability to form colony forming units (CFUs), to express several surface markers such as ($CD44^+$, $CD90^+$, $CD105^+$, $CD34^-$,$CD45^-$) assesed by Flow Cytometry using a FACSCalibur™ (Becton Dickinson, Franklin Lakes, NJ), and capacity to differentiate into either adipocytes, osteoblasts and chondrocytes that were assesed using the Stem Pro Tri-lineage Differentiation Kit™.

Cytotoxicity Assay by MTT

Cell citotoxicity was determined using the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan. Breifly, ASCs were seeded in 96 well plates with medium containing BPA (1uM-10uM) or TCDD (10nM) for 7 days. Cells were then prepared using the MTT kit (*In Vitro* Toxicology Assay Kit, MTT based) and then spectrophotometrically measured absorbance at a wavelength of 570 nm.

Western Blot Assay

ASCs were pooled and cultured for protein collection at day 7 in the following treatment with 1uM-10uM of BPA and 10nM of TCDD. Pelleted cells were lysed with RIPA buffer and centrifuged for lysate collection, and protein concentration was quantified by the Bradford assay. A total of 30ug of protein were loaded on a 10% SDS– polyacrylamide gel and transferred onto nitrocellulose membrane. The blots were blocked with BSA for 1h, then each primary antibody against: ABCG2, SOX2, BAK, AhR and ERβ, were incubated overnight at 4 to 8ºC, washed with PBS with 0.01% Tween 20 (PBST), followed by staining with a secondary antibody, washed with PBST, and visualized with chemiluminescence reagent on QuantStudio™. An antibody against β-actin was used as an internal control and for normalization.

Statistical Analysis

All values are expressed as mean S.E.M. or S.D. The statistical differences among two or more groups were determined by ANOVA, followed by the post-hoc Tukey multiple comparison tests vs the respective control group and t test. Statistical significance was set at $p<0.05$. Analysis was performed using Prism™ (GraphPad Software, San Diego, CA, USA). All studies were performed as n = 3 independent replicates.

Results

Cytotoxicity Assay by MTT

To identify cytotoxic possible concentrations of BPA and TCDD, hASCs were seeded and exposed to 1uM, 10uM (BPA) and 10nM (TCDD) for 7 days. BPA in a concentration of luM reduced mitochondrial dehydrogenase activity compared to control (significantly [0.376 to 0.562] in 95% Cl of difference). TCDD had no significant effect on mitochondrial activity in a concentration of 10nM compared to control as seen in Figure 1.

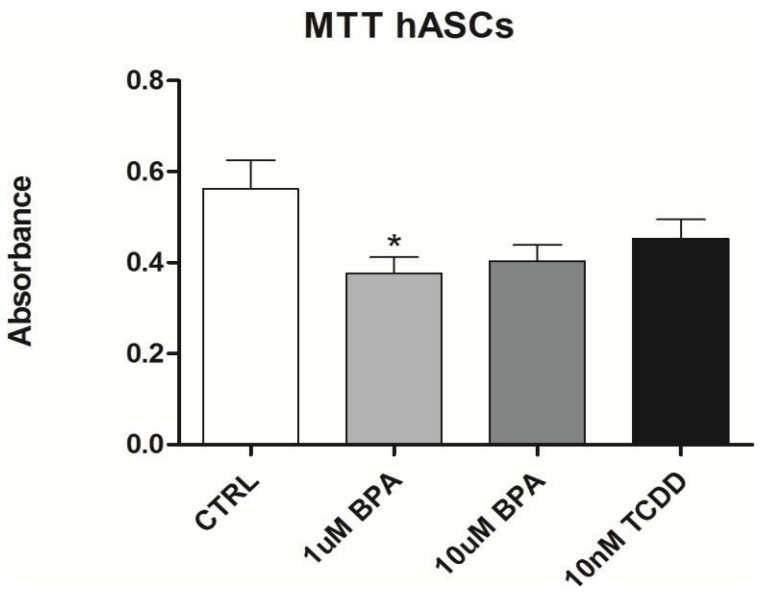

Figure 1. MTT Assay to access citotoxicity on hASCs exposed to 1uM, 10uM of BPA and 10nM of TCDD. All values are given as mean ± SEM; N = 3; $P < 0.05$.

Western Blot Assay

hASCs were exposed to 1uM, 10uM of BPA and 10nM of TCDD during 7 days. After this period, samples were quantified to access some proteins related to: self renew and stem cell maintenance (ABCG2 and SOX2), transporter for xenobiotics (ABCG2), apoptosis (BAK), the receptor that mimics TCDD (AhR) and the receptor that BPA mimics (ERβ). hASCs that received BPA didn´t present statistically different to ABCG2 quantification; but, when cells were exposed to TCDD, it had 1.95 fold higher compared to control as seen in Figure 2. For BAK, SOX2 and ERβ quantifications no difference was seen between the exposures or groups analyzed as Figure 3, 4 and 5 shows, respectively. Figure 6 shows the AhR quantification, that 10nM of TCDD was capable to increase in 1.16 fold compared to control.

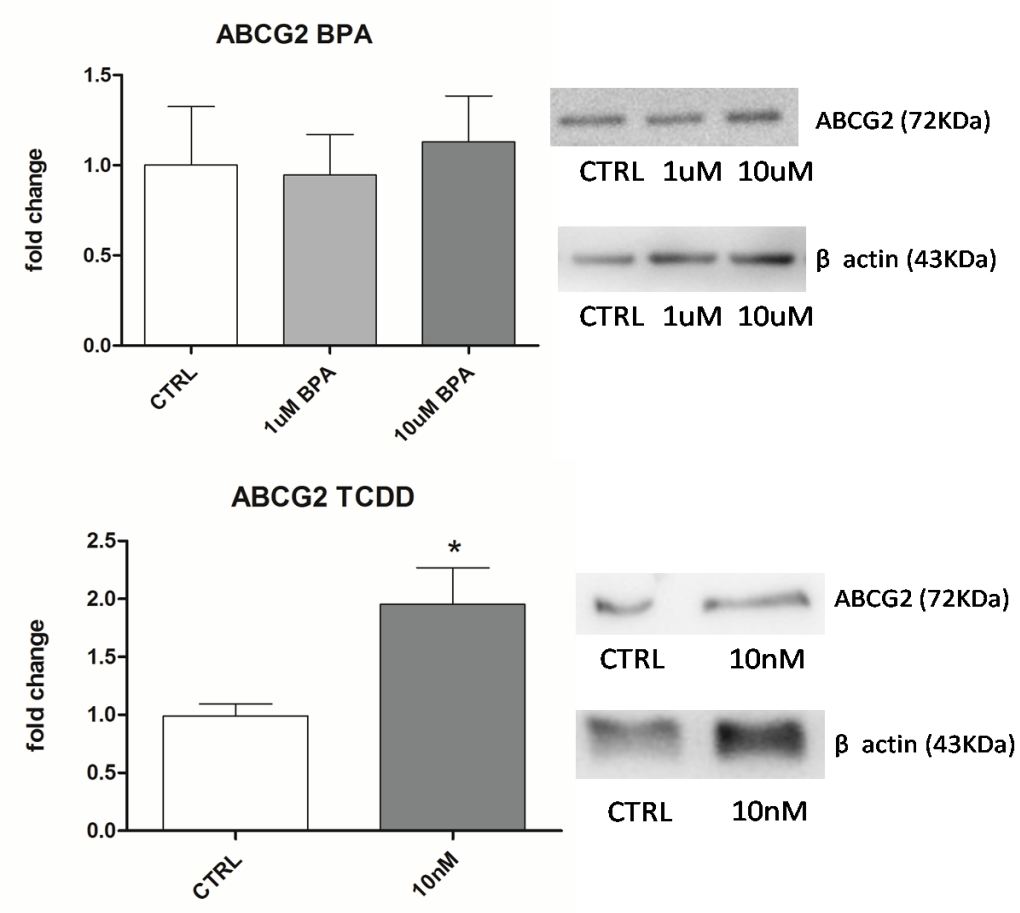

Figure 2. hASCs exposed to BPA and TCDD. Expression of ABCG2 and β-actin analyzed by Western blot. All values are given as mean ± SEM; N = 3; $p < 0.05$.

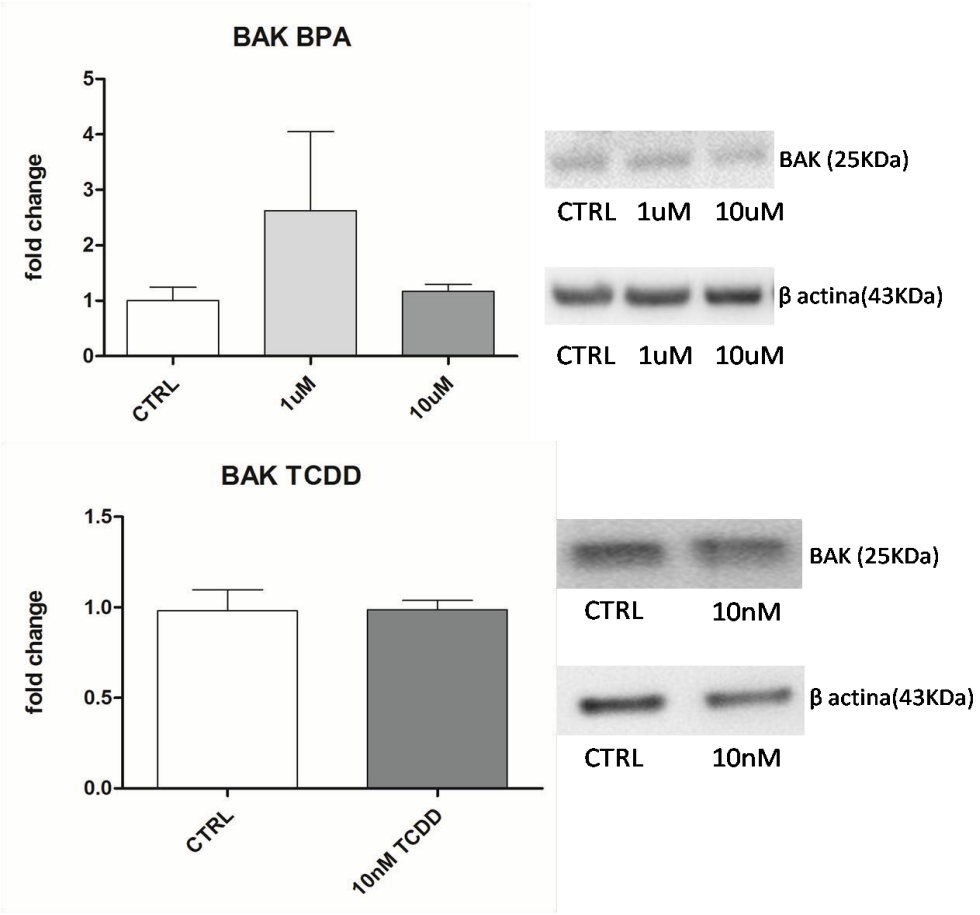

Figure 3. hASCs exposed to BPA and TCDD Expression of BAK and β-actin analyzed by Western blot. All values are given as mean ± SEM; N = 3, $p < 0.05$.

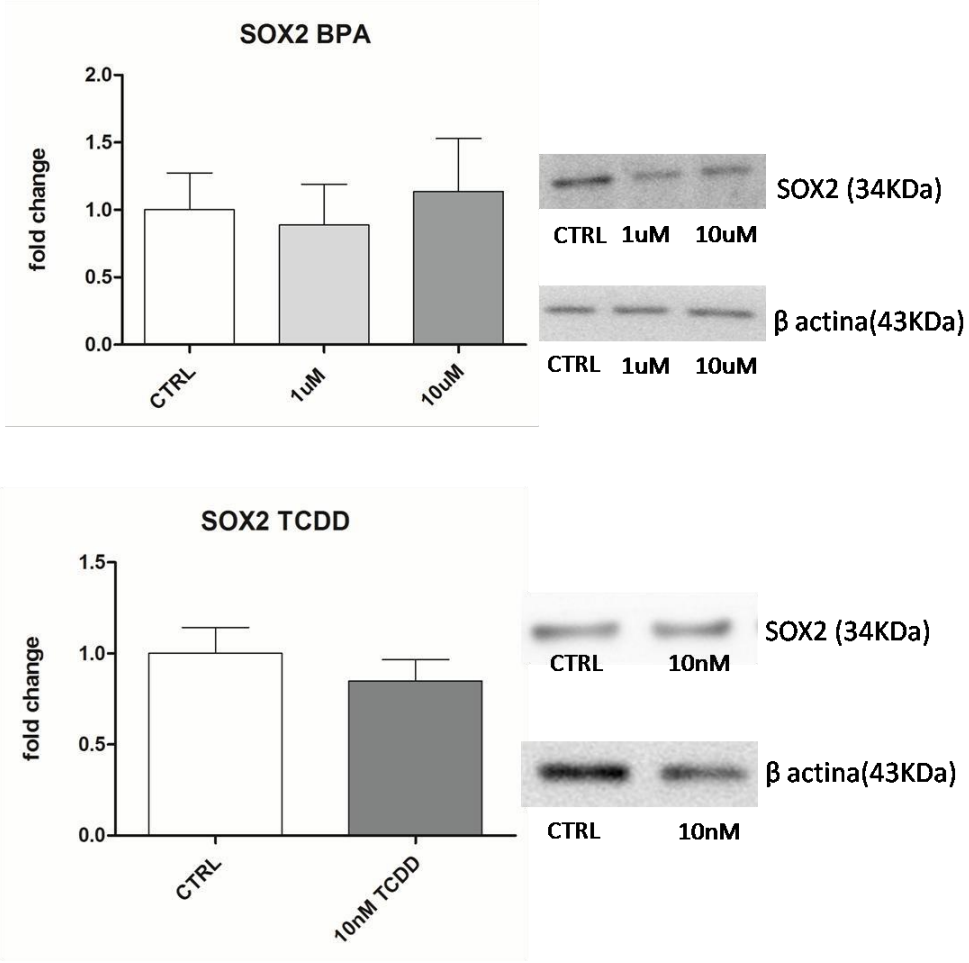

Figura 4. hASCs exposed to BPA and TCDD. Expression of SOX2 and β-actin analyzed by Western blot. All values are given as mean ± SEM; N = 3; ⁄P < 0.05.

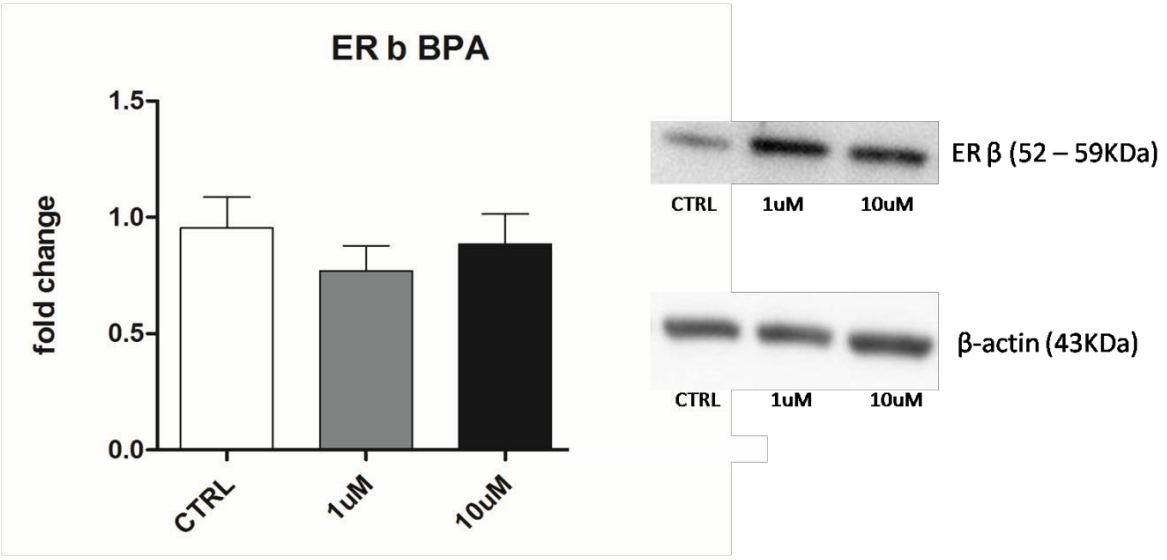

Figure 5. hASCs exposed to BPA and TCDD. Expression of ER β and β-actin analyzed by Western blot. All values are given as mean ± SEM; N = 2; *P < 0.05.

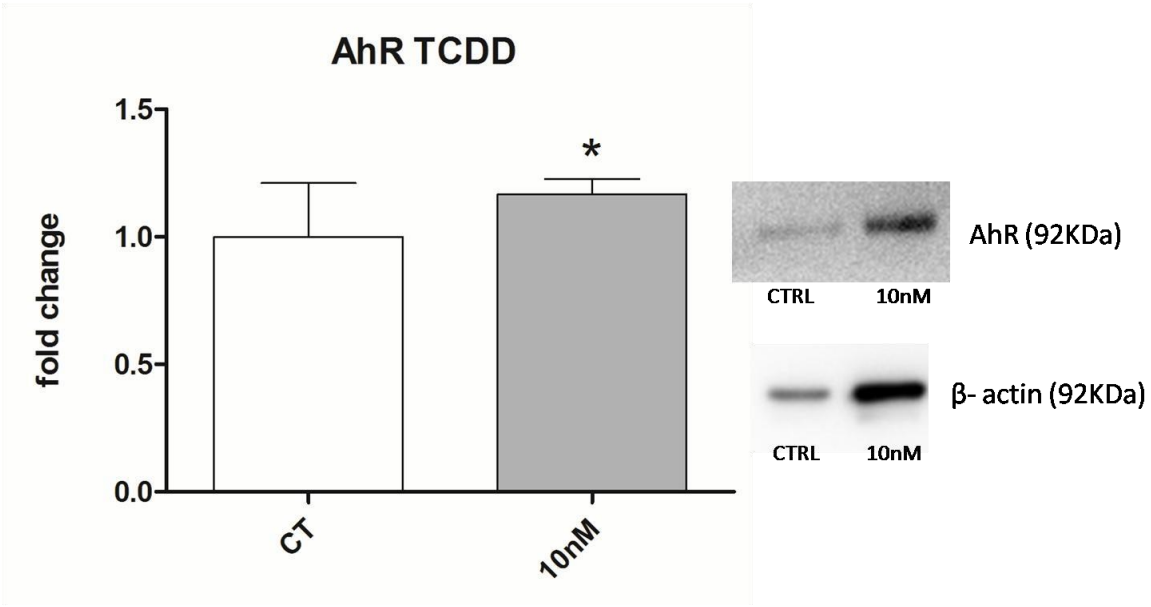

Figure 6. hASCs exposed to BPA and TCDD. Expression of AhR and β-actin analyzed by Western blot. All values are given as mean ± SEM; N = 1; *P < 0.05.

Discussion

Given that hASCs are an attractive source of stem cells for clinical applications **(Pham et al., 2014),** producing them according to Good Manufacturing Practices (GMPs) is a worldwide demand **(Sensebé et al.; 2013).** These cells have been used in clinical trials been mandatory provide safety, efficacy, and reproducibility of its production and compliance with GMPs **(Sensebé et al., 2013).**

Due to this, clinics advertising adipose-derived autologous 'stem cell treatments' are proliferating across the USA. The rapid spread of businesses promoting unapproved stem cell interventions reveals a widening gulf between federal regulations governing stem cell based therapies and a marketplace where few companies selling such interventions are subjected to regulatory action. Fatal outcomes, complications, and lawsuits have not slowed this rush to the marketplace **("State of Florida Department Of Health", 2012).** Therefore, most of the problems refers to different issues regarding cell processing, and specially when talking about environmental controls, it is common to ignore. However, it is well known that POPs are present in our daily routine becoming one of the most harmful substances related to endocrine systems and cancer probabilities. Because of their hydrophobicity, POPs tend to distribute into lipophilic compartments, particularly the adipose tissue **(La Merrill et al., 2013)**, leading to a decrease in their availability to other cells and tissues, thereby limiting their systemic toxicity. Recently, various interactions between AT and POPs have been reported, suggesting that this tissue plays a significant role in the kinetics and the its toxicity **(Kim et al., 2012).** So, in this study, hASCs derived from 3 different subjects were exposed to different concentrations of BPA and TCDD during 7 days. Thus, was possible to demonstrate when we exposed them and performed to MTT citotocity assay, (1uM BPA), a significantly decrease in the the number of viable cells comparing with 10uM (BPA) and the control, corroborating with previous study **(Biemann et al., 2012)**. This was interesting to note, because a higher BPA concentration (10uM) was not capable to cause this toxic activity or even more. This fact can possibly be explained by BPA own behaviour; when low doses are able to cause more detrimental effects than higher ones, producing a phenomenon called non-monotonic behavior **(Welshons et al., 2006; Vandenberg, 2014).** Therefore, these results demonstrate the same outcomes seen in related literature. To TCDD, no cytotoxicity activity was verified in hASCs at 10nM concentration.

The Western Blot analysis showed for ACBG2 quantification, that 10nM of TCDD was able to increase significantly (1.95 fold), leading us to corroborate the findings that show TCDD is closely related to the receptor protein complex ABC **(Halwachs et al., 2014).**

Whereas hASCs quantified to AhR, the binding receptor for TCDD o, showed 1.16 fold higher when compared to control, corroborating an intimate relationship already reported in the literature. For BAK, SOX2 and ERβ quantifications, no difference was seen between the exposures or groups analyzed, what shows that these compounds do not interfere on apoptosis via BAK pathway, as long as SOX2 expression did not appear to be changed when exposed to these DEs and also ERβ receptor, that is the main ligand to BPA, and was not altered.

There are very few studies conducted using hASCs exposed to BPA and TCDD. In this study we found that hASCs were affected somehow by these compounds. So, it is possible to conclude until now that 1uM of BPA has toxic activity to hASCs strengthening the findings that low doses has harmful consequences than higher doses. This is important to note, because BPA in low doses can possibly interefe in intrisic hASCs functions. Besides this, TCDD also exhibit ABCG2 increased expression in these cells, which indicates other hASCs alterations by such compound. In addition, TCDD was able to increase AhR expression, endorsing existing data.

In summary, these results present novel demonstration of BPA and TCDD ligands binding and stem cell phenotype protein expression in human stem cell model. Additionally, cell cytotoxicity for low BPA concentration tested (1uM) was found in our study, corroborating the interesting BPA monotonic behavior reported early by **Cuomo and colaborators (2017)**. So, from now, new studies should been done to access more specifically, which biochemical pathways would be related into the hASCs when exposed to BPA and TCDD from this point of interest. Considering the importance of hASCs in primary cultures to provide novel approaches on cell therapy, BPA and TCDD are good EDCs to be studied because of the relevant occurrences showed in this study.

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Supplementary Material

Here we present the data related to hASCs Characterization by Flow Cytometry, as seen in figure 7.

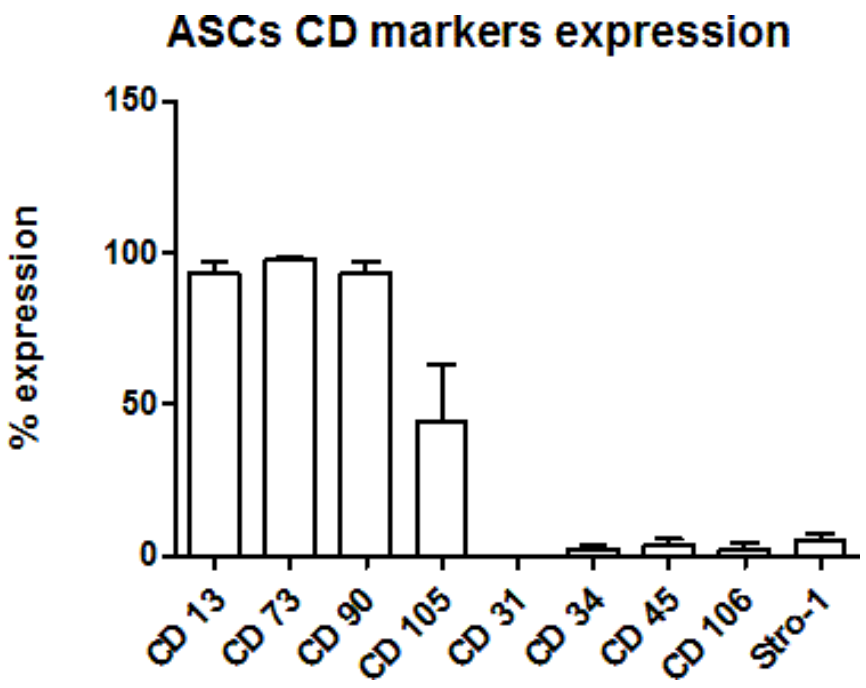

Figure 7. hASCs characterized by flow cytometry. Expression of CD13, CD73, CD90, CD105, CD31, CD34, CD10 and Stro-1. All values are given as mean ± SEM; N = 3; *P < 0.05.

Cells was also characterized by Tri-lineage differention assay, where cells were exposed to three differentiation media to confirm the stemness potencial as seen in figure 8.

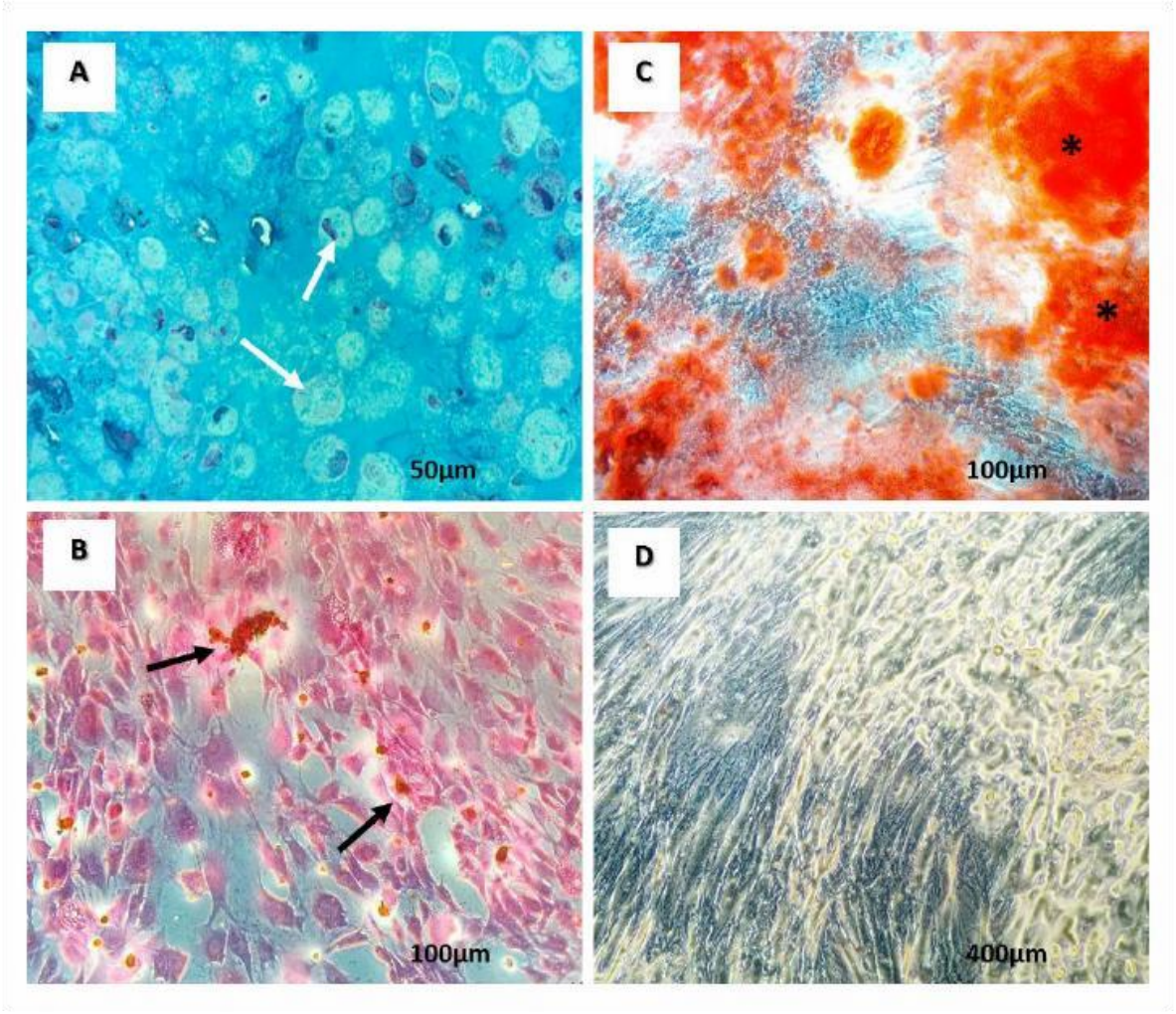

Figure 8. Tri-lineage differentiation of hACs using Stem Pro® (Gibco®). (A) Chondrogenic differentiation: chondrocytes (white arrows) Alcian Blue® staining, 40x magnification. (B) Adipogenic differentiation: lipid droplets (black arrows) Oil Red O® staining, 10x magnification. (C) Osteogenic differentiation: calcium depots (*) Alizarin Red S® staining, 10x magnification. (D) hASCs control not exposed to differentiation media, 5x magnification, confluência 100%.

Capítulo III: Original Article

BPA and TCDD Action on Rat Adipose-derived Mesenchymal Stem Cells (rASCs) Differentiation Potential.

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Abstract

MSCs are a heterogeneous population of cells that proliferate *in vitro* as plastic-adherent cells, have fibroblast-like morphology, form colonie *in vitro* and can differentiate into bone, cartilage and fat cells. ASCs is a stem cell population derived from adipose tissue and due to their isolation from human lipoaspirates, these cells are used for regenerative medicine purposes to prevent and treat diseases occurring across a vast bunch of tissues and organs. The use of experimental tissue-engineered constructs that have been given emergency approval for implantation, and an increase in clinical trial activity in the field of regenerative medicine. When working with cell therapy it is important to take into account the possible contaminations of cells. EDCs are lipophilic substances closely related to adipose tissue. BPA and TCDD are the most known and relevant EDCs studied in the last times. These compounds are also called as obesogens in which they are known to induce adipogenic differentiation. Therefore, this study aimed to access and quantify the differentiation potential of Rat Adipose-derived Mesenchymal Stem Cells (rASCs) exposed to 1uM, 10uM of BPA and 10nM of TCDD and also access other cell behaviours. A cytotoxicity assay by MTT was performed to confirm possible deleterious actitivity of these compounds. Apoptosis/necrosis by Flow Cytometry was perfomed to access this process into the cells and the differentiation potential was also analysed. Comet assay was also performed to verify DNA damage. Results showed that 10uM of BPA and 10nM of TCDD were able to induce proliferation according to MTT analysis. Apoptosis/necrosis assay showed that BPA and TCDD were able to induce apoptosis/necrosis. Comet assay analysis presented DNA damage in rASCs from all experimental groups. When cells were quantified in terms of adipogenic differentiation, 1uM of BPA caused significant oil droplets, while the 10uM of BPA and 10nM of TCDD showed a decrease on adipocyte differentiation. Osteogenic differentiation did not showed significantly results among groups. The present results evidenced that 1uM BPA concentration had the most expressive results in terms of adipocyte differentiation, leading to believe that low doses (1uM) was able to induce this process. Our results showed that rASCs exposed to BPA and TCDD demonstrated alterations in important cellular process, such as cell proliferation, apoptosis rate, adipogenic differentiation, and DNA damage.

Key Words: BPA, TCDD, rASCs, adipocyte differentiation.

Introduction

Mesenchymal Stem Cells (MSCs) can be defined as multipotent mesenchymal stromal cells, that are a heterogeneous population of cells that proliferate *in vitro* as plastic-adherent cells, have fibroblast-like morphology, form colonie *in vitro* and can differentiate into bone, cartilage and fat cells **(Horwitz et al, 2005).** It is known that such cells can be theoretically isolated from all tissues **(Tuan et al. 2003).** They were first identified and isolated from bone marrow more than 40 years ago **(Friedenstein et al. 1966).**

The adipose tissue source was discovered in 2000 by a group from the University of California in Los Angeles (UCLA) that defined as a stem cell population derived from adipose tissue and due to their isolation from human lipoaspirates, they were first termed 'processed lipoaspirate' cells, but they are now called 'adipose-derived stem cells' (ASCs). The current term is more descriptive, as they proposed that ASCs was a multilineage stem cell population that could be isolated from the stromo-vascular fraction of adipose tissue **(Zuk et al., 2001).**

Nowadays, these cells are used for regenerative medicine purposes to prevent and treat diseases occurring across a vast bunch of tissues and organs, including: dermal wounds, such as graft-versus-host disease **(Fang et al., 2007),** autoimmune-induced diseases **(Gonzalez-Rey et al., 2009),** multiple sclerosis **(Riordan et al., 2009),** diabetes mellitus **(Thakkar et al., 2013)**, tracheomediastinal fistulas **(Alvarez et al., 2008)** cardiovascular diseases and traumas, treatment for certain types of cancer, **(Jaklenec et al., 2012; Bailey et al., 2014)** and many others. In this way, cell therapy technologies are translating into more ''routine'' clinical practice. Over the last years, there have been an interesting series of developments that is, treatments becoming more established, the use of experimental tissue-engineered constructs that have been given emergency approval for implantation, and an increase in clinical trial activity in the field (both in publication and in registration) **(Harrison et al., 2014).** However, when talking about cell therapy, one such important issue should be addressed: the environmental contamination. It is well know that human and animals are in contact with a broad number of different chemical substances routinely. One relevant and threatening family of these compounds is the persistent organic pollutants that are endocrine-disrupting chemicals. They include polychlorinated biphenyls (PCBs) and organochlorine pesticides, such as pp-dichloro-diphenyltrichloroethane and its major metabolite dichloro-diphenyl-dichloroethylene (pp-DDE), and β-hexachlorocyclohexane (βHCH) **(Dirinck et al., 2011).** Thus, from a physiological perspective, an endocrine-disrupting substance is a compound, either natural or synthetic, which, through environmental or inappropriate developmental exposures, alters the hormonal and

homeostatic systems that enable the organism to communicate with and respond to its environment **(Diamanti-Kandarakis et al., 2009).** EDCs are lipophilic substances; therefore, they are closely related to adipose tissue. These compounds are also called as obesogens in which they are known to induce adipogenic differentiation **(La Merrill et al., 2013).**

Obesity and related metabolic diseases have boosted dramatically in industrialized countries becoming a global epidemic in the past 20 years. Excessive consumption of unhealthy foods mixed with sedentary lifestyle is the generally accepted causes for this problem. There is a growing hypothesis proposing that exposure to certain EDCs during critical stages in development predispose the exposed individual to weight gain and obesity **(Janesick & Blumberg 2012)**.

As follows, EDCs acts via adipogenesis pathway. They frequently activate peroxisome proliferator activated receptor gamma (PPARγ) pathway **(Rancière et al., 2015),** the nuclear estrogen receptors alpha and beta (ERα, ERβ) **(Watson et al., 2012)**, the aryl hydrocarbon receptor (AhR) among others.

Regarding to specific EDCs that have been linked to obesity, BPA is among the most highly produced and controversial compounds worldwide **(Rubin, 2011)**. BPA is a synthetic monomer used in the production of polycarbonate plastics, epoxy resin linings of canned foods and beverage containers, dental sealants, and thermal receipt paper **(Calafat et al., 2008; Seachrist et al., 2016).** Is a well known chemical compound, and its action occurs mimicking estrogen and also binds to aril hydrocarbon and thyroid hormone receptors; in this way it has broad-ranging health effects and can target multiple endocrine related pathways. The mechanism of action of BPA is associated with its shared homology with estrogen and the upregulation of downstream targets, including peroxisome proliferator-activated receptor gamma (PPARγ) and lipoprotein lipase (LPL) genes, based on results from in vitro experiments on rodents. **(Melzer et al., 2011; Ohlstein et al., 2014).**

Another important and known EDC is the 2,3,7,8-tetrachloro-dibenzopara- dioxin (TCDD). TCDD belongs to the dioxins family, a group of substances which include polychlorinated dibenzo-para-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and a subgroup of polychlorinated biphenyls with dioxin-like properties (dioxin-like PCBs) and is considered the most toxic congener of the group **(Steenland et al., 2004)**. These compounds have a broad variety of effects and have been classified as carcinogen to humans in 1997 **(Steenland et al., 2004)**. The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor with no known endogenous high-affinity ligand. The canonical pathway involves AhR activation following ligand binding, translocation to the nucleus, and heterodimerization with

the AhR nuclear translocator **(Hankinson, 1995; Marinković et al., 2010).** The heterodimer complex then interacts with dioxin response elements in the promoters of target genes and recruits transcriptional coactivators to regulate gene transcription **(Landers & Buncet 1991)**.

Highlighting the novelty of this approach , the main objective of this study is to expose Rat Adipose-derived Mesenchymal Stem Cells (rASCs) to BPA and TCDD, performing differentiation potential quantification and to verify other cell behavior indicators like metabolic activity, apoptosis, and DNA damage.

Methods

Cell Culture and Reagents

rASCs samples used in this study were gently provided by Dr. Deffune´s Laboratory cell bank, that were previously harvested from three healthy adult male Wistar rats at 90 days of age, mean body weight (300g) and BMI± ( ) that were maintained under controlled conditions throughout the period until collection of adipose tissue (animal ethic committee number: CEUA 1009/2013), cells were then isolated and in the fourth passage it were characterized for the adipose derived mesenchymal stem cell population by flow Cytometric and tri-lineage differentiation kit analyses. Cells were seeded in tissue-treated culture flasks (Sarstedt®), maintained with DMEM-F12 medium (Gibco BRL, Grand Island, NY), Media were supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY) and 1% penicillin/streptomycin solution (Gibco BRL, Grand Island, NY). Cells were cultivated at 37ºC in a humidified atmosphere of 5% CO_2. A cell bank was established to provide the total number of cells required for all experiments.

Cell Characterization

The stemness of rASCs was characterized based on their ability to adhere to plastic, fibroblast-like morphology, , expression of cell surface markers ($CD44^+$, $CD71^+$, $CD90^+$, $CD11b^-$, $CD31^-$, $CD45^-$), and capacity to differentiate into either adipocytes, osteoblasts and chondrocytes **(Bourin et al., 2014)**. These results can be seen at supplementary material or unpublised data.

Cytotoxicity Assay by MTT

rASCs were plated at a density of 1000 cells/well in 96-well plates (Nunc™) and exposed with media added by BPA or TCDD and controls. Cytotoxicity was assessed at day 7 of exposition. Cells were washed with PBS and the medium was replaced with fresh PBS containing

100ul of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT; Invitrogen™) stock solution (5mg MTT/1 ml PBS) and the cultures were incubated for 5h at 37°C. Following incubation, optical density absorbance was measured at a wavelength of 570nm.

Apoptosis Assay

rASCs that were exposed to BPA or TCDD were harvested and processed for staining and flow cytometry using FITC Annexin V Apoptosis Detection Kit I (Becton Dickinson, Franklin Lakes, NJ). Briefly, according to the manufacturer, FITC Annexin V staining precedes the loss of membrane integrity, which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore, a staining conjunction with a vital dye such as propidium iodide (PI) or 7-Amino-Actinomycin (7- AAD) is used allowing the identification of early apoptotic cells (PI negative, FITC Annexin V positive). Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. For example, cells that are considered viable are FITC Annexin V and PI negative; cells that are in early apoptosis are FITC Annexin V positive and PI negative; and cells that are in late apoptosis or already dead are are both FITC Annexin V and PI positive. The assay was performed using a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) cytometer and the analysis made on CellQuest (Becton Dickinson, Franklin Lakes, NJ) software.

Genotoxicity by Alcaline Comet Assay

For genotoxicity assay, rASCs were plated at a density of 5000 cells/well in 24-well plates (Nunc™) and exposed with media added by BPA or TCDD and controls. Genototoxicity was assessed at day 7 of exposition, when cells were washed 3 times in PBS solution, collected, counted where the positive control (hydrogen peroxide 0.1%, 20min) for comet assay was incubated until the electroforesis starts. The single-cell gel electrophoresis assay (comet assay) was performed according to Singh et al.,(1988) and Tice et al.,(1995) Each step was performed under indirect light. Briefly, after each treatment, 10uL of cell suspension were mixed with 110 μL of 0.5% low-melting-point agarose at 37°C, layered onto a precoated slide with 1.5 % regular agarose and covered with a coverslip. After agarose solidification (5 min at 4°C), the coverslip was removed and the slide was immersed into cold lysis solution (2.5M NaCl, 100mM EDTA, 10mM Tris–HCl buffer at pH 10 with 1% Triton X-100 and 10% DMSO) for approximately 24h. Slides were then washed with PBS for 5 min and placed into a horizontal electrophoresis chamber filled with freshly prepared alkaline buffer (300mM, NaOH and 1mM EDTA, pH 13) for 20 min. Electrophoresis was conducted for 20 min at 25V and 300mA. Afterwards, the slides were neutralized with

0.4M Tris–HCl (pH 7.5) solution for 15 min, fixed with 100% ethanol and stored at 4°C until analysis. After staining with SYBR Gold (1:10,000 Invitrogen), 50 randomly selected “nucleoids” per slide were

examined at 400X magnification with a fluorescence microscope using an automated image analysis system (Comet Assay IV, Perceptive Instruments, Suffolk, UK). Tail intensity (% of migrated DNA in the tail) was used to estimate the extent of DNA damage. The assays were performed in triplicate, and the coded slides were blindly analyzed.

Differentiation Assay

The concentrations of each EDC used in this study were determined by a literature review considering the most used concentrations for MSCs (Biemman et al., 2012; Chamorro-Garcia et al., 2012; Linehan et al., 2012; Ohlstein et al., 2014; Summarily, cells were exposed to (1uM-10uM) of BPA and 10nM of TCDD diluted in 0.001% DMSO during the entire differentiation protocol.

Multilineage differentiation potential of ASCs was tested for their mesodermal multilineage differentiation potential (i.e., adipogenesis, osteogenesis, and chondrogenesis). Cells were first subcultured and incubated in DMEM-F12 suplemented with 10 % FBS. The next day, differentiation induction was initiated by using the Stem Pro Tri-lineage Differentiation Kit™ (Life Technologies™) added with BPA or TCDD different concentrations.

To assess and quantify differentiation process, at the end of 14 days, cells were stained with Oil-Red O (359 nm) to measure lipid accumulation, Alizarin Red (596 nm) to measure calcium deposition by spectrophotometry analysis.

Statistical Analysis

All values are expressed as mean S.E.M. or S.D. The statistical differences among two or more groups were determined by ANOVA, followed by the post-hoc Tukey multiple comparison tests vs the respective control group. The statistical differences between two groups were analyzed by Student's t-test. Statistical significance was set at $P<0.05$. Analysis was performed using Prism (GraphPad Software, San Diego, CA, USA). Data represent mean ± SE from three independent experiments in duplicate or triplicate repetitions.

Results

We previously characterized the rASCs population and showed that these cells could differentiate into fat, bone, or cartilage *in vitro* and had the properties expected for MSCs (data not showed). In the present study, we previously accessed the citotoxicy potential and differentiation potential quantification rACSs exposed to BPA and TCDD during 7 days.

Cytotoxicity Assay by MTT

To identify cytotoxic concentrations of BPA and TCDD, rASCs were seeded and exposed to 1uM, 10uM (BPA) and 10nM (TCDD) for 7 days. BPA in a concentration of luM showed 0.586nm of absorbance rate compared to control (0.607nm), BPA 10uM increased absorbance rate showing 0.748nm and 10nM of TCDD also had an intense increasing on mitochondrial activity of 1.525nm as seen in Figure 1.

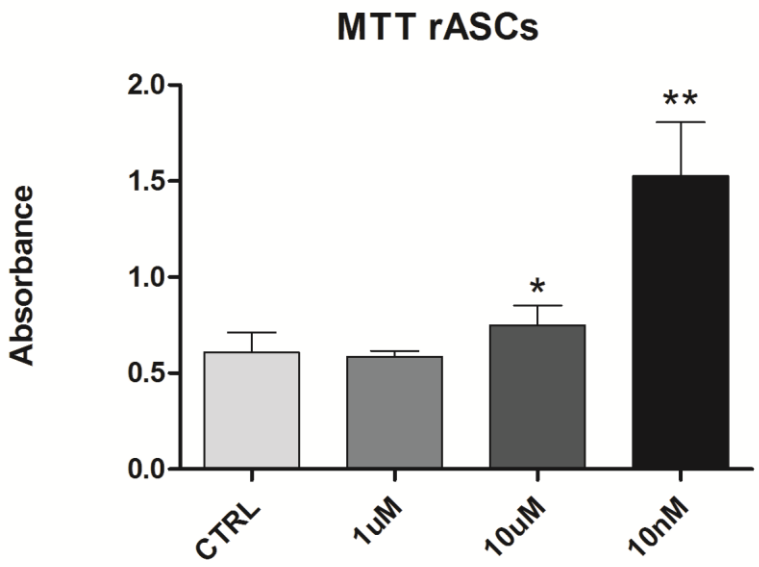

Figure 1. MTT Assay to access cytotoxicity on ASCs exposed to 1uM, 10uM of BPA and 10nM of TCDD. Values are expressed by mean ± SEM. Bars with * are significantly different ($p<0.05$).

Apoptosis/necrosis Assay

Cells exposed to 1uM - 10uM of BPA and 10nM of TCDD were analyzed by Flow cytometry to assess apoptosis and necrosis indications as follows. Figure 2 shows that 1uM of BPA showed a 7.325% of apoptosis/necrosis rate compared to control (0.565%). For BPA 10uM showed 8.7% apoptosis/necrosis rate and for 10nM TCDD: 4.52%.

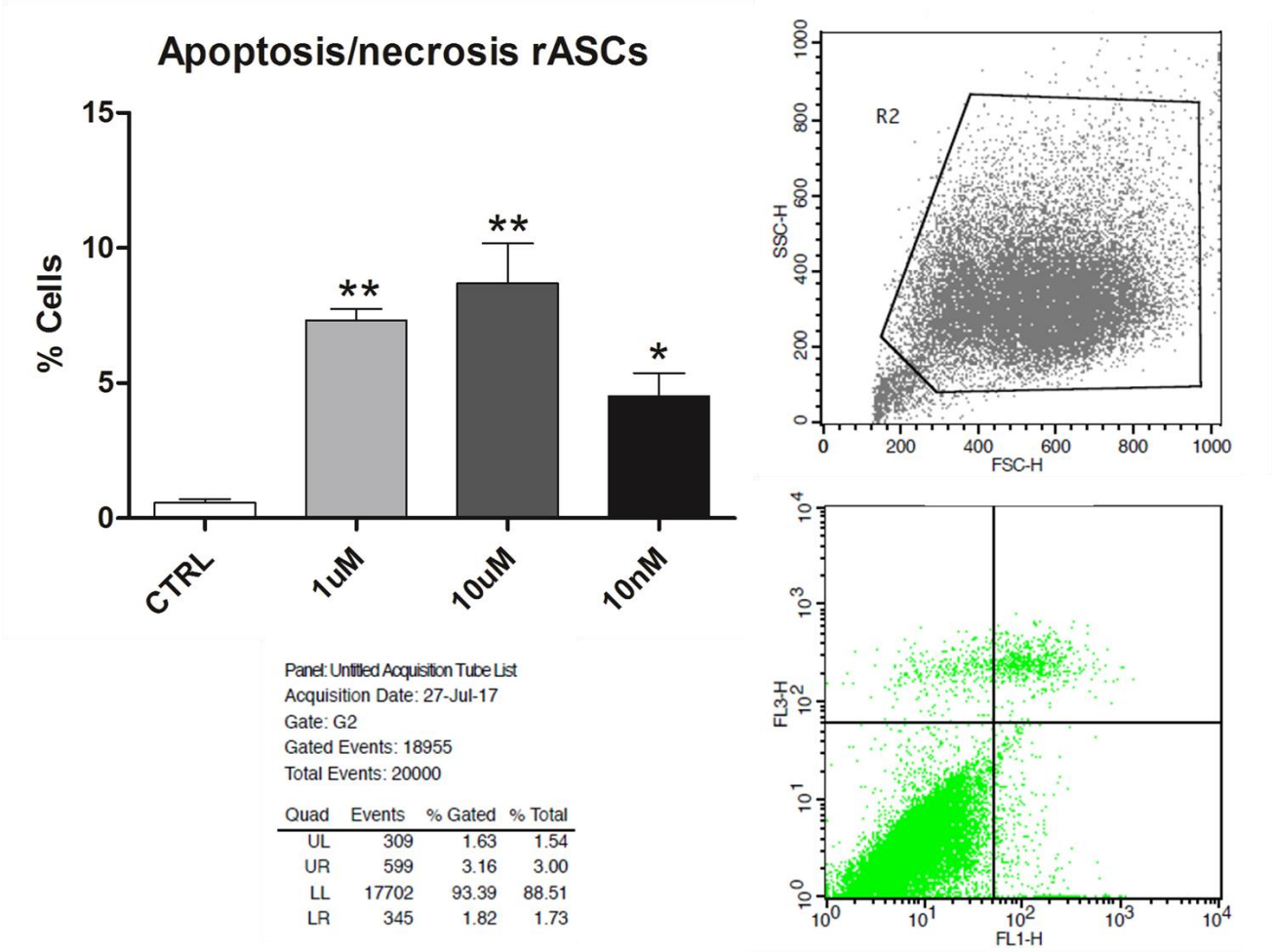

Figure 2. Apoptosis/necrosis Assay to access citotoxicity on rASCs exposed to 1uM, 10uM of BPA and 10nM of TCDD. Representative dot plot showing annexin V/PI cell expression, where control cells were primarily FITC Annexin V and PI negative, indicating that they were viable and not undergoing apoptosis. Cells exposed to BPA and TCDD showed FITC Annexin V positive expression and PI negative represented by the LR quadrant. A population of cells were observed to be FITC Annexin V and PI positive (UR), indicating that they were in end stage apoptosis or already dead and another cell population showed a PI expression, indicating that these cells have already entered necrosis.

Values are expressed by mean ± SEM. Bars with * are significantly different ($p<0.05$).

Genotoxicity by Alcaline Comet Assay

rASCs exposed to BPA and TCDD presented significantly DNA damage in all three groups analyzed, as seen in figure 3. BPA (1uM and 10uM) increased the global comet counting (27.82 and 11.37, respectively) of tail intensity area, compared to control (10.27). TCDD promoted the highest DNA damage in rASCs (36.97 of tail intensity area).

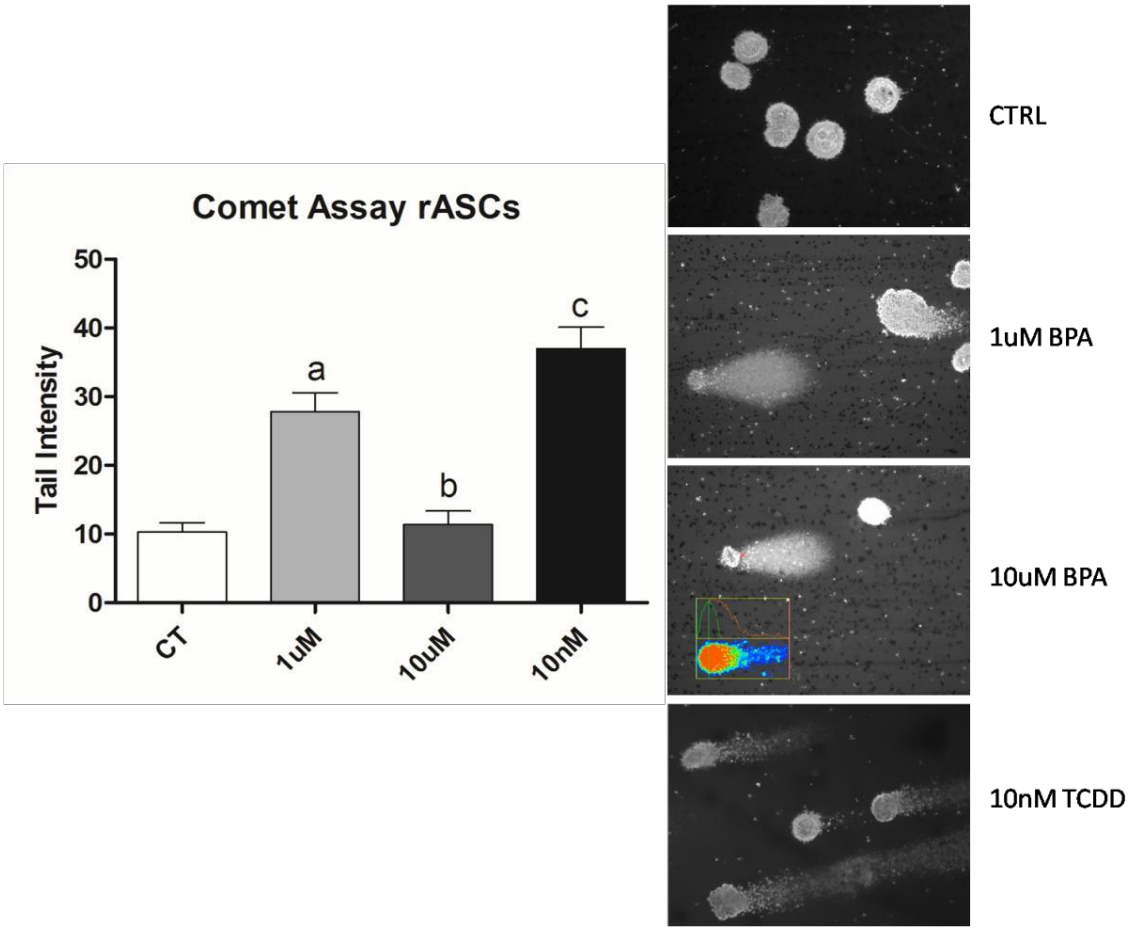

Figure 3. Genotoxicity by Alcaline Comet Assay to access DNA damage citotoxicity on ASCs exposed to 1uM, 10uM of BPA and 10nM of TCDD. Values are expressed by mean ± SEM. Bars with "letters" are significantly different ($p<0.05$). Figures illustrate absence (control group) or presence (BPA and TCDD groups) of DNA tail.

Exposure and Differentiation Assay

To determine if the EDCs analysed here had any effect on adipogenic (Figure 4) and osteogenic (Figure 5) differentiation, cells were exposed to (1uM-10uM) of BPA and 10nM of TCDD diluted in 0.001% DMSO during the entire differentiation protocol (14 days). Quantification by spectrophotometry showed that 1uM of BPA was able to increase dramatically the adipogenic differentiation (0.435 to control and 0.616 for 1uM of BPA, while 10uM of BPA and 10nM of TCDD apparently decreased this process compared to control 0.435 , 0.376 and 0.435,0.218 respectively. Osteogenic differentiation did not showed any difference between the groups analyzed.

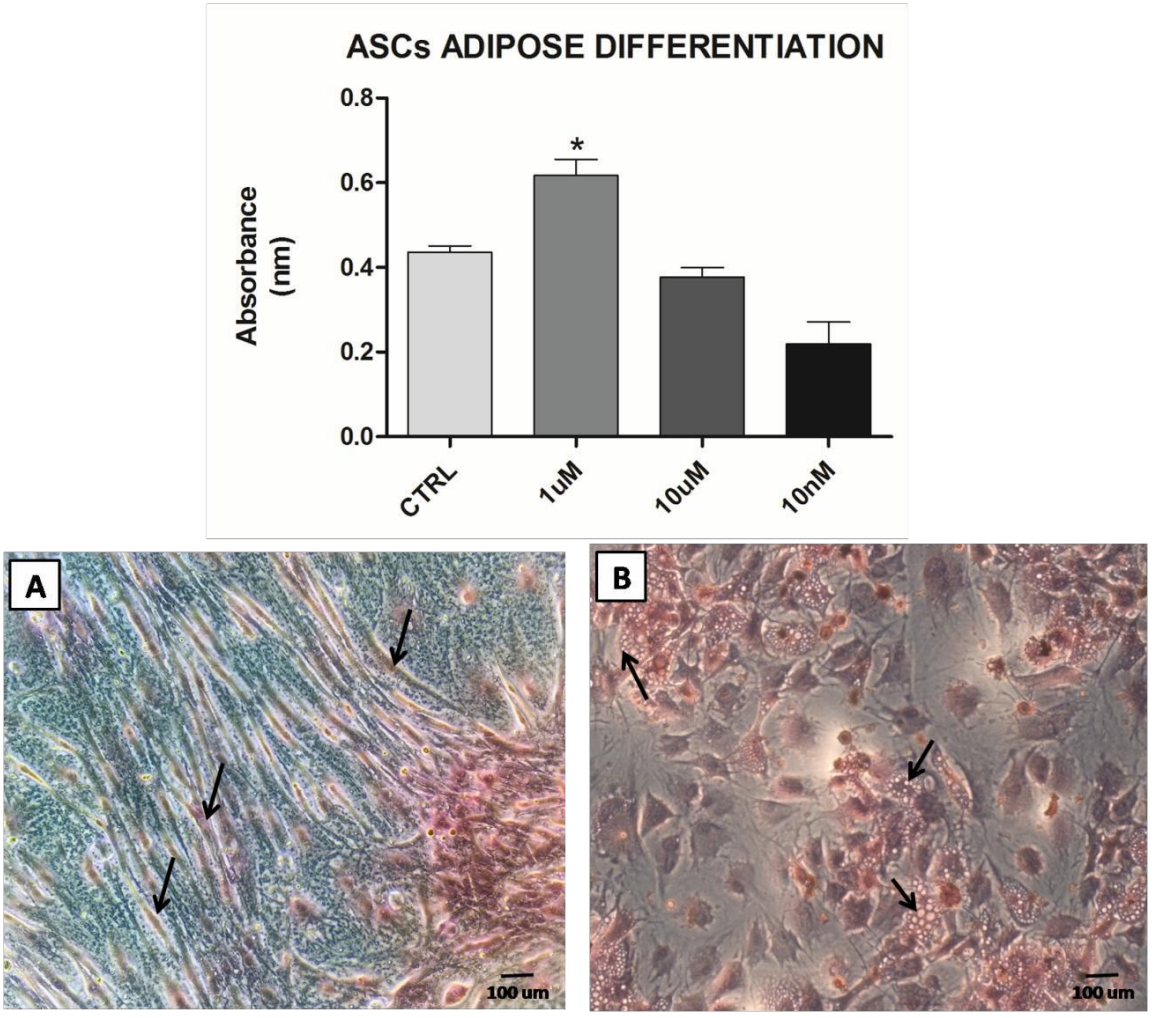

Figure 4. Adipogenic differentiation of rASCs exposed to 1uM - 10uM (BPA) and 10nM (TCDD). Values are expressed by mean ± SEM. Bars with * are significantly different ($p<0.05$). **A.** Fibroblast like morphology of rASCs without adipogenic induction. **B.** Adipocyte morphology of rASCs exposed to 1uM (BPA) commissioned to adipogenic induction.

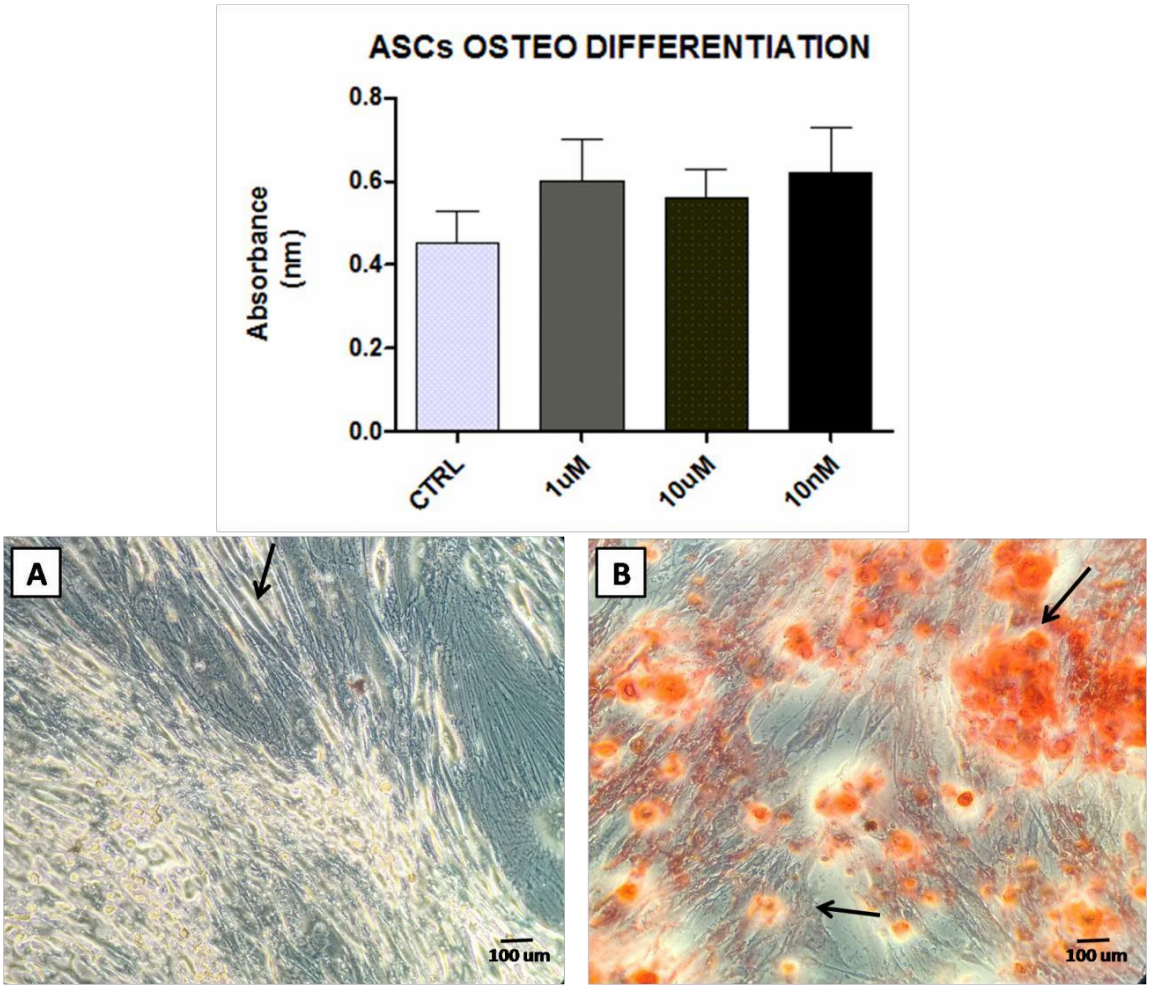

Figure 5. Osteogenic differentiation of rASCs exposed to 1uM - 10uM (BPA) and 10nM (TCDD). **A.** Fibroblast like morphology of rASCs without osteogenic induction. **B.** Osteocyte morphology of rASCs exposed to 1uM (BPA) commissioned to osteogenic induction.

Discussion

MSCs show promise in the field of regenerative medicine because of they can modulate numerous incurable diseases **(Pittenger et al., 2016).** Currently, there are almost 550 published studies conducted with MSCs in clinical trials, such as cardiovascular diseases **(Faiella & Atoui 2016),** diabetic nephropathy **(Liu & Tang, 2016),** diverse brain injuries (including stroke, neural trauma, heatstroke) **(Hsuan et al., 2016)** and many others. The importance of these cells in cell therapy is enormous, mainly by the reason that they are widely employed because of their multiple biological functions, including multilineage differentiation, tissue repair promotion, anti-inflammatory mediation and immunosuppression **(Krampera et al., 2006**

However, it is well known that this new and exciting branch of stem cell biology presents hurdles of unexplained issues that have yet to be overcome. For example, in terms of the mechanisms underlying MSC biological functions, it was originally thought that cells originating from damaged tissue differentiate and replace damaged cells **(Yuet al, 2014).** Nonetheless, subsequent research showed that MSCs engraftment and differentiation at injury sites are very low and transient **(Katsha et al., 2011).** Therefore, for successful cell- based therapies, a significant number of cells are needed, requiring extensive ex vivo cell expansion. Owing to prolonged ex vivo expansion needed in the clinic to obtain a sufficient number of cells for therapy, long-term culture will likely evoke continuous changes in MSCs, including cellular senescence **(Park et al. 2005; Young et al. 2013).** Besides this, other concerns impose limitations in MSCs clinical use such as the safety, efficacy, and reproducibility of its production, donor eligibility and screening, facilities, environmental controls, and storage **(Sensebé et al, 2013).** It is well known that EDCs exert a strong influence on exposed organisms, which can change both organs and systems. This subsequently leads to the development of short or long-term diseases. Therefore, experimental studies are mandatory to access and analyze the effects of EDCs from the cell to the entire organism. For this reason, this study sought to investigate whether the differentiation potential of rASCs can be altered by exposure to BPA and TCDD.

Related to cytotoxicity, MTT analysis of rASCs showed that 10 uM of BPA increased the cell proliferation and 10nM of TCDD had even a more intense increasing in mitochondrial activity This result can be explained by the fact that these concentrations could trigger cell proliferation instead of having

cytotoxic action **(Ohlstein et al., 2014),** showing a possible obesogenic behaviour **.**

For the Apoptosis/necrosis assay, all expositions were significant to cause cell death, These present percentages are significantly passable to induce apoptosis/necrosis verified by Annexin V propidium iodide **(Hingorani et al., 2011).**

For the Comet assay, rASCs exposed to the compounds showed significantly DNA damage in all three groups analyzed, leading us to assume that BPA and TCDD had significant genotoxic action on rASCs **(Fuchs et al., 2012; Froelich et al., 2013).** DNA damage in ASCs exposed to BPA and TCDD is an issue that has no literature reporting these evidence. So, it is possible to affirm that this article presents inedit data in this area.

Adipogenic and osteogenic differentiation quantifications showed that when the rASCs were exposed to these compounds, it was verified that the concentration of 1μM for the adipogenic differentiation obtained the highest mean absorbance .While the other concentrations obtained significantly lower averages; l. However, osteogenic quantifications differentiation did not show a statistically significant difference between the analyzed groups. For these reasons it is possible to conclude that the 1uM of BPA results demonstrated in this study agrees with recent studies on environmentally relevant (the low range), concentration effects showed that BPA often displays a lack of linear dose-dependent relationship typical of many hormones and toxic compounds **(vom Saal & Hughes 2005; Welshons et al., 2006).**

Another issue that is important to be adressed is the proliferative induction of 10uM BPA and 10nM of TCDD, against the lower adipogenic differentiation seen in this type of cells, where 1uM of BPA was capable to highly increase the adipogenic differentiation. One possible reason for this phenomenon is the nonmonotonic behaviour of BPA, where very low doses of natural and synthetic hormones can affect endpoints such as cell proliferation and organ size **(Welshons et al., 2006).**

In summary, the present study examined relevant issues concerning rat adipose mesenchymal stem cells exposed to BPA and TCDD, the main endocrine disrupting chemicals most studied currently and confirms some literature data showing the obesogen characteristic of BPA and also the potencial harmfull of TCDD stated by apoptosis and and with the unpublished results for DNA damage assays.

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Supplementary Material

Here we present the data related to rASCs Characterization by Flow Cytometry, as seen in figure 6.

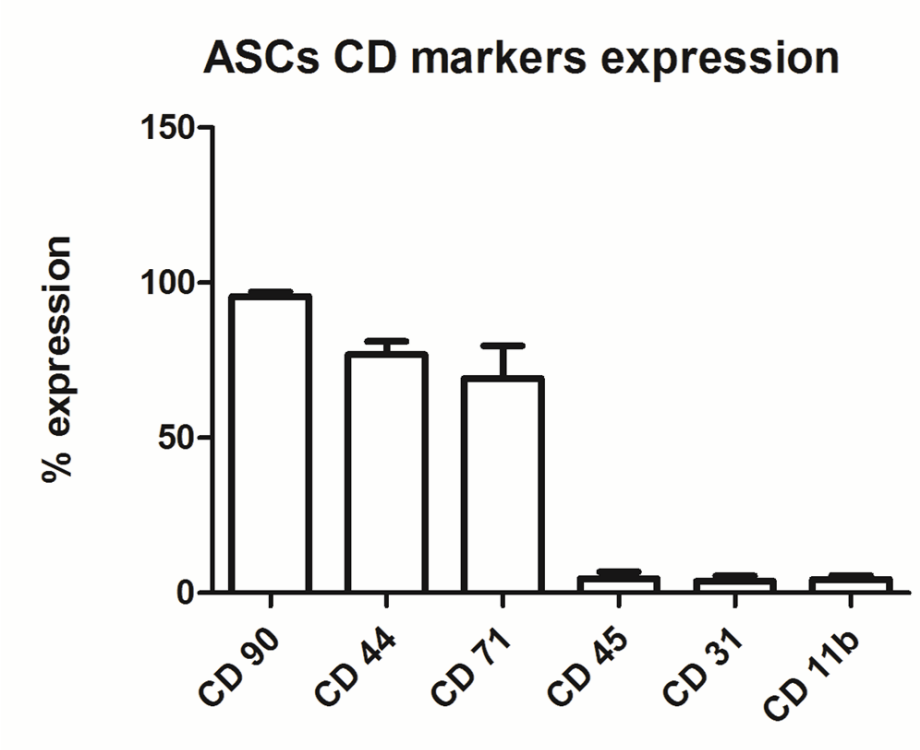

Figure 6. rASCs characterized by flow cytometry. Expression of CD90, CD44, CD71, CD45, CD31 and CD11b. All values are given as mean ± SEM; N = 3; ⁄P < 0.05.

rASCs were also characterized by Tri-lineage differention assay, where cells were exposed to three differentiation media to confirm the stemness potencial as seen in figure 7.

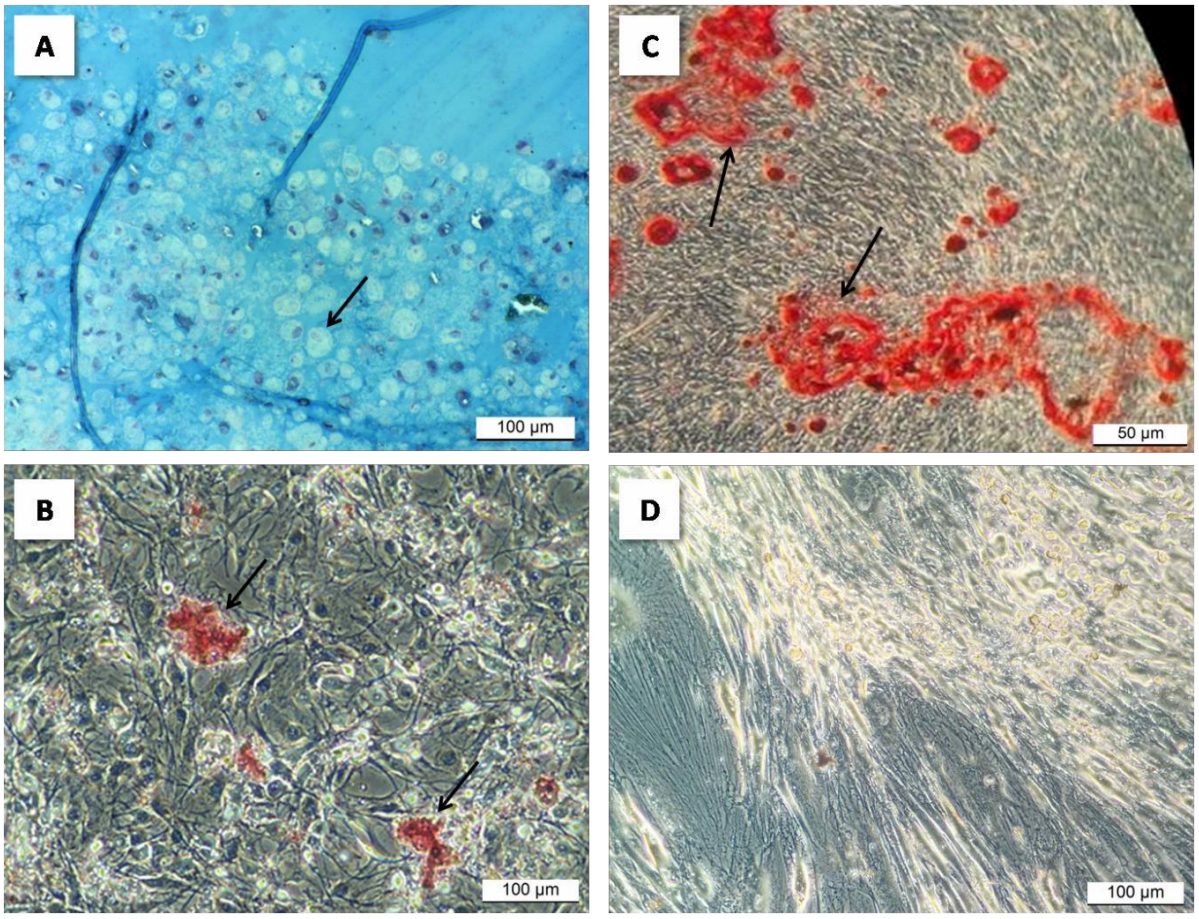

Figure 7. Tri-lineage differentiation of rASCs using Stem Pro® (Gibco®). **(A)** Chondrogenic differentiation: chondrocytes (arrow) *Alcian Blue*® staining, 40x magnification. **(B)** Adipogenic differentiation: lipid droplets (arrows) *Oil Red O*® staining, 40x magnification. **(C)** Osteogenic differentiation: calcium depots (arrows) *Alizarin Red S*® staining, 20x magnification. **(D)** hASCs control not exposed to differentiation media, 40x magnification.

CONCLUSÕES GERAIS DA TESE

A partir dos resultados apresentados anteriormente, conclui-se que:

- ✓ O BPA, na concetração de 1µM, é citotóxico para CTMs-TA humanas e na concentração de 10µM é inductor de proliferação para CTMs-TA de ratos;
- ✓ O BPA e o TCDD promovem apoptose em CTMs-TA de ratos e são genotóxicos, induzindo dano de DNA nessas células;
- ✓ O TCDD altera o perfil fenotípico de CTMs-TA humanas, assim como ativa receptor da família ABCG, que atua como bomba de efluxo e influxo de xenobióticos;
- ✓ Ainda para as CTMs-TA humanas, o TCDD mimetiza moléculas endógenas e ativa a expressão do receptor Aril-hidrocarboneto, corroborando com achados já publicados para CTMs.

ANEXOS DA TESE

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1. Documento paracer consubstanciado do CEP - Uso de animais.

2. Documento paracer consubstanciado do CEP - Uso células humanas.

UNIVERSIDADE ESTADUAL PAULISTA
CAMPUS DE BOTUCATU
FACULDADE DE MEDICINA

unesp

FMB

Ética CEUA

Comissão de Ética no Uso de Animais

Criada através da Portaria DFM nº 611 de 13/12/2012

Certificado

CERTIFICAMOS que o **(Protocolo CEUA 1009/2013)** Bioestimulação de célula-tronco mesenquimal de rato sob a ação da luz contínua e pulsátil de 630 nm utilizando LED, a ser conduzido por Heloísa Vicente Garcia, com a orientação da Profª. Drª. Elenice Deffune, com a colaboração de Woner Mion, com o apoio técnico de Ednelson Henrique Bianchi e Ondina Silvia Cotrim Ferrari, está de acordo com o Conselho Nacional de Controle de Experimentação Animal - **CONCEA**, com a ressalva de que os "ratos" são provenientes de Biotério convencional, sem condições de atestar a Sanidade dos mesmos.

Projeto de Pesquisa aprovado em reunião da CEUA em 04/04/2013

Prof. Dr. Katashi Okoshi
Presidente da CEUA

Alberto Santos Capelluppi
Secretário da CEUA

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PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: POTENCIAL DE DIFERENCIAÇÃO DE CÉLULAS-TRONCO MESENQUIMAIS TECIDO ADIPOSO HUMANAS EXPOSTAS AO 2,3,7,8-TETRACLORODIBENZENO-P -DIOXINA (TCDD) E BISFENOL A (BPA)

Pesquisador: Helga Caputo Nunes

Área Temática:

Versão: 3

CAAE: 32712514.4.0000.5411

Instituição Proponente: Departamento de Morfologia

Patrocinador Principal: MINISTERIO DA EDUCACAO

DADOS DO PARECER

Número do Parecer: 855.412

Data da Relatoria: 02/11/2014

Apresentação do Projeto:

Células-tronco adultas tem sido isoladas e caracterizadas a partir de diferentes tipos de tecidos como medula óssea, cordão umbilical, encéfalo, epitélios, polpa dentária e mais recentemente tecido adiposo. O tecido adiposo subcutâneo representa uma fonte acessível e abundante na obtenção de células-tronco mesenquimais (CTMs). Este tecido possui alta variedade celular, sendo composto principalmente por adipócitos maduros, pré-adipócitos, fibroblastos, células de músculo liso da fração vascular, células endoteliais, monócitos e macrófagos. A adipogênese caracteriza-se como um processo altamente complexo, desde os sinais moleculares orquestrados por genes até a interação células-célula e/ou célula-matriz extracelular, mecanismos importantes para a regulação do processo de diferenciação de adipócitos. Sinais hormonais e nutricionais, assim como disruptores endócrinos, podem afetar tanto positiva como negativamente essa diferenciação. Disruptores endócrinos químicos são compostos naturais ou sintéticos que possuem a capacidade de alterar funções intrínsecas, mimetizando ou bloqueando hormônios endógenos. O TCDD 2,3,7,8-tetraclorodibenzeno-p-dioxina é uma molécula lipofílica pertencente à família dos disruptores endócrinos e acumula-se no tecido adiposo, placenta e leite. É conhecido por interferir nos processos de sinalização do metabolismo do hormônio tireoidiano e no

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