UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

DEPARTAMENTO DE BIOQUÍMICA PROF. TUISKON DICK

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

AVALIAÇÃO DO PAPEL NEUROPROTETOR DO CANABIDIOL FRENTE A TOXINAS REDOX-ATIVAS UTILIZANDO A LINHAGEM DE NEUROBLASTOMA HUMANO SH-SY5Y TERMINALMENTE DIFERENCIADAS E DURANTE A DIFERENCIAÇÃO NEURONAL

PATRÍCIA SCHÖNHOFEN

PORTO ALEGRE, FEVEREIRO DE 2014.

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, como requisito para obtenção do Grau de Mestre em Ciências Biológicas: Bioquímica

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PARTE I

RESUMO

Canabinóides derivados da *Cannabis sativa*, os fitocanabinóides, têm sido alvo de intensa pesquisa nas últimas décadas. Um dos seus compostos mais abundantes, o canabidiol (CBD), tem sido relacionado a efeitos neuroprotetores em neuropatologias, tanto *in vivo* com modelos animais quanto *in vitro* com linhagens de células tumorais humanas ou de origem animal. Até agora, poucos efeitos colaterais indesejado foram associados ao CBD. Neste estudo, avaliou-se o efeito neuroprotetor / neurotóxico do CBD em neurônios terminalmente diferenciados (maduros) e durante a diferenciação neuronal (modelo de toxicidade para o desenvolvimento neuronal) da linhagem de neuroblastoma humano SH-SY5Y. Uma curva dose-resposta foi realizada para estabelecer uma dose sub-letal com atividade antioxidante (2,5 µM). Em células SH-SY5Y terminalmente diferenciadas, a incubação com 2,5 µM CBD não foi capaz de proteger as células contra os efeitos neurotóxicos do glicolaldeído, metilglioxal, 6-hidroxidopamina e H_2O_2. Além disso, não foram observadas diferenças no potencial antioxidante e na densidade de neuritos. Quando as células SH-SY5Y foram expostas ao CBD durante a diferenciação, um protocolo para avaliar a toxicidade durante o desenvolvimento neuronal, também não foram observadas diferenças no potencial antioxidante e na densidade de neuritos. No entanto, o CBD causou um aumento da neurotoxicidade em todos os compostos testados. Os nossos dados indicam que 2,5 µM de CBD, a dose mais elevada tolerada pelas células SH-SY5Y, não apresentou atividade neuroprotetora em SH-SY5Y terminalmente diferenciadas e mostram, pela primeira vez, que a exposição ao CDB durante a diferenciação neuronal poderia sensibilizar as células imaturas para futuros desafios com toxinas.

ABSTRACT

Cannabis sativa-derived cannabinoids, named phytocannabinoids, have been a target of intense research over the last decades. One of its most abundant compounds, Cannabidiol (CBD), has been implicated with neuroprotective effect in many neuropathologies, both *in vivo* with rat models and *in vitro* with human or animal-derived tumoral cell lines. Until now, no undesired side-effect have been associated to CBD. In this study, we evaluated CBD's neuroprotective/neurotoxic effect in terminal-differentiated (mature) and during the neuronal differentiation (neuronal developmental toxicity model) of the human neuroblastoma SH-SY5Y cell line. A dose response curve was performed to establish a sub-lethal dose of CBD with antioxidant activity (2.5 µM). In terminal-differentiated SH-SY5Y cells, incubation with 2.5 µM CBD was not able to protect cells against the neurotoxic effect of glycolaldehyde, methylglyoxal, 6-hydroxydopamine and H_2O_2. Moreover, no difference in antioxidant potential and neurite density was observed. When SH-SY5Y cells were exposed to CBD during the differentiation, a protocol to evaluate neurodevelopmental toxicity, no difference in antioxidant potential and neurite density was also observed. However, CBD caused an increased neurotoxicity in all drugs tested. Our data indicate that 2.5 µM of CBD, the higher dose tolerated by differentiated SH-SY5Y cells, does not provide neuroprotection in terminal-differentiated SH-SY5Y and shows, for the first time, that exposure of CDB during neuronal differentiation could sensitize immature cells to future challenges with toxins.

LISTA DE ABREVIATURAS

2-AG – 2-Aracdonoilglicerol

6-OHDA – 6-hidroxidopamina

AEA – Anandamida, N-aracdonoiletanolamida

AGE – Produtos Finais Avançados de Glicação

AR – Ácido Retinóico

Aβ – Peptídeo β-amiloide

CB1 – Receptor Canabinóide tipo I

CB2 – Receptor Canabinóide tipo II

CBD – Canabidiol

DAT – Transportador de Dopamina

FAAH – Amida Hidrolase de Ácidos Graxos

GA – Glicolaldeído

H_2O_2 – Peróxido de Hidrogênio

LTD – *Long Term Depression,* depressão a longo prazo.

MG – Metilglioxal

MPP+ – 1-metil-4-fenil-piridina

PPAR – Receptor Ativado por Proliferadores de Peroxisomos

THC – Δ^9-tetrahidrocanabinol

TRP – Receptor Catiônico de Potencial Transiente

TRPV-1 – Receptor Catiônico de Potencial Transiente Tipo 1 / Receptor Vanilóide

1. **INTRODUÇÃO:**

1.1. Uso terapêutico de antioxidantes e neurodegeneração

Embora diferentes doenças neurodegenerativas tenham suas causas específicas, a neurodegeneração, ou seja, morte de neurônios, causada por dano oxidativo elevado é um fator em comum na fisiopatologia da maioria destas doenças (Halliwell 2001; Halliwell 2006). Em condições normais, defesas antioxidantes e espécies reativas estão em equilíbrio, embora alguns oxidantes não sejam removidos por participarem de funções biológicas importantes como o combate a infecções e resposta inflamatória (Halliwell 2006). Quando este equilíbrio é desfeito, temos o estresse oxidativo, que por definição, leva ao acúmulo de danos celulares.

O tecido cerebral é especialmente sensível ao dano oxidativo devido ao alto consumo de oxigênio, à presença de aminoácidos citotóxicos como o glutamato, de neurotransmissores auto-oxidáveis, de altos níveis de ferro entre outras especificidades (Halliwell 1992; Halliwell 2006).

Uma vez que, como dito acima, a produção de espécies reativas é importante na neurodegeneração, a busca e descrição de novas moléculas antioxidantes com potencial neuroprotetor são alvos para desenvolvimento de novas terapias (Posser et al. 2008).

Como revisado por Firuzi (2011), assume-se que os antioxidantes compensam os efeitos nocivos das espécies reativas, prevenindo ou tratando doenças relacionadas ao estresse oxidativo. No entanto, apesar do grande entusiasmo das décadas de 1980 e 1990, muitos agentes tidos como antioxidantes, como vitaminas, não apresentaram resultados positivos em testes clínicos. Apesar disto, estudos epidemiológicos pré-clínicos têm obtido

resultados positivos nos quais antioxidantes da dieta se mostraram benéficos, mantendo o interesse da comunidade científica na pesquisa de compostos antioxidantes com potencial terapêutico.

Assim, é possível identificar moléculas antioxidantes baseando-se apenas na sua estrutura química. No entanto, nem todas estas substâncias apresentam esta capacidade em sistemas biológicos ou, segundo Gutteridge e Halliwell (2010), antioxidantes podem levar a citotoxicidade. Por isto, é aconselhável que compostos antioxidantes passem por triagens em modelos experimentais adequados antes de sua utilização *in vivo*.

Como exemplo de compostos com predita atividade antioxidante, os canabinóides (compostos fenólicos endógenos, naturais ou sintéticos ativadores de receptores canabinóides) (Howard et al. 2013) vêm despertando crescente interesse devido a suas propriedades neuroprotetoras em vários modelos de dano neuronal e neurodegeneração, sendo considerados alvos potenciais para o desenvolvimento de novas terapias farmacológicas (Koppel and Davies 2008; Fernández-Ruiz et al. 2010).

1.2. Fitocanabinóides e o Sistema Endocanabinóide

A planta *Cannabis sativa* tem sido usada para fins medicinais há milhares de anos por diferentes culturas, embora hoje saibamos os seus efeitos adversos (Zuardi 2006). A primeira evidência do uso desta planta data de 4000 AC na China (Li 1973), provavelmente era cultivada para obtenção de suas fibras para produção de têxteis, para alimentação (frutas) e como medicamento (Touw 1981).

A *Cannabis* foi introduzida na Medicina Ocidental no século XIX, sendo bastante utilizada até o final deste século. Porém, no início do século XX, esse uso diminuiu devido aos diversos efeitos apresentados (Zuardi 2006).

Na resina de plantas fêmeas da *Cannabis* são encontrados cerca de 80 compostos lipossolúveis, os fitocanabibóides, (Elsohly and Slade 2005; Izzo et al. 2009). Todos fitocanabinóides são encontrados exclusivamente na *Cannabis* e seus maiores componentes, o Δ^9-tetrahidrocanabinol (THC, principal composto psicoativo) e o canabidiol (CBD) são sintetizados a partir de um mesmo precursor, o canabigerol (Galve-roperh et al. 2013).

Os fitocanabinóides foram isolados, identificados e sintetizados pela primeira vez décadas atrás (Gaoni e Mechoulam 1971), mas apenas quando os receptores canabinóides foram descritos no cérebro, o modo de ação da *Cannabis* e dos fitocanabinóides foi esclarecido, levando também à identificação e isolamento de seus homólogos endógenos (Howlett et al. 2010), os endocanabinóides. Os primeiros a serem descobertos e mais bem caracterizados são a N-arachidonoiletanolamina (ou anandamida, AEA) e 2-arachidonoilglicerol (2-AG) (Luchicchi e Pistis 2012).

Os receptores canabinóides, os endocanabinóides (como a anandamida e o 2-AG) e as enzimas que catalisam sua biossíntese, constituem o sistema endocanabinóide, que exerce importantes ações sobre o sistema nervoso central (Pertwee et al. 2010).

Os endocanabinóides são sintetizados e liberados em resposta a estímulos fisiológicos ou patológicos, ligam e ativam seus receptores, causando diversos efeitos biológicos em diferentes tecidos (Pertwee et al. 2010). Os principais receptores alvos para fitocanabinóides e

endocanabinóides são receptores canabinóides acoplados à proteína G tipo 1 (CB1), amplamente expresso no sistema nervoso, e tipo 2 (CB2), expresso principalmente em células do sistema imune embora presentes em algumas células do sistema nervoso (Di Marzo e De Petrocellis 2012; Pertwee 2012). Em células neuronais estes dois receptores apresentam perfis de expressão opostos, com CB1 crescente e CB2 decrescente ao longo diferenciação neuronal (Begbie et al. 2004; Palazuelos et al. 2006; Watson et al. 2008).

Durante o desenvolvimento, CB1 e CB2 regulam várias cascatas de proteínas cinases envolvidas na proliferação e sobrevivência celular, com consequências importantes sobre o destino de células progenitoras (Galve-roperh et al. 2013).

No sistema nervoso, o sistema endocanabinóide modula as sinapses através da liberação de endocanabinóides, que atuam como mensageiros retrógrados, pelo neurônio pós-sináptico ativando receptores CB1 no neurônio pré-sináptico, levando a diminuição da liberação de neurotransmissores na fenda sináptica (Alger 2002; Velasco et al. 2012), como ilustra a figura 1 (Velasco et al. 2012). Este Sistema modula eventos como LTD (*Long Term Depression*) que são responsáveis pela formação da memória e plasticidade sináptica (Han et al. 2012).

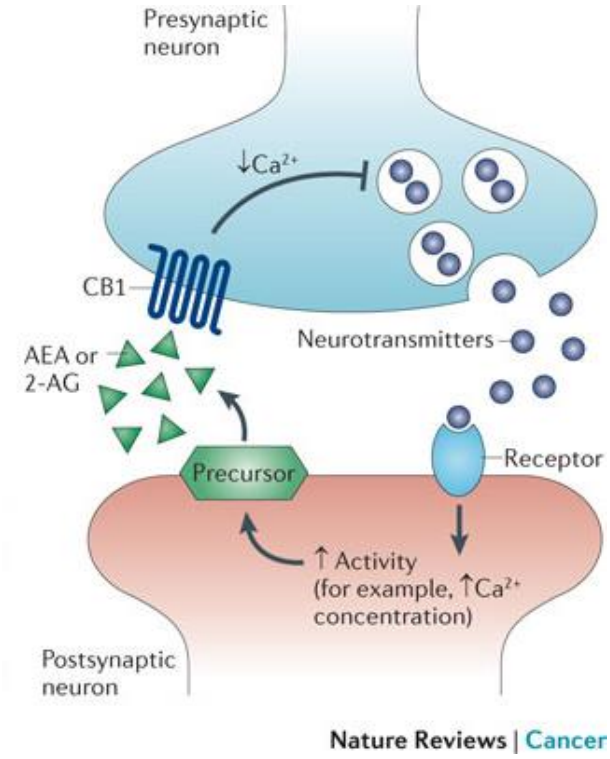

Fig. 1: Sistema endocanabinóide. No sistema nervoso, endocanabinóides são sintetizados sob demanda no neurônio pós-sináptico, em resposta ao aumento de Cálcio intracelular, e liberados na fenda sináptica, onde ativam CB1. Como consequência, no neurônio pré-sináptico ocorre redução do influxo de Cálcio, resultando em uma menor liberação de neurotransmissores (Adaptado de Velasco et al. 2012).

Outros alvos dos canabinóides incluem o receptor vanilóide tipo 1 (TRPV-1), membro da super-família de receptores catiônicos de potencial transiente (TRP) (Di Marzo and De Petrocellis 2010), e receptores ativados por proliferadores de peroxisomos (PPAR), uma família de receptores nucleares capazes de alterar o "*turnover*" lipídico e o metabolismo, que demonstraram ligarem-se fracamente à AEA e 2-AG, bem como a alguns fitocanabinóides sintéticos (Pistis and Melis 2010).

Fitocanabinóides também interagem com enzimas do sistema endocanabinóide. Por exemplo, CBD inibe a FAAH (*fatty acid amide hydrolase*), a enzima responsável pela degradação de AEA, regulando a disponibilidade deste endocanabinóide (De Petrocellis et al. 2011). Ou seja, CBD potencialmente aumenta os níveis teciduais de AEA o que pode mediar alguns efeitos farmacológicos do CBD e seus análogos (Bisogno et al. 2001).

Estudos apontam evidências de que o sistema endocanabinóide tem um papel modulador da atividade dopaminérgica nos gânglios da base, coordenando sinais moduladores da atividade motora da via nigroestriatal (Morera-Herreras et al. 2012; Harvey et al. 2012).

Pesquisas recentes apontam que os efeitos motores causados pela administração de canabinóides, são mediados pelos receptores CB1 localizados principalmente nas redes dopaminérgicas dos gânglios da base, sugerindo que a ação destes compostos sobre o sistema endocanabinóide se deve à modulação da transmissão dopaminérgica (Morera-Herreras et al. 2012). Ainda assim, resultados controversos vêm sendo observados quanto à eficiência dos canabinóides em melhorar estes sintomas, evidenciando a necessidade de maiores estudos sobre a interação entre os canabinóides, como o CBD, e o sistema endocanabinóide e seus efeitos nos sintomas motores de doenças neurodegenerativas.

1.3. Propriedades neuroprotetoras do Canabidiol

O CBD é um dos fitocanabinóides mais relevantes, representando mais de 40% do extrato total da *Cannabis*, sem apresentar os típicos efeitos psicoativos do THC (Karniol et al. 1974; Grlic 1976). O mesmo possui um anel

fenólico que lhe confere potencial antioxidante, conforme sua estrutura química ilustrada na figura 2 (Borges et al. 2013).

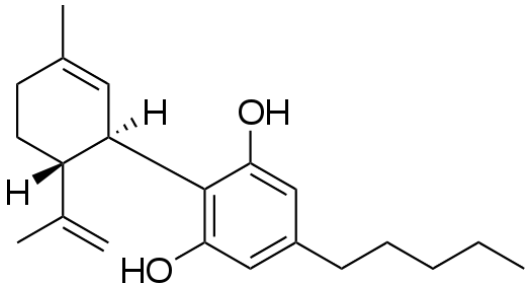

Fig. 2: Fórmula estrutural do Canabidiol.

O CBD tem sido associado a propriedades neuroprotetoras em um grande número de estudos. Mais precisamente, propriedades anti-inflamatórias e antioxidantes foram relatadas indicando possíveis usos terapêuticos em doenças neurodegenerativas como Doença de Parkinson, Doença de Alzheimer, isquemia cerebral, entre outras (Fernández-Ruiz et al. 2013). Além disto, CBD é anti-emético, como a maioria dos canabinóides, e esta capacidade pode estar relacionada à modulação de receptores de serotonina (Parker et al. 2011). O CBD também tem ação antitumoral contra diversos tipos de câncer (Ligresti et al. 2006; Massi et al. 2006; Solinas et al. 2012) e apresenta resultados positivos como antipsicótico, ansiolítico, antidepressivo (Crippa et al. 2010) e anti-epilético (Gordon and Devinsky 2001).

Embora não haja dados sobre segurança e possíveis efeitos adversos do uso de derivados da *Cannabi*s em crianças, há relato de uma pesquisa clínica que reuniu pais de crianças com epilepsia resistente ao tratamento, que passaram a administrar *Cannabis* com altos níveis de CBD a seus filhos.

(Porter and Jacobson 2013). Outro estudo, mais antigo, já havia testado o CBD purificado em pacientes adultos epiléticos e saudáveis (Cunha et al. 1980). Ambos relataram melhora na frequência das crises convulsivas.

Há alguns estudos avaliando o papel neuroprotetor do CBD *in vivo* e *in vitro* frente a toxinas redox-ativas como 6-hidroxidopamina (6-OHDA) (Lastres-becker et al. 2005), peptídeo β-amiloide (Aβ), peróxido de hidrogênio (H_2O_2) entre outros (Harvey et al. 2012; Carroll et al. 2012). Em um estudo recente, CBD reverteu a redução dos níveis de sinaptofisina e o aumento de caspase-3 induzidos em um modelo animal para Doença de Parkinson induzida por ferro (da Silva et al. 2013), melhorando também deficiências de memória associadas a toxicidade do ferro neste mesmo modelo (Fagherazzi et al. 2012). Após a indução de hipóxia-isquemia em ratos recém-nascidos, a administração de CBD reduziu os danos cerebrais e restaurou funções neurocomportamentais dos animais (Pazos et al. 2012). Ainda em recém-nascidos, o tratamento com CBD após a transecção do nervo ciático resultou no reestabelecimento das funções sensoriais e motoras (Perez et al. 2013).

Como o CBD aparentemente não apresenta efeitos adversos importantes e ainda exibe um amplo espectro de possíveis propriedades terapêuticas (Mechoulam et al. 2007; Bergamaschi et al. 2011; Pazos et al. 2012), o seu uso é considerado tão protetor o quanto inócuo (seguro) em modelos animais adultos (Lastres-Becker et al. 2005) e recém-nascidos (Lafuente et al. 2011; Pazos et al. 2012). Medicamentos como o Sativex®, que combina THC e CBD, já estão em uso para o tratamento sintomático da dor e espasticidade causada pela esclerose múltipla e para amenizar efeitos colaterais de tratamentos contra o câncer (Sastre-Garriga et al. 2011).

1.4. Modelos experimentais utilizados nos estudos com CBD

A maioria dos estudos com CBD são realizados com modelos *in vivo*, cultura primária derivada de tecido nervoso de roedores ou linhagens celulares humanas derivadas de tumores (Harvey et al. 2012). Abordagens *in vivo* avaliam principalmente efeitos neurocomportamentais e neuropatológicos e são caros, demorados, inadequados para a rápida triagem (*screening*) de um grande número de compostos. Ainda, outros modelos animais (como cultura primária) não são sensíveis o suficiente para predizer neurotoxicidade em humanos, pois são incapazes de mimetizar o comportamento de células humanas (Bal-Price et al. 2008). Além disto, linhagens tumorais não têm características moleculares e morfológicas de células terminalmente diferenciadas, como neurônios humanos maduros (Radio and Mundy 2008).

Apesar da intensa pesquisa pré-clínica em várias doenças neurodegenerativas (Ramos et al. 2007; Zuardi 2008; Valdeolivas et al. 2012), os mecanismos moleculares de ação do CBD ainda não foram completamente identificados em células neuronais (Fernández-Ruiz et al. 2013). Assim, existe a necessidade de utilização de modelos *in vitro* mais adequados para este tipo de pesquisa, tais como linhagens celulares.

Linhagens têm sido amplamente utilizadas para a avaliação toxicológica rápida de um grande número de compostos (Radio and Mundy 2008). Uma delas, a linhagem de neuroblastoma humano SH-SY5Y, apresenta várias vantagens para os estudos de neurociências, como a sua origem humana, a facilidade para crescimento e manutenção e é um modelo adequado para estudar a neurotransmissão (Bal-Price et al. 2008). Ainda assim, apesar de sua

origem tumoral, a morfologia neuronal pode ser acessada por um processo de diferenciação em neurônios dopaminérgicos induzida por ácido retinóico (AR), o que também leva ao aumento de marcadores bioquímicos de neurônios maduros (Lopes et al. 2010). Assim, células da linhagem SH-SY5Y terminalmente diferenciadas com AR são consideradas como um modelo *in vitro* mais adequado para avaliar o potencial neuroprotetor ou neurotóxico de compostos para posterior aplicação em estudos *in vivo* (Lopes et al. 2010; Lopes et al. 2012). Esta linhagem também pode ser utilizada como um modelo para *screening* de drogas com potencial atividade durante o desenvolvimento neuronal, uma vez que estas drogas sejam administradas durante o processo de diferenciação com AR (Radio e Mundy 2008).

Na verdade, culturas de células derivadas de tecidos do sistema nervoso vêm sendo usadas em estudos sobre a ação de toxinas durante o desenvolvimento do sistema nervoso (Bal-Price et al. 2008; Radio and Mundy 2008), destacando-se como uma forma rápida e eficaz de avaliação do potencial de drogas sobre o desenvolvimento neuronal.

2. OBJETIVOS

2.1 Objetivo geral

Este estudo foi elaborado com o objetivo de avaliar os possíveis efeitos neuroprotetores e antioxidantes do canabidiol em nível celular, em neurônios humanos maduros e durante seu desenvolvimento. Para tal, foram utilizadas doses subletais de CBD (99.9%, THC-Pharm, Frankfurt, Germany) em células da linhagem de neuroblastoma humano SH-SY5Y terminalmente diferenciadas e durante a diferenciação.

2.2 Objetivos específicos

- Definição da dose de CBD com maior efeito antioxidante *in vitro* e menor citotoxicidade.
- Determinação de parâmetros redox em células da linhagem SH-SY5Y tratadas ou diferenciadas com CBD.
- Avaliação de parâmetros morfológicos, como a densidade de neuritos, em células da linhagem SH-SY5Y tratadas ou diferenciadas com CBD.
- Avaliação da neuroproteção em células da linhagem SH-SY5Y tratadas ou diferenciadas com CBD desafiadas com toxinas redox-ativas H_2O_2, 6-OHDA, MG e GA, simulando os mecanismos neurodegenerativos.
- Avaliação dos níveis de expressão de componentes do sistema endocanabinóide em SH-SY5Y diferenciadas em comparação com o fenótipo tumoral, através de bancos de dados e ferramentas de bioinformática.

PARTE II

3. RESULTADOS

Os resultados desta dissertação estão apresentados na forma de artigo científico (Capítulo I).

CAPÍTULO I: Manuscrito a ser submetido para o periódico Neurotoxicity Research.

Title:

Evaluation of the Neuroprotective Role of Cannabidiol Against Redox-Active Toxins Using Terminal-Differentiated and During Neuronal Differentiation of Human Neuroblastoma SH-SY5Y Cell Line

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Abstract

Cannabis sativa-derived cannabinoids, named phytocannabinoids, have been a target of intense research over the last decades. One of its most abundant compounds, Cannabidiol (CBD), has been implicated with neuroprotective effect in many neuropathologies, both *in vivo* with rat models and *in vitro* with human or animal-derived tumoral cell lines. Until now, no undesired side-effect have been associated to CBD. In this study, we evaluated CBD's neuroprotective/neurotoxic effect in terminal-differentiated (mature) and during the neuronal differentiation (neuronal developmental toxicity model) of the human neuroblastoma SH-SY5Y cell line. A dose response curve was performed to establish a sub-lethal dose of CBD with antioxidant activity (2.5 µM). In terminal-differentiated SH-SY5Y cells, incubation with 2.5 µM CBD was not able to protect cells against the neurotoxic effect of glycolaldehyde, methylglyoxal, 6-hydroxydopamine and H_2O_2. Moreover, no difference in antioxidant potential and neurite density was observed. When SH-SY5Y cells were exposed to CBD during the differentiation, a protocol to evaluate neurodevelopmental toxicity, no difference in antioxidant potential and neurite density was also observed. However, CBD caused an increased neurotoxicity in all drugs tested. Our data indicate that 2.5 µM of CBD, the higher dose tolerated by differentiated SH-SY5Y cells, does not provide neuroprotection in terminal-differentiated SH-SY5Y and shows, for the first time, that exposure of CDB during neuronal differentiation could sensitize immature cells to future challenges with toxins.

***Keywords*:** Cannabidiol, CBD, Neuroprotection, Neurodevelopmental toxicity, SH-SY5Y cells, Neurotoxicity, Redox-active Compounds.

Introduction

The plant *Cannabis sativa* has been used for medicinal purposes for thousands years by different cultures (Cassol-jr et al. 2010). The two major components of cannabis extract are Δ^9-tetrahydrocannabinol (THC, the main psychoactive ingredient), and cannabidiol (CBD), that represents up to 40% of its extract and is devoid of the typical psychoactive effects of THC (Karniol et al. 1974; Grlic 1976). These compounds are the major members of the phytocannabinoids, or plant-derived cannabinoids, which include more than 80 lipid-soluble compounds present in the female plants and found exclusively in cannabis (Izzo et al. 2009).

Phytocannabinoids were first identified decades ago (Gaoni and Mechoulam 1971), but only the further discovery of cannabinoid receptors led to identification of their endogenous homologous, the endocannabinoids, (Howlett et al. 2010), of with the best characterized are N-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) (Luchicchi and Pistis 2012). Together with their related enzymes, endocannabinoids and receptors form the endocannabinoid system (Pertwee et al. 2010). Both phytocannabinoids and endocannabinoids targets the G protein-coupled cannabinoid receptors type-1 (CB1), widely expressed in the nervous system, and type-2 (CB2), mainly expressed in immune cells (Di Marzo and De Petrocellis 2012; Pertwee 2012). Along the neural development, CB1 and CB2 regulate protein kinase cascades involved in cell proliferation and survival, with major consequences on progenitor cell fate decisions (Galve-roperh et al.

2013), while CB1 expression increases CB2 decreases during neuronal differentiation (Begbie et al. 2004; Palazuelos et al. 2006; Watson et al. 2008).

The identification of CBD anti-inflammatory and antioxidant potential brought a wide range of possible therapeutic uses of CBD on neurodegenerative disorders including Parkinson's disease, Alzheimer's disease, cerebral ischemia, among others (Fernández-Ruiz et al. 2013). Moreover, CBD is anti-emetic (Parker et al. 2011), has anti-tumoural properties against many types of cancer (Ligresti et al. 2006; Massi et al. 2006; Solinas et al. 2012) and is also referred as an antipsychotic, anxiolytic, antidepressant (Crippa et al. 2010) and antiepileptic (Gordon and Devinsky 2001). Parents are already using CBD-enriched cannabis for treatment resistant epilepsy children, although data of cannabidiol use among children are inconclusive about its safety and tolerability (Porter and Jacobson 2013).

CBD is predicted to have antioxidant properties conferred by the presence of a phenolic ring in its structure (Borges et al. 2013). There are some studies evaluating the neuroprotective role of CBD *in vivo* and *in vitro* against redox-active neurotoxins such as 6-hydroxydopamine (6-OHDA) (Lastres-becker et al. 2005), *β*-amyloid (A*β*) peptide, hydrogen peroxide (H_2O_2) among others (Harvey et al. 2012; Carroll et al. 2012). In a recent study, CBD was able to reverse iron-induced reductions in synaptophysin levels and increases in caspase-3 levels (da Silva et al. 2013), and it has either improved memory impairments associated to iron toxicity in a rat model (Fagherazzi et al. 2012). CBD administration after hypoxia-ischemia in newborn rats can also reduce brain injury and restored neurobehavioral function (Pazos et al. 2012). Still in

newborn rats, treatment with CBD after transection of the sciatic nerve has resulted in both motor and sensory neuron rescue (Perez et al. 2013).

As CBD apparently have no important side effects and exhibits a broad spectrum of possible therapeutic properties (Mechoulam et al. 2007; Bergamaschi et al. 2011; Pazos et al. 2012), it has been predicted as innocuous (or harmless) from adult (Lastres-Becker et al. 2005) to newborn animal models (Lafuente et al. 2011; Pazos et al. 2012). Due to its protection for neuroinflammatory disorders (Costa et al. 2004), a medicine containing CBD combined with THC (Sativex®) has been already licensed for the symptomatic treatment of spasticity and pain associated with multiple sclerosis (Sastre-Garriga et al. 2011).

Most studies with CBD used *in vivo* models, primary cultures derived from rodents or tumoral-derived human cell lines (Harvey et al. 2012). Despite the intense preclinical research into numerous neurodegenerative disorders (Ramos et al. 2007; Zuardi 2008; Valdeolivas et al. 2012), CBD's molecular mechanisms of action are yet to be completely identified (Fernández-Ruiz et al. 2013). For this purpose, *in vivo* neurotoxicity testing evaluating the effects of compounds on neurobehavioral and neuropathological processes are expensive, time consuming, and unsuitable for screening large number of chemical and, as other animal models, is not sensitive enough to predict human neurotoxicity (Bal-Price et al. 2008). Moreover, tumoral cells does not have the molecular and morphological characteristics of human neurons (Radio and Mundy 2008). Furthermore, few studies have been conducted to evaluate the effects of CBD on developing neurons.

Thus, there is the necessity of a more appropriate *in vitro* model to rapidly access these informations. Lineages have been widely used for the rapid toxicological evaluation of large numbers of chemicals (Radio and Mundy 2008). One of them, the human neuroblastoma SH-SY5Y, presents several advantages for neuroscience studies such as its human origin, the facility to grow and maintain and is a suitable model for studying neurotransmission (Bal-Price et al. 2008). Still, regardless of its tumoral origin, the neuronal morphology can be accessed by a differentiation process into dopaminergic phenotype through retinoic acid (RA) (Lopes et al. 2010). Thus, the RA-differentiated SH-SY5Y cells are considered a more suitable *in vitro* model to evaluate neuroprotective/neurotoxicity of compounds (Lopes et al. 2010; Lopes et al. 2012). It also can be a model to screening of drugs during neuronal development when these drugs are administered during the differentiation process, since cell cultures derived from nervous system tissue have been used to understand the action of toxins in the nervous system development (Bal-Price et al. 2008; Radio and Mundy 2008).

In this sense, the aim of this study was to evaluate CBD's effects in terminal-differentiated (mature) as well as during the process of differentiation (neuronal developmental toxicity model), using the RA-differentiated human neuroblastoma SH-SY5Y cell line.

Experimental Procedures

Chemicals

Materials used in cell culture were acquired from Gibco®/Invitrogen (São Paulo, SP, Brazil). Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Cannabidiol (99.9%, THC-Pharm, Frankfurt, Germany) was diluted in ultrapure dimethyl sulfoxide (DMSO).

Cell Culture, Differentiation, and Treatments

Exponentially growing human neuroblastoma SH-SY5Y cell line, obtained from ATCC (Manassas, VA, USA), was maintained at 37ºC in a humidified atmosphere of 5% CO_2. The cells were grown in a mixture of 1:1 of Ham's F12 and Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% of fetal bovine serum (FBS), 2 mM of glutamine, 1000U/mL penicillin, 1000µg/mL streptomycin and 2,5µg/mL of Fungizone® (amphotericin B). Medium was changed each 3 days and cells were sub-cultured once they reached 80% confluence. After 24 h of plating, neuronal differentiation was triggered by lowering the FBS to 1% with the addition of 10 µM RA during 7 days, as previously established by our group (Lopes et al. 2010). In the seventh day of RA-induced differentiation, the SH-SY5Y cells were treated with CBD for 24 h. For evaluation of CBD's effects over neuronal development, CBD was co-administered with RA during the differentiation. In the seventh day, CBD and RA were replaced and experiments were performed 24 h after. For cell viability and reactive species (RS) generation assays, cells were seeded in 96-wells plate at density of 2×10^4 cells/well.

For cell extract preparation, cells were seeded in 75 cm^2 culture flasks and, after differentiation and treatments, the medium was removed and cells were washed with PBS. After that, cells were frozen at -80ºC and thawed twice

in 10 mM PBS. Cells extracts were homogenized and centrifuged to remove cell debris. The supernatant was collected and antioxidant assays were performed immediately.

Neurites Density

The stellate morphology and neurites density were analyzed by immunofluorescence. Cellular treatments were performed in twelve-well plates at a density of 10^5 cell/well. Cells were washed with PBS, fixed with methanol / acetone solution (1:1) for 20 minutes and permebilized with PBS / Tween 0,2%. After washed twice with PBS, blocking was performed with 1% BSA solution for 1 h. Then, cells were incubated overnight with anti-βIII tubulin antibody Alexa 488-conjugated. After that, the nuclear dye Hoescht 33258 was added. Readily, 5 microscopic fields were randomly selected and photographed using an Olympus IX70 inverted microscope and analyzed with NIS-elements software. Neurites density was assessed using the AutoQuant Neurites software (demo version), and expressed as arbitrary units (A.U.).

Neurotoxicity assay

Neurotoxicity was evaluated by the quantification of 3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction to a blue formazan product by cellular dehydrogenases. At the end of the treatment, cells were incubated with 0.5 mg/mL of MTT during 1 h at 37 ºC. Then, medium was discarded and DMSO was added to solubilize the formazan crystals. The absorbance was determined at 560 and 630 nm in a SoftMax Pro Microplate

Reader (Molecular Devices, USA). The results were expressed in percentage of untreated cells (mean ± SD value).

Neuroprotection assay

After treatment or differentiation with selected dose of CBD, cells were washed with PBS and challenged with the different toxins: 6-Hydroxidopamine (6-OHDA, LD_{50} = 15 µM), Methilglyoxal (MG, LD_{50} = 1350 µM), Glycolaldehyde (GA, LD_{50} = 115 µM) and Hydrogen Peroxide (H_2O_2, LD_{50} = 750 µM) to evaluate the neuroprotective features of CBD through MTT assay. The results were expressed in percentage of untreated cells (mean ± SD value).

Reactive species generation

To evaluate if CBD treatment was able to decrease the generation of reactive species (RS), we used the probe DCF-DA (2, 7-dichlorodihydrofluorescein diacetate) (Wang and Joseph 1999; Halliwell and Whiteman 2004). After CBD treatment, the medium was removed and 10 µM DCF-DA was added. After 1 h incubation, medium was changed and the fluorescence was measured in a plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm. The results were expressed in percentage of untreated cells (mean ± SD value).

Total Radical-Trapping Antioxidant Potential (TRAP) and Total Antioxidant Reactivity (TAR)

The non-enzymatic antioxidant capacity of CBD was assessed through the total radical-trapping antioxidant potential (TRAP) assay, which is based on

the measurement of luminescence generated by luminol oxidation by AAPH (2, 20-azobis 2-amidinoprepane) decomposition, in glycine buffer (pH 8.6). After system stabilization (buffer, luminol and AAPH), different concentrations of CBD or cell extracts were added and the luminescence signal decreases proportionately to its antioxidant potential. The luminescence was monitored in a Wallace 1450 MicroBeta TriLux Liquid Scintillation Counter & Luminometer (Perkin Elmer). For data analysis, a time per chemiluminescence curve was obtained and the relative ‘‘area under the curve’’ (AUC) in the recovery phase was used, as previously established (Lissi et al. 1995; Dresch et al. 2009). In order to evaluate not only the quantity of oxidants but also their reactivity, we used the total antioxidant reactivity assay (TAR), (Lissi et al. 1995).

Thiol (–SH) levels

Briefly, samples were dilute in 10 mM of boric acid with 0.2 mM EDTA (pH 8.5). 10 mM of DTNB was added and –SH levels were determined by measuring absorbance at 412 nm. Results are expressed in nmol –SH/mg protein, as described (ELLMAN 1959).

Differential Gene Expression and Enrichment Analysis

Microarray expression profiles were extracted from the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/). Differential gene expression was evaluated using ViaComplex® software (Castro et al. 2009; Castro et al. 2010).Gene Set Enrichment Analysis (GSEA) was used to identify genes that contribute individually to global changes in expression levels in a given microarray dataset (Subramanian et al. 2005).

Protein Quantification

The proteins contents were measured by the Bradford assay (Bradford 1976).

Statistical Analysis

Data are expressed as percentage of untreated cells (control) (mean ± SD) from at least three independent experiments. For statistical analysis, data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test. Differences were considered significant at $P > 0.05$.

Results

Dose selection

Initially, a dose response curve was performed to evaluate basal neurotoxicity and antioxidant potential of CBD (1.0, 2.5, 5.0 and 10.0 μM), aiming to find the concentration of that presents high *in vitro* antioxidant potential with concomitant low neurotoxicity against the RA-differentiated human neuroblastoma SH-SY5Y cells. Although by TRAP assay CBD were able to scavenge the peroxyl radical generated by AAPH decomposition only in the higher concentration, TAR assay shows significant antioxidant reactivity of CBD as low as 2.5 μM (Fig. 1a). MTT assay shows that CBD does not affect cell viability until 2.5 μM (Fig. 1b). As this dose has also presented lower radical production by DCF-DA assay (Fig. 1c), it was selected to all further experiments.

Effects of Cannabidiol in terminal-differentiated SH-SY5Y cells

In order to evaluate the effects of CBD in mature neurons, we used RA-differentiated SH-SY5Y cells treated with CBD for 24 h. No statistically significant results were found by TRAP, TAR (Fig. 2a) or thiol levels (Fig. 2b) of cell extract. Morphological parameters and neurites densities were assessed (Fig. 2c) and no differences were observed between treatments.

To evaluate neuroprotection, cells were challenged with the LD_{50} of 6-OHDA, MG, GA and H_2O_2 for 24 h. In all challenges, there was a significant loss of viability either in cells treated only with the toxins and in the ones pre-treated with CBD (Fig. 2d).

Effects of Cannabidiol during the differentiation of SH-SY5Y cells

Differentiated SH-SY5Y cells with RA and CBD for 7 days were used to assess the effects of CBD during the differentiation process. No statistically significant results were found by TRAP, TAR (Fig. 3a) or thiol levels (Fig. 3b) of cell extract. Morphological parameters were assessed (Fig. 3c), evidencing no significant differences between these cells. Cells were also challenged with sub-lethal doses of 6-OHDA, MG, GA and H_2O_2. None of the toxins were able to induce cell death alone. In fact, similar results were observed in cells differentiated with CBD and those treated only with toxins. However, when these cells differentiated with CBD were challenged with toxins, their viability significantly decreased (Fig. 3d).

Differential gene expression and enrichment analysis show that the endocannabinoid system components presented enriched expression patterns

in RA-differentiated SH-SY5Y cells in comparisson to undifferentiated cells of this lineage (Fig. 4). With emphasis on CB1 receptor gene expression enrichment, but not CB2, in accordance to previous studies reporting an up-regulation of CB1 and down regulation of CB2 along neuronal development (Begbie et al. 2004; Palazuelos et al. 2006; Watson et al. 2008)

Discussion

Despite the large number of studies reporting positive results of CBD as a neuroprotective and neurogenic molecule in several animal models *in vitro* and *in vivo* (in addition to pre-clinical trials), few studies evaluated the effect of CBD over terminal-differentiated human neuron-like cells. Once animal models do not seems to provide accurate and useful information about human neurotoxicity, as they are not able to mimic human cell conditions, alternative tests (as *in vitro* assays) in screening strategies would allow better and faster compound data acquisition, thus, facilitating predictions of neurotoxicity for further use in the refinement of *in vivo* studies (Bal-Price et al. 2008). Additionally, *in vitro* models could be used to screen for chemical effects on neurodevelopment, including effects on differentiation and neurites outgrowth (Radio and Mundy 2008). In this context, the human neuroblastoma SH-SY5Y cell line, which can be differentiated to cells with mature neuronal phenotype (Lopes et al. 2010), emerges as a suitable model for studying the effect of neuroprotective / neurotoxic molecules in maturing neurons.

As CBD is known as a potent antioxidant molecule and since oxidative stress is related to pathophysiologic mechanisms of many neurodegenerative diseases (Schapira 2008), our dose selection was based on cell viability and

antioxidant activity. The dose-response curve was based on previous studies, as reviewed in Fernández-Ruiz et al. 2013. We selected 2.5 μM of CBD for further treatments based on cell viability and on *in vitro* redox parameters. Although this dose has shown the best results in dose selection experiments, other concentrations of CBD could provide different results in our model, since the dose of 2.5 μM may be outside of the reported bell-shaped dose-response curve of CBD (Mishima et al. 2005; Campos et al. 2012; Fernández-Ruiz et al. 2013). Our results also show that, at lower doses than the selected, CBD is not able to decrease oxidative stress (Fig. 1a). Yet, at higher doses, although CBD showed potent antioxidant potential, it was strongly cytotoxic over terminal-differentiated human neuron-like cells. The concept of antioxidant administration to human health should be taking with caution, because any antioxidant compound may alter the intracellular redox balance toward a more reduced state, being as much cytotoxicity as oxidative stress (Gutteridge and Halliwell 2010), as we have seen in our results. The same curve was also tested in not differentiated SH-SY5Y cells without significant changes in cell viability throw the concentration as high as 10 μM (data not show), which is in agreement with previous reports (Harvey et al. 2012).

Hereafter, we challenged SH-SY5Y cells with the redox-active toxins MG, GA, 6-OHDA and H_2O_2. Glycotoxins, such as MG and GA, are used in neurodegenerative models for diabetic neuropathy (Davies et al. 1986; Ohkawara et al. 2012; Sato et al. 2013). These toxins generates protein complexes irreversibly glycated non-enzymatically, the advanced glycation end products (AGE), present at higher levels in individuals exposed to prolonged hyperglycemia (Nagai et al. 2000). 6-OHDA, one of the most used toxin in

Parkinson's Disease experimental models (Gomez-Lazaro et al. 2008; Lopes et al. 2012), is an analog of dopamine with similar structural characteristics and affinity to its transporter (DAT), that accumulates inside the neurons causing oxidative damage (Lehmensiek et al. 2006). Hydrogen peroxide (H_2O_2) is an oxidant used in neurotoxicity models and associated with neuropathologies (Huang et al. 2004; Harvey et al. 2012; Turkez et al. 2013; Huang et al. 2014; Turkez et al. 2014).

Using differentiated SH-SY5Y cells as a model for mature human neurons screening of neuroprotection / neurotoxicity, our results show that CBD is not protective against the toxins assessed, although morphological changes was not observed. It corroborates with another study in which CBD had no protective effect against 7 mM of 1-methyl-4-phenylpyridinium (MPP+) toxicity at 0.01 0.1 and 1.0 µM in differentiated SH-SY5Y (Carroll et al. 2012), however they used lower doses. Cannabidiol also improved cell viability in response to *tert*-butyl hydroperoxide in PC12 rat pheochromocytoma cells and undifferentiated SHSY5Y cells, while it was not able to inhibit *β*-amyloid and H_2O_2 toxicity at 1.0 and 10.0 µM (Harvey et al. 2012).

As reviewed in (Fernández-Ruiz et al. 2013), CBD does not have affinity with CB1, but, depending on its concentration, it can act as agonist or antagonist of this receptor. In the nanomolar range, CBD can antagonise the pharmacological effects of CB1 agonists (THC, AEA), despite having low affinity in the micromolar range for CB1 *in vitro* (Pertwee et al. 2002; Englund et al. 2012). The dose selected in this study seems to antagonize CB1 activity. Phytocannabinoids also modulate the activity of the endocannabinoid system, for example, CBD can inhibits FAAH (fatty acid amide hydrolase) the enzyme

that inactivate AEA (De Petrocellis et al. 2011), increasing is the tissue levels (Bisogno et al. 2001). CB1-mediated neuroprotection occurs through inhibition of adenylyl cyclase, and further decrease of intracellular calcium during a neurotoxic event (Zhuang et al. 2005). This receptor expression is increased in response to toxin exposure in differentiated SH-SY5Y, as an indicative of neuronal damage, which happens in disease process (Carroll et al. 2012). Yet, nigral CB1 activity was also increased in animal model for Parkinson's Disease (Lastres-becker et al. 2005). However, the same study found that protective effect of CBD is unlikely to be mediated by the CB1 (Lastres-becker et al. 2005; Carroll et al. 2012). Therefore, in different conditions, CBD seems to be able to interact directly in different receptors of the endocannabinoid system, or indirectly, through regulation of endocannabinoids levels which have more affinity with these receptors.

In our results, although bioinformatics approach shown that CB1 expression was enhanced in differentiated cells, and it has been proved the up-regulation of CB1 occurs in response to neuronal damage (Carroll et al. 2012), CBD did not provided improvements on cell antioxidant defenses nor neuroprotection against the toxins administered, giving us another evidence of the low direct action of this phytocannabinoid in CB1 receptor.

Despite in mature neuronal cells CBD seems to not have neuroprotective effects in our model, previous studies reported interactions with CB1 receptors in neuronal precursor cells (Wolf et al. 2010). In order to elucidate CBD's actions in human neurons development, under differentiation SH-SY5Y cells was used as a model for screening of neurotoxic / neuroprotective profile of CBD. We found that these cells, besides having no

significant alterations on redox state and morphology, were more susceptible to toxins challenge, enhancing the amount of cell death. The ones that received the toxin alone or only CBD and AR during differentiation, presented similar patterns of cell viability.

Corroborating with our data, in several studies both phytocannabinoids and endocannabinoids detain the development of early embryos through CB1 regulation (Paria et al. 1998; Wang et al. 1999; MacCarrone et al. 2000; Nones et al. 2010). However, CB1, CB2 and endocannabinoids are induced during the formation of embryonic stem cells, and pharmacological blockade of these receptors induces their death, suggesting a role of endocannabinoid system in the survival of embryonic stem cells (Jiang et al. 2007; Oh et al. 2013). Indeed, the endocannabinoid system exerts a regulatory role on neural progenitor cell proliferation, differentiation and migration in the developing nervous system and the restricted neurogenic areas that persist in the adult brain (the hippocampal subgranular zone and subventricular zone) by engaging CB1 (Harkany et al. 2007; Díaz-Alonso et al. 2012). Also, CBD can promote adult hippocampal neurogenesis by activating CB1 (Wolf et al. 2010). These evidences suggest that the effects of CBD in under development neurons might be due to its regulation CB1 receptor activity.

Moreover, administration of CBD to newborn piglets shortly after hypoxia-ischemia has a protective effect on neurons and astrocytes, preserves brain activity, prevents seizures and improves neurobehavioral performance (Alvarez et al. 2008; Lafuente et al. 2011). In newborn rat brains, CBD treatment also mediated prevention of necrotic and apoptotic cell death in an *in vitro* model of hypoxia-ischemia damage (Pazos et al. 2012) and rescued

neuron function after sciatic nerve transection (Perez et al. 2013). As CBD is not often associated with side effects, these previous results indicate that it would be an useful partner for therapy strategies, such as hypothermia in ischemic newborn brains (Pazos et al. 2012). However, our results have shown that CBD administered during the neuronal development enhances the damage of human neurons and there are some reports about adverse effects of isolated CBD use in humans – or even cannabis use, although most of the cannabis effects are assigned to Δ^9-THC. For instance, fetal development is affected by prenatal maternal cannabis use, while in the infancy, there are negative impact in cognitive or behavioral outcomes (Huizink 2013). Moreover, a search on electronic databases for preliminary clinical trials found that high-dose oral CBD, although exert a therapeutic effect for social anxiety disorder, insomnia and epilepsy, may cause mental sedation (Zhornitsky and Potvin 2012). Cannabinoids can also impair all stages of memory as encoding, consolidation, and retrieval (Ranganathan and D'Souza 2006). Cannabis use during adolescence increases the risk of developing psychotic disorders, as schizophrenia, later in life (Bossong and Niesink 2010). As reviewed by Bergamaschi et al. 2011, although CBD does not present toxicity in non-transformed cells and does not induce undesired effects in most organism functions, it can induce some side effects, such as alterations of in vitro cell viability. Thus, besides not being neuroprotector in our human neuronal model, CBD can also presents hazardous unwanted effects in clinical trials and carefully risks evaluation criteria might be applied before its use is recommended to infants and adults. There are already many synthetic cannabinoids in test, designed in order to enhance protective properties, which

might be a better target for new treatment strategies with these compounds (Velez-Pardo et al. 2010; Elsohly et al. 2014; Lax et al. 2014).

This study is the first to use SH-SY5Y as a model for human neuronal development. We presented results about the deleterious effects of CBD in this approach, mainly when compared to previous studies in which it was appointed as protective against brain damage and neurogenesis inductor.

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Conflict of Interest

The authors declare none.

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Figures

Fig. 1 Dose selection of the CBD for the further experiments. a) *In vitro* total radical-trapping antioxidant potential (TRAP) and total antioxidant reactivity (TAR) of CBD. Left figure presents representative TRAP traces of the effect of CBD at the concentrations of 1.0, 2.5, 5.0, 10.0 μM as % of system measurements (AAPH + buffer + luminol). The central figure represent the ‘‘AUC’’ values and is expressed as % of radical produced compared to vehicle

(black bar). Right figure represents TAR profile of CBD, expressed as % of radical scavenging in comparison to vehicle (black bar). b) Cytotoxicity curve of CBD. Cells were treated with the dose curve of CBD during 24 h and cell viability was evaluated by MTT assay. Results are expressed as % of vehicle. c) RS production of CBD at 2.5 μM was evaluated by DCF assay and measurements are expressed as relative fluorescence units (RFU).

Fig. 2 The effect of sub-lethal dose of CBD over differentiated human neuroblastoma SHSY5Y cells. SH-SY5Y cells were treated by 24 h with CBD at the concentration of 2.5 μM. a) Total radical-trapping antioxidant potential (TRAP) of treated cells. Left figure represents TRAP traces of the effect of CBD or vehicle on cells. The central figure represent the ‘‘AUC’’ values and is expressed as % of radical produced compared to vehicle (black bar). Right figure represents TAR profile of treated cells, expressed as % of radical scavenging in comparison to vehicle (black bar). b) Elmann’s reduced thiol levels. c) The effect of sub-lethal dose of CBD over the cellular morphology of differentiated human neuroblastoma SH-SY5Y cells. Representative phase contrast and fluorescent images of nuclei stained with Hoesht dye and cytoskeleton labeled with anti-β III tubulin of cells treated by 24 h with vehicle (first column) or CBD (second column). Right figure represents the quantification of the neuritis density per cell body. c) Evaluation of neuroprotection of sub-lethal dose of CBD against H_2O_2, 6-OHDA, MG and GA. SH-SY5Y cells were pre-incubated by 24 h with CBD or vehicle, washed with PBS, and further treated with LD_{50} of the toxins for 24 h. Cell viability was determined by MTT assay.

Fig. 3 The effect of sub-lethal dose of CBD administered during the differentiation of human neuroblastoma SHSY5Y cells. SH-SY5Y cells were differentiated with CBD (together with RA) during the seven days of differentiation at the concentration of 2.5 μM. a) Total radical-trapping antioxidant potential (TRAP) of treated cells. Left figure represents TRAP traces, representing the effect of differentiation with CBD or vehicle on cells. The central figure represent the ‘‘AUC’’ values and is expressed as % of radical produced compared to vehicle (black bar). Right figure represents TAR profile

of treated cells, expressed as % of radical scavenging in comparison to vehicle (black bar). b) Elmann's reduced thiol levels. c) Morphologic effect of sub-lethal dose of CBD in the differentiation of human neuroblastoma SH-SY5Y cells. Representative phase contrast and fluorescent images of nuclei stained with Hoesht dye and cytosol labeled with anti-β III tubulin of cells differentiated treated with vehicle (first column) or differentiated with CBD (second column). Right figure represents the quantification of the neuritis density per cell body. c) Evaluation of neuroprotection of sub-lethal dose of CBD against H_2O_2, 6-OHDA, MG and GA. SH-SY5Y cells differentiated with CBD or treated with vehicle, was washed with PBS, and further treated with sub-lethal doses of the toxins for 24 h. Cell viability was determined by MTT assay.

Fig. 4 Bioinformatics approach for comparison of endocannabinoid signaling gene network in RA-differentiated and undifferentiated human neuroblastoma SH-SY5Y cells. a) STRING 9.0 gene interactions representation of selected KEGG PATHWAY database. b) Two-state landscape analysis of gene interaction networks from GSE9169 dataset. Coordinates (X- and Y-axis) represent normalized values of the input network topology. Color gradient (Z-axis) represents the relative functional state mapped onto graph according to the data input (differentiated cells-a vs. proliferative cells-b), where z=a/(a+b). The landscape is generated by ViaComplex V1.0. d) Ranked gene list of endocannabinoid system pathway from GSE9169 dataset.

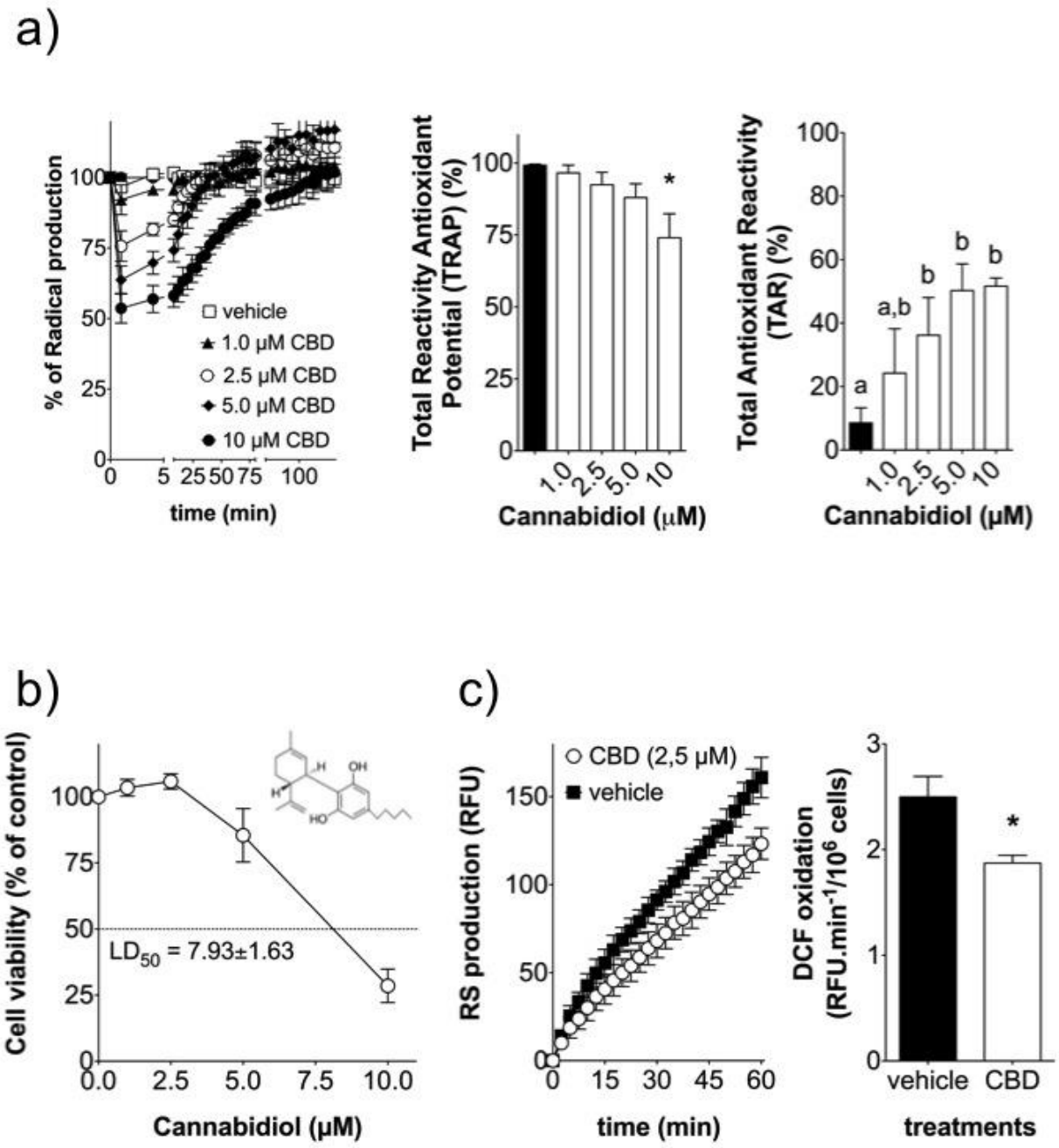

a)
% of Radical production
time (min)
vehicle
1.0 µM CBD
2.5 µM CBD
5.0 µM CBD
10 µM CBD
Total Reactivity Antioxidant Potential (TRAP) (%)
Cannabidiol (µM)
Total Antioxidant Reactivity (TAR) (%)
Cannabidiol (µM)
b)
Cell viability (% of control)
LD50 = 7.93±1.63
Cannabidiol (µM)
c)
CBD (2,5 µM)
vehicle
RS production (RFU)
time (min)
DCF oxidation (RFU.min-1/10^6 cells)
vehicle
CBD
treatments

Figure 1

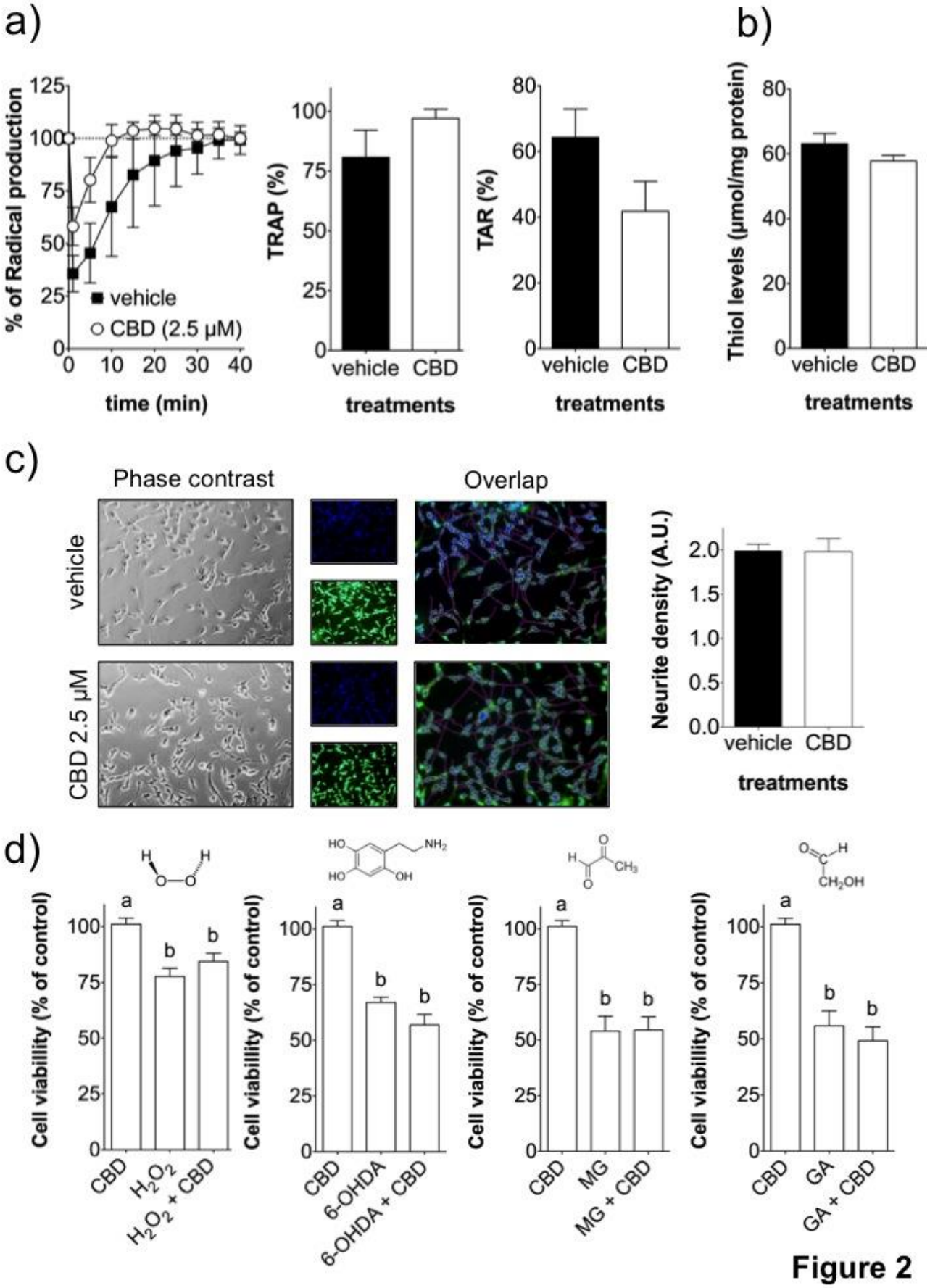

a)
% of Radical production
time (min)
vehicle
CBD (2.5 µM)
TRAP (%)
TAR (%)
vehicle
CBD
treatments
b)
Thiol levels (µmol/mg protein)
c)
Phase contrast
Overlap
vehicle
CBD 2.5 µM
Neurite density (A.U.)
d)
Cell viability (% of control)
CBD
H2O2
H2O2 + CBD
6-OHDA
6-OHDA + CBD
MG
MG + CBD
GA
GA + CBD

Figure 2

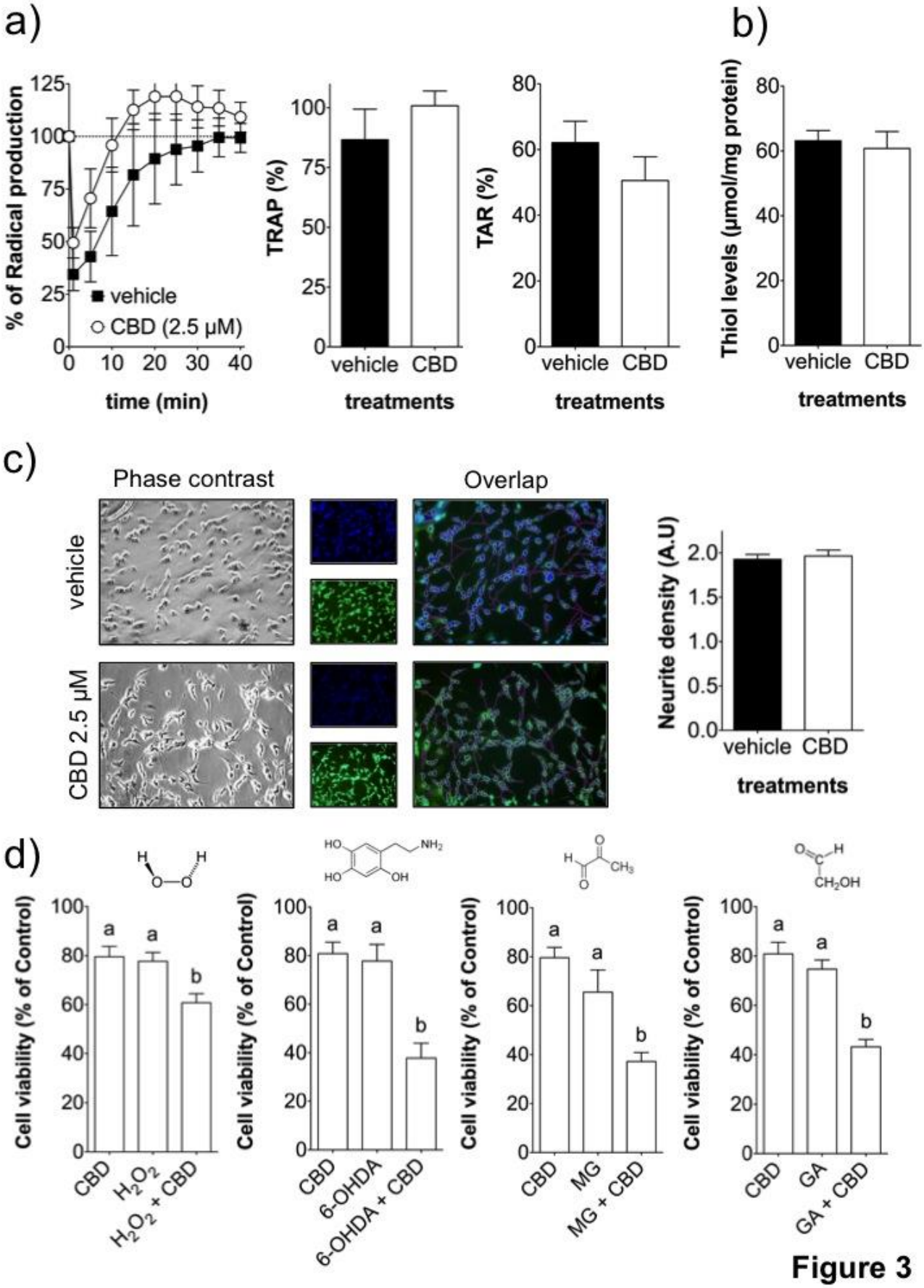
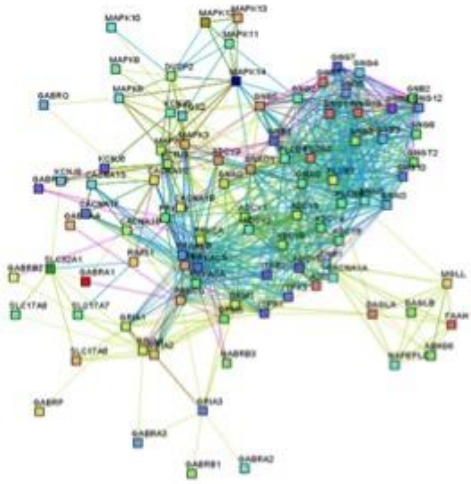
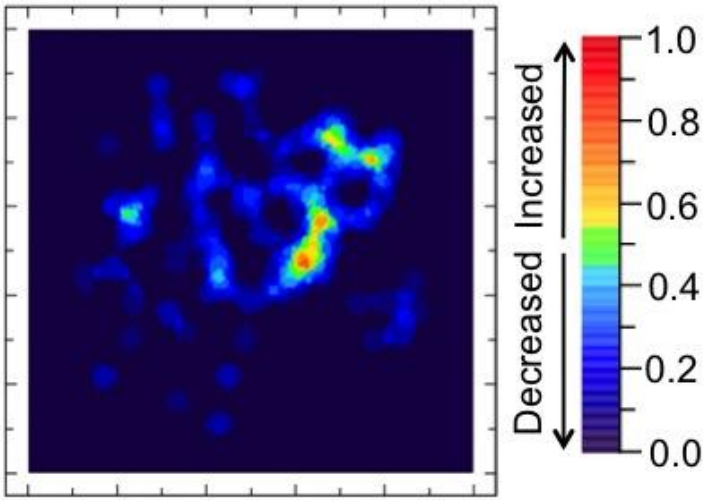

a)
% of Radical production
time (min)
vehicle
CBD (2.5 µM)
TRAP (%)
TAR (%)
treatments
b)
Thiol levels (µmol/mg protein)
c)
Phase contrast
Overlap
vehicle
CBD 2.5 µM
Neurite density (A.U)
d)
Cell viability (% of Control)
CBD
H2O2
H2O2 + CBD
6-OHDA
6-OHDA + CBD
MG
MG + CBD
GA
GA + CBD

Figure 3

a)

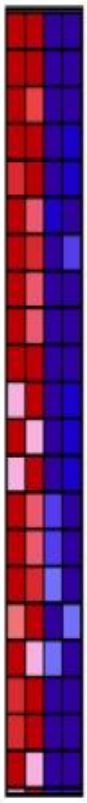


b)



c)

Components of the Endocannabinoid Signaling Gene Network Significantly Enriched during RA-differentiation of Human SH-SY5Y Neuroblastoma Cells.



Gene Symbol	Gene Name
GRIA1	glutamate receptor, ionotropic, AMPA 1
GNG3	guanine nucleotide binding protein (G protein), gamma 3
GNG2	guanine nucleotide binding protein (G protein), gamma 2
CNR1	cannabinoid receptor 1 (brain)
GNG8	guanine nucleotide binding protein (G protein), gamma 8
GRIA3	glutamate receptor, ionotrophic, AMPA 3
ADCY4	adenylate cyclase 4
MAPK12	mitogen-activated protein kinase 12
GNG7	guanine nucleotide binding protein (G protein), gamma 7
CACNA1B	calcium channel, voltage-dependent, L type, alpha 1B subunit
KCNJ6	potassium inwardly-rectifying channel, subfamily J, member 6
GNGT1	G protein, gamma transducing activity polypeptide 1
ADCY5	adenylate cyclase 5
ADCY6	adenylate cyclase 6
PRKACA	protein kinase, cAMP-dependent, catalytic, alpha
ITPR3	inositol 1,4,5-triphosphate receptor, type 3
GNB5	guanine nucleotide binding protein (G protein), beta 5
ADCY7	adenylate cyclase 7
MAPK9	mitogen-activated protein kinase 9
ABHD6	abhydrolase domain containing 6
MAPK3	mitogen-activated protein kinase 3

Data generated by Gene Score Enrichment Analysis (GSEA) with the comparison of RA-differentiated (A) *vs.* undifferentiated (B) human SH-SY5Y neuroblastoma cells transcripts obtained from GSE9169 dataset (Gene Expression Omnibus) ($P<0.05$).

Figure 4

PARTE III

4. DISCUSSÃO

Com base na grande importância do dano oxidativo para a neurodegeneração presente em diversas doenças (Halliwell 2001), este estudo foi elaborando visando avaliar o papel antioxidante e neuroprotetor de um composto fenólico bastante utilizado em pesquisas, o canabidiol, ou CBD.

Apesar do grande número de estudos relatando resultados positivos do CBD como uma molécula neuroprotetora e neurogênica em modelos animais *in vitro* e *in vivo* (além de testes pré-clínicos), poucos estudos avaliaram o efeito do CBD sobre células neuronais humanas maduras.

Os modelos animais não fornecem informações suficientemente precisas e úteis sobre a neurotoxicidade em humanos, por não serem capazes de mimetizar as condições de células humanas (Bal-Price et al. 2008). Por isso, testes alternativos (como modelos *in vitro*) em estratégias de *screening* permitem uma melhor e mais rápida aquisição de dados sobre compostos. Isto tornaria as análises de neurotoxicidade mais convenientes para sua posterior utilização no refinamento de estudos *in vivo* (Bal-Price et al. 2008). Além disso, modelos *in vitro* podem ser utilizados para a avaliação dos efeitos de compostos químicos sobre o desenvolvimento neurológico, incluindo efeitos na diferenciação e crescimento de neuritos (Radio e Mundy 2008).

Neste contexto, a linhagem de neuroblastoma humano SH-SY5Y, que pode ser diferenciada em fenótipo de neurônio dopaminérgico maduro (Lopes et al. 2010), surge como um modelo apropriado para estudos sobre efeitos neroprotetores / neurotóxicos de compostos em neurônios em maturação.

Considerando-se que o CBD é conhecido como um potente antioxidante e o estresse oxidativo está relacionado com os mecanismos

patofisiológicos de doenças neurodegenerativas (Schapira 2008), a seleção da dose de CBD utilizada neste estudo foi baseada na viabilidade celular e atividade antioxidante e em estudos prévios, como revisado por Fernández-Ruiz et al. (2013). Selecionamos a dose de 2.5 μM a ser utilizada nos experimentos seguintes de acordo com os resultados de viabilidade celular e em parâmetros redox.

Os resultados também mostram que em doses mais baixas do que a escolhida, CBD não é capaz de diminuir o stress oxidativo (Fig. 1a). Em doses mais elevadas, embora o CBD tenha se mostrado potencialmente antioxidante, ele foi fortemente citotóxico sobre células humanas com fenótipo neuronal maduro.

A administração de antioxidantes para a saúde humana deve ser considerada com cautela, pois qualquer composto antioxidante pode alterar o equilíbrio redox intracelular em direção a um estado mais reduzido, sendo tão citotóxico quanto o próprio estresse oxidativo (Gutteridge e Halliwell 2010), como nossos resultados demonstraram.

A mesma curva também foi testada em SH-SY5Y não diferenciadas sem mudanças significativas na viabilidade celular mesmo na concentração mais alta, 10 μM (dados não apresentados), corroborando com resultados prévios (Harvey et al. 2012).

Em seguida, células terminalmente diferenciadas da linhagem SH-SY5Y (fenótipo neuronal maduro) foram desafiadas com toxinas redox-ativas Metilglioxal (MG), Glicolaldeído (GA), 6-hidroxidopamina (6-OHDA) e peróxido de hidrogênio (H_2O_2).

Glicotoxinas, como MG e GA, são usadas em modelos de neurodegeneração para neuropatia diabética (Davies et al. 1986; Ohkawara et al. 2012; Sato et al. 2013). Estas toxinas originam complexos com proteínas irreversivelmente glicadas não enzimaticamente, os produtos finais avançados de glicação (AGEs), presentes em maiores níveis em indivíduos expostos à hiperglicemia prolongada, mas que também estão associados ao processo de envelhecimento (Nagai et al. 2000).

A 6-OHDA é uma das toxinas mais usadas em modelos experimentais para Doença de Parkinson (Gomez-Lazaro et al. 2008; Lopes et al. 2012). Esta neurotoxina é um análogo da dopamina com características estruturais semelhantes a este neurotransmissor e com afinidade pelo seu transportador (DAT). A 6-OHDA acumula nos neurônios causando estresse oxidativo e dano celular (Lehmensiek et al. 2006).

Já o peroxido de hidrogênio (H_2O_2) é um oxidante associado a diversas neuropatias e amplamente usado em modelos de neurotoxicidade (Huang et al. 2004; Harvey et al. 2012; Turkez et al. 2013; Huang et al. 2014; Turkez et al. 2014).

Utilizando células da linhagem SH-SY5Y terminalmente diferenciadas como um modelo de neurônio humano maduro para *screening* de neuroproteção / neurotoxicidade, os resultados observados mostram que CBD não oferece proteção frente às toxinas avaliadas, embora alterações morfológicas não tenham sido observadas. Estes dados corroboram com um estudo anterior no qual o CBD, nas doses de 0.01 0.1 e 1.0 µM, não teve efeito protetor sobre a toxicidade do 1-methil-4-fenilpiridinium (MPP+, 7 mM) em SH-SY5Y diferenciada (Carroll et al. 2012), apesar de terem utilizado doses mais

baixas do que as que utilizamos. Em maiores doses, 1.0 e 10.0 µM, o canabidiol foi capaz de aumentar a viabilidade celular em resposta ao *tert*-butil hidroperóxido em células de feocromocitoma murino PC12 e em células da linhagem SH-SY5Y não diferenciadas, apesar de não ter sido capaz de inibir a toxicidade induzida por H_2O_2 e *β*-amiloide (Harvey et al. 2012).

Como revisado por Fernández-Ruiz et al. (2013), CBD não tem grande afinidade por receptores CB1, mas, dependendo da sua concentração, ele pode agir como agonista ou antagonista deste receptor. Em nível nanomolar, o CBD comporta-se como antagonista dos efeitos farmacológicos dos agonistas de CB1, como THC, apesar de apresentar baixa afinidade por CB1 em nível micromolar em experimentos *in vitro* (Pertwee et al. 2002; Englund et al. 2012). A dose selecionada neste estudo parece antagonizar a atividade de receptores CB1.

Os fitocanabinóides também modulam a atividade do sistema endocanabinóide, por exemplo, CBD pode inibir a enzima FAAH (fatty acid amide hydrolase) responsável por inativar a AEA (De Petrocellis et al. 2011), aumentando os níveis teciduais deste endocanabinóide, o que pode ser um dos mecanismos mediadores dos efeitos farmacológicos do CBD e de alguns de seus análogos (Bisogno et al. 2001).

A neuroproteção mediada por CB1 ocorre através da inibição da adenilil ciclase e posterior diminuição dos níveis de cálcio durante um evento neurotóxico (Zhuang et al. 2005). A expressão deste receptor está aumentada em resposta a toxinas em SH-SY5Y diferenciada, como um indicativo de dano neuronal, o que também ocorre em processos patológicos (Carroll et al. 2012). Ainda, a atividade de CB1 na substância *nigra* também se mostrou aumentada

em um modelo animal para Doença de Parkinson (Lastres-becker et al. 2005). No entanto, o mesmo estudo demonstrou que o efeito neuroprotetor do CBD provavelmente não foi mediado pela atividade de CB1 (Lastres-becker et al. 2005; Carroll et al. 2012). Portanto, em diferentes condições, o CBD demonstra ser capaz de interagir diretamente com receptores do sistema endocanabinóide, ou indiretamente, através da regulação dos níveis de endocanabinóides que têm mais afinidade com estes receptores.

Os resultados obtidos neste estudo mostraram que o CBD não aumentou as defesas antioxidantes das células tratadas nem propiciou neuroproteção contra as toxinas administradas, embora ferramentas de bioinformática tenham mostrado que a expressão de receptores CB1 foi aumentada em células terminalmente diferenciadas. Além disto estudos anteriores provaram que receptores CB1 têm seu imunoconteúdo aumentado em resposta a lesões neuronais (Carroll et al. 2012). Esta pode ser mais uma evidência da baixa ação direta deste fitocanabinóide sobre CB1.

Apesar de em células neuronais maduras o CBD não ter apresentado atividade neuroprotetora em nosso modelo, estudos anteriores relataram seus efeitos em células precursoras neuronais (Wolf et al. 2010). Visando esclarecer a atividade do CBD em neurônios humanos em desenvolvimento, células da linhagem SH-SY5Y em diferenciação foram utilizadas como um modelo para *screening* de propriedades neurotóxicas / neuroprotetoras do CBD. Verificamos que essas células, além de não apresentarem alterações significativas no estado redox e em parâmetros morfológicos, foram mais suscetíveis aos desafios com toxinas, aumentando os percentuais de morte celular. As células que receberam apenas as toxinas e as que foram diferenciadas com CBD e AR

sem sofrerem desafios, apresentaram padrões semelhantes de viabilidade celular.

Corroborando com os nossos dados, em vários estudos anteriores, tanto fitocanabinóides quanto endocanabinóides causaram interrupção do desenvolvimento de embriões através da regulação CB1 (Paria et al. 1998; Wang et al. 1999; MacCarrone et al. 2000; Nones et al. 2010). No entanto, CB1, CB2 e os endocanabinóides têm seus padrões de expressão e atividade induzidos durante o desenvolvimento de células tronco embrionárias, e o bloqueio farmacológico desses receptores induz a morte destas células. Isto sugere um papel fundamental do sistema endocanabinóide e de seus reguladores endógenos e exógenos na sobrevivência de células tronco embrionárias. (Jiang et al. 2007; Oh et al. 2013).

De fato, o sistema endocanabinóide exerce um papel importante sobre a modulação da proliferação, diferenciação e migração de células progenitoras neurais durante desenvolvimento do sistema nervoso e em áreas neurogênicas que persistem no cérebro de indivíduos adultos (zona sub-granular hipocampal e zona sub-ventricular) através de interações com receptores CB1 (Harkany et al. 2007; Díaz-Alonso et al. 2012). Além disto, o CBD também promove neurogênese hipocampal em adultos através da ativação de CB1 (Wolf et al. 2010). Essas evidências sugerem que os efeitos do CBD durante o desenvolvimento de neurônios humanos pode ser devido a sua atividade na regulação do receptor CB1.

Além disso, a administração de CBD em um modelo experimental utilizando suínos neonatos submetidos à hipóxia-isquemia teve um efeito protetor sobre os neurônios e astrócitos, preservando a atividade cerebral,

prevenindo convulsões e melhorando o desempenho neurológico e comportamental dos animais tratados (Alvarez et al. 2008; Lafuente et al. 2011). Em um modelo *in vitro* de danos causados por hipóxia-isquemia em cérebros de recém-nascidos, o CBD também mediou a prevenção de morte celular por necrose e apoptose (Pazos et al. 2012). Como o CBD não é geralmente associado a efeitos colaterais, os resultados prévios acima indicam que o mesmo seria útil em estratégias de novas terapias ou como adjuvante em terapias já estabelecidas, como a hipotermia aplicada em cérebros de recém-nascidos isquêmicos (Pazos et al. 2012).

Entretanto, nossos resultados mostraram que o CBD administrado durante o desenvolvimento aumenta o dano a neurônios humanos e existem alguns relatos sobre efeitos adversos do uso da *Cannabis* ou mesmo do CBD isoladamente em humanos. Por exemplo, o consumo pré-natal de *Cannabis* pela mãe afeta o desenvolvimento do feto, enquanto que na infância, há impacto negativo nos resultados cognitivos ou comportamentais (Huizink 2013). Resultados de uma busca por os testes clínicos preliminares em bancos de dados eletrônicos constataram que altas doses orais de CBD, apesar do efeito terapêutico para o transtorno de ansiedade, insônia e epilepsia, podem causar sedação mental (Zhornitsky and Potvin 2012).

Os canabinóides também podem comprometer todos os estágios da memória incluindo codificação, consolidação e recuperação (Ranganathan e D'Souza 2006), devido a efeitos hiperativadores do sistema endocanabinóide que promovem a LTD (depressão de longa duração) que prejudica a formação da memória de trabalho (Han et al. 2012). O consumo de *Cannabis* durante a

adolescência também aumenta o risco de ocorrência de transtornos psicóticos, como a esquizofrenia, na vida adulta (Bossong e Niesink 2010).

Assim, além de não ser neuroprotetor em nosso modelo humano, o CBD também pode apresentar efeitos indesejáveis e perigosos em testes clínicos e devem-se aplicar critérios de avaliação de riscos cuidadosamente elaborados antes que seu uso seja recomendado para crianças e adultos. Já existem muitos canabinóides sintéticos em teste, projetados a fim de melhorar as propriedades protetoras dos fitocanabinóides, que podem ser um melhor alvo para novas estratégias de tratamento com estes compostos (Velez-Pardo et al. 2010; Elsohly et al. 2014; Lax et al. 2014).

Este é o primeiro estudo a utilizar SH-SY5Y como um modelo para o desenvolvimento neuronal humano. Nós apresentamos resultados sobre os efeitos deletérios do CBD nesta abordagem, principalmente quando comparado com estudos anteriores que o apontam como protetor contra danos cerebrais e como indutor de neurogênese.

Em resumo, nossos dados indicam que 2,5 µM de CBD, a dose mais elevada tolerada pelas células SH-SY5Y diferenciadas, não é capaz de fornecer neuroproteção em SH-SY5Y terminalmente diferenciadas e mostram, pela primeira vez, que a exposição ao CDB durante a diferenciação neuronal poderia sensibilizar as células imaturas para futuros desafios com toxinas.

Ainda assim, é importante lembrar que este estudo foi realizado utilizando-se uma linhagem celular que, apesar de humana, dotada de marcadores de neurônios diferenciados e de apresentar morfologia estrelada típica de neurônios (Lopes et al. 2010), representa um único tipo celular. Com isto, não são consideradas relações intercelulares, o que pode ser um fator

importante para a falta de reprodutibilidade *in vitro* de dados observados *in vivo*. Mas, além de estas relações serem extremamente complexas e ainda não totalmente conhecidas, dados *in vivo* são obtidos em modelos animais cuja fisiologia é diferente da dos humanos (Bal-Price et al. 2008).

A utilização de experimentos *in vitro* para identificação e seleção de compostos perigosos ou protetores está baseada na premissa de que se um determinado composto que tem um efeito *in vitro*, ele também tem o potencial de alterar os mesmos parâmetros *in vivo*. Entretanto, para que fosse possível a aplicação direta de dados obtidos *in vitro* avaliação dos possíveis riscos ou potenciais de compostos, seria necessária uma compreensão mais completa dos mecanismos relacionados à expressão de toxicidade *in vivo*, o que ainda não é possível (Radio e Mundy 2008).

Contudo, nossos resultados são um importante contraponto aos achados da maioria das pesquisas anteriores, que apontam as inúmeras propriedades terapêuticas do canabidiol de outros canabinóides discutidas acima.

Sem considerar a polêmica do uso da planta *Cannabis sativa* para fins medicinais – ou mesmo recreativos, os diversos fitocanabinóides e canabinóides sintéticos disponíveis vêm apresentando resultados contraditórios, como exposto na considerável bibliografia utilizada para referenciar este trabalho. Estes resultados, aliados a grande repercussão que estes compostos e a própria planta vêm recendo nos últimos anos, sob o estigma de produto natural sem contraindicações, evidenciam a necessidade de que pesquisas em torno destes compostos sejam intensificadas (Porter e

Jacobson 2013) em modelos adequados, rápidos e confiáveis (Bal-Price et al. 2008).

Com isto, além de efeitos benéficos, possíveis riscos à saúde poderiam ser identificados antes que estes compostos cheguem à utilização por humanos. A relação risco / benefício seria então avaliada de acordo com a necessidade de cada paciente e com os comprovados efeitos terapêuticos do canabinóide em questão.

5. CONCLUSÃO

Os resultados apresentados e discutidos nos permitem afirmar que, no modelo experimental utilizado neste estudo, o CBD não apresentou as propriedades neuroprotetoras e antioxidantes atribuídas a ele pela literatura. Além disto, células diferenciadas com CBD apresentaram percentuais de viabilidade próximos aos valores de células tratadas com toxinas e, quando desafiadas, estas células apresentaram menor viabilidade. Além disto, verificamos que a linhagem de neuroblastoma humano SH-SY5Y é um modelo eficiente para *screening* de compostos e seus efeitos durante a diferenciação neuronal.

Com isto, concluímos que o uso do CBD como adjuvante contra doenças neurodegenerativas em humanos é ainda controverso, principalmente durante o desenvolvimento do sistema nervoso, sendo necessárias mais pesquisas em modelos experimentais adequados.

6. PERSPECTIVAS

Como vimos, este trabalho mostrou que tanto o modelo de célula diferenciada quanto de célula em diferenciação são eficientes para *screening* de compostos, geração de dados importantes para a classificação destes compostos como neuroprotetores / neurotóxicos e posterior aplicabilidade em estudos *in vivo*. Por isso, estes modelos podem ser utilizados para avaliação de diversos compostos com possíveis ações em células neuronais humanas.

Derivados sintéticos do CBD e de outros fitocanabinóides têm sido produzidos com o objetivo de aumentar as propriedades protetoras e reduzir efeitos indesejados de seus precursores naturais (Elsohly et al. 2014). Por isto, apesar de o CBD não ter se mostrado neuroprotetor neste modelo, seus derivados sintéticos e outros fitocanabinóides serão submetidos ao mesmo modelo experimental, aumentando assim a qualidade dos dados disponíveis sobre a ação destes compostos em células humanas.

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