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MARIA NOEL MARZANO RODRIGUES PETRUZZI

AVALIAÇÃO HISTOPATOLÓGICA DO EFEITO DO CANABIDIOL EM UM MODELO EXPERIMENTAL DE CARCINOGÊNESE ORAL

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Pontifícia Universidade Católica
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Orientadora: Profa. Dra. Maria Antonia Zancanaro de Figueiredo

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Aprovada em: ____ de _____ de 2017.

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2017


AD VERVM DEVM

Dedico esta Tese às professoras, funcionários e pacientes do Serviço de Estomatologia e Prevenção do Câncer Bucomaxilofacial do Hospital São Lucas da Pontifícia Universidade Católica do Rio Grande do Sul.

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Epígrafe

Que nada nos limite, que nada nos defina, que nada nos sujeite.
(Simone de Beauvoir, 1908-1986).


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RESUMO

O diagnóstico tardio do carcinoma espinocelular oral está relacionado a um alto índice de morbi-mortalidade e recorrência após o tratamento. Portanto, há um crescente interesse na validação de marcadores biológicos que contribuam para o diagnóstico precoce, novas estratégias de quimioprevenção e recursos adjuvantes para o tratamento dessa neoplasia maligna. A presente Tese centrou-se na avaliação do efeito anticarcinogênico do canabidiol (CBD) em um modelo experimental consagrado para a indução de alterações epiteliais, com risco de malignidade, na mucosa oral de murinos. Para tanto, 15 ratos Fischer 344 foram aleatoriamente divididos em três grupos, onde todos os animais tiveram o ventre de suas línguas expostas ao 7,12-dimetilbenzantraceno (DMBA) três vezes por semana. A partir da segunda semana os grupos receberam por via intraperitoneal, veículo (grupo 1), 5 mg/Kg ou 10 mg/Kg de CBD (grupos 2 e 3, respectivamente). Na décima segunda semana após o início do experimento realizou-se a eutanásia dos animais, dissecção das línguas e processamento dos espécimes. As análises foram realizadas por meio das técnicas histológicas de rotina e imunoistoquímica. Observou-se a inibição do desenvolvimento de displasia oral severa e carcinoma, bem como a modulação dos índices de proliferação celular nos grupos 2 e 3 em relação ao grupo 1. A hipótese nula pôde ser rejeitada, uma vez que os resultados obtidos apresentaram nível de significância de 0,05 (intervalo de confiança = 95%). A seguir, apresentam-se três artigos científicos que descrevem primeiramente o experimento original desenvolvido e, em sequência, as revisões da literatura para subsidiar a discussão do tema proposto.

Descritores: Diagnóstico bucal; Câncer da cavidade oral; Carcinoma espinocelular; Quimioprevenção; Canabidiol; 7,12-dimetilbenzantraceno; Ratos endogâmicos Fischer 344


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SUMMARY

A late diagnosis of oral squamous cell carcinoma is related to high morbidity and mortality rates, as well as recurrence after treatment. Hence, there is an increasing interest in the validation of biological markers, chemoprevention strategies, and adjuvant treatment alternatives for combating oral cancer. The present Thesis evaluated the anticancer effect of cannabidiol (CBD) in a validated experimental murine model of oral carcinogenesis. Fifteen Fischer 344 rats were randomly divided into three groups of five animals each and subjected to a 7,12-dimethylbenzanthracene (DMBA) topical application on the ventral mucosa of their tongues, thrice a week. From the 2nd to the 12th week, they received intraperitoneally-administered vehicle (group 1), CBD 5 mg/kg (group 2), or CBD 10 mg/kg (group 3). After euthanasia, the tongues were dissected, processed and assessed by histological and immunohistochemical analysis. Group 2 and group 3 showed inhibition of severe oral epithelial dysplasia and carcinoma, and exhibited lower cell proliferation as compared to group 1. The null hypothesis was rejected when results showed statistical significance at a 0.05 level (confidence interval = 95%). In sequence are presented three manuscripts, first one regarding the original experiment and the two subsequent ones providing overall theoretical support.

Keywords: Oral diagnosis; Mouth neoplasms; Squamous cell carcinoma; Chemoprevention; Cannabidiol; 7,12-dimethylbenzanthracene; Inbred F344 rats


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SUMÁRIO

INTRODUÇÃO

A terminologia câncer bucal compreende as neoplasias malignas que acometem o vermelhão labial e a mucosa oral (Brasil, 2015). Essa enfermidade é considerada o décimo primeiro tumor maligno em prevalência no mundo e representa 5% de todos os tipos de câncer diagnosticados anualmente. Estima-se que na população mundial a incidência seja de 1:20000 habitantes aumentando para 1:1100 em homens acima dos 75 anos de idade (OMS, 2005). No Brasil, a estimativa do Instituto Nacional de Câncer José Alencar Gomes da Silva (INCA) para o biênio 2016/2017, sugere 15490 novos casos, distribuídos em uma proporção de 2,56 homens a cada mulher afetada. O número de mortes provocado pela referida doença ultrapassou os 5000 no ano de 2010 (Brasil, 2015). Na região sul o câncer bucal é o quinto e décimo quinto tipo de câncer mais frequente entre indivíduos do sexo masculino e feminino, respectivamente; totalizando 1160 novos casos em 2016.

Além da predileção pelo sexo masculino, o câncer intra-oral acomete prioritariamente adultos, a partir da quarta ou quinta década de vida, em sua maioria de classe socioeconômica baixa e expostos cronicamente aos efeitos carcinogênicos do tabaco e álcool (Adeyemi *et al.*, 2011; Oliveira *et al.*, 2015). Porém, evidências sugerem que também pode desenvolver-se em faixas etárias mais precoces, influenciado por fatores predisponentes como a infecção pelo papilomavírus humano (HPV) e quadros de imunossupressão primária ou secundária (Beachler *et al.*, 2014; Bodner *et al.*, 2014; Young *et al.*, 2015). Também, a interação entre polimorfismos genéticos e modificações ambientais tem sido contemplada como um possível fator precipitante para o câncer bucal (Ma *et al.*, 2015; Maurya *et al.*, 2015; Su *et al.*, 2015).

Os tumores malignos do vermelhão labial têm como causa primária a exposição prolongada a radiação ultravioleta e usualmente localizam-se no lábio inferior. Assim como constatado para os tumores intra-orais, o tabagismo, infecções virais e imunossupressão, estão associados ao desenvolvimento desse tipo de lesão que habitualmente, também afeta mais homens que mulheres (Ochsenius *et al.*, 2003; Calcaianu *et al.*, 2015).

A semiologia do câncer bucal inclui a realização de uma anamnese completa, exame físico minucioso e análise anatomopatológica da lesão para o diagnóstico definitivo (Calcaianu *et al.*, 2015). Em indivíduos com alteração clinicamente visível da mucosa oral, recomenda-se como padrão ouro a realização imediata de uma biópsia incisional com auxílio de lâmina de bisturi seguida de exame histopatológico da peça operatória. Outros exames complementares, tais como citologia, espectroscopia de fluorescência e marcadores salivares ou hematológicos ainda não podem ser recomendados como substitutos seguros para a biópsia convencional, conforme resultados de meta-análise publicada por Macey e colaboradores (2015).

As neoplasias malignas orais podem apresentar características clínicas e histológicas variadas. Contudo, os dados reportados na literatura mostram que entre 70% e 90% dos pacientes são acometidos por carcinoma de células escamosas ou espinocelular (Krutchkoff *et al.*,1990; Chidzonga *et al.*, 2006; Effiom *et al.*, 2008).

O sítio anatômico intra-oral mais afetado é a língua, seguido do assoalho bucal. Segundo alguns autores essa característica deve-se a dissolução das substâncias carcinogênicas na saliva e a deposição das mesmas sobre tais regiões devido à força gravitacional. Entretanto, determinados estudos apontam a gengiva maxilar e mandibular como as localizações primárias preferenciais, o que talvez sugira uma relação com outros fatores causais, tais como os oncogenes. Ainda, certos pesquisadores referem que em

lesões abrangendo a face lingual da mandíbula e o assoalho bucal pode haver dificuldade na definição do sítio inicial, causando um aumento espúrio nos índices de frequência estabelecidos para os carcinomas gengivais (Arotiba *et al.*, 2006; Effiom *et al.*, 2008; Adeyemi *et al.*, 2011).

Aproximadamente 60% dos tumores malignos são diagnosticados em fases avançadas da doença. De acordo com estudos realizados em países menos desenvolvidos, o carcinoma espinocelular pobremente diferenciado é o subtipo histológico mais prevalente. Entretanto, essa característica não é unanime, sendo os carcinomas bem diferenciados e moderadamente diferenciados, predominantes em algumas amostras (Arotiba *et al.*, 2006; Effiom *et al.*, 2008; Adeyemi *et al.*, 2011; Gupta *et al.*, 2014).

A presença de metástase em linfonodos cervicais, independentemente, ocasiona uma diminuição de 60% para 40% na probabilidade de haver um índice de sobrevida igual ou maior que 5 anos. Outros achados, tais como o maior volume do tumor, invasão perineural, embolia linfovascular, disseminação extra-capsular e comprometimento da margem cirúrgica, também estão associados a um pior prognóstico. Embora a influência desses fatores na taxa de mortalidade ainda não esteja bem estabelecida, a sua presença tem sido considerada relevante para a indicação de terapias adjuvantes pós-operatórias, como a radio e quimioterapia (Bernier *et al.*, 2004; Cooper *et al.*, 2004; Bradnwein-Gensler, 2005; Kademani *et al.*, 2005; Liao *et al.*, 2008; Thiagarajan *et al.*, 2014; Abu-Serriah *et al.*, 2015).

A morbidade associada ao carcinoma espinocelular é alta, acarretando importante prejuízo à qualidade de vida do paciente. O diagnóstico continua ocorrendo tardiamente e a ressecção cirúrgica do tumor com margem de segurança, tratamento de escolha para essa neoplasia maligna, pode causar extensa mutilação. Em lesões não passíveis de excisão, o uso concomitante de quimio e radioterapia é considerado o plano de tratamento

mais adequado, segundo resultados obtidos por meio de meta-análise (Geum *et al.*, 2013; Akhlaghi *et al*., 2014; Rana *et al.*, 2015).

Ao longo de décadas têm havido extensos debates sobre a possibilidade de realizar-se o rastreamento do câncer bucal em fases pré-malignas como aplica-se ao câncer de colo uterino, por exemplo. Nesse contexto, a Organização Mundial da Saúde propôs o uso dos termos *lesão ou condição cancerizável* (pré-cancer) pressupondo o desenvolvimento de câncer bucal, também, a partir dessas alterações (Warnakulasuriya *et al.*, 2007). A leucoplasia, eritroleucoplasia e as lesões palatais em fumantes reversos, constituíam o grupo de lesões cancerizáveis. As condições cancerizáveis compreendiam a fibrose submucosa, queilite actínica, líquen plano e lúpus eritematoso discoide (OMS, 2005).

A distinção entre ambos os termos pressupõe que a lesão cancerizável represente uma alteração morfológica localizada, a partir da qual há uma maior probabilidade de desenvolvimento de câncer, se comparado ao tecido aparentemente normal, no mesmo indivíduo. Já, a condição cancerizável indica um estado generalizado, onde há risco aumentado para o desenvolvimento do tumor maligno (Warnakulasuriya *et al.*, 2007).

Mais recentemente optou-se por outra nomenclatura devido ao reconhecimento da franca variabilidade anatômica do câncer bucal mesmo nas ditas lesões cancerizáveis e a possibilidade do tumor desenvolver-se em tecido com aparência clínica imutada (Gupta *et al*., 1980; Silverman *et al*., 1984; Schepman *et al*., 1998; Warnakulasuriya *et al.*, 2007). Cunhou-se, então, o termo *desordem potencialmente maligna*, de caráter mais genérico, porém ainda inespecífico quanto ao maior ou menor risco de malignidade apresentado pelas entidades patológicas incluídas no espectro da classificação (Speight, 2007; Naeayan, Shilpashree, 2016).

Na avaliação anatomopatológica dessas desordens é necessário identificar indícios de alterações celulares e da arquitetura tissular. Adicionalmente, averigua-se a extensão de comprometimento morfológico do tecido epitelial – do estrato basal para o granuloso ou córneo – de modo a prover a gradação das displasias em leve (grau I), moderada (grau II) ou intensa (grau III) (Barnes *et al.*, 2005).

O manejo adequado dessas lesões pelo profissional e a possível estimativa de risco para malignidade a ser reportada ao paciente, deve contemplar além do aspecto histopatológico. Evidências epidemiológicas resultantes de estudos com metodologias similares devem sempre ser valorizadas, bem como a contribuição da multifatoriedade e individualidade no processo de carcinogênese (Barnes *et al.*, 2005; Speight *et al.*, 2007).

Embora, nem todas as desordens potencialmente malignas exibam um padrão histopatológico compatível com o de displasia, evidências sugerem que a presença da mesma indique uma natureza probabilística de malignização (Narayan, Shilpashree, 2016). Assume-se, nessa conjuntura, um modelo de carcinogênese tempo-dependente passando por estágios de displasia leve a severa, a qual por seu caráter de irreversibilidade somada à danos genéticos cumulativos, resultaria no câncer. Entretanto, estudos clínicos observacionais ainda não asseguram essa linearidade no curso da doença. A título de exemplo, podem-se citar os resultados da meta-análise de Anderson e Ishak (2015), a qual revelou para a leucoplasia oral um índice cumulativo de transformação maligna altamente variável – 0,13% a 34,0% – em 24 publicações disponibilizadas entre os anos 1960 e 2013.

Possivelmente, a falta de relação entre a displasia epitelial oral diagnosticada nas biópsias incisionais e o subsequente desenvolvimento de neoplasias malignas possa estar vinculada ao elevado número de métodos para classificação dessa condição. Também,

pode ser atribuída à subjetividade inerente à leitura das lâminas que resulta em elevados índices de variabilidade inter-observador (Warnakulasuriya *et al.*, 2008; Manchanda, Shetty, 2012). Ainda, considera-se que possa haver uma falta de representatividade do fragmento coletado para a totalidade da lesão (Holmstrup *et al.*, 2007; Kuribayashi *et al.*, 2015).

As teorias mais aceitas sugerem que o desenvolvimento de um tumor maligno primário resulte do acúmulo de defeitos genéticos e epigenéticos no meio intra e extracelular. Instala-se, assim, um desequilíbrio homeostático permanente que culmina na alteração definitiva dos mecanismos regulatórios de proliferação, sobrevivência e diferenciação celular. Adicionalmente, há uma diminuição progressiva da capacidade imunológica antitumoral do hospedeiro frente às células alteradas que provocam distúrbios microambientais e desenvolvem habilidades que corroboram para a progressão da doença (vide Figura 1) (Hanahan, Weinberg, 2011; Bose *et al.*, 2015; Quan, Fang *et al.*, 2016; Sakakura *et al.*, 2016).

Sabe-se que o carcinoma espinocelular está relacionado a um alto índice de morbi-mortalidade e recorrência após o tratamento (Carreras-Torras, Gay-Escoda, 2009; Gomez *et al.*, 2009). Portanto, é necessário ampliar ou reforçar os conhecimentos sobre a etiopatogênese da doença, determinando fatores que auxiliem a predizer de maneira mais exata, o potencial de malignização de certas lesões (Scully, Kirby, 2014). Apesar de ser um objetivo desafiador, nota-se um crescente empenho da comunidade científica em promover a validação de marcadores biológicos para diagnóstico, bem como estratégias de quimioprevenção. É importante salientar que esse tipo de intervenção precoce pode retardar ou impedir a formação dos tumores malignos em pacientes com maior risco,

como os portadores de desordens potencialmente malignas (Foy *et al.*, 2013; Warner *et al.*,2014; William, El-Naggar, 2016).

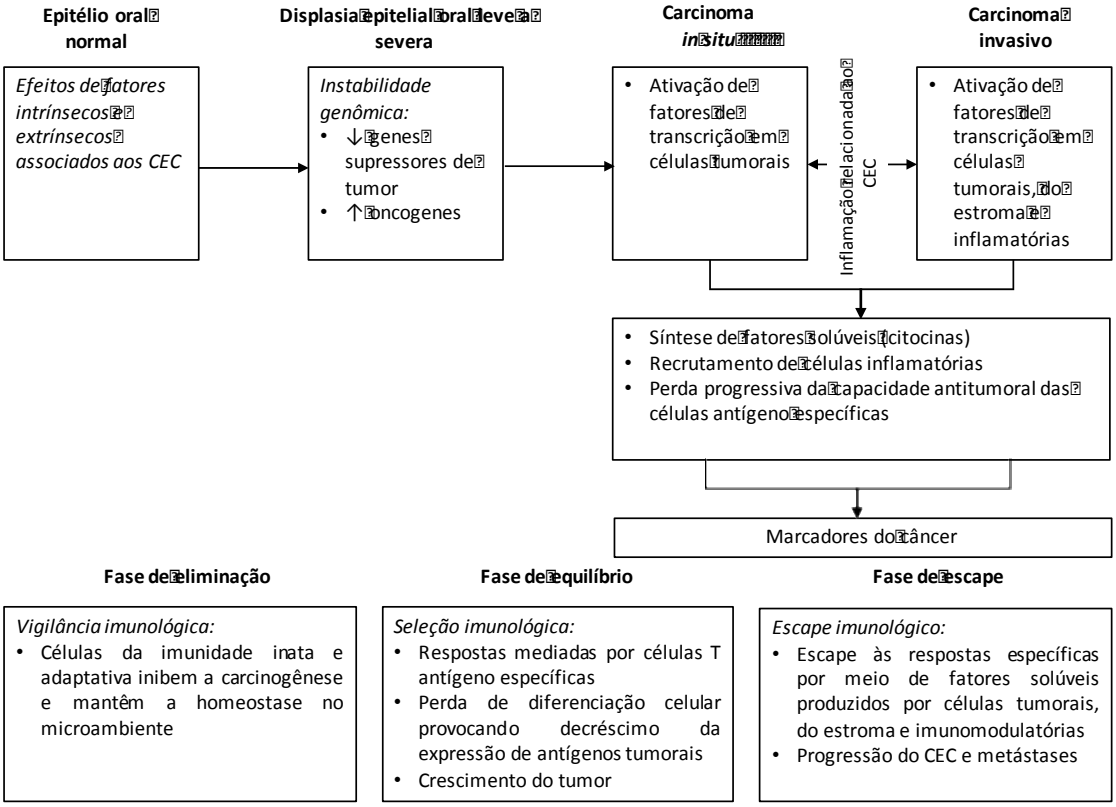

Figura 1: Progressão do câncer e resposta imunológica do hospedeiro. Uma combinação de fatores de risco promove alterações progressivas no epitélio oral. Inicialmente, o hospedeiro monta uma resposta imune eficaz eliminando a maioria das células atípicas (fase de eliminação) (Öhman *et al.*, 2015). No entanto, as células transformadas que resistem à pressão seletiva sofrem alterações epigenéticas que levam à regulação negativa dos genes supressores de tumores e à regulação positiva dos oncogenes (fase de equilíbrio) (Gasche, Goel, 2013; Sarode *et al.*, 2015). Estas células aberrantes sofrem a contínua perda de expressão de antígenos tumorais e proteínas do complexo principal de histocompatibilidade (MHC)-I e II (Leibowitz *et al.*, 2013). Consequentemente, as células T antígeno específicas tornam-se progressivamente ineficazes e o equilíbrio do microambiente é interrompido, promovendo a carcinogênese oral (Bose *et al.*, 2016; Sakakura *et al.*, 2016). Finalmente, as células tumorais, as células do estroma e as células imunitárias residentes ou recrutadas, interagem bilateralmente por meio de citocinas e quimiocinas, para facilitar a invasividade tumoral e a metástase

(fase de escape) (Dunn *et al.*, 2001; Fang *et al.*, 2015; Rani *et al.*, 2015; Pal *et al.*, 2016). CEC - carcinoma espinocelular. Fonte: Elaborado pela Autora (2017).

Os modelos em murinos para o estudo da etiopatogênese do câncer oral induzido por 7,12-dimetilbenzantraceno (DMBA) aplicado topicamente na mucosa oral encontram-se entre os mais utilizados há cerca de 60 anos (Sally, 1954). O DMBA é um hidrocarboneto aromático policíclico cujo potencial carcinogênico deve-se, em parte, a ativação da via de sinalização do receptor aril hidrocarboneto (AhR)/citocromo P4501A1 (CYP1A1) e a sua conversão em DMBA-3,4-diol-1,2-epóxido (Trombino *et al*., 2000).

O AhR não estimulado encontra-se no citosol, em células de diversos tecidos, estabilizado pela sua associação à proteína de choque térmico (HSP)-90, às co-chaperonas p23 e à proteína XAP2. Fisiologicamente, o fator de transcrição AhR exerce funções vinculadas ao desenvolvimento e proliferação celular, contudo o seu ligante endógeno ainda não foi identificado (Mimura, Fujii-Kuriyama, 2003; Kochhar *et al.*, 2014).

A interação do DMBA com o AhR desencadeia uma alteração conformacional resultando em sua translocação para o núcleo, onde dimeriza-se com o seu receptor nuclear (receptor nuclear translocador de aril hidrocarboneto: Arnt), passando a ser um fator de transcrição ativo. O Ahr ativado aumentará a transcrição do CYP1A1 via elementos genéticos móveis, especialmente a proteína XRE (Mimura, Fujii-Kuriyama, 2005). Finalmente, há a regulação positiva do CYP1A1 que, por meio de sua atividade enzimática, promove a biotransformação do DMBA às suas formas mutagênicas (Figura 2) (Nakata *et al.*, 2009; Maayah *et al.*, 2015).

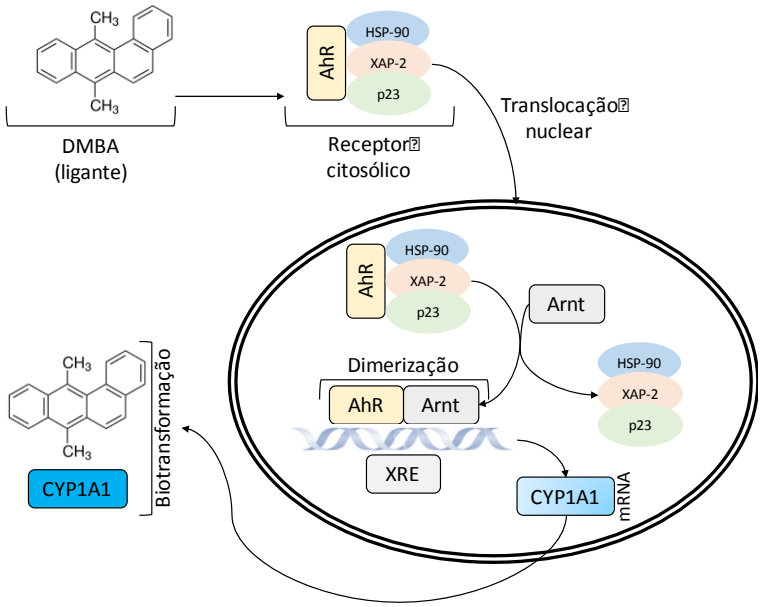

Figura 2: Biotransformação do DMBA. A representação esquematiza a ativação da via de sinalização do receptor aril hidrocarboneto (AhR)/citocromo P4501A1 (CYP1A1) pelo DMBA, promovendo a sua metabolização em DMBA-3,4-diol-1,2-epóxido. AhR - receptor aril hidrocarboneto, Arnt - translocador nuclear de Ahr, CYP1A1 - citocromo P4501A1, HSP-90 - proteína de choque término, (Trombino, Near, Matulka, 2000; Mimura, Fujii-Kuriyama, 2003 e 2005; Nakata, Urano, Fujii-Kuriyama, 2009; Maayah, Ghebeh, Alhaider, 2015). Fonte: Elaborado pela Autora (2017).

O protocolo de indução foi adaptado ao longo dos anos, tornando-se altamente reprodutível. Uma solução padrão de DMBA a 0,5% em acetona, aplicada três vezes por semana, durante 12 a 16 semanas é considerado o método ideal para promover a formação de tumores malignos na mucosa jugal de *hamsters* ou na língua de ratos (Sally, 1954; Morris, 1961).

Além da padronização, o custo do processo é baixo e o DMBA é facilmente aplicado, não requerendo escarificação prévia da mucosa. As desvantagens da metodologia incluem o trabalho laboratorial expressivo do pesquisador, além do manejo constante dos animais e do agente carcinogênico. Diversos autores descrevem, ainda, que apesar de haver graus variados de displasia epitelial oral logo ao final do processo,

mostrando alterações na morfologia celular e arquitetura tecidual, há um longo período de latência para o desenvolvimento efetivo de tumores malignos na cavidade oral (Bampi *et al.*, 2014; Patil *et al.*, 2015). Adicionalmente, sabe-se que pode haver uma limitação da reprodutibilidade quando o modelo é aplicado em roedores transgênicos (Avgoustidis *et al.*, 2012).

A vigilância imunológica dos animais expostos ao DMBA mostra-se limitada na prevenção de mutações nas células epiteliais frente às agressões. Esse carcinógeno habitualmente provoca alterações no genoma com aumento da transcrição de proto-oncogenes pouco imunogênicos, favorecendo o escape das células alteradas aos linfócitos T citotóxicos específicos (Nasti *et al.*, 2015). Defeitos em genes como o HRas, são um claro exemplo dessa propriedade, que tem sido implicada na etiopatogênese do câncer do urotélio da bexiga e folicular da tireoide (Šolman *et al.*, 2015). Outrossim, é um dos proto-oncogenes desregulados com maior frequência no carcinoma espinocelular oral em murinos e humanos (Murugan *et al.*, 2016). Também, a expressão aberrante de certos genes durante todos os estágios de alteração da mucosa bucal, são considerados importantes para a avaliação do processo de malignização dos epitélios orais nesse modelo de estudo (Yang *et al.*, 2010). A Figura 3 exibe as etapas do processo de carcinogênese induzido por DMBA.

A literatura sugere que as proteínas p53 e Ki-67 são os marcadores com maior sensibilidade e especificidade para identificar precocemente o potencial de malignização de lesões cancerizáveis. Também, podem ser considerados preditores independentes para displasias orais e para averiguar as alterações celulares em tecidos epiteliais expostos a carcinógenos químicos como o DMBA (Balakrishnan *et al.*, 2010; Humayun, Prasad, 2011; Yagyuu *et al.*, 2015; Mondal *et al.*, 2016)

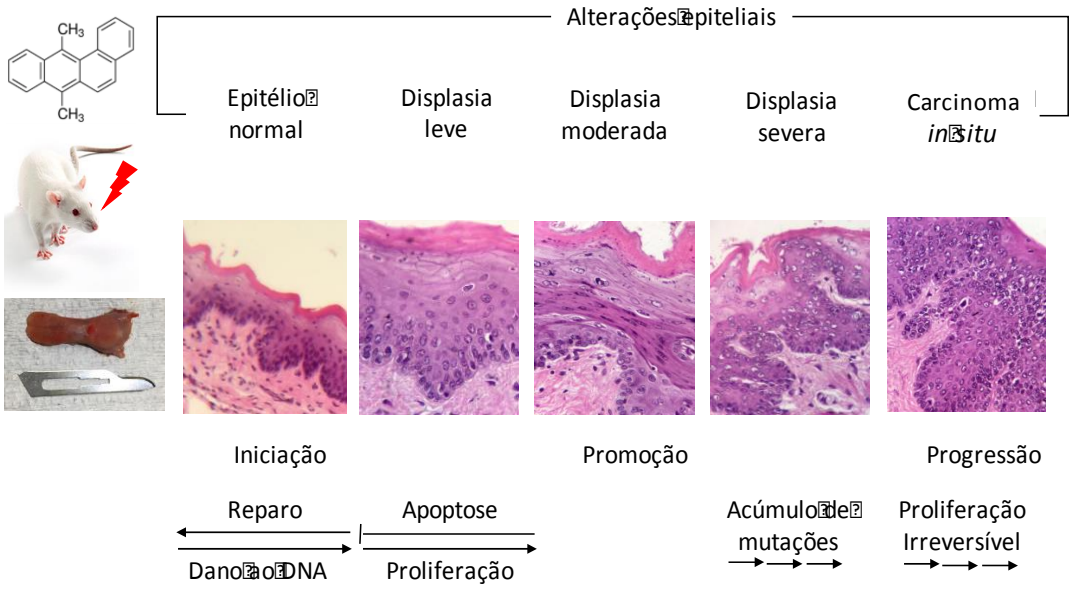

Figura 3: Alterações epiteliais promovidas pelo DMBA na mucosa oral de murinos. O processo de carcinogênese depende da iniciação de um ciclo de danos ao DNA celular, cujo caráter é primeiramente reversível. A persistência do fator nocivo, perpetua o ciclo de alterações cumulativas que em determinado momento passam a ser irreversíveis, implicando na formação do carcinoma espinocelular oral. Fonte: Elaborado pela Autora (2017).

Alterações no gene supressor de tumor *p53* interferem em diversos recursos antiproliferativos tais como interrupção do ciclo celular, senescência, autofagia, apoptose e inibição da angiogênese (Zilfou, Lowe, 2009; Li *et al.*, 2015). Em situação de homeostasia, a proteína p53 é mantida em níveis baixos pelo seu regulador Mdm2 por meio da via ubiquitina-proteassomo (Choi, Myer, 2008; Verma *et al.*, 2014). Logo, em tecidos normais, a imunomarcação para p53 geralmente é negativa, ou restrita a até 10% das células saudáveis na camada basal do epitélio (Nylander *et al.*, 2000). Assim como o carcinoma espinocelular oral, as displasias tendem a ser positivas para p53. A identificação dessa proteína nas camadas basal e suprabasal, bem como o aumento da sua imunomarcação, relaciona-se com a severidade da displasia e, portanto, é considerada um

evento precoce na transformação de condições potencialmente malignas (Brooks, Gu, 2010; Reddy *et al.*, 2012; Verma *et al.*, 2014).

Proteínas que regulam a proliferação celular, também podem estar superexpressas em displasias epiteliais e no carcinoma espinocelular oral (Pigatti *et al.*, 2015). A proteína Ki-67 é expressa durante a fase ativa do ciclo celular, contudo aumenta progressivamente à medida que o epitélio torna-se alterado (Mondal *et al.*, 2016). A Ki-67 favorece um estado de elevada proliferação e capacidade invasiva em neoplasias malignas orais (Bascones-Martínez *et al.*, 2013).

Apesar dos resultados pouco conclusivos até o momento, acredita-se que estratégias quimiopreventivas poderiam ser empregadas para inibir ou controlar o desenvolvimento do carcinoma espinocelular oral (Sulfikkarali, 2013; Scrobota, 2016). As vitaminas A, C e E, bem como o extrato de sementes de uva, a curcumina e o própolis vermelho brasileiro apresentaram resultados preliminares positivos na redução do estresse oxidativo local e na severidade da displasia epitelial oral induzida por DMBA (Athirajan *et al*., 2014; Edefonti *et al*., 2015; Edefonti *et al*., 2015b; Ribeiro *et al*., 2015; Scrobota *et al*., 2016). Entretanto, os resultados reportados por esses autores são considerados inconclusivos. Deixam, ainda uma grande lacuna no que tange às reais estimativas de haver possibilidade concreta de sugerir-se o uso de quimioprevenção para interromper o processo de malignização em células do epitélio oral, por meio da reversão de danos em lesões displásicas ou através da limitação da invasividade do carcinoma *in situ*.

Estudos publicados nos últimos anos sugerem que os extratos padronizados de *Cannabis sativa* com alto teor de canabidiol (CBD) poderiam ser aplicados como agentes anticarcinogênicos para diferentes tipos de tumores, *in vitro* e *in vivo*. Apesar do interesse no estudo dos canabinoides não ser recente, datando da década de 1960, a idealização do

sistema canabinoide-receptor como um possível alvo terapêutico para o câncer ocorreu efetivamente nos últimos 20 anos.

Entre os diferentes canabinoides descritos, o CBD, constituinte não psicotomimético da *Cannabis sativa*, é considerado seguro e bem tolerado por humanos (Martin-Santos *et al.*, 2012). Atualmente, é conhecido por suas propriedades anticonvulsivantes, analgésicas, antipsicóticas e neuroprotetoras (Johnson *et al.*, 2013; Gomes *et al.*, 2014). Primeiramente, essa droga foi utilizada para inibir os efeitos secundários do tratamento antineoplásico quimioterápico, náusea e êmese (Davis, 2016). Mais tarde, as propriedades anticarcinogênicas desse composto começaram a ser testadas. Entretanto, os resultados encontrados ainda são controversos e a compreensão dos mecanismos de ação é limitada (Aviello *et al.* 2012; De Petrocellis *et al*., 2013; Massi *et al.*, 2013; Romano *et al*., 2014; Elbaz *et al*., 2015).

Os resultados preliminares indicam que o CBD interferiu na modulação de diferentes etapas da carcinogênese em tumores malignos da região colorretal (Romano *et al*., 2014), mama (Elbaz *et al*., 2015), próstata (De Petrocellis *et al*., 2013), pulmão (Ramer *et al.*, 2012) e glioblastoma (Deng *et al.*, 2017). Esse canabinoide mostrou-se capaz de proteger o DNA contra danos oxidativos, aumentar a síntese de endocanabinoides e reduzir a proliferação celular através de múltiplos mecanismos (Aviello *et al*., 2012, Pisanti *et al.*, 2013; Romano *et al*., 2014).

Entre os receptores que podem interagir com esse ligante encontra-se o receptor canabinóide 1 (CB1) e CB2, potencial do receptor transiente subfamília vanilóide membro 1 (TRPV1) e TRPV2, receptores desorfanizados GPR55 e receptor gama ativado pelo proliferador de peroxissoma (PPARγ). Sugere-se, também, que o efeito possa ser independente de receptores quando obedecida uma certa dose e aplicando-a a

determinados tecidos alvo (Arnold *et al.*, 2012; De la Ossa *et al.*, 2013, Nabissi *et al*, 2013; McAllister *et al.*, 2015; Nabissi *et al.*, 2015).

Diferentes estudos, *in vitro* e *in vivo* sugeriram que o seu efeito antineoplásico – aumento do índice de morte e redução da proliferação celular, bem como a diminuição da migração de células cancerígenas, pode ser provocado de forma dependente ou independente do CB_1 e CB_2, assim como de outros receptores: TRPV1, receptor Adenosina A2A (A_{2A}) e PPARγ (Gallily *et al.*, 2003; McKallip *et al.*, 2006; Nabissi *et al.*, 2013). Não há estudos, até o momento, que tenham avaliado essa importante interação, ligante (CDB)-receptor, em células derivadas de carcinoma espinocelular oral.

Ligresti e colaboradores (2006) concluíram que o CBD apresentou o efeito inibidor mais potente em cultura de células tumorais mamárias quando comparado a outros canabinoides como o Δ-9-tetraidrocanabinol (THC), canabigerol, canabicromeno e canabidiol ácido.

Massi *et al*. (2008) observaram que o CBD é capaz de promover a produção de espécies reativas de oxigênio (ROS) e ativar a caspase-3 de forma gradual e tempo dependente, assim como a caspase-8 e caspase-9, precedendo a apoptose em células humanas de glioma. McKallip *et al.* (2006) reportaram um processo similar em células humanas leucêmicas, logo após a exposição das mesmas ao CBD. Os resultados compreenderam produção de caspases 3, 8 e 9; perda do potencial da membrana em mitocôndrias, com liberação do citocromo C, incremento na expressão de Nox4 e p22 e, consequentemente, na síntese de ROS.

Em 2011 o experimento de McAllister *et al.* mostrou que a exposição de células tumorais mamárias ao CBD, culminou na redução da massa tumoral e de ocorrência de metástase pulmonar em ratos fêmeos imunocompetentes. Os mecanismos envolvidos foram a modulação diferencial da quinase regulada por sinal extracelular (ERK) e a

produção de ROS, ambos levando a inibição da expressão da proteína inibidora de diferenciação 1 (Id-1), potente regulador de metástases em tumores de mama e demais neoplasias malignas. Ainda no mesmo ano, em outro estudo realizado em células mamárias, Shrivastava *et al.* (2011) tornaram a observar um importante acréscimo na síntese de ROS provocado pelo CBD. Também, como em pesquisas prévias, constataram perda de potencial da membrana mitocondrial e liberação do citocromo C para o citoplasma, culminando, na ativação da via intrínseca da apoptose.

Em estudo realizado em células endoteliais da veia umbilical humana o CBD mostrou-se inibidor da angiogênese de maneira dose-dependente. Essa droga reduziu a expressão de metaloproteinase de matriz 9 (MMP-9), inibidor tecidual de metaloproteinase 1 (TIMP1), ativador de plasminogênio uroquinase (uPA), endotelina 1 (ET-1), fator de crescimento derivado de plaquetas (PDGF-AA) e interleucina 8 (IL-8) (Solinas *et al.*, 2012). Modificações na expressão dessas proteínas têm sido vinculadas ao aumento da angiogênese, remodelação tecidual, alterações na diferenciação celular e na função de células assassinas naturais.

McAllister *et al.* (2011), Shrivastava *et al.* (2011) e Murase *et al.* (2014), entre outros autores previamente citados, concluíram que a produção de ROS é um mecanismo crucial pelo qual o CBD controla a progressão do câncer de mama. Esse agente, promove a inibição de canal aniônico 1 dependente de voltagem, localizado na membrana mitocondrial externa, ocasionando o aumento nos níveis intracelulares de cálcio e promovendo a produção de ROS (Ligresti *et al.*, 2006, Rimmerman *et al.*, 2013). O comprometimento da homeostase redox - se a síntese de ROS alcançar um limiar incompatível com a sobrevivência celular - causa aumento da permeabilidade da

membrana mitocondrial externa e liberação do citocromo C, formação de apoptossomo e ativação de caspases executoras (Wondrak, 2009).

Elbaz *et al.* (2015) demonstraram que o uso de CBD, em modelos *in vitro* e *in vivo* de câncer mamário, diminuiu a proliferação de células tumorais e da vascularização. O agente farmacológico, também inibiu o recrutamento de macrófagos ativados pela via alternativa para o sítio da neoplasia maligna e reduziu a via de transdução de sinais do fator de crescimento epidérmico (EGF) e seu receptor (REGF). Observou-se ainda que o CBD é capaz de modificar as citocinas secretadas pelo tumor, inibindo o fator de crescimento de colônias monocítico-granulocítico (GM-CSF) e a proteína inflamatória de macrógafo CCL3 (Pirilä *et al.*, 2015). Em casos de metástase pulmonar de neoplasias malignas da mama, tanto o GM-CSF quanto a CCL3 apresentaram-se superexpressos.

Sobre o uso de canabinoides como uma alternativa terapêutica para o manejo do carcinoma espinocelular oral ou de condições cancerizáveis, ainda há diversos aspectos a serem esclarecidos. Dada a relevância do tema e a inexistência de estudos *in vitro* ou *in vivo* com a mesma proposição, justifica-se a realização de pesquisas na área.

A presente Tese estrutura-se sob a forma de três artigos. Apresenta-se, inicialmente, o experimento desenvolvido, cujo objetivo foi averiguar o efeito do CBD em línguas de ratos expostas ao DMBA. Em sequência, seguem duas revisões da literatura que abordam temas subsidiários à compreensão da análise proposta.

Artigo 1 | *Novel chemopreventive effects of cannabidiol in an experimental model of 7,12-dimethylbenzanthracene-induced oral carcinogenesis: preliminary results*

O artigo de pesquisa foi formatado de acordo com as normas do periódico BMC Cancer (Qualis A1). As normas para a submissão e publicação do manuscrito podem ser consultadas *on-line* (https://bmccancer.biomedcentral.com/submission-guidelines).

ABSTRACT

Background

A novel potential therapeutic use of cannabidiol (CBD) is being considered for cancer prevention, decreasing tumour growth, and metastasis. In this study, we investigated the inhibitory effect of CBD on the progression of an oral carcinogenesis model experimentally induced with 7,12-dimethylbenzanthracene (DMBA).

Methods

The ventral tongue mucosae of male Fischer 344 rats were treated with 0.5% DMBA thrice weekly for 12 weeks. Then, from week two of the DMBA administration, the groups were intraperitoneally treated with the vehicle or CBD 5 or 10 mg/kg (groups 1, 2, and 3, respectively) twice weekly until the completion of 20 doses. Alterations of the epithelium integrity were assessed using routine histopathological analysis and immunohistochemistry labelling with Ki-67.

Results

A statistically significant difference was found between groups 1 and 2 (P = 0.018) and between groups 1 and 3 (P = 0.014) in the histopathological grading of cellular and tissue architectural alterations. A decrease in the Ki-67 labelling index of groups 2 and 3 compared to that of group 1 ($P < 0.001$) and compared to each other ($P < 0.001$) was observed. In addition, the Ki-67 labelling was restricted to the epithelial basal layers in groups 2 (P = 0.030) and 3 (P = 0.023) compared to that in group 1.

Conclusions

Although the results are preliminary, we concluded that in the studied sample, CBD exerted a chemopreventive effect *in vivo* by limiting DMBA-induced oral carcinogenesis and controlling cell proliferation.

Keywords: *Cannabis sativa*; Cannabidiol; Squamous cell carcinoma; Cell proliferation; Chemoprevention

BACKGROUND

Advances in research on cannabidiol (CBD), a non-psychomimetic cannabinoid, suggest that it could be considered a promising adjunctive resource in cancer management [1-4]. CBD showed positive results in alleviating intractable cancer-related pain [5], prevented chemotherapy-induced alopecia [6], nausea and emesis, and cancer cachexia-anorexia syndrome [7,8]. Furthermore, several authors have reported CBD as a promising agent in the regulation of carcinogenesis. Studies in glioblastoma, colorectal, breast, and prostate cancers have found strong evidence that CBD decreased the expression of several proteins specifically involved in tumour growth, angiogenesis, invasion and metastasis [1-4,9-13]. However, the results of preclinical studies have not been translated into clinical use yet because the exact mechanisms involved in the chemotherapeutic effects of CBD, as well as its indications and dose, have not been fully elucidated [3,14-17].

To date, despite extensive clinical investigations, a chemopreventive strategy to control the incidence of oral squamous cell carcinoma (OSCC) has not been developed [18,19]. The OSCC burden particularly affects developing countries that lack prevention programmes that serve the risk population and services for oral health promotion and attention [20]. Although this pathology is amenable to early detection, most lesion diagnoses are delayed, which is related to disease progression to advanced stages [21], recurrence, and limited patient survival rate after treatment [22].

A great challenge in this field is improving the risk assessment and identification of new targets to accurately predict potentially malignant diseases that would progress to OSCC. In addition, the integration of valuable biomarkers and the use of novel agents is still needed to reduce the rate of second primary tumours [23]. Increased cell proliferation is a reliable marker of the transition of the normal epithelium to dysplasia or malignancy [24], and can be assessed using Ki-67 immunolabelling.

CBD reduced chemical-induced preneoplastic lesions and tumour growth in an animal model of colon carcinogenesis [3]. However, its effects in preventing OSCC development are unknown. Therefore, the aim of the present study was to evaluate the effect of CBD in a 7,12-dimethylbenzanthracene (DMBA)-induced oral carcinogenesis model in male rats using routine histopathological and immunohistochemical (IHC) analyses.

METHODS

Animals

The care and use of laboratory animals were performed in accordance with the guidelines of the National Council for Control of Animal Experimentation (COBEA, Brazil) [25] and Arouca Law [26]. The experimental protocol was approved by the Institutional Ethics Committee for Animal Use (Protocol number: 6971). We used 12-week-old male Fischer 344 rats (*Rattus norvegicus*) with a mean weight of 350 g at the beginning of the experiment. They were housed under a 12-h light/dark cycle (lights turned on and off at 7:00 a.m. and 7:00 p.m., respectively), in temperature controlled cages (22°C) placed in ventilated racks. Nuvilab chow (Nuvital Nutrientes S.A, Colombo, PR, Brazil) and water were provided *ad libitum*.

Experimental groups

After acclimation, the sample of 15 animals was randomly divided into three groups of five rats each that were all exposed to DMBA. Then, the groups were treated as follows: (1) injected with vehicle and (2 and 3) treated with CBD at final doses of 5 mg/kg and 10 mg/kg, respectively.

Carcinogenesis model

The animals were physically restrained manually, and then the ventral mucosa of the tongue was exposed to 100 μL 0.5% DMBA in acetone (Sigma-Aldrich, St. Louis, MO, USA). Disposable regular micro-applicators (Microbrush®, Grafton, WI, USA) were used for each animal. The rats were deprived of food and water for 60 min after DMBA administration and the procedure was repeated thrice weekly for 12 weeks for all the groups. At each dosing, a freshly thawed carcinogen preparation was used. The selected protocol was according to that established in previous studies [27-32].

CBD administration

Synthetic CBD (≥ 99% purity, gently donated by THC Pharm GmbH, Frankfurt, Germany) was prepared by dilution in 98% saline (0.15 M sodium chloride [NaCl]) and 2% Tween 80 (Sigma-Aldrich) immediately before use and administered at 5 mg/kg and 10 mg/kg to groups 2 and 3 in a volume of 1 mL/kg [33]. CBD administration was started after six doses of DMBA were administered to group 2 and group 3 to initiate the deleterious chemical effect on oral epithelial cells. CBD injections were administered intraperitoneally every other day after the DMBA treatment, twice weekly until 20 doses were completed. The animals in group 1 received only the vehicle injected intraperitoneally [3,4]

Euthanasia

The animals were euthanised using isoflurane (Cristalia, Porto Alegre, RS, Brazil) inhalation in an appropriate anaesthesia chamber after the experimental protocols were completed. Then, the tongues were dissected [34].

Histological specimen processing

The tongues were fixed for 24 h in 10% buffered formalin (Top Glass, Porto Alegre, RS, Brazil) [35], each specimen was sectioned into two portions in the sagittal direction over the median sulcus of tongue, and then they were paraffin embedded to obtain 15 blocks (one per animal). Then, 4-µm-thick sections were mounted on slides and routinely stained with haematoxylin and eosin (H&E).

IHC sample processing

For IHC staining, we cut 4-µm-thick sections from the formalin-fixed paraffin-embedded tissue blocks. Then, the sections were dried in an 80°C for 15 min, placed in a BenchMark® XT system (Ventana Medical System Inc., Oro Valley, Arizona, USA), and processed according to the following protocol.

First, the samples were deparaffinised and pre-treated with Cell Conditioner 2 (pH 6.0) for 60 min. After the washing steps, the tissue samples were then incubated with the primary monoclonal antibody anti-Ki-67 (1:150, Clone SP6, Zeta Corporation, Sierra Madre, CA, USA) for 32 min. Subsequently, chromogenic detection was performed with the XT ultraView Universal DAB detection kit (diaminobenzidine, DAB), counterstained with haematoxylin, washed and mounted. Samples of tonsil carcinoma were used as a positive control while the negative control was processed without the primary antibody.

Histological analysis

All evaluations were performed in duplicate by an independent observer who was blinded to the group treatments and the IHC findings. The intra-observer accuracy was analysed using a paired *t*-test and Pearson correlation test, which showed the absence of a significant difference between the groups ($P \leq 0.05$) and a strong correlation ($r = 0.8$), respectively. Each section was examined using a light microscope (Nikon Eclipse E200 LED) under constant lighting conditions using ×10 and ×40 objectives (approximate magnification of 100× and 400×). The tissue specimen site that exhibited the most intense

alterations were then classified in accordance with the World Health Organization (WHO) Classification of Tumours: Pathology and Genetics of Head and Neck Tumours (Table1) [36,37].

Table 1: Diagnosis criteria for histopathological analysis [36,37]

Grade	Level involved	Cytological changes	Architectural changes
Mild OED	Lower third	Cell and nuclear pleomorphism Nuclear hyperchromatism	Basal cell hyperplasia
Moderate OED	Up to the middle third	Cell and nuclear pleomorphism Anisocytosis and anisonucleosis Nuclear hyperchromatism Increased and abnormal mitotic figures	Loss of polarity Disorder maturation from basal to squamous cells Increased cellular density Basal cell hyperplasia Bulbous drop shaped rate pegs
Severe OED	Up to the upper third	Cell and nuclear pleomorphism Anisocytosis and anisonucleosis Nuclear hyperchromatism Increased and abnormal mitotic figures Enlarged nuclei and cells Hypercromatic nuclei Increased number and size of nucleoli Apoptotic bodies	Loss of polarity Disorder maturation from basal to squamous cells Increased cellular density Basal cell hyperplasia Bulbous drop shaped rate pegs Dyskeratosis Secondary extensions on rete tips Acantholysis
Carcinom *in situ*	Full epithelial thickness	All changes might be present	Top-to bottom change Loss of stratification

OED – oral epithelial dysplasia.

IHC analysis

The tissue specimen site that exhibited the most intense IHC labelling was selected using an approximate magnification of 100×. The images were captured using a Nikon Eclipse E200 LED microscope equipped with an image capture system (10MP camera). Five consecutive fields [38] containing non-overlapping, full-thickness epithelium images (approximate magnification, 400×) were captured and stored in the JPEG format. The lighting conditions and magnification were maintained constant during the photography process. Cells were considered immunostained if they exhibited a brown

nuclear colouring regardless of intensity [24,38]. The Ki-67 labelling index (% of immunostained cells in the total area of captured epithelium) was assessed using a computer-assisted image analysis software (ImageJ, Figure 1) [39]. Thereafter, the epithelial layers that exhibited Ki-67 immunostained nuclei were evaluated and classified base on three scores: basal (just above the basement membrane), parabasal (within two layers above the basement membrane), and suprabasal (above the parabasal layer) [24,40,41].

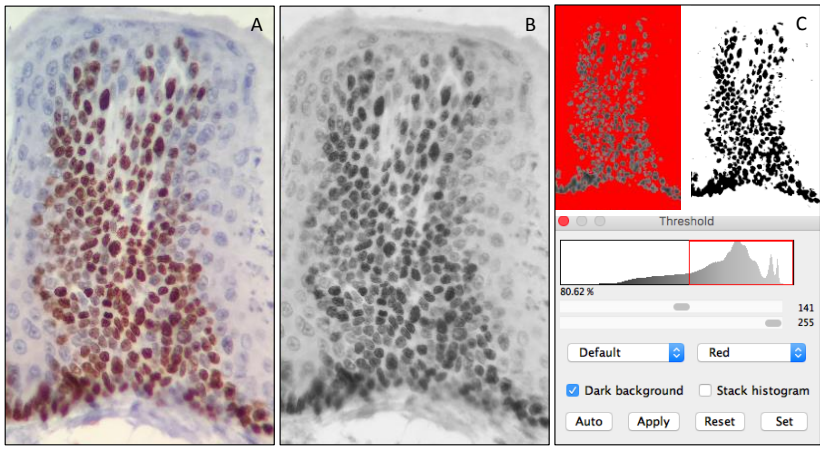

Figure 1: Quantification of immunohistochemical labelling of Ki-67 using an automated technique. (A) Capture selected for analysis (approximate magnification, 400×). (B) Image converted to an 8-bit grey scale with (C) adjusted threshold resulting in a binary version with only two-pixel intensity, allowing cell counting, which is expressed as area (%).

Statistical analysis

Quantitative data were presented as the means ± standard deviation. The Ki-67 scores were expressed as median, minimum, and maximum values. The Ki-67 labelling index data were analysed using a random-effects model (mixed model) that accounted for the nature of correlated observations in the same animal [42,43]. The scores of Ki-67 distribution across epithelial layers were analysed using the Kruskal-Wallis test with adjustments for multiple comparisons using the Hommel method. For all statistical

analyses, the data were considered significant at $P \leq 0.05$, and the data were analysed using the Statistical Package for the Social Sciences (SPSS software) version 22.0.

RESULTS

CBD administration prevented onset of severe oral epithelial dysplasia and OSCC in rat tongues exposed to DMBA

The effects of CBD were investigated in a DMBA-induced model of tongue carcinogenesis in Fischer 344 rats. As expected, the intraperitoneal administration of CBD exerted a chemopreventive effect by inhibiting the progression of DMBA-induced alterations to a severe grade oral epithelial dysplasia and *in situ* carcinoma. A statistically significant difference was observed between groups 1 (vehicle) and 2 (CBD 5 mg/kg, $P = 0.018$), as well as between groups 1 and 3 (10 mg/kg, $P = 0.014$). In group 3, all the rat tongues evaluated exhibited a mild epithelial dysplasia, and in group 2, one (20%) rat presented a moderate epithelial dysplasia; however, statistical significance was not found between the CBD doses tested ($P = 0.317$). Table 2 summarises the findings of the histopathological assessment and Figure 2 illustrates the characteristic findings of epithelial alterations.

Table 2: Descriptive findings of the histopathological assessment

Experimental group	Grading ($N = 15$)									
	Non-dysplastic		Mild OED		Moderate OED		Severe OED		Carcinoma *in situ*	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%
Group 1 (DMBA + vehicle)	-	-	-	-	1	20%	3	60%	1	20%
Group 2 (DMBA + CBD 5mg/kg)	-	-	4	80%	1	20%	-	-	-	-
Group 3 (DMBA + CBD 10mg/kg)	-	-	5	100%	-	-	-	-	-	-

OED – oral epithelial dysplasia.

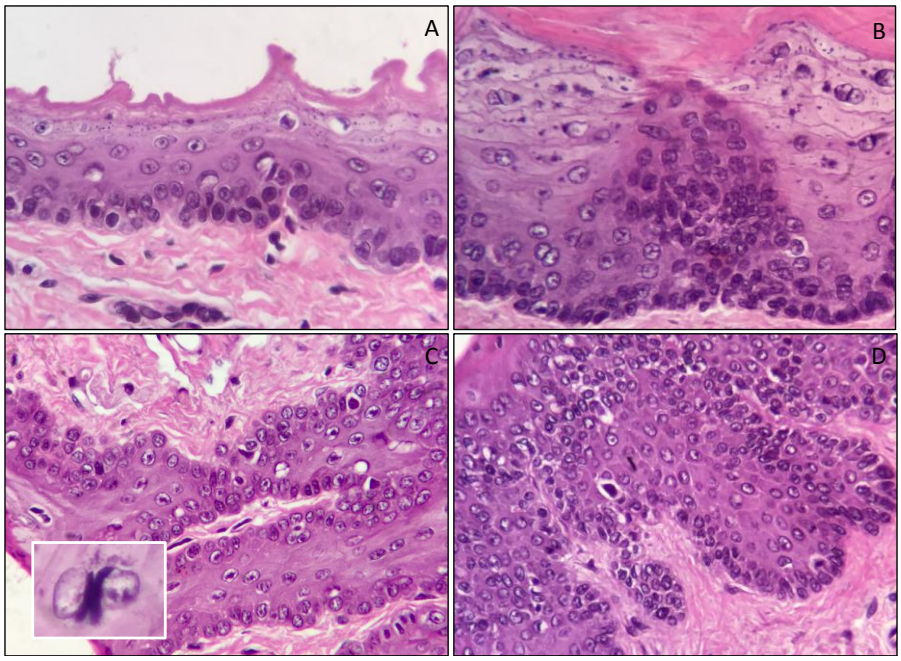

Figure 2: Analysis of 7,12-dimethylbenzanthracene (DMBA)-exposed tongue ventral mucosa (haematoxylin and eosin, H&E). (A) Mild oral epithelial dysplasia exhibiting cellular and nuclear pleomorphism and hyperchromatism, and discrete hyperplasia of the basal layer (approximate magnification, 400×). (B) Moderate oral epithelial dysplasia with anisocytosis and anisonucleosis, nuclear hyperchromatism, mitosis, and loss of polarity (approximate magnification, 400×). (C) Severe oral epithelial dysplasia exhibiting (details, white square) high atypical mitotic figure (approximate magnification, 100× and 1000×). (D) Carcinoma *in situ* with markedly alterations from bottom to top (approximate magnification, 100×).

CBD administration reduced Ki-67 labelling index

Figure 3 summarises the distribution of Ki-67 labelling index values of the three groups and their immunohistochemical aspects. In the established rat model of carcinogenesis, CBD controlled the cell proliferation in a dose-dependent manner (Table 3). This effect decreased the Ki-67 labelling indexes in groups 2 and 3 compared to that of group 1 ($P < 0.001$) while groups 2 and 3 differed significantly from each other ($P < 0.001$).

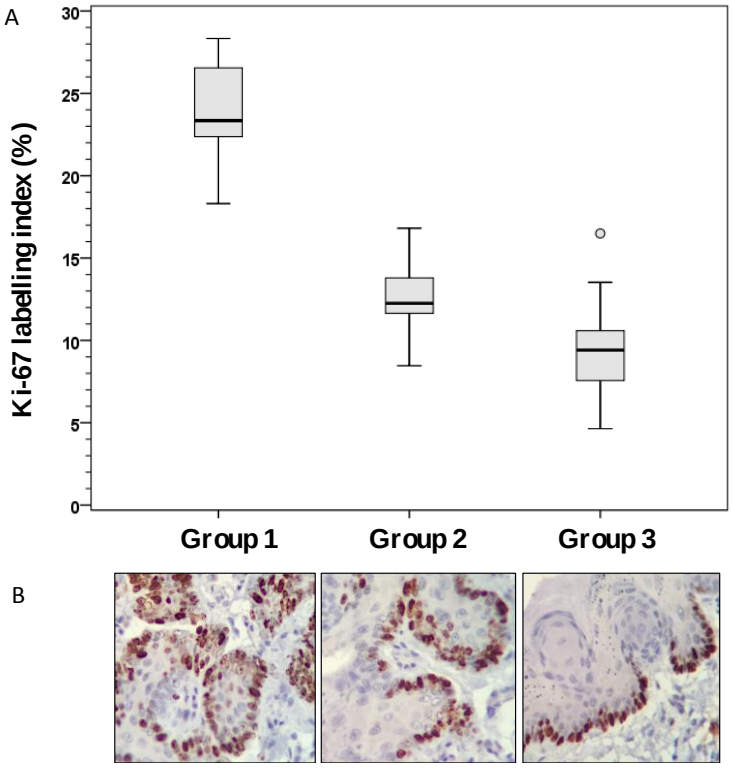

Figure 3: Data distribution of Ki-67 labelling index. (A) Box plot showing the distribution of Ki-67 labelling index values of the three groups. Upper horizontal line of box, 75th percentile; lower horizontal line of box, 25th percentile; horizontal bar within box, median; upper horizontal bar outside box, 90th percentile; lower horizontal bar outside box, 10th percentile. Circles represent outliers. (B) Immunohistochemical findings of the three groups (approximate magnification, 400×).

Table 3: Pairwise comparisons of Ki-67 index labelling (% of area)

Experimental Group	Mean	Mean difference*	95% confidence interval		*P* value**
			Minimum	Maximum	
Group 1 (DMBA + vehicle)	24.091^{α}	$11.359^{\alpha\beta}$	$9.667^{\alpha\beta}$	$13.051^{\alpha\beta}$	<0.001
		$14.850^{\alpha\delta}$	$13.158^{\alpha\delta}$	$16.542^{\alpha\delta}$	
Group 2 (DMBA + CBD 5mk/kg)	12.732^{β}	-11.359	-13.051	-9.667	<0.001
		$3.491^{\beta\delta}$	$1.799^{\beta\delta}$	$5.183^{\beta\delta}$	
Group 3 (DMBA + CBD 10mk/kg)	9.241^{δ}	-14.850	-16.542	-13.158	<0.001
		-3.491	-5.183	-1.799	

*The mean difference is significant at 5% level. **Sidak adjusted *P* values for multiple comparisons.

CBD administration restricted Ki-67 epithelial immunostaining to basal stratum

CBD at 5 mg/kg (group 2, P = 0.030) and 10 mg/kg (group 3, P = 0.023) contributed to the maintenance of cell proliferation restricted to the basal layers of DMBA-exposed mucosae compared to results of tissue samples from the vehicle-treated rats (group 1). These findings indicate a favourable effect of CBD in controlling cell proliferation during the carcinogenic stimulus. However, between groups 2 and 3, no statistically significant differences occurred in the Ki-67 labelling scores of the epithelial layers (P = 0.221). Figure 4 illustrates the scores of Ki-67.

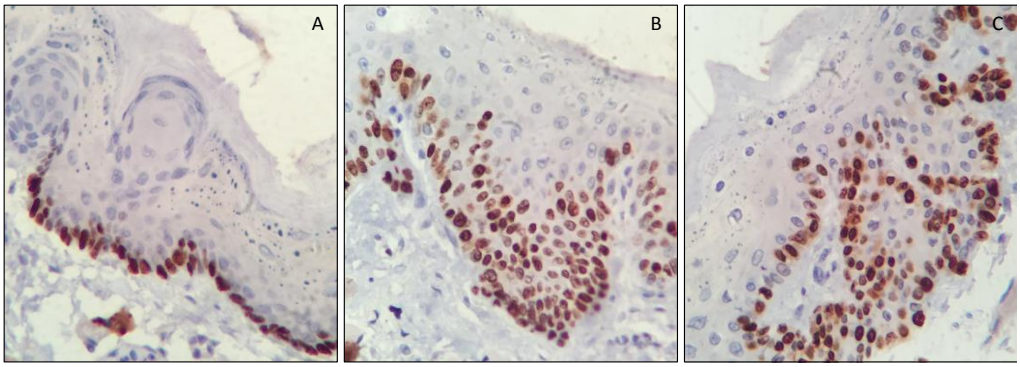

Figure 4: Scores of Ki-67 distribution in (A) basal, (B) parabasal, and (C) suprabasal cell layers (approximate magnification, 400×).

DISCUSSION

In the context of the existing literature, this manuscript presents our results on the novel use of CBD as a chemopreventive agent in oral carcinogenesis [3,4,44]. The use of Fischer 344 rats for the study of chemically induced oral carcinogenesis and chemoprevention has been validated previously [29,45]. The routine histopathological assessment of the rat tongues exposed to DMBA revealed considerable similarities to the cell and architectural tissue alterations observed in human patients [46]. Thus, the criteria

used to classify the oral epithelial dysplasia and carcinoma were considered adequate and reliable for our purpose.

The treated groups were exposed to DMBA for 12 consecutive weeks [29]. In group 1, which was treated intraperitoneally with the vehicle alone, only one rat (20% frequency) developed *in situ* carcinoma and the prevalence of severe epithelial dysplasia was 80%. These findings could be attributed to the latency period for the onset of malignant tumours that occurs in some animals, as proposed previously [47]. Additionally, it is widely known that despite well-established criteria, microscopic evaluations are affected by the subjectivity of the observer's interpretation [48,49]. However, in this study, no significant difference was found between the duplicate intra-observer analysis.

CBD is highly lipophilic and, therefore, exhibits low bioavailability, which presents a challenge in defining optimal drug delivery [50]. In addition, the ideal dose for chemoprevention in animal models of carcinogenesis remains debatable [3,4]. Since there are no published studies evaluating the effects of CBD in oral carcinogenesis, we selected doses of 5 mg/kg and a 10 mg/kg in accordance with experimental studies performed in breast and colorectal cancer [3,4,44].

McAllister and colleagues [44] observed that 1 mg/kg and 5 mg/kg CBD significantly decreased breast tumour lung metastases in a dose-dependent manner. Elbaz and co-workers [4] found similar results using a 10 mg/kg dose, which reduced breast tumour growth and lung metastasis in rats. We observed that both 5 mg/kg and a 10 mg/kg CBD doses prevented the onset of OSCC in all rat tongues exposed to DMBA compared to the vehicle. These findings suggest that the effects of DMBA were inhibited or arrested by CBD administration.

We did not investigate the mechanism underlying the abovementioned results. However, previous reports suggest that CBD is a potent inhibitor of the catalytic activities of cytochrome P450 (CYP) 1 enzymes at intra- and extra-hepatic sites [51]. Although the entire CBD structure is required for complete inhibition, the pentylresorcinol portion of the structure is currently considered to likely have a direct role in the inhibition of CYPA1 [52]. CYP1A1 and CYP1B1 mediate the activation of polycyclic aromatic hydrocarbons such as DMBA, to their carcinogenic forms [52-55].

The selected delivery route (intraperitoneal) ensured that CBD was primarily absorbed into the mesenteric vessels, which drain into the portal vein and pass through the liver. Therefore, CBD would be expected to undergo hepatic metabolism before reaching the systemic circulation. Further, oral or intra-lesion administration would probably exhibit similar results once the drug is also exposed to significant first-pass metabolism in the liver [56,57]. Studies to evaluate the pharmacokinetics and doses-response profile would be expedient and are encouraged. This knowledge is crucial for defining the ideal route of administration and ensuring consistent bioavailability, as well as for the successful translation of results from bench to bedside [8].

Ki-67 is a reliable indicator of cell proliferation in malignant tumours and a useful marker for the early detection of mucosa at high risk of developing oral cancer [41,58-60]. This protein is expressed during the active cell cycle phases of normal cells. However, in malignantly transformed cells, the alpha splice variant of this protein is continuously overexpressed. Apparently, the proteasome-dependent degradative mechanism fails to down-regulate Ki-67 expression in cancer [61].

Ki-67 was found to be reduced in CBD-treated animals in previous experimental models of cancer [3,4]. In our study, CBD administration at 5 mg/kg and 10 mg/kg

reduced the index of Ki-67 labelling in a dose-dependent manner. Accordingly, it was observed that the CBD-treated animals mostly exhibited mild epithelial alterations.

CBD inhibited the cell cycle at the G1 phase and induced cell accumulation in the sub-diploid phase in multiple myeloma [62]. These effects were mediated by the regulatory effects of CBD on extracellular signal-regulated kinase (ERK), serine/threonine kinase 1 (AKT) and nuclear factor (NF)-κB pathways with major effects on the transient receptor potential cation channel subfamily V member 2 (TRPV2). Altogether, TRPV2 and the phosphoinositide 3-kinase (PI3K)/AKT pathway support a novel route by which CBD induced autophagy in glioma stem-like cells [17].

In malignantly transformed cells, a failure of the trafficking of TRPV2 from the endosome to the plasma membrane promotes unchecked proliferation and cell resistance to apoptotic signals [63,64]. TRPV2 channels have been found in the junctional epithelium [65], tongue, buccal mucosa, and palate of rats [66], demonstrating that these sites might be susceptible to CBD-induced anticancer effects. In addition, OSCC lesions in humans overexpress TRPV2, possibly because carcinogenic substances such as alcohol and tobacco can trigger these receptors [67]. In the present study, the receptors activated by CBD and involved in the inhibition of cell proliferation were not evaluated. Therefore, the comprehensive identification of receptors involved in the anticancer effects of CBD is urgently needed.

The Ki-67 IHC staining in groups 2 and group 3 was restricted to the basal layers of the epithelium exposed to DMBA, but the effect was independent of the dose. It has been reported that the suprabasal expression of Ki-67 is a determining indicator of the severity of epithelial dysplasia and the histopathological grading of OSCC [24], corroborated the findings of the present study.

Was observed that CBD exhibited a few beneficial effects in the chemically induced carcinogenesis experimental model, based on the encouraging positive results obtained. However, lower and higher doses of CBD have shown antagonist effects in other fields of research. For example, 3 mg/kg and 30 mg/kg doses of CBD increased and decreased, respectively, the cell proliferation and Ki-67 in neural cells. Nevertheless, the meaning of this finding is still unknown [33].

Therefore, we would like to underscore that although these preliminary results suggest CBD has positive effects in oral cancer chemoprevention, several outstanding related aspects are yet to be elucidated. Despite the limitations inherent to the design of the study, we obtained relevant results that would contribute to defining future research directions of CBD applications in oral cancer prevention and management.

CONCLUSION

We concluded that in the test sample, CBD exerted an *in vivo* chemopreventive effect by limiting DMBA-induced carcinogenesis and controlling cell proliferation. The receptors and pathways activated by CBD in the DMBA-induced oral carcinogenesis model need to be established in future studies.

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Artigo 2 | *Is oral squamous cell carcinoma a potential therapeutic target for cannabidiol? What have we learnt from literature?*

A revisão da literatura foi formatada de acordo com as normas do periódico Anticancer Research (Qualis B1). As normas para submissão e publicação podem ser consultadas *on-line* (http://ar.iiarjournals.org/site/misc/ifora.xhtml).

ABSTRACT

Background

The effect of cannabidiol (CBD) against solid malignant tumours has been widely demonstrated both *in vivo* and *in vitro*. This cannabinoid presented ability to inhibit cell proliferation and migration, tumour growth, invasion and metastasis. There is no evidence in the literature of the use of CBD as a chemopreventive or therapeutic agent against oral squamous cell carcinoma (OSCC). However, the molecular targets involved in CBD-mediated anticancer effects against other kinds of tumours suggest a great potential for it use against OSCC. Actually, surgery is the pillar of OSCC treatment despite the poor results and adjuvant chemo and radiotherapy are associated with a non-significant benefit. Then, attempts to improve patient outcome remain necessary.

Main body

We conducted a literature review highlighting the main findings of CBD use in experimental models of glioblastoma, multiple myeloma, breast, lung, colorectal and prostate cancers. The receptors and signalling cascades that supported the effects of CBD were briefly reviewed. Thereafter, a parallel with the expected outcomes of the use of CBD against OSCC was proposed and potential molecular targets were evidenced.

Conclusion

In general, the mechanisms responsible for CBD anti-tumour properties, as well as effective doses and delivery system remain to be elucidated. However, the available findings encourage us to suggest that CBD use would be beneficial to arrest OSCC progression. Studies for assessment of the effect of CBD in OSCC cells lines or experimental models are urgently required.

Keywords: Oral squamous cell carcinoma; Head and neck cancer; Cannabidiol; Receptor, cannabinoid, CB1; Receptor, cannabinoid, CB2; TRPV cation channels; Reactive oxygen species

BACKGROUND

Plants have been used for a wide range of purposes throughout the vast majority of human history. In particular, *Cannabis sativa* (*Cannabaceae* family) has been globally used for recreation, culinary purposes, empirical treatment of diseases, and as a source of both cellulosic and woody fibres [1,2].

From the XIX century until the present day, the identification and structural elucidation of more than 100 different phytocannabinoids have been reported [3-7]. Wood [8] and Michoulam and Shvo [9] isolated the first and second active compounds of cannabis, cannabinol (CBN) and cannabidiol (CBD), respectively. Lately, Gaoni and Michoulam [10] identified delta-9-tetrahydrocannabinol (THC), the main active compound of *C. sativa*. Another milestone was the discovery of cannabinoid receptor system and its endogenous ligands, which mediate most of the cannabis-induced effects [11-14].

CBD is one of the most studied compounds of the class cannabinoids for medical purposes and is characterised by its non-psychotomimetic properties [15,16]. CBD-mediated effects can be dependent [17-19] or independent of cannabinoid receptors [20-27] or even independent of protein-mediated mechanisms owing to its lipophilic nature [28-30]. This compound demonstrated optimistic effects as an adjunctive therapeutic option in the management of side effects of chemotherapy and radiotherapy and for palliative care in other conditions like emesis, mucositis and chemotherapy induced peripheral neuropathy [31-33].

Accumulating evidence from *in vitro* and *in vivo* studies investigating the effects of CBD against cancer has emerged since the last decade. According to published data, this agent demonstrated positive effects in preventing cancer development in known at

risk mucosa and inhibiting angiogenesis, cell proliferation, invasion, and metastasis in solid malignant tumours [34-40].

To date, the anticancer properties of CBD against oral squamous cell carcinoma (OSCC) have not been tested. However, this malignant tumour presents a few pathogenic mechanisms that are similar to those of other cancers that have been successfully prevented or treated by CBD, making OSCC a suitable target for evaluating the chemopreventive potential of cannabinoids [41].

Thus, the present review focuses on recent data on the effects of CBD against cancer, obtained using a PubMed database search. A brief background on the efficacy of this phytocannabinoid in modulating the different steps of carcinogenesis is provided. Also, a few mechanisms that would be implicated in CBD-mediated prevention and treatment of OSCC are hypothesised and future research directions are considered.

OVERVIEW ON GENERAL FINDINGS

CBD presented a wide range of anticancer effects against different malignant cell lines (*in vitro*) and in xenograft or syngeneic tumour models (*in vivo*). Based on the available literature from 2013 to 2017, it was observed that CBD interacts with multiple pharmacological targets, and certain mechanisms are not completely understood. The key observations are summarized in Figure 1.

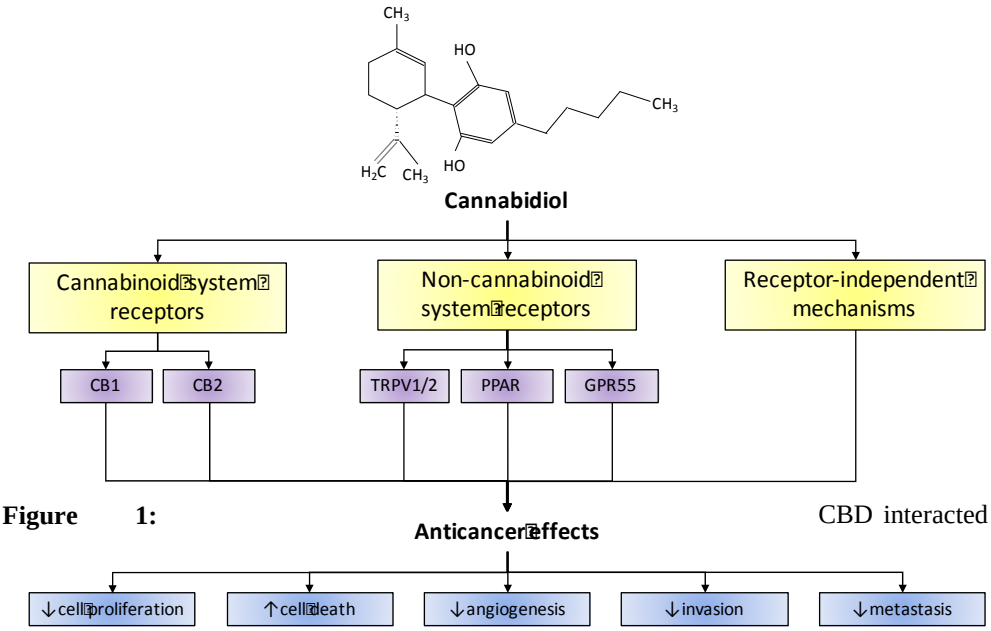

Figure 1: CBD interacted in a receptor dependent or independent-manner to inhibit pro-tumour characteristics in malignant transformed cells. CB1 - cannabinoid receptor 1, CB2 - cannabinoid receptor 2, TRPV1 - transient receptor potential vanilloid-1, TRPV2 - transient receptor potential vanilloid-2, PPARγ - peroxisome proliferator-activated receptor gamma, GPR55 - G protein-coupled receptor 55.

Effect of CBD in glioma/glioblastoma experimental models

Glioblastoma is among the most aggressive cancers in adults with an overall five-year survival rate ranging between 0.05 and 4.7% [42]. There is compelling evidence that CBD would be beneficial for the management of this type of brain tumour. Table 1 summarizes the studies reviewed in this section.

To evaluate the anticancer effect of CBD in animal models of glioma/glioblastoma, different doses (1–15 mg/kg) were used daily or every other day [23,30,35,43]. In all the studies, CBD showed protective effects against malignant cells. Nevertheless, in a few experiments, classic dose-dependent responses were not observed and a very limited dose range was shown, possibly because of the low affinity of CBD to receptors [36].

Intraperitoneal injection is the most commonly used route of drug delivery. However, a previous study reported that CBD-loaded microparticles are as effective as

intraperitoneal injections. The validation of this new delivery route would be of great interest, especially in experimental conditions, because it allows a five-day interval between doses [35,43].

The effect of CBD alone or in combination with other therapeutic modalities was tested. The main advantage of combined therapy is the reduction in concentration of drugs with known adverse effects by means of adjuvants that are free from side effects, consequently leading to improved efficacy [30]. In addition, experimental studies involving different drug combinations are of great relevance as they reflect clinical conditions, especially in gliomas/glioblastomas, where the blood-brain barrier tends to limit the effect of medication.

Cannabinoids licensed for human use usually contain CBD/THC at a 1:1 ratio [23,30,35,43]. Previous studies reported that the concomitant use of both the cannabinoids did not produce loss of activity [35,43]. This could be because THC has great affinity to CB1 and CB2; however, CBD mostly acts independent of the cannabinoid receptor system [24-27,30].

Correspondingly, with regard to mechanisms, Nabissi *et al.* [23,44] showed that CBD binds to transient receptor potential vanilloid 1 (TRPV1) and TRPV2. Moreover, activated the signalling cascades of phosphoinositide 3-kinase/serine threonine kinase 1 (PI3K/AKT) and mitogen activated protein kinase/extracellular signal-regulated kinases (MAPK/ERK).

Table 1: Preclinical studies evaluating the anticancer effects of cannabidiol in gliomas

Experimental model	Effective doses	Receptor-dependent or independent mechanisms	CBD-mediated effect	Reference
Glioma cell (U87MG) and orthotopic tumour models	• 6.7 mg CBD loaded microparticles (MP), every 5 days, for 24 days or 6.1 mg THC MP alone or 1:1 (w:w) THC+CBD mixed MP, 24-days study • CBD/THC 15 mg/kg, i.p, once a day, nearly 3 weeks, alone or in combination	↓Ki-67, ↓CD31	• ↓cell proliferation, ↓tumour weight, ↑apoptosis, ↓angiogenesis • No significant differences in solution were found among cannabinoid-loaded MP and cannabinoids	De la Ossa *et al.*, 2013 [35]
Glioma cell line (U87MG) and MZC primary glioblastoma cells	10 µM CBD	TRPV2	↑cytotoxicity of alkylating agents	Nabissi *et al.*, 2013 [23]
Glioma cell lines (U87MG and T98G)	12 µM CBD	↑ ERK, ↑AKT, ↓HIF-1α, ↓TGF-β1, ↓CXCL-16, ↓PDGF-A, ↓angiogenin, ↓MMP9 and ↓TIMP4	↓ expression of proteins involved in growth, invasion, and angiogenesis	Solinas *et al.*, 2013 [36]
Glioma stem-cell lines (GSC-1, 30 and 83)	14.6-19.4 µM CBD	TRPV2 and PI3K/AKT	↓proliferation, cell cycle arrest (G0/G1) and ↑ autophagy dependent of cell differentiation	Nabissi *et al.*, 2015 [44]
Glioma cell lines (T98G, U87MG, and GL261) and orthotopic tumour models in C57BL/6 mice	• 10 µM CBD (*in vitro*) • 2 mg/kg CBD+THC, i.p, 3 doses in 5 days intervals during 3 weeks, with or without 4Gy irradiation (*in vivo*)	MAPK/ERK	↑cell radiosensitivity, ↑autophagy and apoptosis, ↓tumour volume	Scott *et al.*, 2015 [43]
Glioma stem-cell lines (GSC-3832 and GSC-387) and orthotopic tumour models	• 2.6-3.5 µM CBD (*in vitro*) • 15 mg/kg CBD, i.p, 5 days a week, nearly 3 weeks (*in vivo*)	↑ROS (↓Sox2, Id1, p-STAT3 and ↑phosphorylated (p)-p38 MAPK)	↓inhibited cell renewal and stemness, ↑animal survival	Singer *et al.*, 2015 [45]
Glioblastoma multiforme human cell lines (98G, U251, and U87MG) and orthotopic tumour models (PDGF-GBM)	• 3.2-9.2 µM CBD (*in vitro*) • 1-3 µM CBD + DNA-damaging agents (*in vivo*)	Not evaluated, suggested binding to GPR55, CB1, and TRPV1	CBD + DNA-damaging agents ↑ antiproliferative responses in a synergistic manner	Deng *et al.*, 2017 [49]

Legend: AKT, serine/threonine kinase 1; CB1, cannabinoid receptor 1; CBD, cannabidiol; CXCL-16, C-X-C motif chemokine ligand 16; ERK, extracellular regulated MAP kinase; GPR55, G protein-

coupled receptor 55; HIF-1α, hypoxia inducible factor 1 alpha subunit; Id-1, inhibitor of DNA binding 1; MAPK/ERK, mitogen activated protein kinase/extracellular signal-regulated kinases; MMP9, matrix metallopeptidase 9; PDGF-A, platelet derived growth factor subunit A; PI3K/AKT, phosphoinositide 3-kinase/serine threonine kinase 1; ROS, reactive oxygen species; SOX, SRY box 2; STAT3, signal transducer and activator of transcription 3; TGFβ, transforming growth factor beta 1; THC, Δ^9-tetrahydrocannabinol; TIMP4, tissue inhibitor of metalloproteinase 4; TRPV, transient receptor potential cation channel subfamily V member 1 (TRPV1) and TRPV2.

Acute myeloid leukaemia gene (aml-1a) has been reported to promote the expression of TRPV1 and TRPV2 in glioblastomas, which is associated with reduced patient survival. CBD inhibits gliomagenesis via inhibition of proliferation and cell viability, activation of autophagy, and cell cycle arrest in a TRPV2-dependent manner through the PI3K/AKT pathway [44-47]. AKT is a major proto-oncogene implicated in several cell functions and modulates several genes such as cyclin E1, cyclin-dependent kinase 2 (CDK2), E2F1 transcription factor 1, Raf-1, the anti-apoptotic genes Bcl-XL and beclin-1, Fas, and procaspase-8 [23,44]. Thus, therapeutic agents such as CBD that target PI3K/AKT are of utmost value in fighting malignant tumours [36,43-45].

CBD regimens alone or in combination with THC have been proved to be valuable adjuvants in increased cytotoxicity of alkylating agents [23]. The efficacy of doxorubicin (DOX), temozolomide, and carmustine was increased in a CBD/TRPV2-dependent manner. In this situation, TRPV2 undergoes conformational changes, leading to enhanced Ca^{2+} influx and uptake of alkylating agents, thereby resulting in the improvement of antiproliferative and cytotoxic effects at lower doses [23,48].

Deng *et al.* [49] evaluated synergistic effects among CBD and temozolomide, carmustine, or cisplatin and found that only a range of concentrations resulted in additive

responses. In fact, the authors observed a slight improvement in therapeutic indices and reported that certain dose combinations resulted in antagonism.

Singer *et al.* [45] observed a receptor independent CBD-mediated increase in reactive oxygen species (ROS) production leading to inhibition of stemness and cell viability. Moreover, ROS production regulates the most common signalling pathways in cancer MAPK and PI3K, which are critical in linking oncogenes and regulating transcription factors, like the hypoxia-inducible factor-1 alpha (HIF-1α). HIF-1α induces the overexpression of glucose transporters (GLUT) in hypoxic tumours causing the Warburg effect - a process that helps the optimization of glucose metabolism from the more efficient oxidative phosphorylation to the less efficient glycolytic pathway [50,51]. Moreover, HIF-1α promotes angiogenesis in a vascular endothelial growth factor (VEGF)-dependent manner, which is essential for tumour progression [51,52]. Therefore, the knockdown of HIF-1α after CBD administration, would promote a positive effect, arresting tumour invasion and metastasis [53].

Other ROS-mediated CBD effects included suppression of known predictors of poor outcome, such as inhibitor of DNA binding 1 (Id1), SRY-box 2 (Sox2), and signal transducer and activator of transcription 3 (STAT3). Previous studies reported that these factors orchestrate the invasiveness and mesenchymal differentiation of glioblastomas [54-56].

The downregulation of matrix metallopeptidase (MMPs) by CBD was also observed, and this would represent a crucial resource for the management of glioblastoma multiforme [35,36,45]. MMPs are overexpressed during tumour development and malignantly transformed cells migrate to and invade the surrounding tissues through the dissolution of matrix proteins of the normal tissue [57].

Scott *et al.* [43] evaluated the effect of CBD alone or in combination with THC in a glioma tumour orthotopically implanted into C57BlL/6 mice receiving suboptimal doses of radiotherapy. The authors observed that after 21 days, the animals that received both the cannabinoids showed marked arrest of tumour growth, probably due to the complementation of radiation-induced cell death by means of autophagy and apoptosis. In the same study, immunohistochemical evaluation of angiogenesis (CD31 marker) reduced significantly when compared to that in the control group.

CBD is effectively a promising agent for glioblastoma management; however, before conducting clinical trials, the schedule of CBD administration should be standardised and its long-term effects clarified. In particular, the use of CBD as an adjuvant of DNA-damaging agents and radiotherapy requires further attention.

Effect of CBD against breast cancer

In 90% of cases, breast cancer-related deaths are caused by tumour metastasis to lung [58,59]. This antimetastatic effect is mediated by CBD-modulation of ERK and ROS pathways, resulting in reduced expression of Id-1, which in turn was associated with prolonged survival *in vivo* [58-61]. However, CBD did not inhibit primary tumour growth in the study by Murase *et al.* [58], despite it positive effects against metastasis. The reason of these contradictory findings remain unclear.

The epidermal growth factor (EGF) signalling pathway, which is overexpressed in triple negative breast cancer and associated with poor prognosis, has also been targeted by CBD. In a previous study, it was reported that the proliferation and migration ability of SUM159 cell lineage reduced significantly in a CBD/EGF-dependent manner [61]. In addition, CBD interferes with the nuclear factor kappa B (NF-kB) signalling pathway, which is essential for epithelial-mesenchymal transition and metastasis, via EGF/EGF

receptor (EGFR) pathway. NF-kB promotes bone and lung metastases through GM-CSF and MMP2/MMP9 production, respectively [60,61].

Elbaz *et al.* [61] evaluated the effect of CBD/TRPV2 binding in enhancing the antitumour activity of chemotherapeutic drugs. They observed that breast cancer cells treated with CBD and DOX showed increased DOX intake, reduced viability, and upregulated levels of apoptosis markers, such as cleaved poly ADP ribose polymerase (PARP) and caspase-3, *in vitro*. In addition, *in vivo* study showed that the combined administration of CBD and DOX decreased the volume and weight of tumours, with marked apoptosis, compared to CBD or DOX treatment alone.

In the study by Murase *et al.* [58], a combination of CBD with THC in equal proportions proved to be more effective than CBD alone. Moreover, other agonists of cannabinoid receptors, such as WIN-55 and the novel O-1663, were more effective than CBD against cancer. A multi-target-mediated approach would improve the effect of cannabinoids. Further research is needed to clarify the wide spectrum of combinations.

The overall results indicate the potential of CBD in breast cancer therapy. The details of the experimental studies are presented in Table 2.

Table 2: Preclinical studies evaluating the anticancer effects of cannabidiol against breast cancer

Experimental model	Effective doses	Receptor-dependent or -independent mechanisms	CBD-mediated effect	Reference
Breast cancer cells (MDA-MB231-luc-D3H2LN and MDA-MB231 + Id1) and female BALB/c mice with orthotopic tumour model (4T1) or i.v model of metastasis (MDA-MB231) in female athymic nu/nu mice	• 0.6-4 µM CBD (*in vitro*) • CBD (0. mg/kg^{-1} to 1 mg/kg^{-1}) or CBD+THC (1 mg/kg^{-1}, 1:1), i.p, 5 days a week, for nearly 30 days (*in vivo*)	CB1- and CB2-independent	↓ migration and invasion, ↓tumour growth and metastasis, ↑survival	Murase *et al.*, 2014 [58]
Breast cancer cells (SUM159, 4T1, SCP2, and MVT-1) and female Balb/C and FVB mice with orthotopic tumour models (SUM159)	• 6 µM CBD (*in vitro*) • 10 mg/kg CBD, i.p, every other day, 3 weeks (*in vivo*)	EGF/EGFR (inhibition of the activation of EGFR, AKT, ERK and NF-kB signalling), ↓MMP2 and MMP9 and ↓Ki-67	↓cell proliferation, migration and invasion, ↓tumour growth and metastasis, ↓macrophages in tumour stroma	Elbaz *et al.*, 2015 [39]
Triple negative breast cancer cells (SUM159 and MDA-MB23) and orthotopic tumour models (SUM159)	• 5 µM CBD (*in vitro*) • CBD (5 mg/kg) and doxorubicin (Dox) (5 mg/kg), i.p, alone or combined, once per week for 4 weeks with (*in vivo*)	TRPV2	• ↑TRPV2 expression, ↑ chemotherapeutic agent (Doxorubicin) uptake and apoptosis in tumour cells • CBD+Doxorubicin ↓tumour weight and ↑apoptosis compared to CBD or Doxorubicin alone in mice	Elbaz *et al.*, 2016 [61]

Legend: AKT, serine/threonine kinase 1; CB1, cannabinoid receptor 1 and CB2; CBD, cannabidiol; EGF/EGFR, epidermal growth factor/ epidermal growth factor receptor; ERK, extracellular signal-regulated kinases; MMP, matrix metallopeptidase 2 (MMP2) and MMP9; NF-KB, nuclear factor kappa B; THC, Δ^9-tetrahydrocannabinol; TRPV2, transient receptor potential cation channel subfamily V member 2.

CBD-induced effects in lung cancer

Haustein *et al.* [62] proposed that CBD-induced upregulation of intracellular adhesion molecular-1 (ICAM-1) and increased the susceptibility of lung malignant cells to lymphokine-activated killer (LAK) cells. This suggests that CBD-mediated antitumour immune surveillance is effective in reducing cancer progression in a TRPV1-dependent manner. Both CB1 and CB2 receptors were also implicated in the results. Despite the known low affinity of CBD to cannabinoid receptor, its effects are enhanced through the inhibition of fatty acid amidohydrolase (FAAH) activity. FAAH decreases CBD degradation and may prolong CBD action on CB1 and CB2. Notably, this cannabinoid did not show cytotoxic potential against non-tumour bronchial epithelial cells.

In the study by Ramer *et al.* [37], CBD did not exert antiangiogenic effect against lung cancer cell lines *in vitro*. Moreover, CBD was found to increase tube formation and migration. Therefore, the negative results were attributed to the lack of elements of the tumour microenvironment in the *in vitro* experiment. Because CBD has been proved to exert antiangiogenic activity in other studies at similar doses, further studies are required to evaluate the unexpected pro-cancer characteristics of CBD.

In the same study, the potential of CBD in inducing tissue inhibitors of matrix metallopeptidase inhibitor 1 (TIMP-1) in an attempt to enhance its protective antiangiogenic role during tumour progression was evaluated. CBD was found to show antiangiogenic effects after the addition of recombinant TIMP-1 to human umbilical vein endothelial cells (HUVEC). In addition, antimigratory effects were observed for lung cancer cell lines.

Ramer *et al.* [24] investigated the proapoptotic and tumour-regressive action of CBD. They found that the therapeutic effects were mediated by the nuclear translocation of peroxisome-proliferator-activated receptor γ (PPARγ) by cytochrome c oxidase

subunit II (COX-2)-dependent prostaglandins. There is still a lack of clinical studies to prove efficacy and safety of CBD-based lung cancer therapies; however, the preclinical results are optimistic and should be most extensively studied.

The findings summarised in Table 3 reveal the mechanisms underlying the preventive effect of CBD against human lung cancer cells.

Table 3: Preclinical studies evaluating the anticancer effects of cannabidiol against lung cancer

Experimental model	**Effective doses**	**Receptor-dependent or -independent mechanisms**	**CBD-mediated effect**	**Reference**
Lung cancer cell lines (A549 and H460), human primary lung tumour cells and and orthotopic tumour models in athymic nude mice (A549)	• 3 μM CBD (*in vitro*) • 5 mg/kg CBD, i.p, 3 days interval, 4 weeks (*in vivo*)	COX2, PPARγ	↑apoptosis, tumour regression	Ramer *et al.*, 2013 [24]
Lung cancer cell lines (A549, H460) and metastatic cells (BEAS-2B)	3 μM CBD	CB1, CB2, and TRPV1-induced overexpression of ICAM-1	↑lung cancer cell death by lymphokine-activated killer cells overexpressing ICAM-1, but not LFA-1	Haustein *et al.*, 2014 [62]
Lung cancer cell lines (A549, NCI-H460, NCI-H358), metastatic cells (BEAS-2B) and human umbilical vein endothelial cells (HUVEC)	3 μM CBD	CB1, CB2, and TRPV1-induced overexpression of TIMP-1	CBD + recombinant TIMP-1 promoted ↓cell migration, ↓tube formation, ↓sprout formation, without interfering in cell viability. No effects were found with CBD alone.	Ramer *et al.*, 2014 [37]

Legend: COX2, cytochrome c oxidase subunit II; PPARγ, peroxisome proliferator activated receptor gamma; CB1, cannabinoid receptor 1 and CB2; CBD, cannabidiol; TRPV1, transient receptor potential cation channel subfamily V member 1; ICAM-1, intercellular adhesion molecule 1; LFA-1, integrin subunit beta 2 (ITGB2); TIMP-1, tissue inhibitor of metalloproteinase 1.

CBD-related effects in colon cancer

Epidemiological studies suggest that for fighting colorectal cancer, efforts in screening of at risk population are essential [63]. Also, prevention and targeted treatment are important. In this context, we suggest that CBD should be of great value. Hence, more experimental and clinical studies are necessary to provide new evidence and guidelines for its use.

According to Romano *et al.* [38], CBD presented a positive preventive effect on colon carcinogenesis in a sensitive antagonist manner to cannabinoid receptors *in vitro* and *in vivo*. The authors observed a decrease in cell proliferation and formation of preneoplastic lesions, as well as retarded tumour growth.

It was established that CBD exerted effects on the adhesion and migration of colon cancer cells and served as a ligand for G protein-coupled receptor 55 (GPR55), which is involved in proliferation, invasion, and angiogenesis [64]. Previous studies showed that CBD/GPR55 interaction reduced the formation of F-actin filaments, which resulted in the reduction of cell adhesion and migration. This effect was mediated by Ca^{2+} mobilization and phosphorylation of AKT and ERK1/ERK2 pathways [65,66]. Table 4 summarises the main findings in this section of the manuscript.

Table 4: Preclinical studies evaluating the anticancer effects of cannabidiol against colon cancer

Experimental model	Effective doses	Receptor-dependent or independent mechanisms	CBD-mediated effect	Reference
Colorectal carcinoma cells (DLD-1 and HCT116), healthy colonic cells, azoxymethane (AOM)-induced and HCT116 xenograft models of colon cancer	• 0.3-5 μM CBD (*in vitro*) • AOM-induced tumours: 5 mg/kg, i.p, 3 days a week, 4 weeks • HCT116 tumour: 5 mg/kg CBD, i.p, once a day, 10 days (*in vivo*)	CB1 and CB2	↓proliferation of tumour cells, ↓ formation of preneoplastic lesions and tumours in AOM group and retarded tumour growth in HCT116-induced neoplasms (during 7 days)	Romano *et al.*, 2014 [38]
Colon cancer cells (HCT116, HT-29 and SW480)	• 2.5 μM CBD (*in vitro*) • 5 mg/kg^{-1} CBD (*in vivo*)	GPR55, ERK1/ERK2	↓adhesion and migration, cancer colon cell arrest in the liver	Kargl *et al.*, 2016 [64]

Legend: AOM, azoxymethane; CB1, cannabinoid receptor 1 and CB2; ERK1/ERK2, extracellular signal-regulated kinases 1/ extracellular signal-regulated kinases 2; GPR55, G protein-coupled receptor 55.

CBD in multiple myeloma management

In multiple myeloma cell lines, CBD promoted the regulation of ERK, AKT, and NF-kB pathways, and the effects of the cannabinoid were enhanced in TRPV2-positive cells compared to that in TRPV-negative cells. When used in combination with bortezomib, the effect of the proteasome inhibitor was enhanced [67] suggesting that CBD is advantageous as an adjunctive drug. Nabissi *et al.* [68] observed similar results in their study, wherein CBD and THC, as well as CBD in combination with carfilzomib acted synergistically to induce cell death. In this case, CBD exerted its effects in a CB2-dependent manner. The effective doses used in the reviewed studies are presented in Table 5.

Table 5: Preclinical studies evaluating the anticancer effects of cannabidiol against multiple myeloma

Experimental model	Effective doses	Receptor-dependent or -independent mechanisms	CBD-mediated effect	Reference
Multiple myeloma cell lines (RPMI8226 and U266)	20 µM CBD alone or in combination with bortozemib	ERK, AKT, NF-kB, especially in TRPV2-positive cells	↓growth and arrested cell cycle progression, ↑cell death	Morelli *et al.*, 2014 [67]
Multiple myeloma cell line (U266 and RMPI)	THC+CBD (12.5µM each)	CB2, ↑phosphorylated H2AX, ↓expression of CXCR4 and CD17 and their chemotactic activity	↑cytotoxicity and autophagy, ↓cell migration	Nabissi *et al.*, 2016 [68]

Legend: AKT, serine/threonine kinase 1; CB2, cannabinoid receptor 2; CBD, cannabidiol; CXCR4, C-X-C motif chemokine receptor 4; ERK, extracellular signal-regulated kinases; H2AX, H2A histone family member X; NF-kB, nuclear factor kappa B; THC, Δ^9-tetrahydrocannabinol; TRPV2, transient receptor potential cation channel subfamily V member 2.

Anticancer effects of CBD in other malignant tumours

De Petrocellis *et al.* [69] tested 12 different cannabinoids against prostate cancer cells and observed that CBD presented superior results independent of serum deprivation of cultures. In xenograft tumours, the cannabinol reduced growth and induction of intrinsic apoptotic pathways. Additionally, CBD proved to be a partial antagonist of the transient receptor potential cation channel subfamily M member 8 (TRPM8) and elevated ROS synthesis. However, CBD was ineffective against DU-145 xenografts, although it potentiated the effect of docetaxel.

Against cervical cancer cells, CBD reduced growth and induced apoptosis without cell cycle arrest, promoted marked loss of shape, size reduction, and nuclear fragmentation that were observed in apoptotic cells. In addition, 66% loss of ATP activity was detected in, suggesting that energy depletion could be responsible for cell death. Moreover, increased caspase 3 and 7 activity, as well as enhanced expression of proapoptotic proteins, such as p53 and Bax, were also observed. In this section we

presented studies showing that CBD would also be effective in experimental models of prostate and cervical cancer (see Table 6).

Table 6: Preclinical studies evaluating the anticancer effects of cannabidiol in prostate and cervical cancers

Experimental model	Effective doses	Receptor-dependent or -independent mechanisms	CBD-mediated effect	Reference
Human prostate epithelial cells (PC-3, DU-145, 22RV1, and LNCaP) and xenograft in athymic nude mice (LNCaP and DU-145)	1-10 µM CBD (*in vivo*)	Independent of CB1, CB2, and TRPV1, ↑caspase 3 and 7 activity, ↑p53, ↑PUMA, ↑ROS, ↑intracellular Ca^{2+}	↑apoptosis and ↑markers of intrinsic apoptotic pathways	[69]
Cervical cancer cells (HeLa, ME-180 and SiHa)	SiHa and HeLa IC50= 3.2 µg/mL CBD; ME-180 IC50=1.5 µg/mL CBD	↑caspase 3 and 7 activity, ↑p53 and Bax, ↓RBBP6 and Bcl-2	↓cell growth, apoptosis associated to morphological changes without cell cycle arrest, ↓ATP levels,	[70]

Bax, BCL2 associated X, apoptosis regulator; Bcl-2, apoptosis regulator; CB1, cannabinoid receptor 1 and CB2; PUMA, BCL2 binding component 3 (Bbc3); RBBP6, RB binding protein 6, ubiquitin ligase; ROS, reactive oxygen species; TRPV1, transient receptor potential cation channel subfamily V member 1.

Why we should test CBD against oral cancer?

The effect of CBD against oral cancer has not been tested yet. However, owing to the common mechanism of action observed against a vast majority of malignant tumours, we suggest that CBD would exert beneficial effects on OSCC as well.

Cannabinoid receptors have been detected in human OSCC of the tongue, and are more significantly expressed in older patients than in younger patients and in females than in males. It has been suggested that both, CB1 and CB2, may constitute potential targets for oral cancer treatment [71,72]. The anticancer effects mediated by the interaction of CBD with cannabinoid receptors include modulation of MMPs, urokinase-type plasminogen activator (uPA), endothelin-1 (ET-1), and CXCL16 [34,37,62]. The

recurrence of OSCC lesions with histologically negative margins and oral cancer metastasis has been associated with the expression of MMP2 and MMP9 [73,74]. According to Pavon *et al.* [75], the overexpression of uPA/uPAR enhanced the migratory, invasive, and metastatic potential of OSCC cells, resulting in poor prognosis. ET-1 is an important angiogenic factor involved in the progression of solid tumours. In OSCC, ET-1 acts in an autocrine and paracrine manner to promote cell growth and modulate tumour stroma, thereby favouring the progression of the disease [76,77]. CXC chemokines have been associated with tumour cell invasion and osteolysis in OSCC, and they are considered valuable targets to prevent cancer cell invasion to the bone [78,79]. Therefore, we suggest that exploring the cannabinoid receptor-mediated anticancer effects by means of CBD use would be a new strategy in oral cancer management.

TRPV1 and TRPV2 have been considered to be suitable CBD targets that can induce anti-tumour immune surveillance [62], and play a major role in the intake of chemotherapeutical agents, cell growth and cycle arrest inhibition, and apoptosis of malignant cells [37,61,67]. In the oral cavity (tongue, buccal mucosa, gingiva, and floor of the mouth), the presence of TRPV1-TRPV4 has been detected, especially in population exposed to risk factors, including alcohol and tobacco consumers and OSCC carriers. In addition, CBD regulated ERK, EGF, AKT, and NF-kB pathways in cancer through TRPV2 [80,81]. These signalling cascades are also activated in oral cancer (Lin *et al.*, 2015 and 2017), and their targeting has been proved to induce early apoptosis and limit invasion in OSCC [82-84]. Therefore, we suggest that CBD-mediated targeting of TRPV would positively contribute to controlling tumour progression in the field of oral Oncology. Additionally, the evaluation of chemopreventive effects of CBD via PPARγ, which regulates cell proliferation, differentiation, and apoptosis in OSCC [85], need further attention.

According to the literature, cancer cells possess higher endogenous oxidative stress than normal cells. In OSCC, ROS is involved in the initiation and promotion of multistage oral carcinogenesis. Therefore, the enhancement of the anticancer effects of CBD via ROS-based mechanisms would selectively promote malignant cell death [86,87].

As described, CBD is emerging as a potential therapeutic agent against cancer, as it is devoid of psychoactive effects. The use of CBD in combination with other cannabinoids and chemotherapeutic agents or radiotherapy would be effective in cancer management. In addition, the possibility of reducing the dose of more aggressive therapeutic regimens would improve patient compliance to anticancer treatment.

Since OSCC prognosis has remained unchanged over the past decades with a poor survival rate, further studies on targeted anticancer therapy would improve patient survival. CBD would possibly interfere in the pathogenesis of OSCC through different signalling pathways in a receptor-dependent or -independent manner, as observed in other solid tumours.

CONCLUSIONS

The findings of the present literature review provide clues for the design of novel *in vitro* and *in vivo* methods for the evaluation of CBD-mediated effects on oral cancer. The inhibitory potential of CBD on OSCC cell proliferation, migration, invasion, angiogenesis, and metastasis should be evaluated.

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Artigo 3 | *Role of tumour-associated macrophages in oral squamous cell carcinoma progression: an update on current knowledge*

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ABSTRACT

Background

Oral squamous cell carcinoma (OSCC) accounts over 90% of malignant neoplasms of the oral cavity. This pathological entity is associated to a high mortality rate that has remained unchanged over the past decades. Tumour-associated macrophages (TAMs) are believed to have potential involvement in OSCC progression. However, the molecular networks involved in communication between stroma and cancer cells have not yet been fully elucidated.

Main body

The role of M2 polarized cells in oral carcinogenesis is supported by a correlation between TAMs accumulation into OSCC stroma and poor clinical outcome. Signalling pathways such as the NF-κB and cytokines released in the tumour microenvironment promote a bidirectional cross-talk between M2 and OSCC cells. These interactions consequently result in an increased proliferation of malignant cells and enhances aggressiveness, thus reducing patients' survival time.

Conclusions

Here, we present a comprehensive review of the role of interleukin (IL)-1, IL-4, IL-6, IL-8, IL-10 and the receptor tyrosine kinase Axl in macrophage polarization to an M2 phenotype and OSCC progression. Understanding the molecular basis of oral carcinogenesis and metastatic spread of OSCC would promote the development of targeted treatment contributing to a more favourable prognosis.

Keywords: Oral cancer; Oral squamous cell carcinoma; Head and neck cancer; Macrophage activation

BACKGROUND

Cancer pathogenesis events take place in imbalanced microenvironments, where pathological states do not affect only neoplastic cells [1-3]. Instead, cancerous cells disrupt tissue homeostasis, disturbing different cell types and contributing to disease progression, through interactions with mediators of the immune system [4-6]. Oral squamous cell carcinoma (OSCC) is a solid tumour of epithelial origin that affects more than 400,000 individuals annually worldwide [7]. The mortality rate of this disease has remained largely unchanged for the last decades, with a five-year survival under 50% [8]. There is compelling evidence that tumour-associated macrophages (TAMs) have potential involvement in the progression and metastatic spread of OSCC. The most important features associated with their presence in the lesion stroma include facilitation of angiogenesis, tumour cell invasion, augmentation of cell motility, persistent growth, and suppression of anti-tumour responses [9-13]. The signals involved in communication between tumour cells and macrophages have not yet been completely elucidated. However, the interaction among tumour and inflammatory cells seems to be bidirectional [14]. Here, we present the strategies by which tumour cells influence macrophage physiology to display a pro-tumour phenotype, and the contribution of TAMs to OSCC progression. Is given an overview of potential markers that could provide support for diagnostic, evaluation of clinical outcome, and be used as valuable antineoplastic targets.

Macrophage differentiation

In adults, inflammatory monocytes ($CD64^{+}$/$CD16^{-}$ $CCR2^{+}$ $Ly6C^{+}$) constitutively originate tissue-resident macrophage populations [15, 16]. The exposure of these cells to microenvironmental stimuli results in complex phenotypic modifications in a time- and location-dependent manner [17-19]. The activation of different regulatory mechanisms

and transcription pathways result in a vast spectrum of macrophage subtypes, of which M1 and M2 represent the extreme polarization phenotypes [18, 19]. The M1 polarization state depends on microbial stimulus and a T helper type 1 (T_H1) cytokine profile (classical activation pathway). Whereas M2 polarization depends on a T helper type 2 (T_H2) cytokine profile (alternative activation pathway) [20]. Interferon-gamma (INF-γ) and interleukin (IL)-4 secretion sustain an M1 and an M2 phenotype commitment, respectively [21]. M1 are innate immune effector cells that fight intracellular microbial challenges by means of reactive oxygen species and nitrogen intermediates. Activation of signal transducer and activator of transcription (STAT)-1 in M1 macrophages is important for optimal T_H1 responses [22], such as direct tumour cell death [23, 24]. M2 macrophages block T_H1 and differentiate in the tumour stroma from blood monocytes, or resident macrophages in resting state, after making contact with neoplastic cells presenting aberrant production of certain cytokines [18]. Additionally, they promote cancer progression by STAT-3 activation, inducing and maintaining a pro-carcinogenic inflammatory microenvironment [25].

OSCC cells and TAM interactions

Histopathologically, OSCC presents as fibrous connective tissue with unusual amounts of extracellular matrix rich in fibroblasts, vascular vessels, and inflammatory cells [26]. Among the local milieu of OSCC stromal spaces, rich in perlecans and inflammatory cells, monocytes or resting macrophages are differentiated into $LyC16^{high}$, $CD163^{+}$, $CD204^{+}$, and $CD68^{+}$ expressing TAMs. These cells are considered of utmost biological importance for disease progression and correlate with increased dedifferentiation in primary tumour sites [27-29]. Moreover, TAMs elicit tumour relapse and/or post-operative cervical lymph node metastasis via angiogenesis and suppression

of anti-tumour immunity [9]. An increase in the number of $CD163^{+}$ macrophages occurs in oral leukoplakia. However, they co-express phosphorylated STAT-1, suggesting that in premalignant lesions TAMs possess an M1 phenotype in a dominant T_H1 microenvironment [30]. Polarization to an M2 TAM phenotype probably occurs gradually and early during the onset of cancer. It is suggested that several interleukins (IL-1, IL-4, IL-6, IL-8, and IL-10), and other factors, such as the receptor tyrosine kinase Axl, participate in promoting this phenomenon. In the next sections we propose a topic structured discussion of relevant findings that corroborate this theoretical assumption. Figure 1 briefly reviews the effect of interleukins on TAMs present in OSCC stroma.

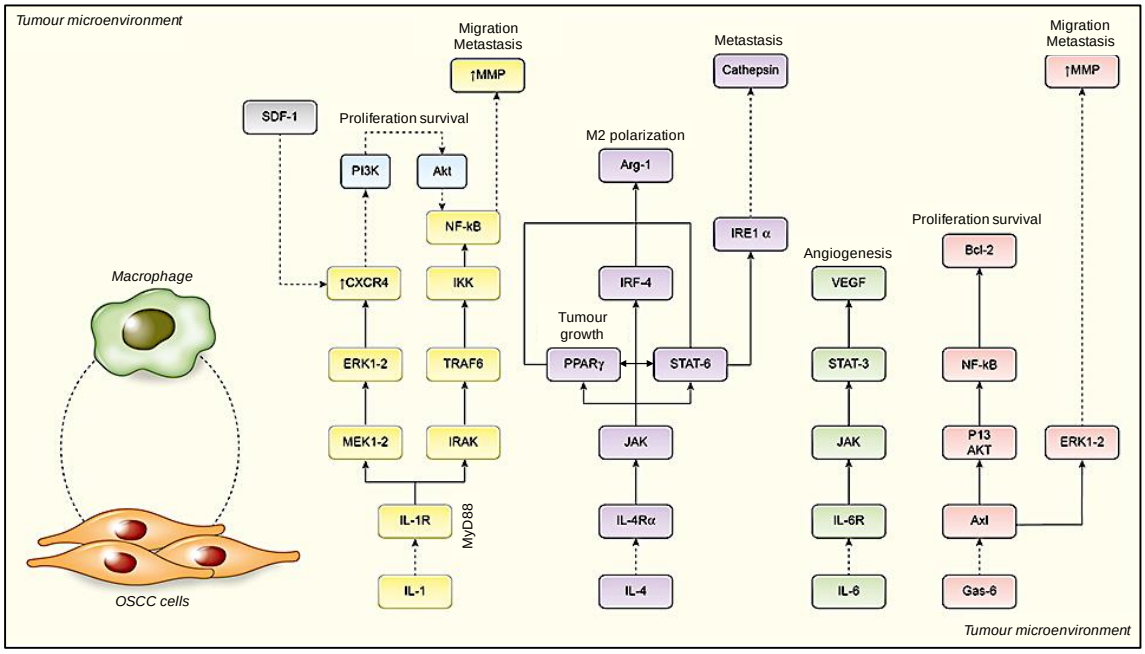

Figure 1: Macrophages in resting state suffer microenvironmental effects coordinated by OSCC cells. Interleukin (IL)-1, IL-4, IL-6, IL-8 and IL-10 (not shown), and Gas-6 are produced by OSCC cells and promote macrophage phenotype switching to an M2 polarization state. In turn, TAMs augment the recruitment of chemotactic receptors to tumour sites, induce tumour proliferation, and favour angiogenesis and invasiveness [31, 32, 36, 40-43, 47, 48, 65, 66].

IL-1

Tumour released IL-1 cross-talks to TAMs and induces M2 polarization to an immunosuppressive phenotype via IL-1 receptor (IL-1R) and myeloid differentiation primary response gene 88 (MyD88), which requires I-kappaB kinase beta (IKKβ)-mediated nuclear factor kappa B (NF-κB) [31,32]. Interleukin-1 beta (IL-1β) is a critical mediator of chronic inflammation and is implicated in OSCC during early and late stages of carcinogenesis. Pro-IL-1β is upregulated in tobacco and betel quid related oral cancer, and is secreted in an inflammasome-dependent manner [33], although it is absent in homeostatic conditions. In the presence of IL-1β TAMs suffer an upregulation of C-X-C motif chemokine receptors (CXCR), especially CXCR4, induced by the activation of extracellular signal regulated MAP kinase (ERK). Macrophages then become attracted by CXCR4 ligands, like stromal cell derived factor-1 alpha (SDF-1α) [34]. SDF-1α is highly inducible in hypoxic and proangiogenic niches, where it reinforces the autocrine/paracrine loop that contributes to an M2 phenotype [35]. Nevertheless, OSCC cells and TAMs together through IL-1β/IL-1R and CXCR4/ SDF-1α and via activation of the ERK signalling pathway produce tumour cell migration and invasion by inducing expression of matrix metalloproteinase (MMP) enzymes MMP-9 and MMP-13 [36]. These important angiogenic modulating enzymes promote the acquisition of vasculature for oxygenation, nutrition, and waste disposal, which are of fundamental importance for tumour growth [2]. Contrarily, the blockade of CXCR4 by the antagonist 1,1′-[1,4-phenylenebis(methylene)]bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride (AMD3100) inhibited SDF-1 mediated lymph node metastasis [37]. Furthermore, a rich IL-1β microenvironment promotes CXCL1 production, and through CXCR2 this induces tyrosine phosphorylation of the endothelial growth factor receptor (EGFR) [33]. As a result, EGFR activates pathways leading to cell growth, DNA synthesis, and the expression of oncogenes like *fos* and *jun* [38]. IL-1α is found in tumour cell membranes

and in intracellular locations, and is produced in larger amounts than IL-1β in highly metastatic tumours. Despite the lack of evidence that demonstrates its direct participation in OSCC progression through macrophage activation, IL-1α interacts with fibroblasts in the stroma. Therefore, IL-1α acts promoting cell proliferation and upregulating the secretion of IL-8, CXCL-1, and chemokine C-C motif ligand (CCL)-7 [39]. Coincidently, these cytokines are also commonly produced by TAMs, rising the hypothesis of a plausible interaction between OSCC and M2 cells by means of IL-1α.

IL-4

IL-4 is an anti-inflammatory and immunomodulatory cytokine that has been identified as a relevant factor for the activation of TAMs, as well as IL-1. Furthermore, an increased expression of IL-4 receptor alpha (IL-4Rα) correlates with increased OSCC recurrence [40]. Regarding this tumour entity, the interaction between malignant cells and TAMs occurs through the plasminogen activator urokinase (uPA) and its specific receptor uPAR, mainly through the activation of ERK1/2 and increase in the production of IL-4. In OSCC cells this receptor modifies several transduction pathways, affecting neoplastic cell behaviours and acts as a promoter of survival, proliferation, and metastasis [41-43]. The high levels of IL-4 produced modifies the tumour microenvironment and facilitates an increase in arginase-1 levels, considered a biomarker of TAMs [43]. Similarly, this citokine induces cathepsin protease activity in TAMs, where they activate proteins including growth factors, transcription factors, and other proteases, such as MMPs [44]. Cathepsin B is considered a reliable marker for OSCC poor prognosis, correlating to higher tumour grade and lymph node metastasis [45].

IL-6

IL-6 expression in OSCC has been related to high lymph node metastatic rates and poor tumour differentiation, especially in male patients [46]. SDF-1alpha increases secretion of IL-6 in cultured human OSCC cells via CXCR4, ERK, and NF-κB pathways [47], in a similar manner to that seen for IL-1β/IL-1R. Moreover, the aberrant synthesis of IL-6 by neoplastic cells may be controlled by the CXCR4-specific inhibitor AMD3100 [47]. The calcium binding protein S100A9, associated with loss of differentiation and recurrence, tends to be deregulated in both tumour and stromal cells. The expression of S100A9 in monocytes exerts a tumour-promoting effect upon co-culture with oral cancer cells, in particular by releasing IL-6 and the activation of NF-κB or STAT-3 that is not achieved in tumour cell monoculture [48]. In response to apoptotic tumour cell supernatants, signalling patterns were identified that contributed to the TAMs phenotype. Two targets, IL-4Rα and cannabinoid receptor 2 (CB2), were validated and confirmed to regulate both IL-6 and IL-10 production in TAMs, contributing to autocrine/paracrine activation of STAT-3 in macrophages and tumour cells [49]. These findings emphasise the relevance of tumour cells and TAMs interactions for disease progression.

IL-8

IL-8 is a pro-angiogenic, pro-inflammatory mediator important for OSCC angiogenesis progression [50]. The mitogen activated protein kinases (MAPK) pathway is used by OSCC IL-8 to activate angiogenic activity in TAMs [51] augmenting, for example, vascular endothelial growth factor (VEGF) production. The receptors CXCR1 and CXCR2 have been detected in both oral normal keratinocytes and OSCC cells, where they exhibit higher expression. The presence of IL-8 CXC receptors in tumour cells increases ERK phosphorylation and MMP-7 and MMP-9 release, representing a tendency

to proliferation, migration, and invasion [52]. Is important to consider that matrix metalloproteinase enzymes are essential for the achievement of a complete angiogenic potential of TAMs. At the same time, the progressive development of the tumour requires vast vasculature. Chronic periodontitis and tobacco consumption have both historically been associated to oral cancer. Then, recent published works propose the following interesting associations that also support the important role of IL-8 in OSCC progression. It is probable that *Porphyromonas gingivalis* contributes to OSCC progression, increasing IL-8 levels in the microenvironment and upregulating MMPs [53]. Nicotine also increases IL-8 release in OSCC, binding to the nicotine acetylcholine receptor (nAChR) and inducing calcium influx, that phosphorylates Ca(2+)/calmodulin-dependent kinase II (CaMK II) and NF-κB [54].

IL-10

In more dedifferentiated tumour niches the microenvironment progressively acquires an immunosuppressive profile [1-4]. IL-10 is a cytokine that modulates immune responses, causing suppressive regulatory T cell differentiation that contributes to tumour cell proliferation [55]. Since persistent viral infection promotes IL-10 upregulation and impaired T-cell responses [56], it is believed that this cytokine plays a critical role in human papilloma virus (HPV)- and Epstein-Barr virus (EBV)-related OSCC progression [57,58]. Moreover, IL-10 indicates poor outcomes in HPV-unrelated OSCC, especially when INF-γ secretion [59] and transforming growth factor beta 1 (TGF-β1) levels [60] are low. Receptors for IL-22, a member of the IL-10 family, are highly expressed in OSCC cells, including in metastatic sites, compared to healthy regions. It was observed that in the OSCC MISK81-5 cell line, IL-22 induced the translocation of phosphorylated STAT-3 and upregulated the expression of Bcl-xL, survivin, and c-Myc, all known anti-

apoptotic genes, as well as suppressor of cytokine signalling 3 (SOCS3) [61]. In this context, diverse pathways for IL-10 production by TAMs have been described, highlighting their contribution to an immunosuppressive state in the tumour stroma. TAMs present a defective TLR response caused by tumour-selective disruption of the MyD88 signalling cascade, and affect the TIR-domain-containing adapter-inducing interferon-β (TRIF)/ TNF receptor associated factors (TRAF3)-dependent pathway in their own favour, leading to favourable transcription at the IL-10 promoter region [62]. In the presence of apoptotic tumour cell-factors like sphingosine-1-phosphate (S1P), TAMs use tyrosine kinase receptor A (TRKA), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt), and MAPK signalling to induce IL-10 [63].

Gas6/Axl

TAMs acquire, possibly by cancer-derived factors like IL-10, the capacity to produce high levels of Gas-6 that promotes tumour development [64]. At the same time, in a bidirectional interaction, OSCC cells, that also produce Gas-6, polarize TAM toward a tumour-promoter phenotype. In OSCC, Gas-6 cooperates with Axl and achieves biological and clinical relevance by triggering the signalling pathways of PI3/Akt and NF-κB [65]. TAMs and OSCC interact in Gas-6/Axl axis-modulated epithelial-mesenchymal transition by upregulating cadherin, n-cadherin, and vimentin expression, and promoting cell invasion and migration. It was found that Axl expression correlates with clinical stage and lymph node status in OSCC patients. Moreover, TAMs count was associated with phosphorylated Axl immunoactivity in OSCC tissues [13,65,66]. Gas-6/Axl and NF-κB may be interesting targets for therapeutic intervention, since NF-κB promotes cancer resistance to apoptosis and production of growth factors in the stroma, which stimulates tumour progression [31].

Role of TAMs in OSCC histopathological diagnosis

Although further clinicopathological studies are needed before interactions between stromal cells and malignant cells can be defined as a key process for OSCC progression, evidence suggests that TAMs play several tumour-promoter roles during carcinogenesis [10,28]. The presence of these polarized cells should be used as a potential marker to distinguish incipient OSCC from invasive lesions, avoiding underdiagnoses. As indicated by Matwaly *et al.* [67], the oral mucosa lacks an objective, standard-like structure that is found in other anatomical regions like the oesophagus, which makes the detection of invasiveness in oral cancer demanding. For a better understanding of TAMs in OSCC, more studies are necessary to define, by means of gene profiling, macrophage subpopulations with different tumour promoting abilities. A better indicator of the dynamic regulation of macrophage phenotype may be cellular cytokines, evaluated by means of tests conducted over multiple time points [67]. However, this methodology is time and cost demanding and probably unfeasible in clinical situations, especially in less developed countries where the prevalence of OSCC is higher. However, from the available evidence, it is possible to suggest that screening for TAM markers in oral biopsies certainly may contribute to accurate assessment of OSCC behaviour, being a valuable tool for the estimation of prognosis in cases related and unrelated to viral infection [57-60, 68].

New diagnostic alternatives

Weber *et al.* [27] propose that even trauma from incisional biopsies might influence tumour biology leading to a worse prognosis and increased risk of developing lymph node metastases in OSCC patients. A wound-healing reaction consecutive to tissue trauma probably provides a microenvironmental stimulus that affects macrophage

polarisation [69]. Until the present, diagnostic procedures and therapeutic planning for OSCC have been supported mainly by histopathological findings. Despite being inviable at present, mostly due to the lack of standardized techniques, interpretation, and validation of parameters, the development of new minimal invasive diagnostic strategies should consider the screening of salivary and serum markers that reflect tumour behaviour, associated or not with the improvement of classical techniques like exfoliative cytology. Several studies have demonstrated valuable associations among OSCC clinical stages and prognosis, and salivary or serum markers associated with TAM's dynamic participation in the tumour stroma [58,68,70-73]. Although salivary markers associated to TAM polarization are not yet used as parameters for definitive diagnoses, they should be taken into consideration to evaluate patients with potent malignant disorders, like proliferative verrucous leukoplakia [74], as well as for recurrence in OSCC treated patients.

Targeting TAMs in OSCC therapeutics

TAMs are potential targets for combination therapy in cancer treatment [75]. As we move forward, comprehension of the role of stromal cells in OSCC progression, suggest that therapies that only target TAMs may be possible, leading to an imbalance in tumour growth and invasiveness [75,76]. However, despite its conceivable relevance, essentially mostly from positive clinical implications, the research in this field is incipient among cancer researchers. Recently a few studies have proposed targeting TAMs pathways to block cancer development [77,78]. Signalling pathways such as the NF-κB and cytokines released in the tumour microenvironment through OSCC cells and TAMs interactions are attractive targets [79]. Inhibitors of cytokines involved in tumour signalling present potential for use to combat cancer, specially those implicated in

promoting a malignancy cycle between OSCC cells and TAMs. Considering that chirurgical approaches are gold standard procedures for OSCC treatment, chemical interventions would be considered of lesser importance. However, it is relevant to underscore that during the last 30 years the disease-free survival and overall survival rates of OSCC patients have remained unchanged, perhaps due to limited care access or professional failures in performing early diagnoses, which is of the utmost relevance for prognosis. For these cases in particular, new therapeutic options are urgently needed.

CONCLUSIONS

Impaired tumour-preventive responses in OSCC are promoted by malignant cells and by soluble factors of the microenvironment that attract and polarize macrophages to a tumour-promoting state. Besides, macrophages reinforce the loop that promotes cancer growth and metastasis. This link between inflammation and cancer regulate OSCC progression and signalling pathways that provide a cross-talk between cancer cells and TAMs should be taken into consideration as valuable antineoplastic targets.

LIST OF ABBREVIATIONS

Akt – Protein kinase B

AMD3100 – 1,1′-[1,4-phenylenebis(methylene)]bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride

Axl – Axl receptor tyrosine kinase

CaMK II – Ca(2+)/calmodulin-dependent kinase II

CB2 – Cannabinoid receptor 2

CCL – Chemokine C-C motif ligand

CXCR – C-X-C motif chemokine receptors

EBV – Epstein-Barr virus

EGFR – Endothelial growth factor receptor

EMT – Epithelial-mesenchymal transition

ERK – Extracellular signal regulated MAP kinase

Gas-6 – Growth arrest specific gene-6

GM-CSF – Granulocyte macrophage colony stimulating factor

HPV – Human papilloma virus

IKKβ – I-kappaB kinase beta

INF-γ – Interferon-gamma

IL – Interleukin

IL-1R – Interleukin-1 receptor

MAPK – Mitogen activated protein kinases

MMP – Matrix metalloproteinase

MyD88 – Myeloid differentiation primary response gene 88

nAChR – Nicotine acetylcholine receptor

NF-κB – Nuclear factor kappa B

OSCC – Oral squamous cell carcinoma

PI3K – Phosphatidylinositol 3-kinase

S1P – Sphingosine-1-phosphate

SDF-1α – Stromal cell derived factor-1 alpha

SOCS3 – Suppressor of cytokine signalling 3

STAT – Signal transducer and activator of transcription

TAMs – Tumour-associated macrophages

TGF-β1 – Transforming growth factor beta 1

T_H1 – T helper 1

T_H2 – T helper 2

TLR – Toll-like receptors

TNF – Tumour necrosis factor

TRAF3 – TNF receptor associated factors

TRIF – TIR-domain-containing adapter-inducing interferon-β

Tyrosine kinase receptor A – TRKA

uPA – Plasminogen activator urokinase

uPAR – Plasminogen activator urokinase receptor

VEGF – Vascular endothelial growth factor

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DISCUSSÃO COMPLEMENTAR

Em janeiro do presente ano, a Agência Nacional de Vigilância Sanitária (ANVISA) aprovou o registro da primeira droga contendo THC e CBD. O mesmo tipo de medicamento é utilizado no Canadá desde o ano 2004, contudo o seu uso popularizou-se no mundo a partir de 2010 (Etges *et al.*, 2016).

Localmente, conhecido como Mevatyl®, mas referido na literatura internacional como Sativex®, o fitofármaco de uso controlado, possui a cada 100 μL 2,7 mg de THC e 2,5 mg de CBD (Etges *et al.*, 2016). O Marinol® ou Dronabinol®, contendo exclusivamente THC também tem sido utilizado nos Estados Unidos na última década (OMS, 2016).

A principal indicação do Mevatyl®/Sativex® é para o manejo de casos severos de espasticidade em portadores de esclerose múltipla, que somam cerca de 2,3 milhões de indivíduos no mundo. Segundo a literatura, o medicamento minimiza os sintomas, proporciona melhora significativa na qualidade de vida e é considerado uma alternativa terapêutica economicamente viável (Meuth *et al.*, 2015; Maccarrone *et al.*, 2017). Gradativamente, o espectro de indicações do Sativex tem se tornado mais abrangente, incluindo dor neuropática crônica não decorrente de tumores malignos (Ottawa, 2016) e doença de Huntington (López-Sendón Moreno *et al.*, 2016).

A compreensão da população geral sobre a utilização de derivados da *C. sativa* para fins médicos ainda é restrita e, aparentemente, permeada por equívocos. Os fitocanabinoides com alto grau de pureza, origem e qualidade estritamente monitoradas, empregados para fins de pesquisa ou tratamento (THC e CBD), diferem do produto consumido ilicitamente na maioria dos países. Há também, os canabinoides sintéticos produzidos em laboratórios mundialmente reconhecidos, como o que foi utilizado na

presente pesquisa. Estes se caracterizam por um maior grau de pureza que aqueles extraídos da *C. sativa*.

Segundo a OMS (2016) 147 milhões de indivíduos fazem uso abusivo da droga conhecida popularmente como maconha ou haxixe. O uso das mesmas gera danos agudos e crônicos à saúde e ganhos econômicos aos exploradores desse mercado, representando uma mazela à sociedade (OMS, 2016). Embora, haja a milênios, relatos do uso empírico dessas drogas com a finalidade de tratar doenças, *a priori* não há razão para esperar efeitos biológicos positivos do seu uso indiscriminado.

Outro ponto importante a ser esclarecido é que as pautas para a legalização do consumo recreativo de drogas e para o uso de substâncias com finalidade experimental ou de prescrição, diferem entre si e envolvem contextos e interesses divergentes (Bridgeman, Abazia, 2017).

Devido aos entraves legais e burocráticos enfrentados pelos pesquisadores para importar derivados da *C. sativa* e ao alto custo do produto, as pesquisas nacionais nesta área ainda são incipientes. Contudo, grandes avanços são esperados para os próximos anos, tendo em vista que a Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo (USP) em breve sediará o primeiro Centro de Pesquisas em Canabinoides, no território brasileiro.[1] O grupo de pesquisadores dessa instituição já acumula um grande número de publicações, principalmente voltadas ao estudo dos efeitos do THC e CBD para a saúde mental (Breuer *et al.*, 2016; Song *et al.*, 2016; Lee *et al.*, 2017; Lisboa *et al.*, 2017).

O conhecimento sobre as potencialidades do CBD como agente terapêutico ainda é limitado. O efeito anticarcinogênico desse canabinoide tem sido investigado

[1] Notícia veiculada em http://ribeirao.usp.br/?tag=centro-de-pesquisas-em-canabidiol (2017). Acesso em: 19 de maio de 2017.

principalmente ao longo das últimas duas décadas. Entretanto, a compreensão dos mecanismos de ação e alvos terapêuticos recentemente começou a ser ampliada e está em constante evolução (De la Ossa *et al.*, 2013; Scott *et al.*, 2014; Elbaz *et al.*, 2015*;* Nabissi *et al.*, 2015).

O uso do CBD aplicado à Odontologia ainda é bastante restrito (Napimoga *et al.*, 2009). Até o presente momento, não foram encontradas publicações sobre a utilização do mesmo para o tratamento do câncer bucal na área da Estomatologia, Patologia Oral, ou na especialidade médica da Oncologia aplicada à região de cabeça e pescoço. Portanto, a concepção metodológica do estudo contemplado nesta Tese amparou-se em subsídios teóricos disponibilizados por pesquisas realizadas em outros modelos experimentais de tumores malignos, principalmente os de origem epitelial, como mama e cólon (Aviello *et al.*, 2012; Romano *et al.*, 2014; Elbaz *et al.*, 2015; Elbaz *et al.*, 2016). Contudo, também foram contemplados estudos em glioblastomas, mieloma múltiplo, câncer pulmonar e de próstata (De Petrocellis *et al.*, 2013; Morelli *et al.*, 2014; Scott *et al.*, 2015; Nabissi *et al.*, 2016; Deng *et al.*, 2017).

A maior dificuldade com relação ao uso do CBD no presente trabalho foi a definição da dose adequada para quimioprevenção, visto que a maioria dos estudos publicados foram realizados em tumores xenogênicos induzidos em ratos atímicos. Dessa maneira, os animais já possuíam a neoplasia maligna desenvolvida ao início da administração do CBD (Elbaz *et al.*, 2015; Elbaz *et al.*, 2016). Nessas situações o canabinoide foi utilizado como agente terapêutico ou adjuvante. Contudo, a metodologia entre esses estudos mostrou-se altamente variável.

Romano *et al.* (2014) submeteram parte de sua amostra a um processo de cancerização quimicamente induzida. O carcinógeno utilizado foi o azoximetano que promove a formação de adutos de DNA O^6-metilguanina, culminando em tumores no

cólon. Similarmente, o DMBA utilizado na presente pesquisa, promove a transformação gradual do epitélio oral sadio para o carcinoma espinocelular. Ambos os modelos, são considerados ideais para o estudo dos mecanismos envolvidos na progressão do câncer e para quimioprevenção. Assim, a dose mínima estabelecida para cada animal foi de 5 mg/Kg de CBD, como sugerido por Romano *et al.* (2014). A dose máxima estabelecida foi de 10 mg/Kg de CBD conforme proposto por Elbaz *et al.* (2015) em seu modelo de carcinoma mamário, não induzido quimicamente.

A administração do CBD em dias alternados aos da aplicação do DMBA foi estabelecida por razões práticas referentes ao manejo e bem-estar animal. Romano *et al.* (2014) aplicavam o CBD por via intraperitoneal três vezes na semana, já Elbaz *et al.* (2015) o faziam duas vezes. No ano 2016, Elbaz e colaboradores em um segundo trabalho, utilizaram 5 mg/Kg em única aplicação semanal. Resultados positivos foram observados nos três estudos, a saber, redução da proliferação celular, da formação de lesões preneoplásicas, da invasão, do crescimento dos tumores e das metástases (Romano *et al.*, 2014; Elbaz *et al.*, 2015; 2016). O canabinoide utilizado no presente estudo foi gentilmente cedido pelo Prof. Dr. Francisco Silveira Guimarães.

A avaliação do potencial anticarcinogênico do CBD na cavidade oral era o principal objetivo do presente estudo. O tempo de aplicação de 12 semanas do DMBA resultou exclusivamente em um carcinoma *in situ* no grupo 1 (veículo). Inicialmente uma frequência maior de neoplasias malignas era esperada. Entretanto, esse desfecho não comprometeu a análise dos resultados e justifica-se pelo fato de não ter sido respeitado um período de latência em que os danos cumulativos, mesmo após a cessação das aplicações, continuariam a promover alterações epiteliais resultando no câncer (vide discussão do artigo 1).

As avaliações histológicas foram realizadas seguindo-se critérios bem estabelecidos, por um avaliador independente, em duplicata e, os índices de correlação entre as análises apresentarem-se adequados. Também, os resultados da avaliação imunoistoquímica mostram complementar os achados da observação histológica. A seleção do marcador Ki-67 (clone SP6) deveu-se ao fato do mesmo ter sido previamente validado em diversos estudos. Uma imunomarcação nuclear por Ki-67 acima de 5% é considerada um indicador precoce para detectar alterações no ciclo celular, com prognóstico de evolução para malignidade (Simionescu *et al.*, 2008; Motta *et al.*, 2009; Humayum, Prasad, 2011; Ganesan, Keating, 2014; Bianco *et al.*, 2015; Pigatti *et al.*,2015; Mondal *et al.*, 2016; Xie *et al.*, 2016; Zargoun *et al.*, 2017; Xu, Yang, Hu, 2017).

Os resultados gerais da presente pesquisa mostraram diferenças estatisticamente significativas sugerindo que o CBD nas doses utilizadas teria um potencial de virtualmente neutralizar ou reduzir os efeitos deletérios do carcinógeno utilizado. Também, observou-se um efeito dose-dependente do CBD sobre a imunomarcação por Ki-67. Ou seja, estabeleceu-se um efeito superior vinculado a dose de 10 mg/Kg, em comparação a 5 mg/Kg ou veículo, com relação ao controle do índice de proliferação celular. Salienta-se que esse resultado foi obtido por avaliação estatística de modelo de efeitos randômicos ou modelo misto (*mixed model*) levando em consideração a natureza das observações correlacionadas no mesmo animal (Detry *et al.*, 2016; Maurissen *et al.*, 2017).

O modelo de análise mostrou-se adequado ao estudo pois considera simultaneamente ambos os efeitos, fixos e randômicos (aleatórios). Os sujeitos, neste caso as médias de Ki-67 correspondentes a cada uma das cinco análises efetuadas na avaliação imunoistoquímica, representam os níveis de efeitos randômicos, enquanto o tratamento representa os efeitos fixos. Assim, cada animal contribuiu com cinco medidas

para a análise, totalizando-se 25 médias de Ki-67 por grupo, permitindo a otimização dos resultados da amostragem.

Medidas múltiplas por sujeito geralmente resultam em correlações que são explicitamente omitidas pelos modelos ANOVA, ANCOVA, de regressão e pela análise clássica de modelos repetidos. No modelo misto, todos os dados disponíveis podem ser usados e os dados ausentes (*missing data*) não causam efeitos nos outros escores do mesmo sujeito. Apesar de não ser aplicável neste caso específico, as variáveis tempo e a simetria, tampouco impedem a realização deste tipo de processamento estatístico que pode ser considerado vantajoso em muitos aspectos. Neste estudo, a análise dos resultados por ANOVA e *post Hoc* Dunnet, também mostrou diferença significativa entre o grupo 2 ($P < 0,001$) e grupo 3 ($P < 0,001$) com relação ao grupo 1 e, entre os tratamentos ($P = 0,025$).

Embora os resultados sugiram potencial quimiopreventivo para o CBD no modelo utilizado, qualquer inferência sobre o seu uso em condições clínicas, seria precipitada e imprudente, devido as limitações do estudo. Parece ser recomendável a reprodução da metodologia aqui proposta em novos experimentos, com a finalidade de acessar outras variáveis. A compreensão mais abrangente dos achados desta pesquisa dependerá de futuros trabalhos que visem avaliar os mecanismos de ação subjacentes aos efeitos do CBD observados microscopicamente.

Recomenda-se também, pesquisar o efeito do CBD em linhagens celulares de carcinoma espinocelular, pois em outros tipos de tumores os resultados encontrados foram elucidativos. Também, conta-se com a vantagem de tempos experimentais bastante reduzidos nesse tipo de metodologia (Hausstein *et al.*, 2014; Scott *et al.*, 2015; Nabissi *et al.*, 2016; Deng *et al.*, 2017).

A seleção de outros agentes carcinogênicos também pode contribuir para a análise da eficácia do CBD *in vivo*. Um substituto adequado para o DMBA pode ser o 4-nitroquinolona 1-óxido (4NQO) que possui a vantagem de ser administrado na água dos animais reduzindo significativamente a manipulação dos mesmos e produz, principalmente, tumores na língua (Kitano, 2000).

Outro modelo animal recomendável para averiguar o efeito do CBD *in vivo*, é o *hamster* (cricetulus) sírio dourado, cuja mucosa jugal é altamente suscetível aos efeitos dos carcinógenos químicos. Também, estudos em modelos de tumores obtidos por xeno ou isoenxertos, que são menos difundidos na Odontologia poderão ser de grande valia (Romanini *et al.*, 2012; Gock *et al.*, 2016).

Atualmente não há um método validado para a quimioprevenção do câncer bucal. Portanto, acredita-se que as pesquisas com CBD devam continuar a ser aperfeiçoadas em nível pré-clínico e interpretadas por meio de técnicas de microscopia e biologia molecular, com esse objetivo. A definição dos alvos terapêuticos e a compreensão das cascatas de sinalização decorrentes de sua ativação, são essenciais para validar a busca por novas estratégias de prevenção e tratamento. Assim, mais tarde, ensaios clínicos poderão ser realizados aproveitando-se os conhecimentos que estão sendo amealhados para a definição de terapias-alvo.

Assim sendo, com relação as atuais diretrizes para a prevenção e tratamento do câncer bucal na população, cabe reforçar a necessidade de informações epidemiológicas sistemáticas sobre a prevalência do câncer bucal e fatores de risco, principalmente nos países menos desenvolvidos. Também, deve-se estimular a realização de pesquisa visando a compreensão dos fatores biológicos e comportamentais envolvidos no câncer bucal. Ainda, considera-se essencial o amplo treinamento dos profissionais da saúde para

o diagnóstico precoce, com envolvimento ativo dos mesmos na prevenção primária, por meio do controle do acesso populacional aos fatores de risco (OMS, 2005).

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SIPESQ

Sistema de Pesquisas da PUCRS

Código SIPESQ: 6971 Porto Alegre, 1 de novembro de 2016.

Prezado(a) Pesquisador(a),

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou o Projeto de Pesquisa "AVALIAÇÃO DO EFEITO ANTITUMORAL DO CANABIDIOL EM MODELO ANIMAL DE CARCINOMA ESPINOCELULAR ORAL" coordenado por MARIA ANTONIA Z DE FIGUEIREDO.

Sua investigação, respeitando com detalhe as descrições contidas no projeto e formulários avaliados pela CEUA, está autorizada a partir da presente data.

Informamos que é necessário o encaminhamento de relatório final quando finalizar esta investigação. Adicionalmente, ressaltamos que conforme previsto na Lei no. 11.794, de 08 de outubro de 2008 (Lei Arouca), que regulamenta os procedimentos para o uso científico de animais, é função da CEUA zelar pelo cumprimento dos procedimentos informados, realizando inspeções periódicas nos locais de pesquisa.

Nº de Animais	Espécie	Duração do Projeto
30	Rattus Norvegicus	01/11/2016 - 01/03/2017

Atenciosamente,

Comissão de Ética no Uso de Animais (CEUA)

Pelotas, 06 de junho de 2016

Certificado

Certificamos que a proposta intitulada "**Avaliação do efeito do canabidiol em modelo animal de carcinoma espinocelular oral**", registrada com o nº23110.003663/2016-18, sob a responsabilidade de **Anelize de Oliveira Campello Felix** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), recebeu parecer **FAVORÁVEL** a sua execução pela Comissão de Ética em Experimentação Animal, em reunião de 09/05/2016.

Finalidade	(X) Pesquisa ( ) Ensino
Vigência da autorização	20/05/2016 a 31/12/2016
Espécie/linhagem/raça	*Rattus norvegicus*/F344/NTacUnib
Nº de animais	30
Idade	5 semanas
Sexo	Machos e Fêmeas
Origem	Biotério Central - UFPel

Solicitamos, após tomar ciência do parecer, reenviar o processo à *CEEA*.

Salientamos também a necessidade deste projeto ser cadastrado junto ao *COBALTO* para posterior registro no *COCEPE* (código para cadastro nº **CEEA 3663-2016**).

Prof. Dr. Éverton fagonde da Silva
Vice-Presidente da CEEA