UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
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Efeitos da capsaicina na etapa de iniciação da carcinogênese de cólon em ratos

Dissertação apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Mestre em Patologia.

Orientador: Prof. Dr. Luís Fernando Barbisan

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Palavras-chave: Capsaicina; Carcinogênese colorretal; Quimioprevenção.

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Provérbios de Salomão, Cap. 2 vs. 3-6.

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Resumo

A capsaicina (8-Metil-N-vanilil-(trans)-6-nonamida) é um composto alcaloide lipofílico e o principal componente responsável pela pungência em pimentas vermelhas, consumidas mundialmente. Estudos sobre o potencial mutagênico e genotóxico da capsaicina apontam resultados inconsistentes e conflitantes. Neste estudo, avaliamos o potencial genotóxico e os mecanismos moleculares envolvidos nos efeitos anti-proliferativos e pró-apoptóticos da capsaicina na carcinogênese de cólon induzida pela 1,2-dimetilhidrazina (DMH) em ratos. Ratos Wistar machos com sete semanas de idade foram randomicamente alocados em seis grupos (n=16). Durante as quatro primeiras semanas do experimento, os grupos 1 e 6 receberam doses intragástricas de óleo de milho (veículo da capsaicina), enquanto a capsaicina foi administrada nas doses de 5mg/kg aos grupos 2 e 4 e 50 mg/kg aos grupos 3 e 5, três vezes por semana. Durante a terceira e quarta semanas, todos os animais receberam quatro injeções subcutâneas de DMH (grupos 1-3, 40mg/kg) ou EDTA (grupos 4-6, veículo do DMH), duas vezes por semana. Os animais foram sacrificados 24 horas (n=6) e 22 semanas (n=10) após o tratamento com a DMH. Vinte e quatro horas após o tratamento com a DMH, a administração de capsaicina diminuiu significativamente a genotoxicidade induzida pela DMH em leucócitos do sangue periférico, bem como a genotoxicidade da água fecal em células tumorais CaCO-2. A capsaicina também reduziu o índice de proliferação de Ki-67 e aumentou a expressão de caspase-3 ativada no cólon dos animais tratados com DMH. A administração de capsaicina promoveu o aumento da expressão de genes associados às vias de resposta adaptativa a químicos, apoptose, desenvolvimento tecidual e diferenciação celular no cólon. Ao fim da vigésima segunda semana, a capsaicina na maior dose reduziu o número de focos de criptas aberrantes (FCA) e aumentou o número de tumores pequenos, bem diferenciados e não-invasivos. Estes resultados sugerem que a capsaicina foi capaz de suprimir a proliferação celular e induzir a apoptose através da regulação das vias do NF-κB e do estress do retículo endoplasmático, assim como modular os genes envolvidos no desenvolvimento tecidual e diferenciação celular, reduzindo a formação de lesões pre-neoplásicas e tumores.

+ **Palavras-chave:** carcinogênese colorretal; capsaicina; quimioprevenção.

Abstract

Capsaicin (8-Methyl-N-vanillyl-(trans)-6-nonenamide), a lipophilic alkaloid compound, is the major pungent ingredient found in red peppers consumed worldwide. Most reports on capsaicin potential mutagenicity and genotoxicity have yielded inconsistent findings. In this study, we evaluated capsaicin putative genotoxicity and molecular mechanisms underlying anti-proliferative and pro-apoptotic effects of capsaicin on DMH-induced rat colon carcinogenesis. Seven-weeks old male Wistar rats were randomly assigned into six experimental groups (n=16 each). During the first four weeks, corn oil was given to groups 1 and 6, while intragastric capsaicin was administered at 5mg/kg to groups 2 and 4, and at 50mg/kg to groups 3 and 5, three times/week. On weeks 3 and 4, the animals received subcutaneous injections of either DMH (groups 1-3, 40mg/kg) or EDTA (groups 4-6, vehicle), twice a week. The animals were sacrificed 24 hours (n=6) and 22 weeks (10) after DMH treatment. Capsaicin significantly decreased DMH-induced genotoxicity in peripheral blood leukocytes and fecal water genotoxicity in CaCO-2 cells, 24 hours after the last DMH administration. Capsaicin also reduced Ki-67 proliferation index and increased caspase-3 apoptosis in the colon from the DMH-treated animals. Evaluation of differential gene expression showed that capsaicin administration up-regulated genes associated with adaptive response to chemicals, apoptosis, tissue development and cell differentiation. High dose of capsaicin reduced the number of aberrant crypt foci (ACF) and increased the number of small, well differentiated and non-invasive tumors, 22 weeks after DMH-treatment. These findings revealed that capsaicin was able to suppress cell proliferation and to induce apoptosis via NF-κB regulation and endoplasmic reticulum (ER)-stress induction, as well as to modulate genes involved in tissue development and cell differentiation, reducing the formation of ACF preneoplastic lesions and tumors.

+ **Keywords:** colorectal cancer; capsaicin; chemoprevention.

Capítulo 1

Revisão da Literatura

1. Câncer colorretal

1.1 – Etiologia, fatores de risco e epidemiologia

O cólon é uma das sedes mais frequentes de neoplasias no homem (Siegel, Desantis, and Jemal 2014). O câncer colorretal (CCR) é um conjunto de doenças heterogênicas resultantes do acúmulo progressivo de alterações genéticas e epigenéticas que culminam no crescimento descontrolado de células epiteliais colônicas (Yamagishi et al. 2016). O CCR é tradicionalmente classificado como tipo hereditário, onde há susceptibilidade genética, e tipo esporádico, resultante de mutações somáticas adquiridas (Stigliano et al. 2014). Estima-se que os componentes hereditários representem cerca de 15 a 30% dos novos casos de CCR ao ano (Mundade et al. 2014). Os casos hereditários são atribuídos a síndromes como polipose adenomatosa familiar (PAF) e câncer colorretal hereditário não-polipoide (HNPCC), também denominado síndrome de Lynch (Del Vecchio Blanco et al. 2015). A maioria dos casos de CCR (70 a 85%) é representada pela forma esporádica da doença, em que não há fatores de risco genéticos identificados (Yamagishi et al. 2016). O desenvolvimento do CCR esporádico é influenciado por hábitos alimentares, estilo de vida, fatores ambientais e mutações somáticas adquiridas (Arnold et al. 2016).

Em termos etiológicos, não há uma causa específica para o desenvolvimento do CCR (Haggar and Boushey 2009). Diversos componentes multifatoriais podem estar envolvidos em interação com a predisposição hereditária e influências ambientais (Slattery et al. 1999; Johnson et al. 2013). O desenvolvimento econômico e a adoção do estilo de vida ocidental levaram à exposição a fatores ambientais e sociais, aumentando o risco de desenvolvimento da doença (Wu et al. 2016). As taxas de incidência e mortalidade do CCR correlacionam-se com estes padrões caracterizados pelo consumo excessivo de alimentos processados e aquisição de hábitos sociais como tabagismo, alcoolismo e sedentarismo (Hannan, Jacobs, and Thun 2009; Pericleous, Mandair, and Caplin 2013; Cong et al. 2014). Outros fatores etiológicos que contribuem para o desenvolvimento do CCR incluem histórico de ocorrência de pólipos intestinais, doenças inflamatórias intestinais (colite ulcerativa e doença de Crohn) e fatores hereditários associados com o aumento da incidência do CCR (Kim and Chang 2014; Axelrad, Lichtiger, and Yajnik 2016).

O CCR é o terceiro tipo mais comum de câncer e a quarta maior causa de mortes entre homens e mulheres no mundo (Favoriti et al. 2016). De acordo com as últimas estimativas mundiais e projeções demográficas do projeto GLOBOCAN (International

Agency for Research on Cancer, IARC 2012), estima-se um aumento de 60% no impacto global do CCR, o que representará cerca de 2,2 milhões de novos casos e 1,1 milhão de mortes em 2030 (Ferlay et al. 2015). A compreensão dos padrões atuais de distribuição geográfica e evolução da doença numa perspectiva global é imperativa para a contextualização regional, bem como para prospecções futuras que envolvam ações de prevenção e intervenção para o CCR (Arnold et al. 2016). Em termos nacionais, o CCR é o terceiro tipo de câncer mais prevalente entre homens e o segundo entre mulheres (Tabela 1), estimando-se uma incidência de 34.280 novos casos para o ano de 2016 (INCA, 2016).

Tabela 1 – Distribuição proporcional dos dez tipos de câncer mais incidentes estimados para 2016 por sexo, exceto pele não melanoma (retirado de INCA, 2016).

Localização primária	casos novos	%			Localização primária	casos novos	%
Próstata	61.200	28,6%	Homens	Mulheres	Mama Feminina	57.960	28,1%
Traqueia, Brônquio e Pulmão	17.330	8,1%			Cólon e Reto	17.620	8,6%
Cólon e Reto	16.660	7,8%			Colo do Útero	16.340	7,9%
Estômago	12.920	6,0%			Traqueia, Brônquio e Pulmão	10.890	5,3%
Cavidade Oral	11.140	5,2%			Estômago	7.600	3,7%
Esôfago	7.950	3,7%			Corpo do Útero	6.950	3,4%
Bexiga	7.200	3,4%			Ovário	6.150	3,0%
Laringe	6.360	3,0%			Glândula Tireoide	5.870	2,9%
Leucemias	5.540	2,6%			Linfoma não Hodgkin	5.030	2,4%
Sistema Nervoso Central	5.440	2,5%			Sistema Nervoso Central	4.830	2,3%

O aumento vertiginoso na incidência e mortalidade do câncer de colón é observado nos países em desenvolvimento, particularmente na Europa Oriental, Ásia e América do Sul (Bray and Soerjomataram 2015). Em contrapartida, as taxas de incidência e mortalidade do CCR demonstram-se estáveis ou em declínio em países com alto índice de desenvolvimento humano, como Estados Unidos, Austrália, Nova Zelândia e países da Europa Ocidental (Favoriti et al. 2016). As razões pelo recente declínio nas taxas de incidência nestes países refletem em grande parte, o aumento da detecção precoce e prevenção através de procedimentos como a polipectomia (Welch and Robertson 2016). Concomitantemente com os fatores que contribuíram para a redução da incidência, o aprimoramento de técnicas no cuidado pré-operatório, bem como significativos avanços nos tratamentos quimioterápicos e radioterápicos, levaram a uma redução uniforme nas taxas de mortalidade do câncer de colón (Rahal et al. 2014; Ananthakrishnan et al. 2015).

O CCR apresenta bom prognóstico quando detectado em estágios iniciais embora cerca de 40% dos casos sejam diagnosticados tardiamente, com sobrevida média estimada de

5 anos (INCA, 2016). Intervenções baseadas na análise dos fatores de risco e a detecção precoce constituem a melhor estratégia para prevenção do avanço da incidência e mortalidade do CCR (Haggar and Boushey 2009).

1.2 – Carcinogênese de cólon

A carcinogênese é um processo longo de múltiplas etapas nas quais modificações genéticas (mutações pontuais, amplificações e deleções gênicas) e epigenéticas (metilação do DNA e metilação e acetilação de histonas) são progressivamente acumuladas no genoma das células (Irigaray and Belpomme 2010; Singh et al. 2015). Este processo é caracterizado pelo acúmulo de mutações em genes que regulam o crescimento, proliferação e diferenciação celulares, gerando instabilidade genômica (Herman 2005). Desta forma, o processo de carcinogênese é dividido classicamente em três etapas: iniciação, promoção e progressão (Vincent and Gatenby 2008; Vineis, Schatzkin, and Potter 2010).

A etapa de iniciação é caracterizada pela exposição de células progenitoras a agentes mutagênicos, resultando na formação de adutos de DNA (Wilson 2013). Esta interação não evoca mudanças observáveis na morfologia celular, pois apenas confere um aumento permanente na suscetibilidade ao desenvolvimento neoplásico (Pitot and Dragan 1991). As células-alvo que sobrevivem ao estímulo mutagênico e contem alterações no DNA são denominadas células iniciadas (Zhou et al. 2009). Estas mutações não reparadas são fixadas no DNA após o processo de replicação, gerando mutações intrínsecas que são transcritas as gerações subsequentes (Lodish et al. 2000). A persistência de um insulto mutagênico pode gerar um acúmulo progressivo de mutações no genoma celular (Reuter et al. 2010).

A promoção tumoral corresponde a etapa subsequente e é caracterizada pela expansão clonal e seletiva de células iniciadas, originando tumores não-malignos (Pitot and Dragan 1991). Nesta etapa, a presença de agentes promotores é essencial para desencadear o desenvolvimento de lesões proliferativas, displásicas e anaplásicas (Weston and Harris 2003). Os agentes promotores apresentam mecanismos de ação não-genotóxicos, evocando resposta proliferativa desencadeada por lesões teciduais ou processos inflamatórios (Hyndman 2016). O estímulo proliferativo resulta no acúmulo progressivo de mutações e aumento da instabilidade genética (Singh et al. 2015).

A etapa de progressão tumoral é marcada pela transformação maligna das células neoplásica (Yokota 2000). O fenótipo maligno é caracterizado pela autossuficiência a fatores de crescimento, insensibilidade aos sinais de inibição do crescimento celular, evasão da apoptose, potencial replicativo ilimitado, angiogênese sustentada, invasão tecidual e

metástase (Hanahan and Weinberg 2011). O acúmulo progressivo de novas mutações associadas ao processo de seleção clonal possibilita a infiltração vascular e linfática (Farnsworth et al. 2014). A metástase corresponde ao estágio final do processo de carcinogênese (Steeg 2016).

Durante as últimas décadas, estudos moleculares identificaram diversas alterações cruciais para o desenvolvimento do CCR esporádico (Worthley and Leggett 2010; Colussi et al. 2013). Na carcinogênese colorretal são identificados três mecanismos distintos e bem definidos, representados pelas vias de instabilidade cromossômica (CIN), via de instabilidade de microssatélites (MSI) e o fenótipo metilador de ilhas CpG (CIMP) (Al-Sohaily et al. 2012). A maioria dos CCR esporádicos são decorrentes de eventos que resultam de aberrações descritas na via de instabilidade cromossômica (Figura 1) (Orsetti et al. 2014).

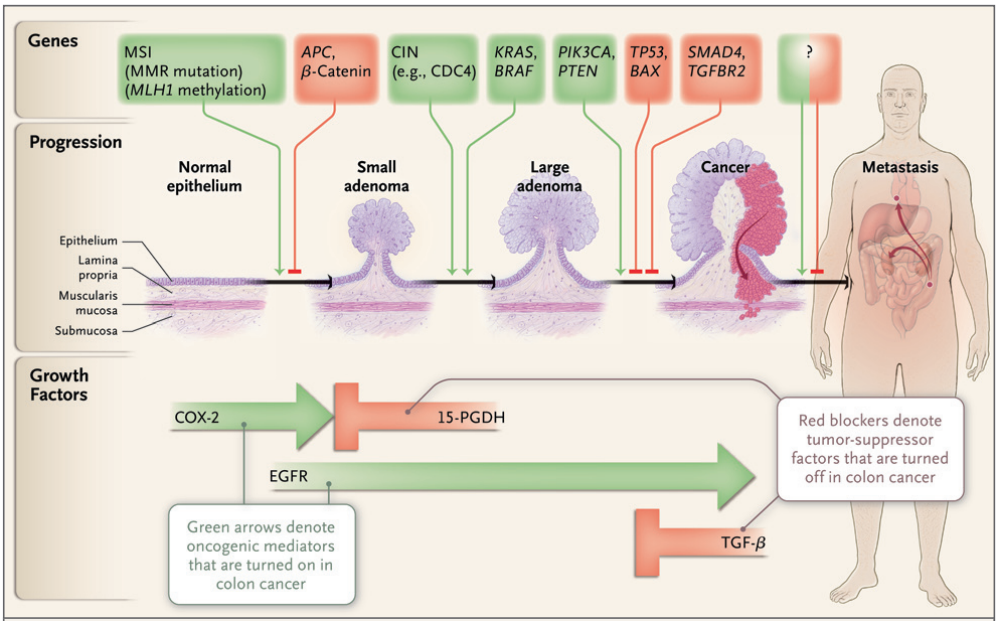

Figura 1 – Bases moleculares da carcinogênese colorretal. A instabilidade cromossômica (ou via clássica) é caracterizada pelo acúmulo progressivo de mutações em oncogenes e supressores tumorais. Retirado de Markowitz and Bertagnolli, 2009.

A via de instabilidade cromossômica está associada com cerca de 70% dos casos de CCR e apresenta a sequência clássica adenoma-adenocarcinoma (Rajagopalan et al. 2003). Esta sequência reflete um padrão de ativação mutacional de oncogenes e inativação de supressores tumorais que resultam em defeitos na segregação dos cromossomos, reparo do DNA e função dos telômeros (Tariq and Ghias 2016). As alterações genômicas desta via incluem a ativação dos proto-oncogenes KRAS, c-Src, c-Myc e inativação dos supressores

tumorais APC e TP53 (Vogelstein and Kinzler 1993; Vogelstein and Kinzler 2004; Markowitz and Bertagnolli 2009).

A inativação do gene APC está entre os eventos mais recentes na progressão do CCR esporádico (Tariq and Ghias 2016). A proteína codificada por este gene está envolvida no complexo de degradação da β-catenina, mediadora central da via de sinalização Wnt (Najdi, Holcombe, and Waterman 2011). A via canônica Wnt é altamente conservada em vertebrados e controla processos fundamentais de proliferação, diferenciação e motilidade celular (Schneikert and Behrens 2007). Mutações no gene APC promovem a estabilização da β-catenina que é acumulada no citoplasma e translocada para o núcleo, onde em associação com complexos de transcrição promove a expressão de genes que favorecem a proliferação celular (Kwong and Dove 2009). Outra importante via que contribui para este estado hiperproliferativo é a via do KRAS, encontrada mutada em cerca de 50% dos carcinomas colônicos (Carethers and Jung 2015). A mutação do KRAS bloqueia a ação de enzimas GTPases permitindo ativação constitutiva da cascata de sinalização RAS, resultando na inibição da apoptose e aumento da proliferação celular (Armaghany et al. 2012). Essas alterações genéticas promovem criptas aberrantes hiperplásicas e displásicas que progridem para adenomas colônicos (Pretlow and Pretlow 2005; Tan and Du 2012).

Os adenomas colônicos são lesões benignas caracterizadas por diferentes graus de displasia celular, podendo apresentar hipercromasia e pseudoestratificação nuclear, redução de secreção de muco e perda de polaridade celular (Colucci, Yale, and Rall 2003). As lesões adenomatosas podem ser sésseis ou pedunculadas, classificadas quanto a sua arquitetura em tubular, vilosa ou tubovilosa (Shussman and Wexner 2014). Os adenocarcinomas são lesões infiltrativas com alto grau de displasia e malignidade, classificados em tubulares ou mucinosos (Bujanda et al. 2010; Fleming et al. 2012).

A perda do supressor tumoral TP53 marca o limite da transição adenoma-adenocarcinoma (Rivlin et al. 2011; Carethers and Jung 2015). O gene TP53 é comumente mutado no CCR e está envolvido no controle do ciclo celular e apoptose, contribuindo para o estado de potencial replicativo ilimitado (Naccarati et al. 2012). Estas alterações genéticas promovem um ambiente de instabilidade cromossômica caracterizadas por acúmulos de aneuploidias e deleções (Liu et al. 2015).

1.3 – Carcinogênese experimental do cólon

Um modelo pode ser definido como uma versão simplificada e mais acessível de uma entidade complexa, compartilhando diversas similaridades com o fenômeno original

(Greek and Menache 2013). O objetivo de utilizar modelos experimentais animais no estudo do CCR é recapitular os eventos moleculares, etiologia, patologia e progressão clínica da doença (Rosenberg, Giardina, and Tanaka 2009). Um modelo ideal deve apresentar características histológicas e moleculares similares, além de representar a complexidade de interações celulares que são relevantes ao processo de carcinogênese em humanos (Johnson and Fleet 2013). Nesta perspectiva, um grande número de compostos químicos possui potencial mutagênico e são utilizados para induzir tumores em animais. Dentre os carcinógenos químicos mais utilizados destaca-se a 1,2-dimetilhidrazina (DMH) e seu metabólito, o azoximetano (Perše and Cerar 2011). O modelo de indução pela DMH é um modelo bem estabelecido e largamente empregado na carcinogênese experimental de cólon, por induzir especificamente lesões colônicas com diversas similaridades morfológicas e moleculares ao câncer de colón esporádico humano (Perse and Cerar 2005).

A DMH é um procarcinógeno completo capaz de induzir as fases de iniciação e promoção da carcinogênese (Rosenberg, Giardina, and Tanaka 2009). A ativação metabólica da DMH ocorre através de uma série de etapas oxidativas no fígado. Os metabólitos são transportados para o intestino através da bile ou do sistema sanguíneo, onde induzem a formação de adutos (*i.e.*, introdução de grupos metil no DNA) (Femia et al. 2010). As alterações no DNA induzidas pela DMH podem ser revertidas pela ação de enzimas de reparo ou pela indução de apoptose, ou podem ainda instalar mutações específicas que levam a vantagem de crescimento com aumento na proliferação celular, levando à formação de criptas aberrantes (Glauert and Bennink 1983; Perše and Cerar 2011). Estudos clássicos demonstram que as células epiteliais colônicas de ratos e sua microflora intestinal também são capazes de metabolizar a DMH em íon metildiazônio, metabólito carcinogênico e altamente reativo, através do recrutamento de múltiplas enzimas com ações similares as oxidases (Wargovich and Felkner 1982; Oravec et al. 1986). O íon metildiazônio é um agente alquilante responsável pela metilação de bases de DNA de células de vários órgãos, incluindo as células epiteliais da zona proliferativa das criptas colônicas, resultando em hiperproliferação e aumento de mutações (Perše and Cerar 2005; Perše and Cerar 2011).

Em roedores, o câncer de cólon é precedido pelo desenvolvimento de focos de criptas aberrantes (FCA), uma lesão pré-neoplásica onde as criptas colônicas apresentam-se com diversos graus de hiperplasia e displasia, com abertura da fenda luminal e com epitélios visivelmente espessados, apresentando-se únicas ou na forma de focos, podendo progredir para pólipos seguidos de adenomas e adenocarcinomas (Bird 1987; Bird and Good 2000; Ochiai et al. 2014). Esta sequência de eventos hiperplásicos/displásicos é uma consequência

do acúmulo de múltiplas alterações genéticas e epigenéticas no epitélio colônico (Sakai, Nakajima, and Kaneda 2014). Embora nem todos os FCA progridam para uma lesão neoplásica, diversos estudos apontam que todas as neoplasias malignas surgem a partir de um FCA (Thorup 1997; Humphries and Wright 2008).

Os FCA são identificados com maior frequência no cólon medial e distal dos roedores, apresentando índices de proliferação celular maiores que os da mucosa normal e mudanças no padrão de atividade enzimática, tais como redução de expressão das hexosaminidases e de mucinas com aumento de sialomucinas, fenômeno geralmente associado ao grau de displasia e multiplicidade das criptas aberrantes (Pretlow et al. 1991; Orlando et al. 2008; Femia, Dolara, and Caderni 2004). São ainda relatadas alterações genéticas nos genes K-Ras, Apc e Tp53, relacionados à proliferação celular e presença de instabilidade de microssatélites, além de alterações em genes associados à inflamação tais como o iNOS e COX-2 (Cheng and Lai 2003; Takahashi and Wakabayashi 2004). Em alguns FCA com maior displasia há acúmulo citoplasmático e nuclear de β-catenina, um marcador potencial de progressão neoplásica (Yamada and Mori 2003; Mori et al. 2004).

Em humanos, os FCA também são encontrados nas porções distais da mucosa do cólon, especialmente em pacientes portadores de polipose familiar (Roncucci et al. 1998; Stevens et al. 2007). Embora consenso em modelos com roedores, a utilização do FCA como biomarcador do câncer de cólon e em estudos de quimioprevenção humana permanece circunstancial (Gupta and Schoen 2009; Takahashi et al. 2012). Contudo, um subconjunto de FCA apresentando displasia e caracterizados por alterações de vias genéticas que controlam a proliferação celular, são potencialmente úteis como marcadores para avaliar indivíduos de alto risco e, portanto, potenciais alvos para agentes quimioterápicos e quimiopreventivos (Shpitz et al. 1998; Wargovich, Brown, and Morris 2010).

2. Quimioprevenção do câncer

O termo quimioprevenção foi cunhado por Michael Sporn em 1976 para conceituar a *"inibição ou reversão da carcinogênese através da utilização de nutrientes não-tóxicos ou compostos farmacológicos capazes de inibir ou reverter o desenvolvimento e progressão de clones mutantes de células malignas"* (Sporn 1976). Em termos mais específicos, a quimioprevenção do CCR envolve o uso ao longo prazo de uma variedade de agentes que podem retardar, impedir ou mesmo reverter o desenvolvimento de neoplasias colônicas, sendo relevante para pacientes predispostos geneticamente e para aqueles que são sensíveis

às causas ambientais do CCR (Cooper et al. 2010; Manzano and Pérez-Segura 2012).

Como revisado em Tanaka (2009) e Riscuta (2016), diversos estudos têm sido conduzidos para elucidar a eficiência e ação de agentes quimiopreventivos, principalmente dos compostos bioativos presentes nos alimentos. As múltiplas etapas do processo de carcinogênese possibilitam diversas fases de intervenção para inibição, reversão ou supressão dos processos da carcinogênese antes do desenvolvimento de neoplasias malignas (Rajamanickam and Agarwal 2008).

Agentes capazes de suprimir estas múltiplas etapas possuem grande potencial para quimioprevenção. Um quimiopreventivo ideal deve exibir pouca ou nenhuma citotoxicidade, boa eficácia em múltiplos alvos, capacidade de consumo oral, mecanismos de ação conhecidos, baixo custo e boa aceitação humana (Benetou, Lagiou, and Lagiou 2015). Recentemente, produtos naturais têm recebido grande atenção prospectiva para a prevenção do câncer, devido aos vários benefícios para saúde, a notável baixa toxicidade e reduzidos efeitos colaterais e as limitações dos agentes quimioterapêuticos convencionais (Hou et al. 2016). O tratamento quimiopreventivo pode iniciar-se antes e durante a exposição ao carcinógeno químico na fase de iniciação, durante as fases de promoção e progressão, ou ainda, ao longo das duas fases de iniciação e promoção da carcinogênese. Estes protocolos de carcinogênese química são utilizados para avaliar efeitos protetores ou promotores do composto testado e fornecem dados bastante reprodutíveis (Femia and Caderni 2008; Steward and Brown 2013).

Na perspectiva da carcinogênese química, compostos que exibem potencial aplicação para quimioprevenção são classificados em duas principais categorias: agentes bloqueadores e agentes supressores (Manson et al. 2000). Essa classificação foi proposta por Wattenberg em 1985 e leva em conta apenas a natureza funcional destes compostos durante o processo de carcinogênese (Wattenberg 1985). Os agentes bloqueadores são conceituados como agentes químicos ou biomoléculas capazes de inibir o estágio de iniciação da carcinogênese, bloqueando, por exemplo, a interação de agentes mutagênicos com o DNA ou aumentando a eficiência do reparo do DNA (Shu et al. 2010). Compostos que afetam os estágios posteriores da carcinogênese (promoção e progressão) são classificados como agentes supressores, devido à habilidade destes compostos de diminuir a capacidade proliferativa de células iniciadas (Shu et al. 2010; Landis-Piwowar and Iyer 2014).

Compostos que exibem atividades inibitórias quando administrados em um curto período antes da exposição ao composto envolvido na etiologia do câncer apresentam implicações que demandam considerações a parte. Alguns agentes bloqueadores são efetivos

quando administrados durante um curto intervalo de tempo, entre alguns minutos ou até algumas horas antes da exposição ao agente carcinogênico (Landis-Piwowar and Iyer 2014). Essas considerações se tornam particularmente importantes na interpretação de estudos epidemiológicos e nas deliberações para estratégias de intervenção, uma vez que efeitos de curto intervalo de tempo podem ser de grande importância (Wattenberg 1985).

Diversos compostos bioativos encontrados em frutas, vegetais e plantas medicinais são capazes de reduzir o risco de desenvolvimento de doenças crônicas, incluindo o câncer (Sales, Pelegrini, and Goersch 2014). Frutas e vegetais são importantes constituintes da dieta humana e exercem papéis fundamentais na manutenção da homeostase, provendo fibras, antioxidantes e uma grande variedade de compostos fitoquímicos biativos (Liu 2013). Desta forma, uma nutrição adequada e uma dieta rica em frutas e vegetais são estratégias essenciais para a prevenção do CCR (Baena and Salinas 2015).

3. Capsaicina

3.1 – *Capsicum:* taxonomia e notas etnobotânicas

Capsicum é um gênero da família Solanacea constituído de espécies herbáceas representado pelas pimentas e pimentões originário da América Central e do Sul (Rêgo et al. 2011). A altura e forma de crescimento destas plantas variam de acordo com as condições de cultivo, apresentando frutos na forma de baga com grande variabilidade morfológica (Zhigila et al. 2014). O gênero *Capsicum* é composto por aproximadamente 35 *taxa* distribuídos em 5 espécies domesticadas (*C. annuum var. annuum, C. chinense, C. baccatum var. pendulum, C. frutescens* e C. *pubescen*), 10 semidomesticadas e cerca de 20 espécies silvestres, sendo as espécies mais conhecidas o pimentão (*Capsicum annum L.)* e as variedades de pimentas malagueta e tabasco (*Capsicum frutescens L*) (Zonneveld et al. 2015). Estima-se que a domesticação das pimentas ocorreu desde o início do povoamento humano nas Américas, há cerca de 12.000 anos (Perry et al. 2007; Kraft et al. 2014).

A principal propriedade das pimentas do gênero *Capsicum* é a pungência, atributo explorado e relacionado aos diversos usos humanos, sejam eles condimentares, repelentes, medicinais ou ritualísticos (Tewksbury et al. 2008; DeWitt and Bosland 2009). Os compostos alcaloides denominados capsaicinóides, são responsáveis pelos efeitos sensoriais associados a pungência (Szolcsányi and Jancsó-Gábor 1975). Encontradas predominantemente no septo dos frutos, a capsaicina e dihidrocapsaicina representam cerca de 90% dos capsaicinóides encontrados em plantas *Capsicum*, sendo os alcaloides mais potentes (Chapa-Oliver and

Mejía-Teniente 2016).

Os relatos dos usos das propriedades pungentes das pimentas são ricos e diversos (Govindarajan 1985). Uma das primeiras fontes históricas da vida entre os Incas, *Commentarios Reales*, escrito por Garcilaso de la Faz em 1609, menciona o uso comum e até diário de pimentas vermelhas na culinária (Bosland 1999). A fumaça irritante resultante da queima de pimentas também foi utilizada pelos ameríndios como forma de punição (Figura 2) ou arma de guerra, sendo efetiva estratégia para repelir invasores (De 2004). Usos similares da pimenta foram relatados por outros viajantes que percorreram os diversos territórios da Hispano-América (Pickersgill 1969; Bosland 1999; Perry and Flannery 2007)

Figura 2 – A fumaça irritante resultante da queima de pimentas era utilizada como forma de punição pelos maias. Historia general de las cosas de Nueva España: The Florentin Codex - Bernardino de Sahagún. 16th Century, em: *World Digital Library.*

As pimentas são empregadas na medicina tradicional há milhares de anos, precedendo até mesmo o uso condimentar durante o processo de domesticação (Bosland 1999). Os estudos da medicina ameríndia revelam que as pimentas possuem uma grande

diversidade de aplicações terapêuticas. Castiglioni menciona que o conhecimento das propriedades antiblenorrágicas de plantas *Capsicum* pelas populações ameríndias é anterior ao contato com os europeus (Castiglioni 1947). Chauca, um dos médicos presentes na segunda expedição de Colombo ao novo mundo, observou aplicações medicinais de plantas do gênero *Capsicum* para o tratamento da dor (Cordell and Araujo 1993). Povos maias e astecas utilizavam extratos de pimentas misturadas com milho para tratar resfriados comuns. Também há relatos de usos de extratos de pimenta para tratamento de queimaduras, asmas, tosses, dores de dente e garganta (Cichewicz and Thorpe 1996; DeWitt and Bosland 2009; Roman et al. 2011).

Na perspectiva regional, o conhecimento das aplicações terapêuticas de plantas *Capsicum* na Amazônia brasileira também é ricamente documentado. Em estudo com os índios Yanomami, Milliken e colaboradores registram a utilização de pimentas para tratar de infecções respiratórias, oftalmias e malária (Milliken and Albert 1997; Milliken and Albert 1999). Quilombolas do Amapá utilizam pimentas para aliviar cólicas menstruais, reumatismo e problemas intestinais (Pereira et al. 2007). Diversos outros estudos destacam usos particulares de frutos e folhas das plantas *Capsicum* no Brasil (van den Berg and da Silva 1998; Barbosa et al. 2002; Roman et al. 2011).

3.2 – Aspectos químicos e farmacológicos da capsaicina

A capsaicina (8-metil-N-vanilil-trans-6-nonamida) é um composto alcaloide cristalino e lipofílico, com fórmula molecular $C_{18}H_{27}NO_3$ e peso molecular de 305,40 g/mol (Reyes-Escogido, Gonzalez-Mondragon, and Vazquez-Tzompantzi 2011). É o principal alcaloide acumulado na epiderme secretora do septo dos frutos do gênero Capsicum, com estrutura química formada pela condensação da vanililamina e do ácido metilnonanóico (Stewart et al. 2005). A capsaicina foi primeiramente cristalizada por Tresh em 1846, caracterizada como molécula apolar solúvel em lipídeos, álcoois e óleos derivados (Tresh 1846). A molécula apresenta isomeria *cis*/*trans*, embora o isômero *trans* seja a forma mais estável e predominante (Nelson 1919) (Figura 3).

Embora as vias para síntese dos capsaicinóides sejam propostas por Leete e Iwai, muitas enzimas envolvidas na biossíntese e regulação destes alcaloides em plantas *Capsicum* ainda são desconhecidas (Leete and Louden 1968; Iwai et al., 1979). Sabe-se que a cadeia aromática vanililamina é formada pela via do fenilpropanoide, que tem como precursor a L-fenilalanina e seus derivados (ácidos cafeico, cumáricos e ferúlicos). A vaniliamnina é subsequentemente condensada com o produto da via dos ácidos graxos ramificados, o 8-

methyl-6-nonenoil-CoA, catalisada pela enzima capsaicina sintase (Aza-González, Núñez-Palenius, and Ochoa-Alejo 2011). As concentrações de capsaicina, assim como outros metabólitos secundários das plantas, podem ser elevadas em casos de stress, mediados por moléculas estáveis que induzem a produção de metabólitos secundários, denominadas elicitores (Mazourek et al. 2009). Experimentos sugerem que é possível aumentar a síntese da capsaicina através da manipulação de concentrações do substrato e da disponibilidade hídrica ofertada as plantas (Narasimha Prasad et al. 2006).

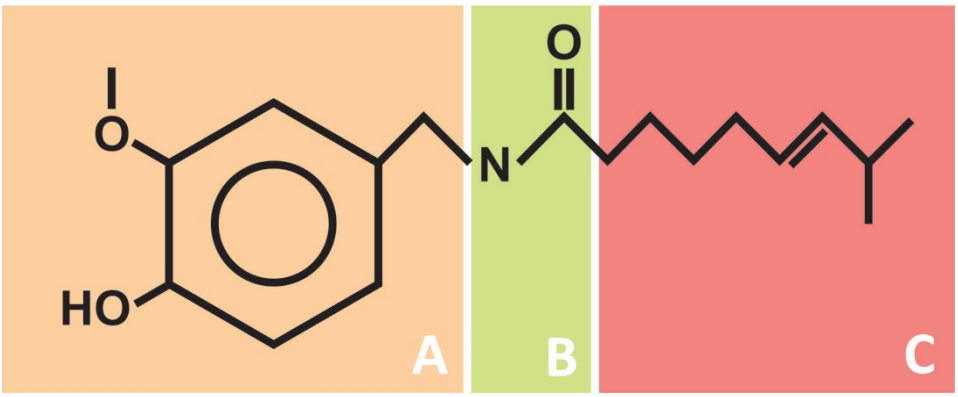

Figura 3 – Regiões da molécula de capsaicina. A (anel aromático); B (ligação amida) e C (cadeia hidrofóbica). Fonte: elaborada pelo autor.

A capsaicina pode ser administrada por diferentes rotas, incluindo oral, sistêmica, tópica e intradérmica. As exposições mais comuns são através do consumo alimentar, em ações de autodefesa (*e.g.,* sprays de pimenta) e no uso de analgésicos tópicos (Suresh and Srinivasan 2010). A absorção da capsaicina pelo trato gastrointestinal ocorre por processo passivo, apresentando uma capacidade total de absorção que varia entre 50 a 90%, em diferentes estudos animais (Leelahuta, Glinsukon, and Wangpanish 1983; Kawada et al. 1984; Donnerer et al. 1990). O pico de concentração plasmática em ratos é observado cerca de 1 hora após a administração oral (Suresh and Srinivasan 2010).

O metabolismo da capsaicina após administração oral ocorre majoritariamente no fígado. Um experimento *in vitro* utilizando frações microssomais humanas demonstrou que a capsaicina foi rapidamente metabolizada, produzindo três principais subprodutos: 16-hidroxicapsaicina, 17-hidroxicapsaicina e 16,17-hidroxicapsaicina (Chanda et al. 2008). Diversas isoenzimas do citocromo P450 participam diretamente do metabolismo da capsaicina (Reilly et al. 2003). As CYPs são enzimas oxidativas pertencentes à família das

monooxigenases, responsáveis pela degradação e eliminação de uma grande gama de xenobióticos (Korzekwa 2014). O metabolismo de xenobióticos é uma complexa rede bioquímica com diferentes interações no organismo, envolvendo a inibição, indução e competição por diferentes vias enzimáticas (McDonnell and Dang 2013).

Há uma série de isoenzimas CYPs que são importantes no metabolismo de compostos farmacológicos e medicinais, mediando reações adversas causadas por interações droga-droga (Preissner et al. 2010) A capsaicina é metabolizada por diversos isotipos de CYPs (CYP1A1, 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 e 3A4) e tem sido correlacionada com a inibição dos isotipos CYP3A1, 2A2, 2B1, 2B2, 2C6, 2C11 e 2E1 no fígado de roedores (Takanohashi and Bernard 2006). A inibição da enzima CYP2E1 está relacionada com o bloqueio da ativação metabólica de diversos carcinógenos, razão pela qual a capsaicina tem sido hipotetizada como potencial agente bloqueador da carcinogênese (O'Neill et al. 2012). A capsaicina é excretada principalmente pelos rins, na forma livre ou de metabólito glucorinado. Uma pequena fração intacta é comumente detectada nas fezes e na urina até 48 horas após a administração oral de capsaicina (Kawada et al. 1984).

O mecanismo de ação da capsaicina tem sido extensivamente estudado. A capsaicina se liga ao receptor de potencial transiente vanilóide do tipo 1 (TRPV1), um receptor ionotrópico excitatório não-seletivo, principalmente expresso em neurônios sensoriais, porém amplamente distribuído em outros tecidos do corpo como rins, intestinos e fígado além de diversas células como granulócitos, polimorfonucleares, mastócitos e macrófagos (Gees, Colsoul, and Nilius 2010). O TRPV1 contém 838 aminoácidos e possui peso molecular de 95 kDa, consistindo de seis domínios transmembranares com uma pequena região de poro entre o quinto e o sexto domínio (Figura 4). O receptor regula os níveis intracelulares de íons através de um canal catiônico não-específico permeável a sódio e cálcio, localizado na membrana plasmática e no retículo endoplasmático (Liao et al. 2013). Este canal é regulado e ativado por substâncias endógenas denominadas endovanilóides (*e.g.* anandamida, NADA/OLDA) e diversos estímulos exógenos incluindo agonistas químicos como a capsaicina e resinoferotoxina, ligantes altamente lipofílicos que compartilham similaridades estruturais com os ácidos graxos endógenos identificados como agonistas do TRPV1 (Reyes-Escogido, Gonzalez-Mondragon, and Vazquez-Tzompantzi 2011; Bourinet et al. 2014).

O TRPV1 está envolvido em diferentes processos fisiológicos dependendo de sua localização no organismo (O'Neill et al. 2012). Uma das principais funções está relacionada com a detecção aguda do calor nocivo e agentes químicos em nociceptores do sistema nervoso periférico (Jung et al. 2004). No sistema nervoso central, o TRPV1 está envolvido no

processamento e modulação da dor, embora estes mecanismos sejam poucos conhecidos (Edwards 2014). Este receptor também está relacionado em certas condições de dor crônica, como em casos de em neuropatias e osteoartrites (Alawi and Keeble 2010).

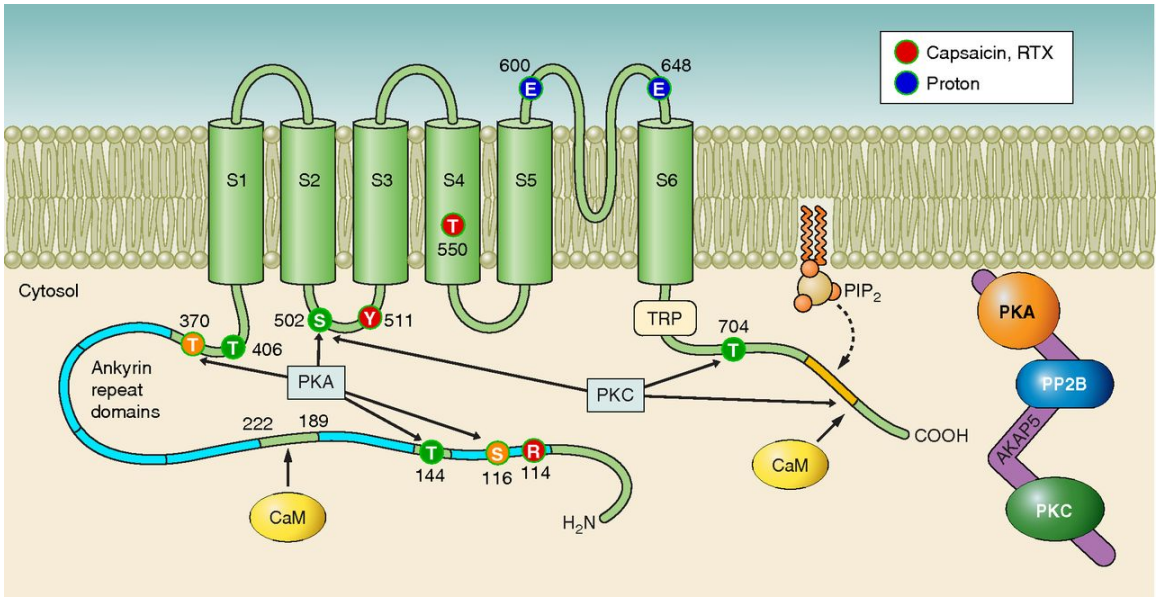

Figura 4 – Topologia do TRPV1 demonstrando múltiplos sítios regulatórios para proteína kinase C e A (PKC e PKA), calmodulina (CaM), fostatidilinositol 4,5-bifosfato (PIP2), capsaicina e resinoferotoxina. *Retirado de Bourinet et al., 2014.*

O TRPV1 contém uma subunidade sensível ao calor responsável pela sensação de pungência causada pela capsaicina (Bourinet et al. 2014). A ligação da capsaicina ao TRPV1 aumenta a liberação do cálcio intracelular, promovendo a liberação de neuropeptídios como a substância P e o peptídeo relacionado ao gene da calcitonina (CGRPA) (Bautista and Julius 2008). A ativação deste receptor pela capsaicina em neurônios sensoriais produz dor, inflamação e sensação local de calor. Este estado de excitação neuronal evocado pela ativação destes canais é seguido por um estado refratário durante o qual os neurônios previamente excitados deixam de responder ao estímulo (Premkumar and Sikand 2008). Este fenômeno de analgesia é causado pela depleção de substância P sendo conhecido como dessensibilização, cujo potencial terapêutico tem sido explorado no contexto da dor (Bautista and Julius 2008; Üçeyler and Sommer 2014).

3.3 – Aspectos controversos da capsaicina na carcinogênese

Considerando o consumo frequente de capsaicina como aditivo alimentar e as atuais aplicações terapêuticas, uma avaliação precisa de quaisquer efeitos nocivos deste composto constitui-se de grande relevância na perspectiva de saúde pública (Surh and Lee 1995). Embora muitos estudos sugiram diversos potenciais terapêuticos, a efetividade e segurança do consumo deste composto não foram ainda elucidados, sobretudo no contexto da carcinogênese (Bode and Dong 2011; Mózsik 2014; Clark and Lee 2016). Estudos revelam que a capsaicina pode atuar na prevenção do câncer inibindo o crescimento de células tumorais leucêmicas, de hepatoma, de gliobastoma e do cólon através da parada do ciclo celular ou indução de apoptose (Qiao et al. 2005; Hassan et al. 2012; Huang et al. 2013; Bozok Cetintas et al. 2014; Jin et al. 2014). Em contraste alguns estudos demonstram o efeito co-carcinogênico da capsaicina visto que extratos de pimenta promoveram o câncer de estômago e fígado em modelos murinos (Díaz Barriga Arceo et al. 1995; Hwang et al. 2010; Liu et al. 2015). Dados epidemiológicos também sugerem que o consumo de grandes quantidades de condimentos a base de pimenta pode estar correlacionado com o risco de desenvolvimento câncer de bexiga e estômago (López-Carrillo et al. 2003; Bley et al. 2012).

3.3.1 – Ensaios toxicológicos: uma questão de pureza

Os dados sobre a citotoxicidade e genotoxicidade da capsaicina na literatura são abundantes e contraditórios. Diversos estudos clássicos sugerem uma potencial atividade mutagênica na presença ou ausência de metabolismo oxidativo (Nagabhushan and Bhide 1985; Lawson and Gannett 1989; Azizan and Blevins 1995), enquanto outros estudos falharam em demonstrar evidências robustas para um potencial efeito mutagênico da capsaicina (Muralidhara and Narasimhamurthy 1988; Chanda et al. 2004; Bley et al. 2012). O consumo de altas concentrações de capsaicina também foi correlacionado com alterações histopatológicas e bioquímicas, incluindo erosão da mucosa gástrica e necrose hepática em ratos (Monsereenusorn, Kongsamut, and Pezalla 1982).

As atividades citotóxicas e/ou genotóxicas da capsaicina reportadas em estudos clássicos de toxicidade podem estar associadas a impurezas e contaminações dos extratos comerciais utilizados (Kuzma et al. 2014). As diferenças de perfis genotóxicos encontradas em diversos estudos com a forma bruta ou purificada da capsaicina sugerem que a fonte e pureza deste composto são importantes fatores a se considerar nos ensaios toxicológicos (Chanda et al. 2004). Em um estudo sobre o potencial mutagênico do consumo de pimentas

associado ao desenvolvimento de câncer de bexiga, a contaminação por aflatoxinas foi reportada em todas as amostras de extrato de pimentas utilizadas (Tsuchiya et al. 2011). Estudos experimentais que analisaram a capsaicina com alto grau de pureza não observaram efeitos citotóxicos ou genotóxicos da capsaicina nos diferentes ensaios toxicológicos (Park and Surh 1997; Chanda et al. 2004; Proudlock, Thompson, and Longstaff 2004).

3.3.2 – Carcinógeno ou co-carcinógeno?

Evidências epidemiológicas e experimentais sugerem que a capsaicina pode exercer atividade carcinogênica ou co-carcinogênica, atuando principalmente durante a fase de promoção tumoral (Surh and Lee 1996). Uma associação positiva entre a incidência de câncer de estômago e o consumo de dietas ricas em pimentas vermelhas foi encontrada em estudo epidemiológico conduzido no México (López-Carrillo et al. 2003). Embora poucos estudos relatem uma associação direta de carcinogenicidade da capsaicina (Toth, Rogan, and Walker 1984; Nagabhushan and Bhide 1985; Toth and Gannett 1992), estudos experimentais demonstram uma possível interação co-carcinogênica em diversos modelos de indução química (Malagarie-Cazenave et al. 2009; Hwang et al. 2010) A ingestão oral de extrato de pimenta apresentou efeito promotor no desenvolvimento de tumores de fígado e estômago em modelo de camundongos BALB/c iniciados com metil-aceto-metilnitrosamina e benzeno hexacloreto (Agrawal et al. 1986). A ingestão de 0,002% de capsaicina na água de beber também apresentou efeitos promotores no desenvolvimento de lesões pre-neoplásicas no fígado de ratos iniciados com dietilnitrosamina (Jang et al. 1991).

Estudos experimentais em outros sistemas e órgãos-alvo identificaram achados similares. A exposição a capsaicina aumentou o número de metástases em modelo de câncer de mama, suprimindo a expressão de genes associados a apoptose (Erin et al. 2004). Em estudo *in* vitro com células tumorais de próstata LNCaP, a capsaicina induziu a proliferação aumentando a expressão de receptores de andrógeno pela ativação da via das ERKs e Akt (Malagarie-Cazenave et al. 2009). As evidências de carcinogenicidade e co-carcinogenicidade da capsaicina ainda são escassas e limitadas, necessitando de modelos experimentais robustos e fundamentação mecanicistica que suporte esta hipótese (Bode and Dong 2011).

3.3.3 – Potencial quimiopreventivo

A capsaicina tem sido hipotetizada como potencial agente bloqueador devido as atividades inibitórias que exerce em diversas enzimas do metabolismo oxidativo, com

especial destaque para a CYP2E1 (O'Neill et al. 2012). Dentre as diversas classes de enzimas CYPs, o isotipo 2E1 é de particular interesse devido ao seu envolvimento com a ativação metabólica de muitos procarcinógenos de baixo peso molecular, sendo responsável pela etapa de iniciação de diversas neoplasias associadas a carcinogênese química (Chanda et al. 2008). Embora amplamente citada na literatura, a atividade inibidora da CYP2E1 pela capsaicina é descrita em apenas um artigo (Reilly and Yost 2006), enquanto diversos outros trabalhos questionam esta atividade (Zhang et al. 1993; Babbar et al. 2009; Zhang et al. 2012).

Em contrapartida, a atividade supressora da capsaicina tem sido amplamente relatada na literatura (O'Neill et al. 2012; Clark and Lee 2016). Estudos demonstram que a capacidade de supressão do crescimento tumoral pela capsaicina pode ser mediada primariamente pela indução da apoptose (Amantini et al. 2009; Ip et al. 2012; Bozok Cetintas et al. 2014) Adicionalmente, outras atividades supressoras associadas aos efeitos supressores da capsaicina incluem a inibição da progressão do ciclo celular, regulação da expressão de fatores de transcrição e supressão dos sinais de crescimento. (Brown et al. 2010; Bozok Cetintas et al. 2014). A apoptose mediada pelo TRPV1 envolve as vias extrínseca e intrínseca da apoptose (Clark and Lee 2016).

A via extrínseca da apoptose é iniciada pelo estímulo dos receptores de morte celular que induzem a formação do complexo DISC (*death-inducing signaling complex*) que, subsequentemente, promove a ativação da caspase 8 (Hassan et al. 2014). Em um estudo que utilizou células tumorais uroteliais, a ativação do receptor TRPV1 pela capsaicina foi capaz de induzir a apoptose mediada pela Faz/CD95. Os autores observaram que a exposição à capsaicina aumentou significativamente a expressão de mRNAs da Faz/CD95, induzindo uma distribuição e agrupamento destes receptores em co-localização com o TRPV1. Estes achados sugerem que os clusters formados pela interação entre Faz/CD95 e TRPV1 resultam na indução do estímulo apoptótico deflagrado pela formação do complexo de sinalização (Amantini et al. 2009). Outro estudo corrobora com esta hipótese ao demonstrar que a porção N-terminal do TRPV1 é capaz de se ligar a FAF-1 (*FAS-associated factor-1*), uma proteína pró-apoptótica associada ao Faz/CD95 (Kim et al. 2006).

A via intrínseca é iniciada por vários sinais intracelulares que causam injúria na célula (e.g. dano de DNA, estresse do retículo endoplasmático) e envolve a resposta mitocondrial (Elmore 2007). A ativação do receptor TRVP1 pela interação com a capsaicina promove a abertura do canal catiônico levando ao aumento do cálcio intracelular, alterando a permeabilidade da membrana mitocondrial e promovendo a liberação proteínas pró-apoptóticas como o citocromo c e o fator indutor de apoptose (AIF) (Lin et al. 2013).

Consequentemente, ocorre a ativação da procaspase-9 pelo complexo ativador denominado apoptossomo, resultante da interação entre o citrocromo c e o AIF (Hassan et al. 2014). Outros mecanismos alternativos que corroboram para as vias extrínseca e intrínseca foram observados, como a capacidade de inibição atividade enzimática de superóxido dismustases, catalases e glutationaperoxidases (Taylor, Cullen, and Martin 2008). A capsaicina também foi capaz de induzir a inibição dos complexos I e III da cadeia transportadora de elétrons em células neoplásicas, porém, curiosamente nenhum efeito de inibição foi observado quando administrada em células normais (Pramanik, Boreddy, and Srivastava 2011).

Com base nestas premissas, é possível que a administração de capsaicina suprima os efeitos carcinogênicos da DMH no cólon, atuando como um agente quimioprotetor. De fato, em artigo prévio, já foi demonstrado que a administração da capsaicina (500 ppm) na ração antes e durante a fase de iniciação induzida pelo AOM reduz o desenvolvimento de FCA e tumores colônicos em ratos Fischer 344 (Yoshitani et al. 2001). Nesta perspectiva, nosso estudo avaliou os efeitos da potencial interação entre a capsaicina e a DMH, quando administrada na fase de iniciação da carcinogênese de cólon.

4 – Hipóteses

Nossas hipóteses baseiam-se em três principais proposições: (i) a capsaicina possui potencial antigenotóxico através da inibição de enzimas do metabolismo oxidativo relacionadas a ativação de carcinógenos; (ii) a capsaicina possui capacidade anti-proliferativa, através da regulação de fatores transcricionais e inibição do ciclo celular; (iii) a capsaicina possui propriedade pró-apoptótica, pela ativação das vias extrínseca e intrínseca.

5 – Objetivo

O objetivo deste estudo foi investigar os efeitos da administração de capsaicina nas doses de 5 mg/kg e 50 mg/kg na etapa de iniciação da carcinogênese de cólon induzida pela 1,2-dimetilhidrazina em ratos.

5.1 – Objetivos específicos

- Avaliar o potencial efeito genotóxico da ingestão de capsaicina em leucócitos de sangue periférico e da água fecal em células CaCO-2.

- Avaliar a expressão imunoistoquímica de Ki-67 e caspase-3 ativada no cólon dos animais tratados com capsaicina.

- Investigar o potencial efeito protetor da capsaicina sobre a expressão de genes que regulam as vias de biotransformação, atividade antioxidante, proliferação celular e apoptose e de dano e reparo de DNA na mucosa colônica, após a administração da DMH ou EDTA.

- Analisar o efeito da ingestão da capsaicina na iniciação da carcinogênese, no desenvolvimento de lesões pré-neoplásicas (focos de criptas aberrates, FCA) e tumores colônicos induzidos pela DMH nas doses de 5 e 50mg/kg.

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Capítulo 2

Article | **Capsaicin modulates cell proliferation, apoptosis and suppress colonic pre-neoplastic lesions induced by 1,2-dimethylhydrazine in rats**

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Abstract

Capsaicin (8-Methyl-N-vanillyl-(trans)-6-nonenamide), a lipophilic alkaloid compound, is the major pungent ingredient found in red peppers consumed worldwide. Most reports on capsaicin potential mutagenicity and genotoxicity have yielded inconsistent findings. In this study, we evaluated capsaicin putative genotoxicity and molecular mechanisms underlying anti-proliferative and pro-apoptotic effects of capsaicin on DMH-induced rat colon carcinogenesis. Male Wistar rats were randomly assigned into six experimental groups (n=16 each). During the first four weeks, corn oil was given to groups 1 and 6, while intragastric capsaicin was administered at 5mg/kg to groups 2 and 4, and at 50mg/kg to groups 3 and 5, three times/week. On weeks 3 and 4, the animals received subcutaneous injections of either DMH (groups 1-3, 40mg/kg) or EDTA (groups 4-6, vehicle), twice a week. The animals were sacrificed 24 hours (n=6) and 22 weeks (n=10) after DMH treatment. Capsaicin significantly decreased DMH-induced genotoxicity in peripheral blood leukocytes and fecal water genotoxicity in CaCO-2 cells, 24 hours after the last DMH administration. Capsaicin also reduced Ki-67 proliferation index and increased caspase-3 apoptosis in the colon from the DMH-treated animals. Evaluation of differential gene expression showed that capsaicin administration up-regulated genes associated with adaptive response to chemicals (Mapk1, Map14 and Smad-4) apoptosis (Aifm1, Caspase-4, Dffb), tissue development and cell differentiation (Foxa1 and Cdh1). High dose of capsaicin reduced the number of aberrant crypt foci (ACF) and increased the number of small, well differentiated and non-invasive tumors, 22 weeks after DMH-treatment. These findings revealed that capsaicin was able to suppress cell proliferation and to induce apoptosis via NF-κB regulation and endoplasmic reticulum (ER)-stress induction, as well as to modulate genes involved in tissue development and cell differentiation, reducing the late formation of ACF preneoplastic lesions and tumors.

+ **Keywords:** colorectal cancer; capsaicin; chemoprevention.

1. Introduction

Colorectal cancer (CRC) is the third most common type of cancer and a leading cause of death among men and women worldwide (Torre et al. 2015). Based on the last World Health Organization (WHO) GLOBOCAN estimates for 2015, CRC burden represented up to 9.7% of all incident malignancies, accounting 746,000 new cases in men and 614,000 in women (Ferlay et al. 2015). The incidence and mortality rates of CRC vary greatly across the world (Kamangar, Dores, and Anderson 2006). However, a demographic concentration is found in developed countries and correlates with western diet and lifestyle patterns, such as smoking, alcohol consumption, obesity and physical inactivity (Stewart 2014). Many of these risk factors are potentially modifiable and avoidable with specific public strategies for cancer prevention. In this regard, reducing consumption of refined starches, saturated fat, and processed or red meat, as well as increasing intake of fruits and vegetables have been associated with lower risk of developing CRC (Dahham and Majid 2016; Carr et al. 2016).

Several bioactive compounds found in foods, vegetables and medicinal plants can reduce the risk of developing chronic diseases, including cancer (Sales, Pelegrini, and Goersch 2014). Many classes of plant-derived bioactive compounds have been major sources for drug discovery used in traditional, complementary and alternative medicine (Shukla and Mehta 2015). Recently, natural bioactive compounds with anticancer activity have been acknowledged with great public enthusiasm. Fruits and vegetables play an essential role in human nutrition and health, providing natural fibers, antioxidants, and a broad range of bioactive phytochemicals (Liu 2013). Therefore, evidence from many pre-clinical and interventional studies support the concept that nutrition and appropriated diet stand as a promising strategy for CRC chemoprevention (Hou, Huo, and Dignam 2013; Baena and Salinas 2015).

The chili pepper is the fruit of herbaceous plants from the genus *Capsicum*, members of the family *Solanacea* and native to the Americas. Chilies have long been domesticated by Mesoamerican civilization and appreciated worldwide for culinary and medicinal purposes (Heiser and Smith 1953). In fact, chili peppers represent a fair amount of total vegetables consumed in daily diets around the world (Kantar et al. 2016). Capsaicin (8-Methyl-N-vanillyl-trans-6-nonenamide) is the major pungent alkaloid ingredient found in chili peppers (Bosland, Votava, and Votava 2012). Recently, capsaicin has emerged as a potential therapeutic drug to treat many human diseases, including pain, obesity, diabetes, cardiovascular conditions, airway disease and cancer (Fattori et al. 2016). Experimentally,

capsaicin has been show to exert anti-proliferative and pro-apoptotic activities in many cancer cell lines (Díaz-Laviada 2010; Brown et al. 2010; Lau et al. 2014; Garufi et al. 2016). Capsaicin has also been hypothesized to interact with a number of cytochrome P450 enzymes (CYPs), altering the metabolism of chemical carcinogens (Zhang et al. 2012).

Nonetheless, most scientific reports on capsaicin potential carcinogenicity have yielded inconsistent findings (Bode and Dong 2011). Capsaicin adverse effects at high dosages have been associated with mutagenicity and carcinogenicity as reviewed by Lee and Park (2003). Furthermore, several preclinical studies have suggested that chili extract or capsaicin alone can exert co-carcinogenic effects in stomach, liver, colon and skin in different chemically-induced carcinogenesis models (Agrawal et al. 1986; Díaz Barriga Arceo et al. 1995; Johnson 2007; Liu et al. 2015). Based on this controversy, the putative effects of capsaicin administration on the initiation stages of 1,2-dimethylhydrazine(DMH)-induced colon carcinogenesis in rats were evaluated. The ability of capsaicin to induce DNA damage, change cell proliferation, apoptosis and the expression of genes involved in oxidative metabolism, antioxidant activity, cell cycle, DNA repair and cell death were analyzed.

2. Material and Methods

2.1 – Chemicals

Capsaicin (8-Methyl-N-vanillyl-trans-6-nonenamide, purity ≥95%, PubChem CID:1548943) and DMH(1,2-dimethylhydrazine hydrochloride, PubChem CID: 1322) were purchased from Sigma-Aldrich (Darmstadt, Germany). All other reagents were of the highest grade available commercially.

2.2 – Study Design

Four-week-old male Wistar rats weighing 125g (ANILAB, Paulínia-SP, Brazil) were housed in polypropylene cages under standard conditions (21 ± 2 °C temperature, 55 ± 10% humidity, and 12h/12h light-dark cycle) with food (NUVILAB-CR-1, Curitiba, Brazil) and tap water *ad libitum*. The animals used in this study were handled in accordance with the principles of laboratory animal care adopted by the Brazilian College of Animal Experimentation (COBEA). This study was approved by the institution's Ethics Review Board (1153/2015-CEUA, Supplementary Material - SM1).

After a 3-week acclimation period, the animals (7-week old) were randomly assigned into six experimental groups of 16 animals each. During the first four weeks, groups received

intragastric doses of corn oil (capsaicin vehicle, G1 and G6), capsaicin at 5mg/kg body weight (bw) (G2 and G4) and 50mg/kg bw (G3 and G5) three times a week. The capsaicin dosages used in this study were based on previous reports (Saito and Yamamoto 1996). On weeks 3 and 4, groups received a subcutaneous injection of DMH (G1, G2 and G3, 40 mg/kg bw) twice a week or EDTA (DMH vehicle, G4, G5 and G6). Body weight and food consumption was recorded weekly. Following capsaicin treatment, the animals were sacrificed 24 hours (short term assays, n=6) and 22 weeks (medium term assays, n=10) after the last DMH administration, respectively (Figure 1).

- Short term assays:

2.3 – DNA damage in peripheral blood leukocytes

Capsaicin genotoxic potential was assessed by DNA damage in peripheral blood leukocytes using the single cell gel electrophoresis (comet) assay. The comet assay on whole blood was performed 24 hours after the last DMH administration under alkaline conditions following the procedure described by Nandhakumar et al. (2011). Peripheral blood samples were collected by retroorbital venipuncture, mixed with 100 µL of low melting point agarose (0.75% in PBS, Invitrogen, USA.), spread on slides pre-coated with normal point agarose (1.5% in PBS, Invitrogen, USA), and coverslipped. Following agarose solidification (4°C for 10 min), coverslips were carefully removed and the slides were incubated overnight into cold lysis solution (2.5M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, 1% sarkosyl, pH 10) at 4°C. Subsequently, the slides were washed three times in PBS and immersed in fresh cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 min. Electrophoresis was conducted at room temperature of 21°C for 20 min at electric field strength 1 V/cm (300mA) for 20 min. The slides were then neutralized with 0.4 M Tris (pH 7.5), dehydrated in 100% ethanol and stained with Sybr Gold (Invitrogen, USA). Readings were performed under an epi-fluorescence microscope (Olympus BX-50, Japan) coupled to a CCD camera. Fifty random nucleoids/sample were scored using Comet Assay IV Image Analysis System (Perceptive Instruments, UK). All experiments and analysis were performed in duplicate.

2.4 – Fecal water genotoxicity

Cecal feces were collected at sacrifice and kept frozen at -20°C prior to use. Fecal water was prepared as described by Klinder et al. (2007) with minor modifications. Briefly, fecal slurry was prepared by mixing feces with ice-cold PBS in a 1:1 proportion (1g of fecal

content + 1 mL of PBS) and homogenizing it for 3 min. Fecal debris was removed by centrifuging homogenates at 35,000 g for 30 min. The supernatant was filtered with an Ø 0.22 µM sterile filter unit (Millipore, Germany), aliquoted and frozen until analysis.

Caco-2 (human colon adenocarcinoma) cells were obtained from Rio de Janeiro Cell Bank (BCRJ, Brazil) and grown in 75cm² culture flaks in DMEM high-glucose medium supplemented with 10% fetal bovine serum, 0.1 nM non-essential amino acids, 50 µg/mL streptomycin in a humid 5% CO_2 atmosphere at 37°C. CaCO-2 cells between passages 38 and 39 were used in the analysis of fecal water genotoxicity. Upon reaching the confluence, cells were harvested with Accutase cell detachment solution (Sigma Aldrich, USA), split into 1-mL centrifuged tubes and spun at 1,200 g for 1 min. The supernatant was removed and cells were directly incubated with 100% fecal water at 37°C for 30min. Cell viability was determined by the trypan blue exclusion assay. The remaining cell pellet was then mixed with 100 µL of low melting point agarose (0.75% in PBS) and spread on slides pre-coated with normal point agarose (1.5% in PBS), and coverslipped. Fecal water genotoxicity in CaCO-2 cells was determined by the comet assay performed as described in the previous section.

2.5 – Serum biochemistry and tissue collection

Six animals from each group were sacrificed 24 hours after the last DMH injection. Blood samples were collected by cardiac puncture under xylazine and ketamine anesthesia (10mg/kg and 80mg/kg bw, respectively). Determination of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity was performed on COBAS 6000 (Roche Diagnostics, USA) using commercial kits. After laparotomy, colon and liver tissue fragments were collected and either stored at –80°C for RNA extraction, or fixed in 4% buffered formalin and stored in 70% ethanol for histopathological and immunohistochemistry analyses.

2.6 – RNA isolation and reverse transcription

Total RNA was extracted from frozen colonic samples using Rneasy Mini kit (Qiagen, Hilden, Germany) followed by on-column DNA digestion. RNA samples were solubilized in nuclease-free water (Qiagen, Hilden, Germany) and their concentration and integrity were evaluated on the NanoVue™ Plus (GE Healthcare) and the Agilent 2100 bioanalyzer (Agilent Technologies, Boeblingen, Germany), respectively. Equal quantities (60 ng/µl) of total RNA from each sample were reverse-transcribed to first-strand cDNA using

SuperScript IV First Strand SuperMix (Invitrogen™, Life Tech, USA) according to manufacturer's instruction.

2.7 – Quantitative real-time PCR

RNA expression profiles were compiled using a 96-well TaqMan® Array Cards (TAC)-based real-time polymerase chain reaction (PCR). The custom TAC assessed 91 genes involved in oxidative metabolism, pro- and antioxidant activity, cell proliferation, DNA damage, DNA repair and apoptosis (Supplementary Data, SD1). β-Actin, Gapdh, Gusb and Hprt1 were used as housekeeping genes to normalize mRNA expression. Target genes were amplified using the TaqMan® Universal Mastermix II (Life Technologies, USA) by a cycling protocol of heat activation at 50°C for 1 min and denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Fluorescence detection was performed on QuantStudio™ 12K Flex Real-Time PCR System (Life Technologies, USA). The relative expression of target genes was analyzed by comparative Ct method performed using ExpressionSuite™ software (Life Technologies, USA). Functional enrichment analysis was conducted using Gene Ontology annotation tool (Ashburner et al. 2000).

2.8 – Immunohistochemistry analysis

Paraffin-embedded colon sections of 5μm-thick were deparaffinized and rehydrated in a graded xylene-alcohol series. Antigen retrieval was performed using a 10nM sodium citrate buffer solution by pressure-cooker heating (Pascal, Dako). Endogenous peroxidase was quenched with 10% hydrogen peroxide solution for 10 min. Tissues sections were incubated with blocking solution (7% skimmed milk in PBS) for 1h and then immune-stained with primary antibodies for Ki-67 (Abcam no. 15580) and active Caspase-3 (Abcam no. ab2302) overnight. Thereafter, sections were washed three times in PBS and incubated with one-step universal HRP polymer (Easy Path, USA) for 25min. Tissue sections were stained for 5 min using DAB as chromogen and counter-stained with Harry's hematoxylin for 1 min. For immunohistochemistry analysis, 6 rats from each group were analyzed and 25 crypts scored per animal. Ki-67 and active Caspase-3 labeling indexes (LI) were scored by the ratio derived from the number of positive-stained cells out of the total number of cells per crypt.

- Medium term assays:

2.9 – Tumor volume and histopathological analysis

Ten animals from each group were sacrificed at the end of the week 22. After laparotomy, their colon specimens were removed, opened longitudinally and pinned flat. Colon specimens were fixed in 10% phosphate-buffered formalin for 24h and kept in ethanol 70% prior to analysis. Macroscopic tumors were counted, removed, and their sizes were measured *ex vivo* using a digital caliper. Tumor volumes were calculated using an prolate spheroid formula 4/3 x 3.14 x (length/2) x (width/2) x (depth/2) (Schiavon et al. 2012). Colon specimens were paraffin embedded and sectioned for histopathological analysis. Macroscopy and microscopy colon adenocarcinomas were classified into invasive (tubular or mucinous) and non-invasive (carcinoma *in situ*) categories according to the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (Nolte et al. 2016).

2.10 – Identification and Quantification of ACF

For identification of aberrant crypt foci (ACF) pre-neoplastic lesions, formalin-fixed colon specimens were divided into 3 segments (proximal, medial and distal) and stained in 0.2% methylene blue for 5 min. The total number of ACF and the number of aberrant crypts (AC) were counted under light microscopy. The ACF were identified topographically according to Bird's morphological criteria (Bird 1987): (i) increased size; (ii) thickened epithelial cell lining; (iii) increased pericryptal space and (iv) irregular lumens. Since ACF size is closely related to the risk of developing colon tumors, ACF were divided into 3 categories: 1-3 crypts/focus, 4-8 crypts/focus and ≥ 9 crypts/focus (Corpet and Taché 2002).

2.12 – Statistical Analysis

Data were statistically evaluated using Prism v6 (GraphPad). One-way ANOVA analysis was performed for statistical comparison among groups followed by *post hoc* Tukey's test. Fisher's exact test was used to compare tumor incidence and histopathological categories. To identify significant differences in gene expression, the normalized expression means were compared using Student's t-test. $p<0.05$ was regarded as statistically significant.

3. Results

- Short term assays:

3.1 – DNA Damage in peripheral blood leukocytes

Figure 2A e D shows the DNA damage in peripheral blood leukocytes of capsaicin-treated and controls groups. The levels of DNA damage in DMH-treated rats were significantly higher than in the respective control groups (G4, G5 and G6, p=0.0001). Capsaicin administration at 50mg/kg (G3) significantly decreased DMH-induced genotoxicity when compared to G1 and G2 groups (p=0.0001). Capsaicin treatments alone did not induce DNA damage when compared to G6.

3.2 – Fecal water genotoxicity

Figure 2C and D shows the effects of capsaicin administration on the fecal water genotoxicity of DMH and EDTA-treated rats. Exposure to fecal water did not change CaCO-2 cell viability in the trypan blue exclusion assay, in all groups (data not shown). The fecal water genotoxicity of DMH-treated rats was significantly higher (p=0.004) than in the respective untreated control groups (G4, G5 and G6, Figure 2C). Capsaicin administration at 50mg/kg (G3) significantly reduced fecal water genotoxicity (p=0.004) induced by DMH when compared to the respective DMH-treated groups (G1 and G2, Figure 2C). Capsaicin administration alone at both doses, 5 and 50 mg/kg (G4 and G5) did not increase fecal water genotoxicity, when compared to the untreated group (G6).

3.3 – Body parameters, liver weight, serum biochemistry and histopathology

Table 1 shows the body parameters, liver weight and serum biochemistry of the rats from the different groups during the 4 and 22-week periods of study. In the first four weeks, a reduction in body weight was observed in all DMH-induce rats, but not statistically significant. DMH-induced rats exhibited a significant elevation in ALT and AST serum levels ($p<0.0005$), consistent with the toxic effects of DMH in the liver. No differences in food intake and liver relative weight were observed.

At the end of week 4, histopathological assessment of the colonic mucosa showed toxic lesions exhibiting crypt distortions, depletion of goblet cells, and increased apoptosis were also found in the colon of DMH-treated groups (Figure 3A). Capsaicin administration did not induce or enhanced toxicity in the colon of DMH-treated and control animals (Figure

3B). No differences in body weight and liver relative weight among the groups were found at the end of the week 22 (Table 1).

3.4 – Evaluation of differential gene expression

Table 2 shows differentially expressed genes in the colonic mucosa from capsaicin-treated groups as compared with the control groups (G1 and G6). We found three genes differentially expressed in both groups receiving capsaicin 5mg/kg (G2 and G4, Table 4) when compared to G1 and G6, respectively. Moreover, capsaicin administration at 50mg/kg induced the differential expression of fifteen genes in the DMH-treated group (G3, Table 2). Functional enrichment analysis performed with Gene Ontology annotation database demonstrated that these groups of upregulated genes belonged to relevant functional categories involved in adaptive response to chemicals, apoptosis and tissue development (Table 3).

3.5 – Ki-67 and active caspase-3 immunohistochemistry

As shown in Figure 4A, capsaicin administration at 50mg/kg (G3) significantly reduced (20%, p=0.0001) Ki-67 proliferation index when compared to the respective DMH-treated groups (G1 and G2). For active caspase-3, a trend towards increasing apoptosis index was observed in the capsaicin 50mg/kg DMH-treated group (G3) in comparison with the untreated groups (Figure 4C, p=0.059). Ki-67 and caspase-3 labeling indexes were similar among DMH-untreated groups (G4-G6).

- Medium term assays:

3.6 – Tumor volume and histopathological analysis

Tumor volumes were classified as small (< 30 mm^3), medium (30-60 mm^3) and large (>60 mm^3) are presented in Figure 6. There was an increase of small tumors (35%) in rats receiving capsaicin at 50mg/kg (G3) when compared with the G1, even though not statistically significant (p=0.147). The average tumor volume in control animals was 105 ± 95 mm^3, while those of rats receiving capsaicin 5 and 50mg/kg were 34 ± 38 mm^3 and 59 ± 47 mm^3, respectively.

The incidence of colon tumors was similar in DMH and capsaicin-treated groups (Table 4). Histopathological analysis showed that DMH-induced tumors proved to be well-differentiated tubular adenocarcinomas (Figure 6C and 6D) or poorly-differentiated mucinous adenocarcinomas (Figure 6E and 6F). There was no difference in tumor multiplicity or

incidence among the groups. However, no mucinous tumors were found in the capsaicin 50mg/kg group (G3), confirming an increased tendency towards well-differentiated and *in situ* carcinomas when compared to the remaining groups.

3.7 – ACF formation

Table 5 summarizes the effects of capsaicin administration on DMH-induced ACF formation. All DMH-treated animals developed ACF in the colon 22 weeks after the last DMH administration, while no ACF was observed in the EDTA-vehicle treated groups. Capsaicin administration at 50mg/kg (G3) significantly suppressed ($0.0008 < p < 0.0209$) the number of ACF consisting of 1-3, and ≥ 10 crypts/focus, as well as the total number of AC and ACF ($0.0209 < p < 0.0244$), when compared to the respective control group (G1). Capsaicin administration at 50mg/kg (G3) was significantly more effective ($0.0008 < p < 0.0244$) than 5mg/kg (G2). Figure 5 shows light-micrographs of normal crypts (5A) and an ACF (5B) with 7 AC in methylene blue stained colonic mucosa.

4. Discussion

In the present study we evaluated capsaicin putative genotoxicity and molecular mechanisms underlying anti-proliferative and pro-apoptotic effects of capsaicin on DMH-induced rat colon carcinogenesis. We provided mechanistic insights on the suppressive role of capsaicin in colon carcinogenesis, when administered before and during DMH regimen. In the short-term analysis, capsaicin 50 mg/kg suppressed the noxious stimuli of DMH-induced cytotoxicity and genotoxicity in the colonic mucosa, resulting in anti-proliferative and pro-apoptotic response through up-regulated expression of genes involved in apoptosis, cell cycle suppression and differentiation. In the medium-term analysis, capsaicin 50 mg/kg suppressed ACF development, multiplicity and modulated tumor volume and invasiveness.

Previous studies have shown that capsaicin possesses substantial antimutagenic and antigenotoxic activities against different chemical mutagens (Huynh and Teel 2005; Hassan et al. 2012; Fernández-Bedmar and Alonso-Moraga 2016). Our findings indicated that capsaicin at 50mg/kg decreased DMH-induced genotoxicity in peripheral blood leukocytes and fecal water genotoxicity in CaCO-2 cells. Moreover, we found that capsaicin regimen and doses used in the present study did not exhibit genotoxicity, as evaluated by the comet assay in peripheral blood leukocytes. In a similar fashion, fecal water obtained from the untreated groups receiving capsaicin did not increase DNA damage in CACO-2 cells.

Together, these findings suggest that capsaicin possess a potent anti-oxidant activity and may inhibit free-radicals involved in double- and single-stranded DNA breaks (De et al. 1995; Proudlock, Thompson, and Longstaff 2004; Melgar-Lalanne et al. 2017). Most studies on capsaicin potential genotoxicity and mutagenicity have evaluated chili extracts or capsaicin with different levels of purity, as reviewed by Bley et al. (2012). Besides, analytical studies have reported capsaicin contamination with organic phosphates, pesticides, fusarium and aflatoxin (Proudlock, Thompson, and Longstaff 2004; Johnson 2007; Kuzma et al. 2014). Therefore, evidence supporting capsaicin genotoxicity remains circumstantial, while it may have an important potential to promote metabolic health (Bley et al. 2012; McCarty, DiNicolantonio, and O'Keefe 2015).

Methyldiazonium ion is the ultimate DMH carcinogenic metabolite responsible for methylation of DNA bases that induce genotoxic stress and trigger NF-κB activation in colonic epithelial cells. (Tanwar, Vaish, and Sanyal 2009; Perše and Cerar 2011). Nuclear factor kappa B (NF-κB), an important mediator for cellular responses to DNA damage, has been shown to facilitate cell escape from letal effects of DNA damage, stimulate cell growth, and induce cell proliferation (Hoesel and Schmid 2013). In our study, we found that capsaicin administration increased the expression of NF-κB and Ikbkg gene, a regulatory subunit of the kappaB kinase (NEMO/IKKγ) complex that phosphorylates and activatesNF-κB (Salminen, Kauppinen, and Kaarniranta 2012). Conversely, capsaicin has also induced the expression of NF-κB inhbitors, such as Mapk3, Mapk14 and Smad4 genes. Mitogen-activated protein kinase (Mapk)-14 gene encodes the protein p38α, a member of a family of serine/threonine stress-activated protein kinases. Activation of p38α has been reported in response to a variety of extracellular stimuli, including genotoxic stress induced by chemicals (Igea and Nebreda 2015). Several studies have shown that p38α expression promotes apoptosis and regulates NF-κB activity (Olson et al. 2007; Gil-Araujo et al. 2014; Igea and Nebreda 2015). Although Mapk3, an extracellular signal-regulated kinase (ERK)-1, has been often regarded as an oncogenic driver, studies have demonstrated that ERK1 pathway can also play a role regulating cell proliferation and differentiation of colonic cells (Sun and Sinicrope 2005; Baba et al. 2010; Urosevic, Nebreda, and Gomis 2014).

Smad4 encodes a protein that is a central regulator of the TGFβ pathway, and its downregulated expression is associated with poor prognosis in a number of gastrointestinal carcinomas (Davison et al. 2014; Zhang et al. 2014). In the colon, alterations in Smad4 expression results in resistance to growth inhibition and uncontrolled cell proliferation (Handra-Luca, Olschwang, and Fléjou 2011; Dienstmann et al. 2017). Therefore, our results

showed that capsaicin 50mg/kg suppressed Ki-67 proliferation indexes under carcinogen exposure. This result is consistent with the concomitant expression of Mapk3, Mapk14 and Smad-4 genes, involved in the suppression of NF-κB activation, cell growth and proliferation. Our results are in agreement with the literature, showing that capsaicin exerts a potent anti-proliferative activity in many tumor cell lines (Aggarwal and Shishodia 2004; Brown et al. 2010; Qian et al. 2016).

Oral administration of capsaicin before and during DMH treatment markedly induced the expression of apoptosis-related genes in the colonic mucosa, including Casp-4, Sp1, Aifm1 and Dffb. These results are in line with the immunohistochemical findings in which an increase of caspase-3 expression was observed. Based on these results, it is possible that increased expression of Casp-4, Sp1 and Aifm genes can be related to endoplasmic reticulum (ER) stress-induced apoptosis (Gong et al. 2017). Evidences from different human tumor cell lines suggest that ER-stress and mitochondria-mediated death pathways are involved in capsaicin-induced apoptosis (Ip et al. 2012; Krizanova et al. 2014). ER stress occurs when the cellular demand for ER function exceeds its capacity, accumulating unfolded protein aggregates that triggers a cascade of key events called unfolded protein response (UPR) (Sano and Reed 2013). ER stress plays critical roles in many cellular functions, including protein synthesis, folding, storage and calcium releasing (Schröder 2008).

The caspase-4 gene (Casp4) is ubiquitously expressed in various tissues and play a key role in both apoptosis and inflammation (Man and Kanneganti 2016). Caspase-4 is mainly located to the ER and is closely associated with a number of ER-stress induced proteins (Bian, Elner, and Elner 2009). Capsaicin administration was reported to elicit activation of caspase-4 expression and to promote ER-stress induced cell death in human breast cell lines (Lee et al. 2009; Choi, Jung, and Oh 2010). In this regard, specificity protein-1 (Sp1) is an important mediator for the induction of the gene Cat-1, an essential amino acid transporter that plays a central role in the induction of UPR (Huang et al. 2010). Moreover, Sp1 overexpression has also been shown to inhibit cell cycle and induce p53-dependet apoptosis in many *in vitro* studies with tumor cell lines (Chuang et al. 2009; Deniaud et al. 2009; Li et al. 2014).

Capsaicin-induced apoptosis has been reported to cause calcium releasing from the ER and to increase transcriptional activation of pro-apoptotic and DNA-damage inducible genes (Thomas et al. 2011; O'Neill et al. 2012; Srivastava 2013). Among them, Aifm gene is regarded as an important effector for caspase-independent cell death (Tica Sedlar et al. 2016). In the present study we have found up-regulation of Aifm gene in the DMH-treated group

receiving capsaicin 50mg/kg. Aifm encodes an apoptosis-inducing factor that functions as an oxidoreductase in the inner mitochondrial membrane (H. Sun et al. 2016). Upon cell death stimuli, mitochondrial membrane disruption by increased cytoplasmic calcium concentration causes AIF release from the mitochondria and translocation to the nucleus (Daugas et al. 2000). AIF is capable of binding to DNA, causing chromatin condensation and DNA fragmentation independent of caspase activation (Cregan, Dawson, and Slack 2004).

In addition to the over-expression of genes involved in ER stress-induced apoptosis and mitochondria-mediated cell death, our results showed that capsaicin, at both doses has increased the expression of Dffb gene in the colonic mucosa. Dffb gene encodes the active subunit of the apoptotic nuclease DNA fragmentation factor (DFF), a heterodimeric protein consisting of two subunits (DFFA, 45-kD and DFFB, 40-kD) that triggers both DNA fragmentation and chromatin condensation during apoptosis (Samejima and Earnshaw 2005). DFFA is an inhibitor subunit that acts as substrate for caspase-3 activation. During apoptosis, activated caspase-3 induces DFFA cleavage, which releases DFFB that is subsequently translocated to the nucleus to trigger DNA fragmentation (Liu et al. 1997; Kitazumi and Tsukahara 2011). It has been recognized that DNA fragmentation factors such as DFF greatly contribute to genomic stability by ensuring the removal of DNA-damaged cells (Yan et al. 2006; Ohyashiki, Kuroda, and Ohyashiki 2017). Therefore, the increased expression of Dffb gene induced by capsaicin sheds light on a potential therapeutic target, as the chromosomal instability pathway underlies the majority of sporadic colon cancers (Pino and Chung 2010; Orsetti et al. 2014).

In the present study, functional enrichment analysis revealed that capsaicin administration up-regulated the expression of gene clusters associated with tissue development and cell differentiation pathways. These findings may be the molecular clue to the chemopreventive effect of capsaicin against the toxic effects of DMH in the colonic mucosa. Histopathological analysis showed that DMH treatment induced apoptosis, loss of goblet cell differentiation and crypt distortions. Following chemical insult and significant cell death, tissue loss is replaced via compensatory cell proliferation (Meier and Banreti 2016). In this scenario, capsaicin administration has been shown to modulate DMH-induced cell proliferation by increasing the expression of anti-proliferative and cell differentiation genes.

Our results showed an increased expression of Foxa-1 and Cdh1 genes in the 50mg/kg capsaicin-treated group. This finding is consistent with a cellular response towards cell differentiation. Forkhead box A1 (Foxa-1) is a transcription factor belonging to the forkhead family, known to play a pivotal role in the postnatal development and cell

differentiation (Bernardo and Keri 2012). Foxa-1 has a unique ability to act as a "pioneer transcription factor", increasing local chromatin accessibility and facilitating recruitment of other transcription factors (Yang et al. 2016). In the colon, Foxa-1 has been shown to modulate the secretory activity and control the differentiation of goblet cells (Ye and Kaestner 2009). Another important gene in cell differentiation pathway is Cdh1. Cdh1 encodes E-cadherin, a cell-cell adhesion glycoprotein that plays an important role in cell growth and invasion suppression. E-cadherin loss is an integral step in epithelial-mesenchymal transition (EMT) and has been associated with tumor progression, invasion and metastasis in CRC (Yun et al. 2014; Heerboth et al. 2015). Conversely, increased E-cadherin expression has been shown to decreased ERK1/2 phosphorylation, suggesting a suppressor role in the oncogenic KRAS pathway (Satow et al. 2014).

Aberrant crypt foci (ACF) are preneoplastic lesions easily identified in whole-mount colon stained with methylene blue, displaying genetic, epigenetic and morphological alterations similar to those observed in humans (Bird 1987; Rodrigues et al. 2002; Mori et al. 2004; Orlando et al. 2008). ACF have been adopted as biomarkers for screening studies of preventive agents in different models of colon carcinogenesis (Wargovich, Brown, and Morris 2010). In our study, capsaicin has been shown to reduce total AC and ACF formation, as well as ACF multiplicity. This result is in agreement with Yoshitani et al. in which dietary capsaicin at 500 ppm during the initiation phase of colon carcinogenesis significantly inhibited ACF formation and tumor development in azoxymethane (AOM)-induced male Fischer rats (Yoshitani et al. 2001). It has been shown that ACF with a high number of aberrant crypts are more likely to progress to adenomas and adenocarcinomas during colorectal carcinogenesis. In this regard, we observed that capsaicin 50mg/kg increased the number of non-invasive, well differentiated tumors, as well the number of small tumors. These results may reflect the suppression pathways in cell proliferation and increased apoptosis observed during the initiation phase of DMH-induced colon carcinogenesis.

5. Conclusion

In conclusion, our study revealed that capsaicin was able to suppress cell proliferation and induce apoptosis via NF-κB regulation and ER-stress induction, as well to modulate genes involved in tissue development and cell differentiation, reducing the formation of ACF preneoplastic lesions, tumor volume and aggressiveness.

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Figures and Tables

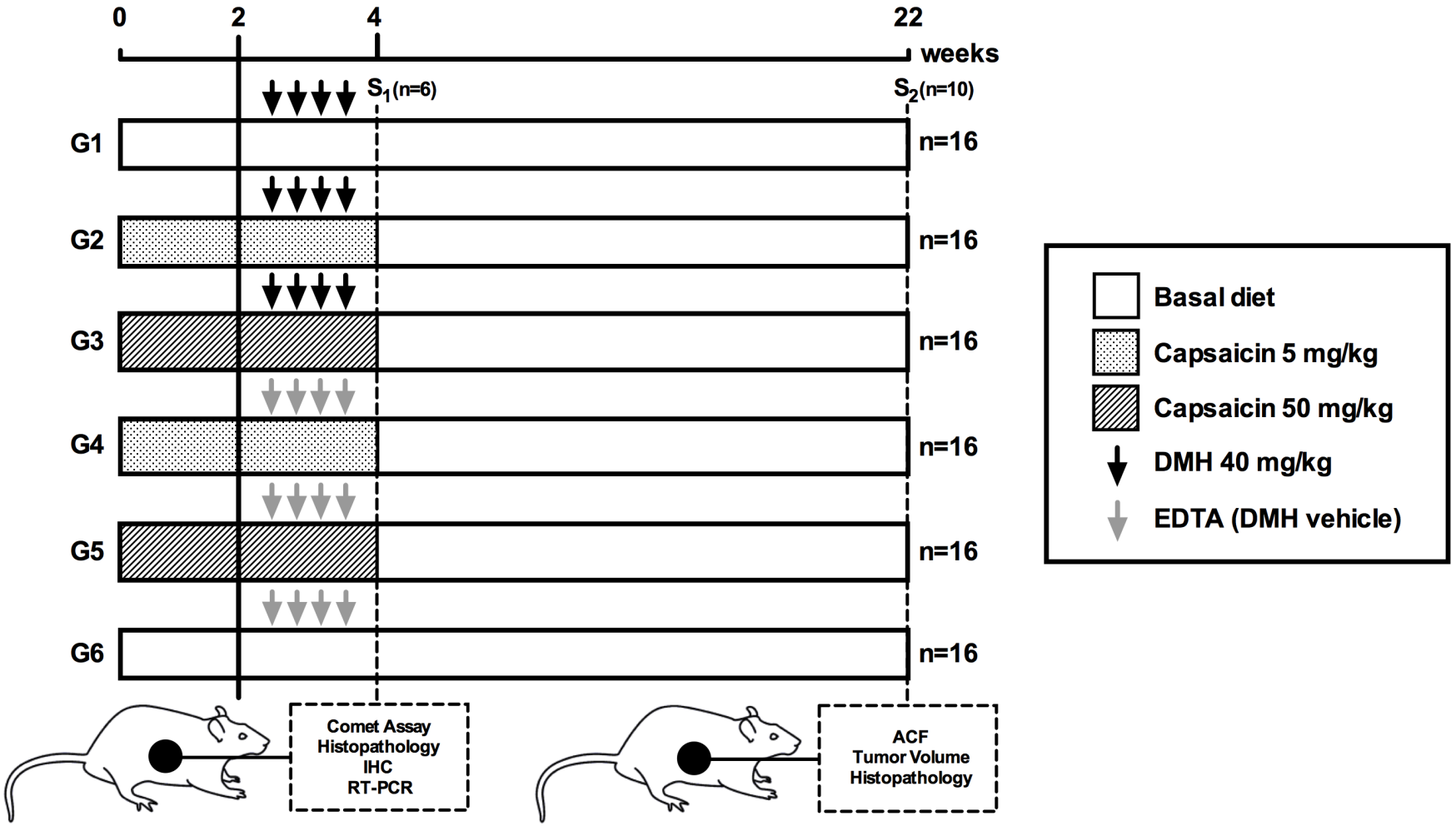

Figure 1 – Schematic diagram of the experimental protocol. G1: DMH + corn oil (capsaicin vehicle); G2: DMH + capsaicin 5 mg/kg bw; G3: DMH + capsaicin 50 mg/kg bw; G4: EDTA (DMH vehicle) + capsaicin 5 mg/kg bw; G5: EDTA + capsaicin 50 mg/kg bw; G6: EDTA + corn oil. S_1: sacrifice, 24h after DMH initation; S_2: sacrifice, 22 weeks after DMH initiation; DMH: 1,2-dimethylhidrazine; EDTA: ethylenediamine tetraacetic acid; IHC: immunohistochemistry; ACF : aberrant crypt foci.

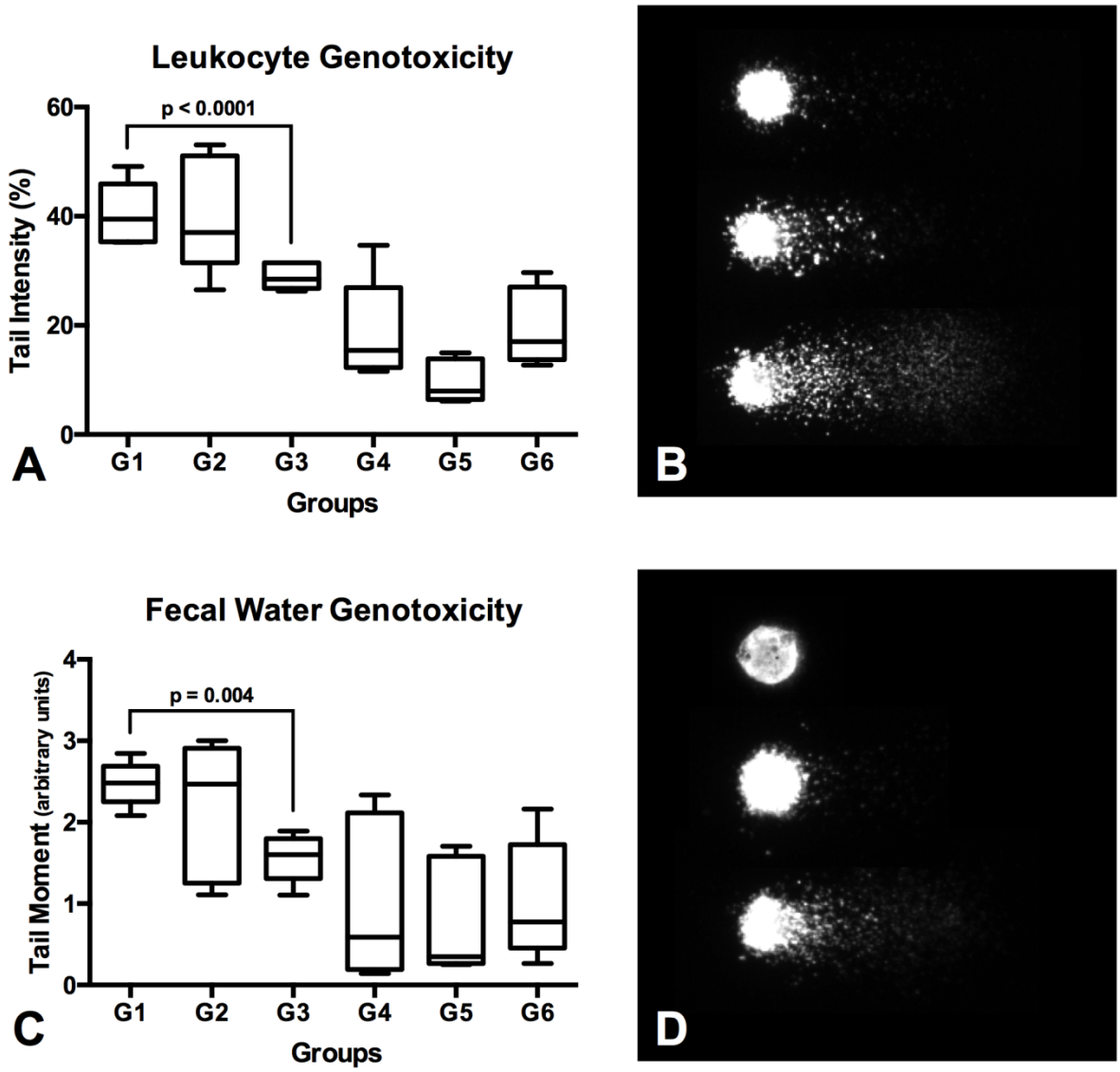

Figure 2 – Detection of DNA damage by the comet assay[1]. **(A)** Suppressing effects of capsaicin administration on DMH-induced genotoxicity in peripheral blood leukocytes. (**B**) Representative comet images showing different levels of DNA damage in peripheral blood leukocytes. (**C**) Suppressing effects of capsaicin administration on DMH-induced fecal water genotoxicity in CaCO-2 tumor cells. (**D**) Representative comet images showing different levels of DNA damage in CaCO-2 cells. [1]Data presented as box plot with median and interquartile ranges, compared by One-way ANOVA followed by *post hoc* Tukey's test. G1: DMH + corn oil (capsaicin vehicle); G2: DMH + capsaicin 5 mg/kg bw; G3: DMH + capsaicin 50 mg/kg bw; G4: EDTA (DMH vehicle) + capsaicin 5 mg/kg bw; G5: EDTA + capsaicin 50 mg/kg bw; G6: EDTA + corn oil. S: sacrifice; DMH: 1,2-dimethylhidrazine; EDTA: ethylenediamine tetraacetic acid.

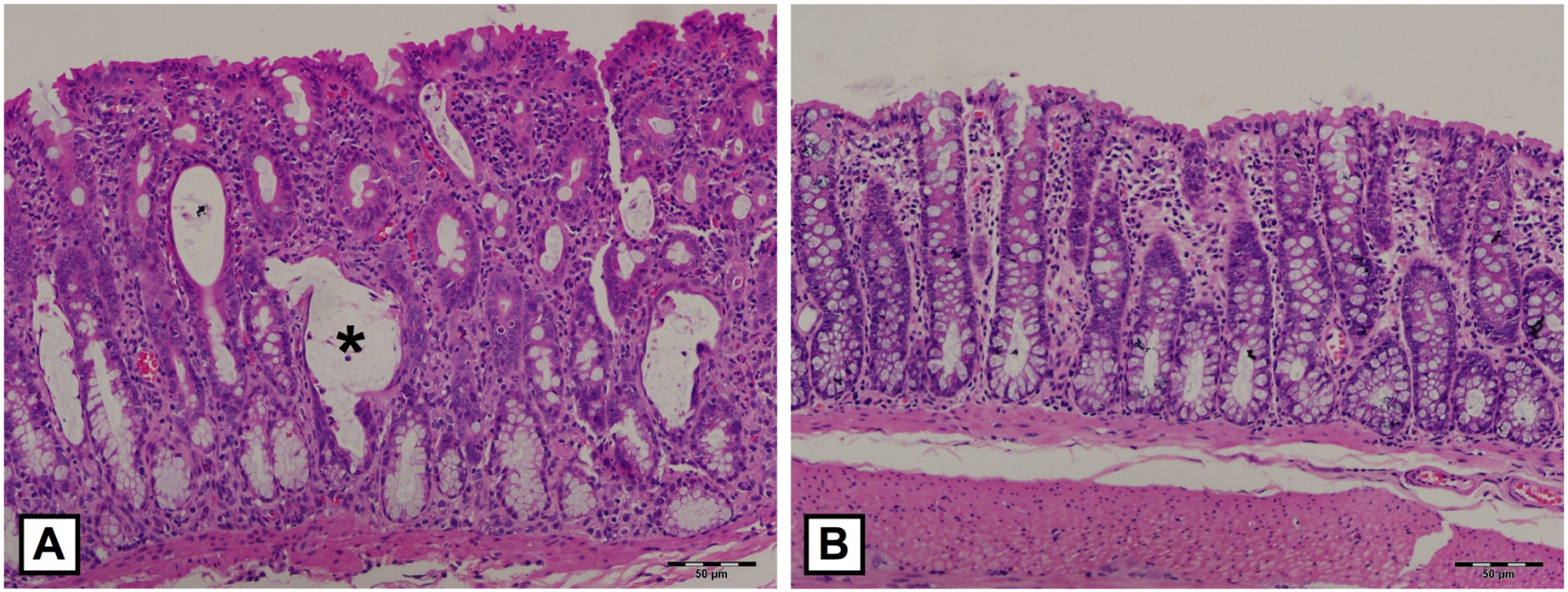

Figure 3 – Histopathology of colonic mucosa in the short-term (4 weeks) assay. (**A**) DMH-induced toxic lesions in the colonic mucosa, exhibiting crypt distortions (*), depletion of goblet cells, and increased apoptosis. (**B**) Normal histology features of the colon in EDTA-treated group.

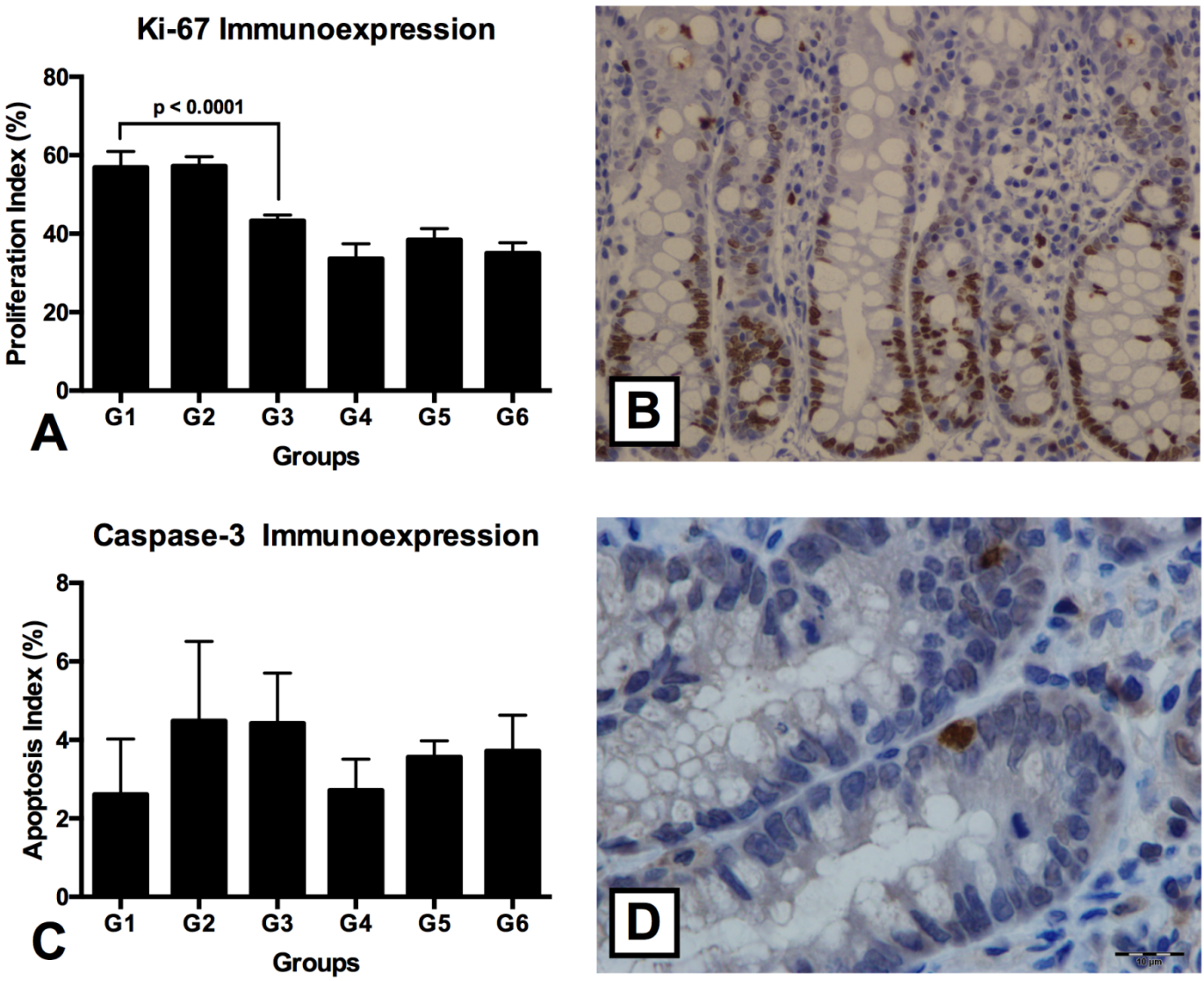

Figure 4 – Effects of capsaicin administration on Ki-67 and Caspase-3 labeling indexes (%) in the colonic crypts of DMH-treated and untreated animals.[1] (**A**) Ki-67 proliferation labelling indexes. (**B**) Representative immunohistochemical images of Ki-67 expression in colonic crypts of DMH-treated animals. (**C**) Active caspase-3 apoptosis labelling indexes. (**D**) Representative immuhistochemical staining of active caspase-3 in the rat colon. [1]Data present as mean ± SD for 6 rats/group. Differences between groups were determined using by One-way ANOVA followed by *post hoc* Tukey's test. G1: DMH + corn oil (capsaicin vehicle); G2: DMH + capsaicin 5 mg/kg bw; G3: DMH + capsaicin 50 mg/kg bw; G4: EDTA (DMH vehicle) + capsaicin 5 mg/kg bw; G5: EDTA + capsaicin 50 mg/kg bw; G6: EDTA + corn oil. S: sacrifice; DMH: 1,2-dimethylhidrazine; EDTA: ethylenediamine tetraacetic acid.

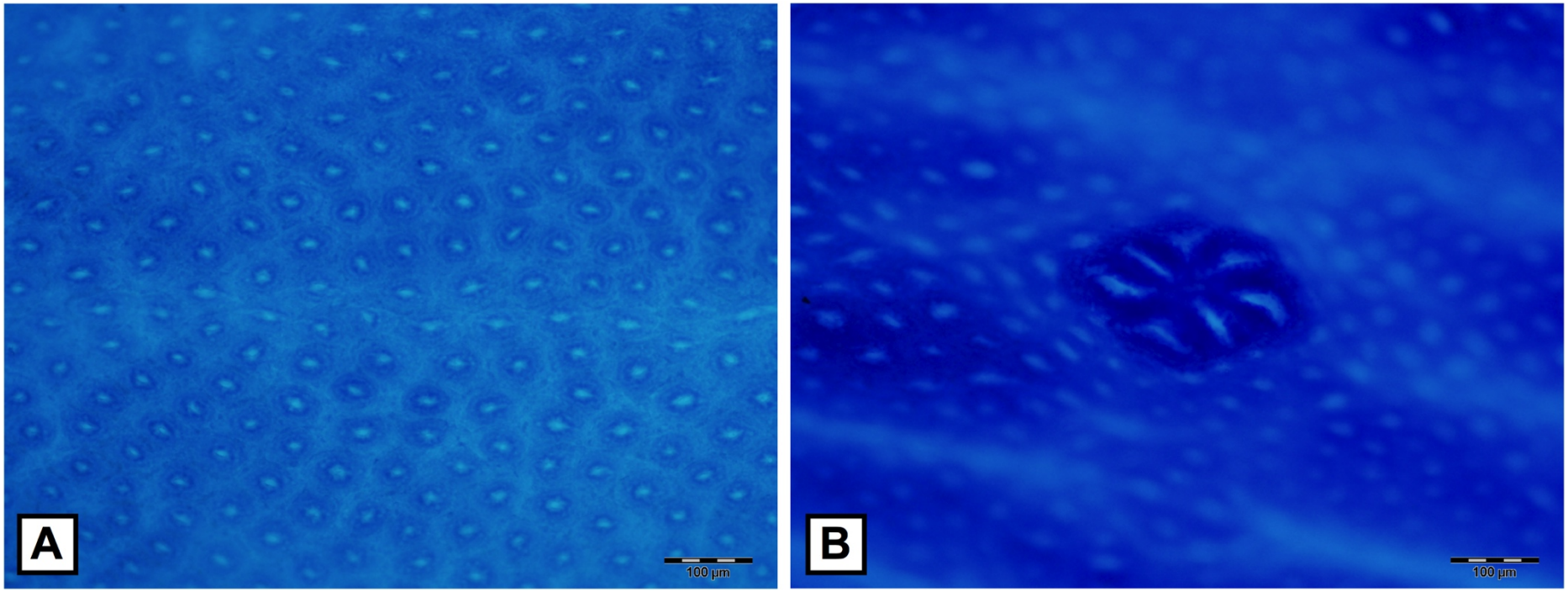

Figure 5 – DMH-induced aberrant crypt foci (ACF) in rats. **(A)** Normal-appearing colonic mucosa stained with methylene blue. (**B**) Methylene blue-stained ACF consisting of seven large, elliptical crypts with thickened epithelial cell lining and increased pericryptal space.

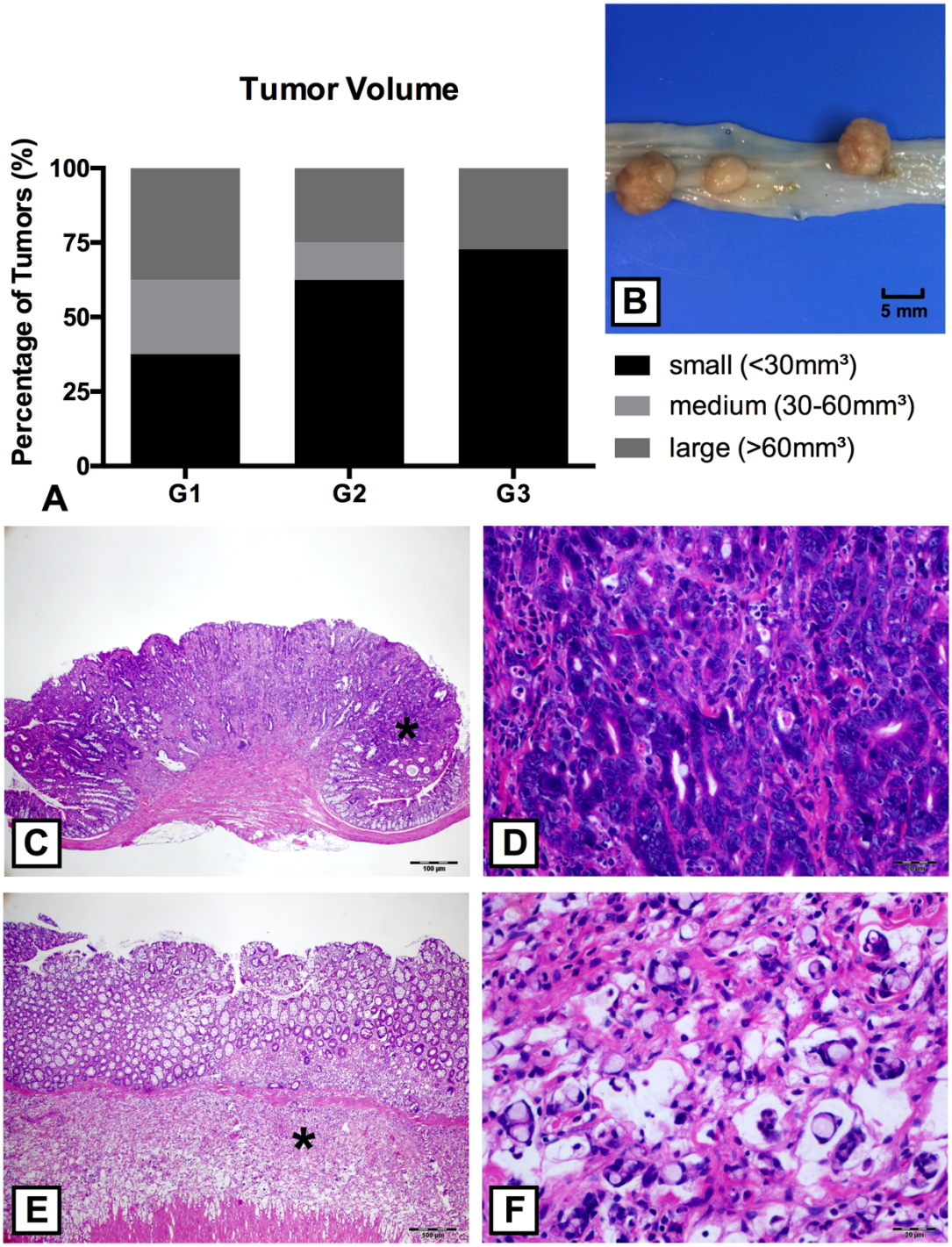

Figure 6 – Volume and histopathology of tumors induced by DMH in control and capsaicin-treated rats. (**A**) Percentage of small, medium and large tumor volumes in the DMH-treated groups. (**B**) Macroscopic image of colon carcinomas in the medial region of the colonic mucosa. (**C**) Sessile, exophytic tumor mass arising from the colonic mucosa. (**D**) Tubular adenocarcinoma. (**E**) Endophytic tumor with extensive submucosal spread. (**F**) Mucinous adenocarcinoma with signet ring cells. G1: DMH + corn oil (capsaicin vehicle); G2: DMH + capsaicin 5 mg/kg bw; G3: DMH + capsaicin 50 mg/kg bw; DMH: 1,2-dimethylhidrazine.

Table 1 – Body weight, food intake and liver parameters in control and capsaicin-treated rats[1].

4 weeks (n=6)						
	G1	**G2**	**G3**	**G4**	**G5**	**G6**
Parameters	**DMH**	**DMH + CAP 5**	**DMH + CAP 50**	**CAP 5**	**CAP 50**	**Control**
Initial body weight (g)	232.25 ± 19.34	225.25 ± 21.08	243.75 ± 20.65	220.75 ± 20.75	222.88 ± 24.17	236.63 ± 34.90
Final body weight (g)	299.94 ± 21.75	281.75 ± 35.05	311.88 ± 39.95	304.54 ± 20.65	307.31 ± 27.85	325.75 ± 41.16
Weight gain (g)	67.69 ± 10.06	61.29 ± 21.41	68.13 ± 28.22	83.92 ± 20.15	89.00 ± 17.02	89.13 ± 21.38
Food intake (g/rat/day)	19.91 ± 4.66	19.04 ± 4.82	21.50 ± 5.29	21.51 ± 2.70	21.79 ± 6.65	22.75 ± 2.66
Liver relative weight (g)	2.91 ± 0.25	2.99 ± 0.20	2.96 ± 0.44	2.91± 0.31	3.06 ± 0.28	2.75 ± 0.07
ALT (IU/L)	99.80 ± 28.69[†]	72.80 ±18.77	79.20 ± 12.73	47.40 ± 9.48	55.60 ± 8.31	51.20 ± 16.34
AST (IU/L)	269.40 ± 85.95[†]	207.60 ± 28.88	262.80 ± 99.61[†]	131.80 ± 6.05	119.60 ± 24.18	126.20 ± 28.10
22 weeks (n=10)						
Parameters	**DMH**	**DMH + CAP 5**	**DMH + CAP 50**	**CAP 5**	**CAP 50**	**Control**
Initial body weight (g)	226.40 ± 18.22	223.20 ± 21.98	244.70 ± 25.51	221.57 ± 24.08	212.71 ± 15.93	241.33 ± 28.04
Final body weight (g)	445.60 ± 30.62	449.50 ± 51.08	468.70 ± 51.97	446.00 ± 35.02	438.00 ± 26.58	467.40 ± 34.66
Weight gain (g)	218.22 ± 19.88	220.38 ± 38.39	221.75 ± 37.21	198.40 ± 26.54	230.00 ± 34.91	228.00 ± 22.83
Liver relative weight (g)	1.97 ± 0.15	2.27 ± 0.37	2.04 ± 0.15	2.21± 0.28	2.11 ± 0.13	2.12 ± 0.25

[1]Values represent the mean ± SD for 6-10 rats/group. Differences between groups were determined using one-way ANOVA followed by Tukey's test. [†]Different from G4, G5 and G6, $p<0.0005$. ALT: alanine aminotransferase; AST: aspartate aminotransferase; DMH: 1,2-dimethylhydrazine; CAP 5: capsaicin 5mg/kg bw; CAP 50: capsaicin 50mg/kg bw.

Table 2 – Genes differentially expressed in the colon samples of capsaicin-treated rats[1].

Comparisons	Gene	Ensembl ID	Fold Change	P value
G2 vs G1	Dffb	ENSRNOG00000025030	1.532	0.022
	Gsk3b	ENSRNOG00000002833	1.627	0.041
	Raf1	ENSRNOG00000010153	1.781	0.021
G3 vs G1	Dffb	ENSRNOG00000025030	1.548	0.016
	Casp4	ENSRNOG00000033697	2.094	0.010
	Aifm1	ENSRNOG00000006067	1.689	0.030
	Wee1	ENSRNOG00000010017	1.553	0.006
	Sp1	ENSRNOG00000014084	1.769	0.004
	Foxa-1	ENSRNOG00000009284	1.761	0.002
	Cdh1	ENSRNOG00000020151	1.951	0,032
	Smad4	ENSRNOG00000051965	1.549	0.034
	Grb2	ENSRNOG00000037360	1.940	0.006
	Raf1	ENSRNOG00000010153	2.046	0.036
	Mapk3	ENSRNOG00000053583	2.229	0.028
	Mapk14	ENSRNOG00000000513	1.867	0.032
	Nfkb1	ENSRNOG00000023258	1.851	0.032
	Stat5b	ENSRNOG00000019075	1.583	0.020
	Ikbkg	ENSRNOG00000060936	1.786	0.016
G4 vs G6	Igfr1	ENSRNOG00000014187	0.569	0.032
	Akt1	ENSRNOG00000028629	0.652	0.019
	CdkN1a	ENSRNOG00000000521	1.933	0.018

[1]Relative expression levels were determined by normalization to beta-actin (Actb), glyceraldeyde-3-phosphate dehydrogenase (Gapdh), beta-glucuronidase (Gusb) and hypoxanthine-guanine phosphoribosyltransferase (Hprt1). Experimental groups were compared using the Student's t-test. Fold change boundary of 1.5 (1.5-fold change) and a P value of < 0.05 were used. G1: DMH + corn oil (capsaicin vehicle); G2: DMH + capsaicin 5 mg/kg bw; G3: DMH + capsaicin 50 mg/kg bw; G4: EDTA (DMH vehicle) + capsaicin 5 mg/kg bw; G5: EDTA + capsaicin 50 mg/kg bw; G6: EDTA + corn oil. S: sacrifice; DMH: 1,2-dimethylhidrazine; EDTA: ethylenediamine tetraacetic acid.

Table 3 – Significantly enriched gene ontology (GO) annotated terms in up-regulated genes of capsaicin 50mg/kg (G3) treated rats.

S. No.	GO term	Fold Enrichment	No. of Genes	P value
1	GO:0070887 - cellular response to chemical stimulus	7.68	13	0.00002
2	GO:0033554 - cellular response to stress	9.10	10	0.00016
3	GO:0006915 - apoptotic process	17.43	9	0.00004
4	GO:0050790 - regulation of catalytic activity	7.00	11	0.00310
5	GO:0070848 - response to growth factor	23.13	9	0.00001
6	GO:0080134 - regulation of response to stress	9.02	8	0.00812
7	GO:0006974 - cellular response to DNA damage	13.32	6	0.02930
8	GO:0010941 - regulation of cell death	9.30	11	0.00018
9	GO:0009888 - tissue development	7.30	9	0.00760
10	GO:0030154 - cell differentiation	5.57	14	0.00015

Table 4 – Incidence and multiplicity of various tumors induced by DMH in control and capsaicin-treated rats[1].

[1]Multiplicity is the average number of all tumors in each tumor-bearing mouse. Multiplicity values are represented as the mean ± SD. Tumor incidence is the percentage of mice bearing the indicated type of tumor. Incidence values are represented as percentage, compared by the Fisher's exact test (p=0.20). DMH:

Groups/Treatments		Number of animals	Number of tumors	Multiplicity	Incidence (%)		
					Tubular Adenocarcinoma	Carcinoma *in situ*	Mucinous Adenocarcinoma
(G1)	DMH	10	15	1.67 ± 1.32	72.72	9.10	18.18
(G2)	DMH + CAP 5	10	11	2.20 ± 0.84	61.53	15.40	23.07
(G3)	DMH + CAP 50	10	13	1.86 ± 1.07	71.43	28.57	0

1,2-dimethylhydrazine; CAP 5: capsaicin 5mg/kg bw; CAP 50: capsaicin 50mg/kg bw.

Table 5 – Inhibitory effects of capsaicin treatment on the number of aberrant crypt foci pre-neoplastic lesions[1].

Groups/Treatments[2]		No. of animals	Number of ACF			Total Number		
			1-3 crypts	4-9 crypts	≥ 10 crypts	AC[3]	ACF	AC/ACF
(G1)	DMH	10	170.10 ± 55.47	110.70 ± 43.47	17.90 ± 13.54	1230.00 ± 375.74	311.30 ± 57.80	3.91 ± 0.71
(G2)	DMH + CAP 5	10	156.70 ± 54.05	140.10 ± 39.63	13.40 ± 7.99	1260.60 ± 370.90	309.90 ± 89.91	4.06 ± 0.35
(G3)	DMH + CAP 50	10	106.90 ± 35.04[a]	75.00 ± 18.98[b]	2.60 ± 1.65[a,b]	660.60 ± 147.52[a,b]	184.60 ± 44.92[a,b]	3.62 ± 0.36
(G4)	CAP 5	7	0	0	0	0	0	0
(G5)	CAP 50	7	0	0	0	0	0	0
(G6)	Control	7	0	0	0	0	0	0

[1]Values represent the mean ± SD for 7-10 rats/group. Differences between groups were determined using one-way ANOVA followed by Tukey's test. [a]Different from G1, $0.0008 < p < 0.0209$. [b]Different from G2, $0.0008 < p < 0{,}0244$. ACF: aberrant crypt foci; AC: aberrant crypt; DMH: 1,2-dimethylhydrazine; CAP 5: capsaicin 5mg/kg bw; CAP 50: capsaicin 50mg/kg .

Supplemetary Data

Supplementary Dataset 1 – List of selected genes for qRT-PCR analysis of colonic samples and associated biological processes.

	Unigene	Gene ID	Symbol	Description	Process*
01	Rn01527840_m1	24465	Hprt1	catalyzes the conversion of IMP and diphosphate to hypoxanthine and 5-phospho-alpha-D-ribose 1-diphosphate	ENDO
02	Rn01775763_g1	24383	Gapdh	Key glycolytic enzyme that converts D-glyceraldehyde 3-phosphate (G3P) into 3-phospho-D-glyceroyl phosphate.	ENDO
03	Rn00667869_m1	81822	Actb	mRNA expression increases following axon injury; may play a role in acceleration of axonal outgrowth	ENDO
04	Rn01789812_g1	50522	Ubc	may play a role in muscle protein catabolism	ENDO
05	Rn00566655_m1	24434	Gusb	catalyzes the conversion of a beta-D-glucuronoside	ENDO
06	Hs99999901_s1	100861533	Rn18s	sequence present as rDNA repeating units distributed on the short arms of chromosomes 3, 11, and 12, precursor for the 18S rRNA	ENDO
07	Rn01418021_g1	24296	Cyp1a1	monooxygenase that plays a role in dioxin metabolism and detoxification	1
08	Rn00561082_m1	24297	Cyp1a2	a monooxygenase that may play a role in xenobiotic metabolism	1
09	Rn01457876_g1	24300	Cyp2b1	p450 xenobiotic-inducible member involved in hydroxylation of decanoic and other fatty acids	1
10	Rn00582954_m1	83790	Cyp2c23	catalyzes the NADPH-dependent conversion of arachidonic acid	1
11	Rn03417171_gH	293989	Cyp2c6v1	may play a role in drug metabolism	1
12	Rn00597330_m1	24303	Cyp2d3	member of the p450 xenobiotic-inducible superfamily	1
13	Rn00580624_m1	25086	Cyp2e1	may play a role in xenobiotic metabolism	1
14	Rn00598500_m1	313375	Cyp2j3	monooxygenase; responsible for the oxidation of endogenous arachidonic acid pools	1
15	Rn00576482_m1	65210	Cyp2j4	cytochrome p450 monooxygenase enzyme	1
16	Rn00595752_m1	25642	Cyp3a1	steroid-inducible member of p450 subfamily 3A	1
17	Rn00568733_m1	29680	Cyp11a1	monooxygenase that catalyzes synthesis of cholesterol and steroids	1
18	Rn00562516_m1	25098	Foxa1	may act as a transcription factor for hepatocyte specific gene expression	1,6
19	Rn01415600_m1	25099	Foxa2	regulates expression of growth hormone regulated CYP genes	1,6
20	Rn00514509_m1	25311	Dcc	transmembrane protein of the immunoglobulin superfamily that plays dual roles as a receptor for other signalling pathways	4
21	Rn02114316_gH	297893	Hdac1	may be involved in chromatin rearrangement during cellular differentiation	4,6
22	Rn01193634_g1	84577	Hdac2	may be involved in chromatin rearrangement during cellular differentiation	4,6
23	Rn00579159_m1	81685	Mlh1	may be involved in DNA mismatch repair	4
24	Rn01437268_m1	494322	Pms1	mismatch repair system component	4
25	Rn00563462_m1	25332	Mgmt	enzyme involved in DNA repair of O(6)-alkylguanine which is the major mutagenic and carcinogenic lesion in DNA	2,4
26	Rn00566938_m1	24786	Sod1	catalyzes the conversion of superoxide to hydrogen peroxide and molecular oxygen; involved in response to oxidative stress	2
27	Rn00690588_g1	24787	Sod2	manganese superoxide dismutase; intramitochondrial free radical scavenging enzyme	2
28	Rn00563570_m1	25352	Sod3	catalyzes the conversion of superoxide to hydrogen peroxide and molecular oxygen	2
29	Rn00584772_m1	84485	Ccs	binds copper; acts as a chaperone to insert copper into the Cu, Zn superoxide dismutase Sod1	4,5
30	Rn00560930_m1	24248	Cat	functions as a hydrogen peroxide: hydrogen peroxide reductase	2
31	Rn00586652_m1	114243	Nox1	structural maintenance involved in superoxide generation and serum-stimulated growth chromosomes (SMC) protein	3
32	Rn00585380_m1	85431	Nox4	enzyme involved in the production of reactive oxygen species in vascular smooth muscle cells	3

33	Rn00577357_m1	79129	Cyba	alpha subunit of cytochrome b558, a component of NAD(P)H oxidase, may play a role in production of reactive oxygen species	3
34	Rn00591196_m1	170841	Mutyh	an DNA repair enzyme that excises adenine residues misincorporated opposite the oxidized base	4
35	Rn00578409_m1	81528	Ogg1	DNA repair enzyme that cleaves out mutagenic 7,8-dihydro-8-oxoguanine (8-oxoG)	4
36	Rn01421973_m1	300711	Atm	serine/threonine protein kinase; critical regulator of the cellular DNA damage response	4
37	Rn00589669_m1	140583	Chek1	protein kinase that can bind to single-stranded DNA; may function in DNA replication and repair	4,6
38	Rn00586616_m1	114212	Chek2	kinase involved in ATM-dependent DNA damage checkpoint pathway	4
39	Rn00490624_m1	63996	Smc1a	structural maintenance of chromosomes (SMC) protein, one of 2 component proteins forming the cohesin multiprotein complex	4,5
40	Rn00565018_m1	25591	Parp1	catalyzes poly (ADP-ribose) protein modification; plays a role in DNA repair and genome stability	4,5
41	Rn00594589_m1	25019	Xrcc6	DNA targeting component of the DNA-dependent protein kinase complex DNA-PK; plays a role in DNA double strand break repair	4
42	Rn01425130_g1	25112	Gadd45a	may mediate a delay in G2 to M cell cycle progression; may induce DNA repair	4
43	Rn01279391_m1	308937	Wee1	G2 checkpoint kinase	5,6
44	Rn00562562_m1	25125	Stat3	transcription factor that plays a role in induction of gene expression during acute phase response	5,6
45	Rn00574281_m1	24918	Stat5	member of the STAT family of transcription factors; may mediate the biological actions of several interleukin factors	5,6
46	Rn00579198_m1	81709	Msh2	mismatch repair protein	4
47	Rn01457299_m1	63879	Xiap	involved in directly inhibiting key apoptotic proteases, caspase 3 and caspase 7	6
48	Rn00755717_m1	24842	Tp53	This gene encodes tumor protein p53, which responds to diverse cellular stresses to regulate target genes that induce cell cycle arrest, apoptosis, senescence and DNA repair.	4,5
49	Rn99999125_m1	24224	Bcl2	an anti-apoptotic protein; involved in inhibiting cell death in many different cell types	6
50	Rn01480161_g1	24887	Bax	Bcl2-related gene; involved in the regulation of apoptotic cell death	5,6
51	Rn00754988_g1	25308	Cstb	inhibits the activity of cathepsins B, H, L, and S; may protect against apoptosis that occurs in response to seizures	5,6
52	Rn01459517_m1	64625	Bid	involved in inducing Bax oligomerization in isolated mitochondrial outer membranes	5
53	Rn00583429_m1	84027	Gsk3b	mediates Par6-atypical protein kinase C (aPKC) complex regulation of cell polarity; may induce apoptosis	5,6
54	Rn00685720_m1	246097	Fas	Tnfsf6/Fasl receptor	5
55	Rn00563754_m1	25385	Faslg	ligand that binds the Fas receptor; plays a role in induction of apoptosis	5
56	Rn00563902_m1	25402	Casp3	apoptotic cysteine-aspartic acid protease that may play a role in neuronal cell death regulation and other apoptotic processes	5
57	Rn00586960_m1	114555	Casp4	cysteine-aspartic acid protease (caspase); involved with the terminal stage of apoptosis; may be involved with inflammation	5
58	Rn00574069_m1	64044	Casp8	member of the cysteine-aspartic acid protease (caspase) family that mediates the terminal stage of apoptosis	5
59	Rn00584462_m1	84359	Dffb	endogenous endonuclease; mediates caspase-3-dependent internucleosomal DNA degradation and related nuclear alterations	5
60	Rn00492098_g1	29467	Ddit3	plays a role in the ER stress response	5
61	Rn00671828_m1	116590	Mapk1	kinase involved in intracellular signaling; component of Mapk signaling pathway	6
62	Rn00820922_g1	50689	Mapk3	kinase involved in intracellular signalling; component of Mapk signalling pathway	5,6
63	Rn00578842_m1	81649	Mapk14	mitogen-activated protein kinase; involved in intracellular signalling, inhibition of apoptosis and gene activation	5,6
64	Rn00442540_m1	83533	Aifm1	may facilitate apoptotic chromatin condensation and DNA degradation	6
65	Rn00580460_m1	24525	Kras	oncogene and member of the small GTPase superfamily	6

66	Rn00589996_m1	114851	Cdkn1a	a Cdk inhibitor; involved in negative regulation of the cell cycle	6
67	Rn00582195_m1	83571	Cdkn1b	inhibits the activity of cyclin-CDK complexes and plays a role in cell cycle control	6
68	Rn00584431_g1	84353	Ctnnb1	involved in mediating the interaction between cadherins and the actin cytoskeleton	6
69	Rn00580109_m1	83502	Cdh1	cell-cell adhesion molecule; may play a role in axonal growth and synase formation	6
70	Rn00572010_m1	59086	Tgfb1	binds the TGFbeta receptor; plays a role in regulation of cell growth and proliferation; induces synthesis of extracellular matrix proteins and may play a role in fibrosis	6
71	Rn00570083_m1	29455	Gdf15	member of the TGF-beta superfamily of growth factors [RGD, Feb 2006]	6
72	Rn01761354_g1	295052	Ccna1	The protein encoded by this gene belongs to the highly conserved cyclin family	6
73	Rn01451446_m1	291234	Mki67	marker of proliferation Ki-67	6
74	Rn01529389_g1	288778	Pa2g4	human and rat homologs may be involved in coordinating ribosome biosynthesis and cell proliferation	6
75	Rn00580398_m1	24329	Egfr	promotes cell proliferation and differentiation; mediates GPCR regulated induction of protein synthesis	6
76	Rn00466507_m1	24703	Raf1	acts as a mitogenic protein kinase; mutant forms may play a role in transformation	6
77	Rn01471333_g1	81504	Grb2	acts as a link between tyrosine kinase receptors and Ras signaling	6
78	Rn00583646_m1	24185	Akt1	inhibits JUN kinase activation and mediates inhibition of apoptosis	6
79	Rn00690900_m1	25233	Akt2	may be involved in phosphatidylinositol 3-kinase (PI3-K) mediated signaling	6
80	Rn01527109_m1	29357	Smad2	transcriptional mediator for both activin and TGFbeta	6
81	Rn00570593_m1	50554	Smad4	transduces signal from TGF-beta; deletion of human homolog gene is associated with pancreatic carcinomas	6
82	Rn00627297_m1	114487	Wnt2	member of a family of secreted glycoproteins that play a role in regulation of cell growth, differentiation, and tumorigenesis	6
83	Rn00583837_m1	25718	Igf1r	receptor for Igf-1; involved in induction of cell cycle progression and survival in many cell types	6
84	Rn00580462_m1	24553	Met	proto-oncogene, functions as a heterodimeric tyrosine kinase	6
85	Rn00566673_m1	24446	Hgf	plays a role in positive regulation of cell proliferation; may promote entry into the cell cycle	6
86	Rn01399572_m1	81736	Nfkb	Rel protein-specific transcription inhibitor	6
87	Rn01640116_m1	309295	Ikbkg	regulatory subunit of the I kappa B kinase complex, which phosphorylates and activates NF kappa B	6
88	Rn01496565_m1	29541	Nthl1	DNA glycosylase activity on DNA substrates containing oxidized pyrimidine residues and has apurinic/apyrimidinic lyase activity.	7
89	Rn00566496_m1	24247	Casr	a G protein-coupled receptor that senses extracellular calcium; involved in regulating the secretion of parathyroid hormone, bile and intestinal fluid	6,7
90	Rn00565867_m1	25706	Cckbr	Cholecystokinin receptor, member of the G protein-coupled receptor superfamily, stimulating phosphatidylinositol turnover and intracellular calcium mobilization.	6,7
91	Rn01758633_m1	25496	Notch1	transmembrane receptor; involved in cell-cell interactions important for development and pattern formation	6
92	Rn01534371_m1	25496	Notch2	transmembrane receptor; involved in cell-cell interactions important for development and pattern formation	6
93	Rn00569647_m1	29146	Jag1	ligand responsible for activating Notch1	6,7
94	Rn00561953_m1	24790	Sp1	transcription factor that recognizes 5'-CCGCCC promoter sequence; plays a role in transcriptional regulation of many genes	5,7
95	Rn01639120_m1	161452	Lef1	This gene encodes a transcription factor belonging to a family of proteins that share homology with the high mobility group protein	7
96	Rn00583117_m1	83810	Trpv1	ion channel that demonstrates heat-evoked membrane currents	7

ENDO: endogenous genes, [1]Oxidative metabolismo enzymes; [2]antioxidant enzymes; [3]prooxidant enzymes, [4]DNA damage and repair; [5]Apoptosis; [6]Cell proliferation and cell cycle; [7]capsaicin pathway.

Anexo

UNIVERSIDADE ESTADUAL PAULISTA
CAMPUS DE BOTUCATU
FACULDADE DE MEDICINA

unesp

FMB

Comissão de Ética no Uso de Animais

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

CERTIFICADO Nº 1108/2014-CEUA

CERTIFICAMOS que o Projeto de Pesquisa **(Protocolo CEUA 1108/2014)** **Efeitos da capsaicina na etapa de iniciação da carcinogênese química de cólon em ratos**, a ser conduzido por Brunno Felipe Ramos Caetano, orientado pelo Prof. Dr. Luis Fernando Barbisan, co-orientado pela Profª. Drª. Maria Aparecida Marchesan Rodrigues Kobayasi, está de acordo com o Conselho Nacional de Controle de Experimentação Animal - CONCEA, com a ressalva de que os **"ratos"** são provenientes de Biotério Convencional, sem condições de atestar a Sanidade dos mesmos.

CERTIFICAMOS que foi autorizada a utilização de **"85 ratos"**. Caso seja necessária a utilização de mais animais deverá ser comunicado a esta CEUA.

Projeto de Pesquisa aprovado em reunião da CEUA em 27/11/2014

Prof. Adjunto Katashi Okoshi
Presidente da CEUA

Kleber Messias de Camargo
Secretário da CEUA

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