UNIVERSIDADE ESTADUAL DE MONTES CLAROS

Felipe Alberto Dantas Guimarães

Potencial antineoplásico e efeito terapêutico adjuvante da crotoxina extraída do veneno da serpente *Crotalus durissus terrificus*: revisão sistemática e estudo *in vitro* em células de carcinoma epidermoide de boca

Montes Claros

2020

Felipe Alberto Dantas Guimarães

Potencial antineoplásico e efeito terapêutico adjuvante da crotoxina extraída do veneno da serpente *Crotalus durissus terrificus*: revisão sistemática e estudo *in vitro* em células de carcinoma epidermoide de boca

Dissertação apresentada ao Programa de Pós-Graduação em Ciências em Saúde da Universidade Estadual de Montes Claros - Unimontes, como parte das exigências para a obtenção do título de Mestre em Ciências da Saúde.

Área de Concentração: Mecanismos e Aspectos Clínicos das Doenças

Orientadora: Prof.ª Dra. Lucyana Conceição Farias

Coorientador: Prof. Dr. André Luiz Sena Guimarães

Montes Claros

2020

G963p

Guimarães, Felipe Alberto Dantas.

Potencial antineoplásico e efeito terapêutico adjuvante da crotoxina extraída do veneno da serpente *Crotalus durissus terrificus* [manuscrito] : revisão sistemática e estudo *in vitro* em células de carcinoma epidermoide de boca / Felipe Alberto Dantas Guimarães. – Montes Claros, 2020.

90 f. : il.

Inclui Bibliografia.

Dissertação (mestrado) - Universidade Estadual de Montes Claros - Unimontes, Programa de Pós-Graduação em Ciências da Saúde/PPGCS, 2020.

Orientadora: Profa. Dra. Lucyana Conceição Farias.

Coorientador: Prof. Dr. André Luiz Sena Guimarães.

1. Antineoplásico. 2. Crotoxina. 3. *Crotalus durissus terrificus*. 4. Carcinoma epidermoide de boca. 5. Radioterapia. 6. Radiação ionizante. I. Farias, Lucyana Conceição. II. Guimarães, André Luiz Sena. III. Universidade Estadual de Montes Claros. VI. Título. V. Título: Revisão sistemática e estudo *in vitro* em células de carcinoma epidermoide de boca.

Catalogação: Biblioteca Central Professor Antônio Jorge.

UNIVERSIDADE ESTADUAL DE MONTES CLAROS-UNIMONTES

Reitor: Antônio Avilmar Souza

Vice-reitora: Ilva Ruas de Abreu

Pró-reitor de Pesquisa: Profª. Clarice Diniz Alvarenga Corsato

Coordenadoria de Acompanhamento de Projetos: Vigílio Mesquita Gomes

Coordenadoria de Iniciação Científica: Sônia Ribeiro Arrudas

Coordenadoria de Inovação Tecnológica: Dario Alves de Oliveira

Pró-reitor de Pós-Graduação: André Luiz Sena Guimarães

Coordenadoria de Pós-Graduação *Lato sensu*: Marcos Flavio D'Ângelo

Coordenadoria de Pós-Graduação *Stricto sensu*: Marcelo Perim Baldo

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

Coordenador: Prof. Dr. Alfredo Maurício Batista de Paula

Subcoordenadora: Profa. Dra. Marise Fagundes Silveira

UNIVERSIDADE ESTADUAL DE MONTES CLAROS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

Unimontes

MESTRANDO(A): FELIPE ALBERTO DANTAS GUIMARÃES

TÍTULO DO TRABALHO: "Potencial antineoplásico e efeito terapêutico adjuvante de substância extraída de veneno da serpente Crotalus Durissus Terrificus: uma abordagem em células de carcinoma epidermoide de boca"

ÁREA DE CONCENTRAÇÃO: Mecanismos e Aspectos Clínicos das Doenças

LINHA DE PESQUISA: Etiopatologia e Fisiologia das Doenças

BANCA (TITULARES)	**ASSINATURAS**
PROFª. DRª. LUCYANA CONCEIÇÃO FARIAS/ ORIENTADORA	Lucyana C. Farias
PROF. DR. ANDRÉ LUIZ SENA GUIMARÃES /COORIENTADOR	
PROF. DR. GERALDO ACLÉCIO MELO	
PROFª. DR. THALLYTA MARIA VIEIRA	

BANCA (SUPLENTES)	**ASSINATURAS**
PROF. DR. SÉRGIO HENRIQUE SOUSA SANTOS	
PROFª. DRª. CARLA SILVANA DE OLIVEIRA E SILVA	

[X] **APROVADO** [ ] **REPROVADO**

Hospital Universitário Clemente Farias – HUCF
http://www.unimontes.br / ppgcs@unimontes.br
Telefone: (0xx38) 3224-8372 / Fax: (0xx38) 3224-8372
Av. Cula Mangabeira, 562, Santo Expedito, Montes Claros – MG, Brasil – Cep: 39401-001

Dedico este trabalho primeiramente a Deus, por ser essencial em minha vida, autor do meu destino, meu guia, presença forte em todos os momentos da minha existência, ao meu pai Ademir Pereira Guimarães, minha mãe Mercina Dantas Guimarães, meus irmãos Rodrigo Dantas Guimarães e Victor Hugo Dantas Guimarães e a minha namorada Kamilla Mota Fernandes, todos os familiares e amigos.

AGRADECIMENTOS

Agradeço a **Deus**, pelo dom da vida, sabedoria e determinação, e por todos os momentos da minha existência, sejam eles bons ou ruins. Momentos estes que fizeram da minha pessoa retomar o sentido da vida, para que as batalhas fossem vencidas e conquistas por mérito meu merecidas.

A minha família, em especial a minha mãe **Mercina Dantas Guimarães** e meu pai **Ademir Pereira Guimarães** dedico estes agradecimentos, sem o apoio, carinho e a força de vocês eu não poderia ter chegado até aqui, me proporcionaram ensinamentos que vou guardar para sempre comigo, valores estes, que foram me passados e que me acompanharam por toda pelo resto da minha vida.

Aos meus irmãos **Rodrigo Dantas Guimarães** e **Victor Hugo Dantas Guimarães** agradeço pelo apoio e amizade atribuídos a mim, cada um expressando da sua maneira. Os ensinamentos no âmbito laboratorial, biotério, na vida, são conhecimentos que de tal forma me fizeram pensar que ao longe posso chegar e que todo dia é dia de aprender, sempre mais e mais.

A minha namorada **Kamilla Mota Fernandes** que esteve ao meu lado em todos os momentos da minha vida, que sempre me apoiou nas minhas decisões, sendo esta companheira maravilhosa a quem eu tanto amo.

A minha sogra **Marinei Soares Mota** e meu sogro **Ildeu Ribeiro Fernandes** agradeço pelo apoio e amizade nesta jornada tão importante da minha vida.

A todos os **meus familiares** que de alguma forma contribuíram para que este dia se concretizasse, por meio das palavras amigas, apoio e também pelos ensinamentos que foram de grande valia.

A minha orientadora e amiga **Lucyana Conceição Farias** os meus humildes agradecimentos pela oportunidade, compreensão, dedicação, confiança, tenho tamanho apreço pela sua pessoa. Obrigado por tudo!!!

Aos **professores** e **funcionários** da secretaria do Programa de Pós-Graduação em Ciências da Saúde – PPGCS, pelo suporte e convivências diárias.

Aos meus **amigos** e **colegas** do Laboratório de Pesquisa que puderam me ensinar grandemente os conhecimentos que me foram passados, pela convivência, profissionalismo, momentos de alegria e tristeza, aprendizado diário e aqui fica a minha gratidão por cada um de vocês: Amanda Lacerda, Renata, Magda, Rogério, Amanda Rodrigues, Andréia, Emisael, Marcela, Sabrina, Luiz Paulo, Daniele, Dani de Paola, Janaína, Valeria, Erivelton, Marileide e todos os outros que aqui não foram listados.

À FAPEMIG e Unimontes pelo auxilio, incentivo e fomento à pesquisa.

A todos os outros que contribuíram de alguma forma para que este trabalho viesse a dar certo. Obrigado!

“Por isso, vos digo que tudo o que pedirdes, orando, crede que o recebereis e tê-lo-eis”.

Marcos 11:24

RESUMO

O carcinoma epidermoide de boca (CEB) é a sexta neoplasia mais comum no mundo, e representa mais de 90% de todos os tumores malignos da cavidade bucal. Em indivíduos acometidos pelo CEB, a radioterapia é uma estratégia terapêutica importante para promover a morte das células neoplásicas e controlar a progressão da doença. Apesar dos avanços obtidos nesta modalidade de tratamento, observam-se, ainda, quadros de radiorresistência, podendo levar um prognóstico desfavorável. É crucial a realização de pesquisas voltadas para o desenvolvimento de terapêuticas complementares, visando favorecer a eficácia da radiação ionizante. Os venenos são fontes promissoras para a descoberta de novos agentes antineoplásicos. A crotoxina (CrTX), uma toxina isolada do veneno da cascavel sul-americana *Crotalus durissus terrificus* possui algumas atividades biológicas, que incluem ações neurotóxicas, imunomoduladoras, anti-inflamatórias, antimicrobianas, analgésicas e antineoplásicas. Diante do exposto, esse estudo engloba duas vertentes de trabalho. A primeira teve como objetivo verificar o potencial antineoplásico da CrTX em diferentes tipos de câncer, através de uma revisão sistemática. O objetivo da segunda vertente foi avaliar o efeito terapêutico adjuvante da CrTX em células de carcinoma epidermoide de boca. A revisão sistemática do primeiro estudo envolveu a busca de estudos *in vitro* e clínicos relacionados ao objetivo proposto. O estudo foi registrado na Plataforma internacional Prospero (CRD42019137665) e seguiu os critérios de busca da Plataforma PRISMA, utilizando as bases de dados PubMed, Scopus, Web of Science e EBSCO. Foram identificados 71 artigos sobre a proposta. A partir da análise destes, adotando critérios de inclusão e exclusão, foram selecionados como elegíveis para a leitura do texto completo um total de 11 artigos. Os resultados da revisão sistemática mostraram que a crotoxina promoveu ação antiproliferativa, apoptose, parada do ciclo celular e ativação de genes para promoção da morte de linhagens cancerígenas, em estudos *in vitro*. Não foi identificado na literatura estudo em humanos adequado à proposta da revisão. Na segunda vertente deste estudo, foram realizados ensaios *in vitro* de proliferação, morte celular, e níveis de espécies reativas de oxigênio (ERO). Células imortalizadas de CEB, SCC-9, foram tratadas com CrTX na concentração de 100 µg/ml, por 72h, seguida da exposição a 2, 4 e 6 Gy de irradiação, em um acelerador linear. A CrTX foi capaz de aumentar

a sensibilidade das células de CEB à radiação ionizante, diminuindo a atividade proliferativa, aumentando significativamente a morte celular e a formação de EROs. Os achados deste estudo demonstraram que a crotoxina potencializou o efeito da radiação ionizante terapêutica, especialmente, em menores doses de radiação. Assim, a CrTX exerceu efeito antineoplásico em diferentes tipos de neoplasias e efeito adjuvante à radiação em células de CEB, podendo ser apontada como uma promissora estratégia terapêutica para futuras pesquisas clínicas para o tratamento de pacientes com câncer bucal.

Palavras-chave: Antineoplástico. Crotoxina. *Crotalus durissus terrificus.* Carcinoma epidermoide de boca. Radioterapia. Radiação ionizante.

ABSTRACT

Oral squamous cell carcinoma (OSCC) is the sixth most common neoplasm in the world and represents more than 90% of all malignant tumors of the oral cavity. In individuals affected by OSCC, radiotherapy is an important therapeutic strategy to promote the death of neoplastic cells and to control the progression of the disease. Despite the advances obtained in this treatment modality, there are still cases of radioresistance, which can lead to an unfavorable prognosis. It is crucial to carry out research aimed at developing complementary therapies, aiming to favor the effectiveness of ionizing radiation. The poisons are promising sources for the discovery of new antineoplastic agents. Crotoxin (CrTX), a toxin isolated from the poison of the South American rattlesnake *Crotalus durissus terrificus*, has some biological activities, which include neurotoxic, immunomodulatory, anti-inflammatory, antimicrobial, analgesic and antineoplastic actions. Given the above, this study encompasses two strands of work. The first aimed to verify the antineoplastic potential of CrTX in different types of cancer, through a systematic review. The objective of the second approach was to evaluate the adjuvant therapeutic effect of CrTX in OSCC cells. The systematic review of the first study involved the search for *in vitro* and clinical studies related to the proposed objective. The study was registered on the international platform Prospero (CRD42019137665) and followed the search criteria of the PRISMA Platform, using the PubMed, Scopus, Web of Science and EBSCO databases. 71 articles on the proposal were identified. From the analysis of these, adopting inclusion and exclusion criteria, a total of 11 articles were selected as eligible for reading the full text. The results of the systematic review showed that crotoxin promoted antiproliferative action, apoptosis, cell cycle arrest and activation of genes to promote the death of cancerous lines, in *in vitro* studies. A human study suitable for the review proposal was not identified in the literature. In the second part of this study, *in vitro* assays of proliferation, cell death, and levels of reactive oxygen species (ROS) were performed. Immortalized cells of OSCC, SCC-9, were treated with CrTX at a concentration of 100 µg/ml for 72 hours, followed by exposure to 2, 4 and 6 Gy of irradiation, in a linear accelerator. The CrTX was able to increase the sensitivity of CEB cells to ionizing radiation, decreasing proliferative activity, increasing cell death and the formation of ROS. The findings of this study

demonstrated that the crotoxin enhances the therapeutic effect of ionizing radiation, increasing its therapeutic effect of radiation on OSCC cells. Thus, CrTX exerted an antineoplastic effect on different types of neoplasms and an adjuvant effect to radiation on OSCC cells and can be appointed as a promising therapeutic strategy for future clinical research for the treatment of patients with oral cancer.

Keywords: Antineoplastic. Crotoxin. *Crotalus durissus terrificus*. Oral squamous cell carcinoma. Radiotherapy. Ionizing radiation.

LISTA DE ILUSTRAÇÕES

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

ALDH	Acetaldeído Desidrogenase
ATM	Ataxia Telangiectasia Mutado
CCE	Carcinoma de Células Escamosas
CCEO	Carcinoma de Células Escamosas Orais
CEB	Carcinoma Epidermoide de Boca
CrTX	Crotoxina
DNA	Ácido desoxirribonucleico
ERO	Espécies Reativas de Oxigênio
GST	Glutationa S-Transferase
HPV	Papiloma Vírus Humano
INCA	Instituto Nacional Do Câncer
miRNAs	Micro ácido ribonucleico
MMP-2	Metaloproteinases-2
MMP-9	Metaloproteinase-9
PLA2	Fosfolipase A2
pRb	Proteína do Retinoblastoma
RNA	Ácido ribonucleico
RT	Radioterapia
SOD	Superóxido Dismutato
TEM	Transição Epitélio-Mesenquimal
VEGF	Fator de Crescimento Endotelial Vascular

SUMÁRIO

1 INTRODUÇÃO

O câncer é a segunda principal causa de morte no mundo, sendo precedido apenas pelas mortes ocasionadas por doenças cardiovasculares (1). Considerando os tipos de cânceres mais prevalentes, o câncer de boca representa um grave problema de saúde pública mundial associado a elevadas taxas de morbimortalidade; é a sexta neoplasia mais comum no mundo, principalmente nos países em desenvolvimento (2-5).

O câncer de boca é uma neoplasia maligna que acomete a cavidade oral ou lábios. A língua é o sítio mais afetado, correspondendo a aproximadamente 60% de todos os cânceres bucais (6, 7). Cerca de 90% dos casos representam o tipo histológico epidermoide, sendo por isso, denominado carcinoma de células escamosas orais (CCEO) ou carcinoma epidermoide de boca (CEB) (2, 8).

As estimativas apontam que a incidência do câncer de boca varia de um a dez casos por 100.000 pessoas na maioria dos países (9). Os fatores de risco responsáveis pelo surgimento do câncer de boca incluem o tabagismo, consumo do álcool, e infecções ocasionadas pelo papiloma vírus humano (HPV), sendo que 74% deste risco são atribuídos ao tabaco e álcool (10, 11).

No ano de 2018, foram identificados 354.864 novos casos de câncer de lábio e cavidade bucal e 177.384 pessoas morreram em decorrência deste tipo de câncer no mundo (12). Estimativas apontam que dois terços dos casos de carcinoma de células escamosas ocorrem no sul e sudeste da Ásia (13).

Dentre os cânceres de cabeça e pescoço, o câncer bucal afeta indivíduos, em sua maioria, na faixa etária de 50 a 70 anos (14). Alguns achados na literatura apontam que 5% destes casos de câncer oral são representados por adultos jovens com faixa etária de 25 a 40 anos, estando parcialmente relacionado ao elevado uso de tabaco e outras drogas, bem como as infecções virais transmitidas sexualmente, como o HPV (13, 15, 16).

O estágio do tumor e o sítio anatômico são os principais fatores que determinam a escolha da modalidade de tratamento para os pacientes com câncer de boca, sendo necessária uma abordagem multidisciplinar para um melhor planejamento do tratamento e avaliação da

resposta pós-tratamento. Os tratamentos para indivíduos acometidos pelo CEB incluem cirurgia, radioterapia (RT), quimioterapia ou combinações dessas modalidades. Os tratamentos combinados podem ser entregues simultaneamente ou em diferentes sequências temporais (13, 17, 18). As modalidades terapêuticas de cirurgia, radioterapia, quimioterapia ou terapia combinada podem favorecer danos teciduais à região peritumoral na cavidade oral, lesionando diretamente os tecidos ou interferindo na produção de células hematopoiéticas. Estas complicações incluem mucosite oral, disgeusia, doenças infecciosas, osteorradionecrose, xerostomia associadas à perda da função glandular (18, 19).

Apesar dos avanços relacionados à detecção, terapêutica do câncer, e sobre o conhecimento científico sobre os fatores de risco atribuíveis ao aparecimento do câncer, o câncer bucal apresenta uma baixa taxa de sobrevida de 50 % a 60% em 5 anos (20).

A crotoxina (CrTX) é uma neurotoxina isolada do veneno da cascavel sul-americana, *Crotalus durissus terrificus* (21, 22). Foi isolada e descrita pela primeira vez por Slota e Fraenkel-Sonrat (1938), pesquisadores do Instituto Butantã, no Brasil (23). A CrTX exerce atividades biológicas, tais como neurotoxicidade, miotoxicidade e nefrotoxicidade; além disso, está associada a ações imunomodulatórias, anti-inflamatórias, antimicrobianas e analgésicas (24, 25).

A atividade antitumoral e antiproliferativa do veneno da cascavel é atribuída a crotoxina (CrTX), que é descrita em diversos estudos como tendo uma atuação sobre as mais diversas linhagens celulares de câncer, incluindo leucemia, colo do útero, ovário, pulmão, cólon, rim, melanoma e cérebro, sendo estes estudos *in vitro* e *in vivo* (24-27).

No entanto, a ação da CrTX sobre o comportamento neoplásico, mecanismos moleculares e potencial terapêutico adjuvante não foi explorada na carcinogênese de boca. Sendo assim, esse estudo tem como foco principal a investigação do efeito adjuvante da CrTX à radiação ionizante terapêutica em células de carcinoma epidermoide de boca.

2 OBJETIVOS

2.1 Objetivo Geral

Avaliar o potencial antineoplásico e o efeito terapêutico adjuvante da crotoxina extraída de veneno da serpente *Crotalus durissus terrificus* em células de carcinoma epidermoide de boca.

2.2 Objetivos Específicos

- Revisar a literatura acerca da ação da crotoxina em diversos tipos de câncer.

- Avaliar o potencial adjuvante da crotoxina sobre o efeito terapêutico da radiação ionizante sobre os parâmetros fenotípicos de proliferação, morte celular e espécies reativas de oxigênio em linhagem celular de carcinoma epidermoide de boca.

3 REVISÃO DE LITERATURA

3.1 Câncer de boca: Aspectos Gerais

O câncer de boca é uma neoplasia que acomete, preferencialmente, as células escamosas do epitélio da mucosa, denominado carcinoma epidermoide de boca (CEB) ou carcinoma de células escamosas orais (CCEO). É o sexto tipo de neoplasia mais comum em todo o mundo, e corresponde por 2 a 4% de todos os casos de câncer no âmbito global (28-32).

O CEB corresponde por mais de 90% de todos os tumores malignos que atingem a cavidade bucal, afetando principalmente a região anatômica da língua, bochecha, assoalho da boca, gengiva (33, 34). Das 6,4 milhões de neoplasias malignas diagnosticadas na população mundial no ano de 2006, cerca de 10% destas estão localizadas na boca (35). Este tipo de câncer vem ganhado destaque nas últimas duas décadas, tornando-se, pois, uma das neoplasias mais pesquisadas (29).

O câncer de boca é mais prevalente nos países em desenvolvimento, sendo considerado uma problemática crescente em várias regiões do mundo. Devido às mudanças no estilo de vida nos últimos tempos, algumas alterações na prevalência do câncer de boca em alguns países foram observadas. Por exemplo, nos países do sul da Ásia, como Índia, Srilanka, Paquistão e Bangladesh, o câncer de boca passou a ser o tipo mais comum de neoplasia, e contribui com quase um quarto de todos os novos casos de câncer (5, 36).

No ano de 2012, na população mundial o câncer de cavidade oral e lábio acometeu 300 mil indivíduos dos quais 145 mil foram a óbito (31, 37). A incidência global do câncer de boca no ano de 2018, foi de 2,8 casos para cada 100 mil homens e de 1,2 casos para cada 100 mil mulheres, e para ambos os sexos são 2 casos para cada 100 mil indivíduos (38). De acordo com Instituto Nacional do Câncer, Brasil (INCA), nas estimativas dos dez tipos de câncer mais incidentes para o ano de 2018 na população brasileira, o câncer de boca representou um total de 14.700 casos, sendo mais prevalente no sexo masculino, com 11.200 casos ocupando a 5ª posição. No sexo feminino, são 3.500 casos, estando na 12ª posição, com um risco estimado 10,86 novos casos a cada 100 mil homens e de 3,28 novos casos para cada 100 mil mulheres (Tabela 1) (39).

Tabela 1 - Distribuição proporcional dos dez tipos de câncer mais incidentes estimados para 2018 na população brasileira, por sexo, exceto pele não melanoma*

Homens				Mulheres			
Localização Primária	Casos	%		Localização Primária	Casos	%
Próstata	68.220	31,7%		Mama Feminina	59.700	29,5%
Traqueia, Brônquio e Pulmão	18.740	8,7%		Cólon e Reto	18.980	9,4%
Cólon e Reto	17.380	8,1%		Colo do Útero	16.370	8,1%
Estômago	13.540	6,3%		Traqueia, Brônquio e Pulmão	12.530	6,2%
Cavidade Oral	11.200	5,2%		Glândula Tireoide	8.040	4,0%
Esôfago	8.240	3,8%		Estômago	7.750	3,8%
Bexiga	6.690	3,1%		Corpo do Útero	6.600	3,3%
Laringe	6.390	3,0%		Ovário	6.150	3,0%
Leucemias	5.940	2,8%		Sistema Nervoso Central	5.510	2,7%
Sistema Nervoso Central	5.810	2,7%		Leucemias	4.860	2,4%

*Números arredondados para múltiplos de 10.

Fonte: INSTITUTO NACIONAL DO CÂNCER (39).

A avaliação clínica da mucosa bucal deve ser realizada minuciosamente por inspeção visual e palpação dos tecidos, examinando principalmente regiões como borda lateral e margens póstero-laterais da língua, orofaringe e assoalho da boca, visando detectar precocemente lesões suspeitas de CEB. As lesões podem apresentar como úlcecras de coloração vermelha ou branca, nódulo endurecido, fissura ou linfonodo cervical aumentado (32).

A carcinogênese bucal resulta de múltiplas modificações genéticas e epigenéticas, onde o acúmulo destas alterações é a base para a iniciação e progressão de uma célula normal para uma célula cancerígena. Uma série de eventos moleculares favorecem para as desregulações do comportamento proliferativo, apoptose, angiogênese e metástases (40-42).

Apesar dos avanços associados às modalidades de tratamento para os indivíduos acometidos pelo CEB, a resposta terapêutica ainda guarda casos de baixa responsividade, estima-se que a sobrevida global em 5 anos permaneça estática em 50% (43). Na grande maioria dos pacientes que apresentam lesões potencialmente malignizáveis ou câncer em estágio inicial, a taxa de cura a sobrevida é alta, mas a ampla maioria dos casos em estágio III e IV tende a ser letal, em parte por apresentar taxas de recorrências locorregionais relativamente altas (44). A detecção precoce de lesões potencialmente malignizáveis na

mucosa bucal é de extrema importância para obtenção de melhores taxas de sobrevida e aumento da qualidade de vida dos pacientes acometidos pelo câncer de boca (45).

3.2 Etiopatogênese do Carcinoma Epidermoide de Boca

A iniciação e progressão do CEB são atribuídas a uma soma de alterações genéticas, epigenéticas, fatores ambientais e estilo de vida, questões ligados principalmente ao uso do tabaco e consumo de álcool (46, 47).

As alterações do genoma estão entre as principais implicações para a carcinogênese, sendo observadas alterações no DNA, como a inserção, exclusão ou mudança de nucleotídeos ou irregularidades cromossômicas, levando à manifestação de um fenótipo defeituoso. A superexpressão de oncogenes está intimamente associada ao desenvolvimento do CEB (48).

As alterações na sinalização intracelular oncogênica, como a proteína quinase ativada por mitogênio (MAPK), fosfatidilinositol-3-quinase (PI3K)/AKT/alvo de rapamicina em mamíferos (mTOR) e transdutor de sinal e ativador de transcrição (STAT) favorecem a patogenia e potencial metastático do CEB (49).

Desregulações em genes supressores de tumor são eventos frequentes na carcinogênese de boca, tais como a inativação por mutações pontuais, deleções e rearranjos em ambas as copias do gene. Cerca de 70% dos tumores sólidos adultos ocorre devido a ocorrência de alterações no gene supressor *TP53*. A proteína p53 impede a divisão celular da fase G1 para a fase S no ciclo celular, estimulando a reparação do DNA após danos no DNA, e também induz a apoptose (50-52). No mecanismo da doença, o gene *TP53* apresenta perda da sua funcionalidade, estando presente nas lesões displásicas e neoplásicas de mucosa bucal; a progressão do quadro histopatológico está ligado à presença dessas mutações (53, 54).

As modificações epigenéticas também são conexas à etiopatogênese do CEB. Mecanismos epigenéticos podem influenciar a desregulação da expressão gênica, a partir de metilação de DNA, modificações de histonas e expressão alterada de micro-RNAs (miRNAs) oncogênicos. Tais eventos epigenéticos desempenham um papel crucial nos

eventos de silenciamento gênico de genes supressor de tumor, que favorece desenvolvimento e progressão da doença (55).

O polimorfismo na metaloproteinase-9 (MMP-9) está associado a um risco aumentado de desenvolver os estágios iniciais do câncer bucal, principalmente em pacientes com elevado uso do tabaco e consumo de álcool. Nota-se níveis elevados de mRNA da MMP-9 nas displasias orais que evoluíram para o CEB (56). Além das alterações genéticas e epigenéticas, os hábitos relacionados ao estilo de vida, como o tabagismo e consumo do álcool, são fatores de risco importante na etiopatogenia do CEB, e que estão presentes em 90% dos casos, promovendo um efeito sinérgico (57). O álcool gera uma alteração na taxa de penetração de substâncias do ambiente oral através da mucosa e essas modificações na permeabilidade da mucosa podem favorecer a carcinogênese de boca (58).

A fumaça do cigarro possui substâncias cancerígenas, dentre elas estão os hidrocarbonetos aromáticos policíclicos, aminas heterocíclicas e nitrosaminas. Além do consumo de álcool por um período prolongado, este pode levar a um efeito combinado da ação de espécies reativas de oxigênio (ERO) juntamente com o acetaldeído, podendo promover alterações no DNA e à carcinogênese. Alterações na função de enzimas metabolizantes, como glutationa S-transferase (GST), superóxido dismutase (SOD), proteínas da família SOD e acetaldeído desidrogenase (ALDH), também pode estar relacionada a uma maior susceptibilidade para o desenvolvimento do CEB (59).

Existe uma importante relação entre o câncer de boca e o tabagismo. A cavidade oral recebe a fumaça do cigarro, onde o seu principal componente é a nicotina, que é absorvida pela membrana da mucosa oral, induzindo a alterações no DNA, aumento do estresse oxidativo e promoção de desregulações do comportamento celular, como proliferação exacerbada e escape da morte celular por apoptose (Figura 1) (60).

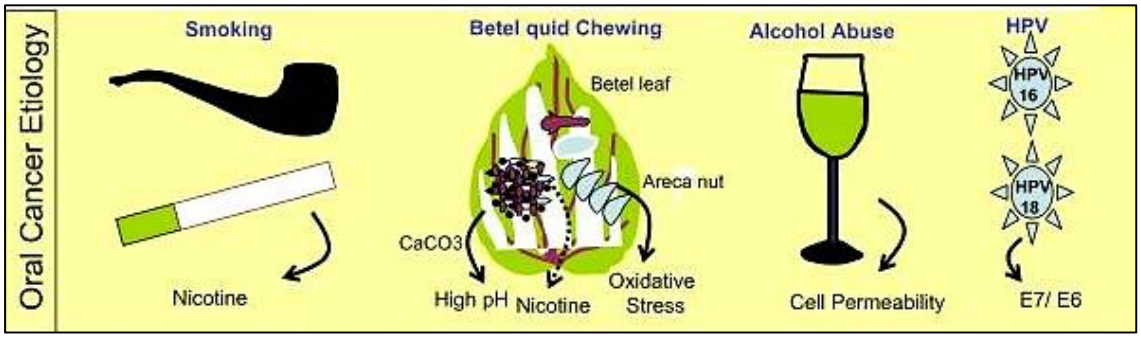

Figura 1 - Principais fatores e seus mecanismos de ação, envolvidos na etiopatogênese do câncer de boca. Fonte: Adaptado de MISHRA, 2010 (61).

O papiloma vírus humano HPV está associado ao desenvolvimento do CEB, principalmente o HPV tipo 16 e 18, que são vírus oncogênicos que mais causam câncer (62). As oncoproteinas E6 e E7 são os tipos de HPV com risco aumentado; induzem principalmente a degradação e inativação de p53 e a proteína do retinoblastoma (pRb). Além disso, as oncoproteinas interagem com um enorme número de proteínas celulares, alterando suas funções normais e facilitando a transformação celular (63).

3.3 Proliferação, migração, invasão, angiogênese e morte celular: mecanismos envolvidos no carcinoma epidermoide de boca.

A célula neoplásica incorpora características que permitem sua sobrevivência e manutenção da proliferação desregulada. A carcinogênese é composta por várias etapas, das quais as alterações moleculares e na morfologia e funções celulares precedem o aparecimento da lesão. Tais alterações estão associadas a características fenotípicas que inclui a migração celular e transição epitélio-mesenquimal (TEM), em regiões com baixa oxigenação onde promovem a sobrevivência, crescimento de células tronco-teciduais e a angiogênese (64, 65).

A proliferação celular desregulada em tumores sólidos é favorecida, especialmente, pela fosforilação em um complexo formado por ciclinas e quinases dependentes de ciclina, sendo estas proteínas consideradas o ponto chave do ciclo celular. E o controle da proliferação está ligado a ativação e inibição do ciclo celular. O mecanismo de desativação do processo proliferativo é baseado em sistemas de transdução de sinais negativos podendo agir sobre a parada do ciclo celular, até que sejam restabelecidas

condições favoráveis para a mitose. Algumas proteínas impedem temporariamente, a iniciação do ciclo celular ou a sua progressão, inibindo as quinases dependentes de ciclinas, quando o equilíbrio mitogênico está regulado negativamente (66, 67). Contudo, o rearranjo do gene da ciclina D1, que é identificada como um oncogene humano derivado de uma superexpressão de proteínas, tem sido associado ao prognóstico em uma abundância de tumores malignos, associada inclusive com o desenvolvimento do CEB e revelando um valor prognóstico (68, 69).

A metástase do câncer tem como características a intensificação dos processos de migração e invasão das células neoplásicas, através de eventos que são facilitados pela perda de adesão celular e propriedades epiteliais, rearranjo do citoesqueleto e degradação de matriz colágena, dentre outros mecanismos que permitem ativar a migração e invasão neoplásica (70, 71).

O desenvolvimento do CEB pode ser caracterizado pelo desenvolvimento inicial de uma lesão potencialmente malignizável se poderá progredir para uma lesão neoplásica. Estudos relatam que o aumento significativo da angiogênese é um fator preponderante que ocorre na transição da mucosa normal para os mais diferentes graus de displasia, carcinoma invasivo e progressão para metástases (72, 73). A angiogênese é a formação de novos vasos sanguíneos que crescem a partir de vasos sanguíneos já existentes. Em geral, o fator de crescimento endotelial vascular (VEGF) é considerado um dos fatores pro-angiogênicos mais importantes envolvido nesse processo (72). As características ligadas ao potencial invasivo das neoplasias estão associadas à degradação da matriz extracelular pelas células neoplásicas, proliferação e migração de células endoteliais, diferenciação e formação de anastomoses capilares (74-76). As metaloproteinases-2 e metaloproteinases-9 (MMP-2, -9), estão associadas à degradação da matriz extracelular e envolvidas no processo de invasão celular (77). A capacidade das células malignas destruírem a membrana basal e componentes da Matriz extracelular relaciona-se ao potencial invasivo e metastático das neoplasias (78) (Figura 2).

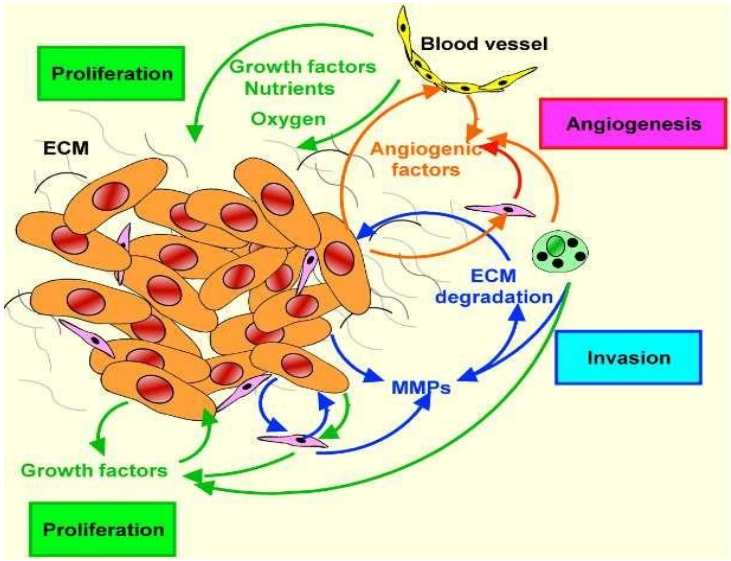

Figura 2 - Principais características do microambiente tumoral, fatores de proliferação, angiogênese e invasão, aspectos de degradação de matriz extracelular. Fonte: Adaptado de KOONTONGKAEW, 2013 (57).

A morte celular pode ocorrer por apoptose ou outras formas não apoptóticas, como por necrose. As características morfológicas da morte celular são dependentes de vários fatores, que incluem o tipo de célula e vias de sinalização. As células neoplásicas possuem a capacidade de evitar a ativação de seu programa apoptótico e escape dos mecanismos de defesa do sistema imunológico. Nas células normais, alguns mecanismos apoptóticos são capazes de eliminar células danificadas por um abundância de estresse, como falta de nutrientes, hipóxia, danos ao DNA e terapia antineoplásica. Já nas células tumorais, podem evitar a ativação da apoptose por meio da inativação de genes indutores de apoptose ou pelo aprimoramento da atividade dos genes antiapoptóticos. O gene *TP53* é importante na parada do ciclo celular e na apoptose, exercendo seus efeitos nos múltiplos estágios da progressão do câncer quando estes se encontram inativos. Além da inativação da proteína p53, outras proteínas indutoras de apoptose BAX e BAD se encontram desreguladas nas células de carcinoma bucal (79).

3.4. Radiação ionizante como estratégia terapêutica para o carcinoma epidermoide de boca

A radioterapia (RT) ou radiação ionizante é uma das modalidades terapêuticas utilizadas no tratamento do câncer de boca, tendo um papel importante no controle do crescimento neoplásico, em especial quando o indivíduo é acometido pela enfermidade e não apresenta condições clínicas para ser submetido ao tratamento cirúrgico, ou não aceita as possíveis mutilações faciais no qual a intervenção cirúrgica poderá acarretar (80).

Apesar do efeito terapêutico, que visa promover a morte das células neoplásicas e controlar a progressão da doença, a radioterapia pode gerar danos aos tecidos normais e consequentes efeitos colaterais, tais como mucosites, osteorradionecrose, xerostomia e disgeusia. Devido os avanços das técnicas de radioterapia, esses efeitos colaterais agudos e crônicos vem sendo reduzidos, especialmente em pacientes expostos à radiação na região de cabeça e pescoço (81, 82).

A radioterapia causa danos nas células-alvo por meio de diversas vias do genoma, tendo como principal efeito a morte direta das células. As ionizações induzidas por radiação podem atuar diretamente sobre moléculas celulares e causar danos, mas também podem agir indiretamente, induzindo o estresse oxidativo e produção de espécies reativas de oxigênio (EROs), derivados da ionização ou excitação da água. As quebras de fita dupla induzidas por radiação representam os tipos mais letais de danos ao DNA, levando à morte celular por apoptose (Figura 3) (83, 84). As quebras nas fitas simples ou duplas do DNA induzidas pela radiação promovem parada do ciclo celular e apoptose, comprometendo a capacidade de multiplicação. A radiossensibilidade de células neoplásicas está associada à ativação de vias de sinalização, envolvendo principais protéinas, como p53, p21, Bax e Puma. No entanto, vários mecanismos de reparo de DNA nas células tumorais interferem nos danos induzidos pela radiação, levando à radiorresistência das células neoplásicas. A inibição de proteínas de reparo de DNA, como ATM ou proteína quinase dependente de DNA (DNA-PK), pode favorecer o aumento da sensibilidade das células neoplásicas ao tratamento por radiação. (Figura 4) (83).

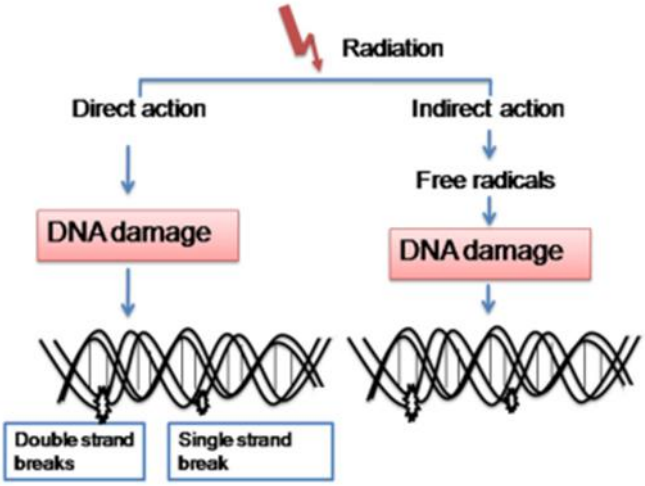

Figura 3 - Principais mecanismos de ação da radioterapia: As radiações ionizações agem diretamente nas moléculas celulares e causam danos; também atuam indiretamente, produzindo radicais livres, que levam à morte celular. Fonte: BASKAR, 2014 (83).

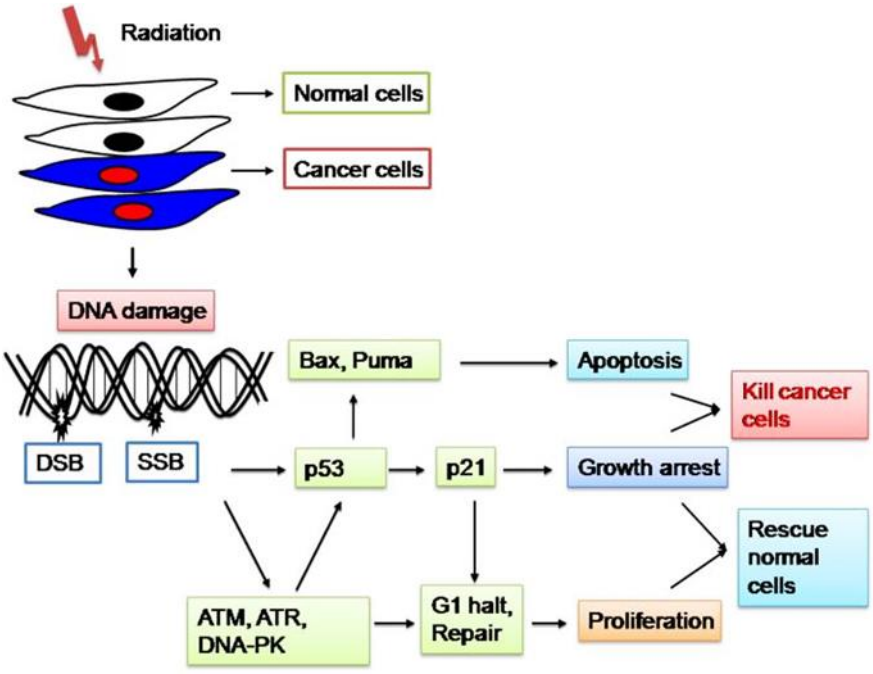

Figura 4 - Principais proteínas ativadas pela radiação ionizante terapêutica para promoção da morte de células neoplásicas. Fonte: BASKAR, 2014 (83).

O gene Ataxia-telangiectasia mutado (ATM) é de extrema relevância para a radiorresistência de células neoplásicas. Trata-se de uma proteína-quinase que funciona como um gene supressor de tumor, desempenhando um papel fundamental na resposta celular apropriada a danos no genoma resultantes de exposição química a carcinógenos ou radiação ionizante, na parada do ciclo celular, reparo do DNA e apoptose (84, 85).

Apesar dos avanços nesta modalidade terapêutica, os tumores podem recorrer dentro de um campo irradiado devido à baixa responsividade do organismo a terapia, levando a um mau prognóstico (86). Assim, é de fundamental importância a realização de pesquisas buscando um maior entendimento sobre os fatores que interferem sobre a sensibilidade de células neoplásicas à radiação ionizante terapêutica (87), bem como a investigação de tratamentos alternativos para aumentar a eficácia da radiação ionizante. Uma abordagem promissora para aumentar a eficácia da radioterapia em pacientes com CEB é a descoberta e uso de drogas que favoreçam a radiossensibilidade de células neoplásicas, a fim de melhorar as taxas de resposta à radioterapia e o controle e/ou progressão da doença.

3.5 Ação biológica e potenciais terapêuticos de substâncias isoladas do veneno da serpente *Crotalus durissus terrificus*

Os componentes diversos presentes em venenos de cobras possuem diferentes efeitos terapêuticos, inclusive retardando a proliferação de células neoplásicas (88).

No mundo, são reconhecidas mais de 10.700 espécies de répteis. O Brasil está em 3º lugar considerando a sua diversidade admirável de espécies de répteis, contando com 795 espécies, sendo 36 *Testudines*, 6 *Crocodylia* e 753 *Squamata* (72 *anfisbenas*, 276 "lagartos" e 405 serpentes). Considerando as subespécies, são 6 *Crocodylia*, 37 *Testudines* e 799 *Squamata* no Brasil (75 *anfisbenas*, 282 "lagartos" e 442 serpentes), totalizando 842 espécies e subespécies de répteis no país (89).

Três gêneros de serpentes da família *Viperidae* são encontradas no Brasil: *Lachesis*, *Bothrops* e *Crotalus*. As serpentes do gênero *Bothrops* estão vastamente distribuídas na Mata Atlântica, inclusive em áreas habitadas pela atividade humana. Por outro lado, as cascaveis brasileiras geralmente vivem em áreas abertas e secas conhecidas como Cerrado, mas também podem estar associadas a áreas de transição (90-92).

Foram identificadas 6 subespécies de *Crotalus durissus* que habitam o território brasileiro: *Crotalus durissus terrifícus, Crotalus durissus collilineatus, Crotalus durissus cascavela, Crotalus durissus marajoensis, Crotalus durissus ruruima* e *Crotalus durissus trigonicus* (93). Destas seis subespécies de *Crotalus durissus* encontradas no Brasil, a *Crotalus durissus terrificus* é a mais frequente. Através do fracionamento do veneno dessa espécie realizado por cromatografia de exclusão molecular foram evidenciadas quatro principais toxinas enzimáticas, que são: convulxina, giroxina, crotoxina e crotamina (94).

A crotoxina (CrTX) é uma Fosfolipase A2 (PLA2), toxina isolada do veneno da cascavel da América do Sul, *Crotalus durissus terrificus* (95, 96). É o componente majoritário presente no veneno, constituindo aproximadamente de 65% do veneno total (97). O isolamento de tal substância foi, inicialmente, descrito, em 1938, por SLOTA e FRAENKEL-SONRAT (98). É um complexo composto por duas subunidades distintas, subunidades A e B. A subunidade B possui atividade da PLA2 e contribui para a citotoxicidade da CrTX (Figura 5) (99).

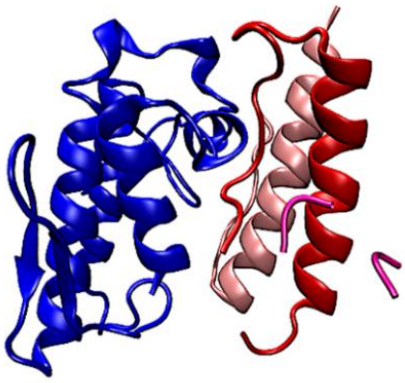

Figura 4 – Estrutura tridimensional da crotoxina (CrTX).
Fonte: Adaptado de SHIMIZU, 2017 (100).

Os principais efeitos tóxicos da CrTX consistem em atividades miotóxicas, neurotóxicas, nefrotóxicas, cardiotóxicas, indutoras de edema, disruptivas lipossômicas e anticoagulantes (101, 102). A despeito de sua toxicidade, a CrTX possui potenciais efeitos terapêuticos, devido a suas ações imunomodulatória, antiinflamatória, antimicrobiana e antinociceptiva (102). Além disso, a CrTX demonstrou efeitos

citotóxicos em alguns tipos de neoplasias, tais como câncer de mama, pulmão, leucemia e esôfago (21, 27, 96, 103). No entanto, os mecanismos moleculares envolvidos no potencial efeito antineoplásico da CrTX não são bem elucidados. Apesar de incipientes, pesquisas científicas têm voltado o olhar para investigar o efeito terapêutico antineoplásico da CrTX. No câncer de pulmão, a CrTX exerceu efeito sinérgico ao quimioterápico gefinitib, amplamente utilizado na terapia do câncer de pulmão (95).

No carcinoma epidermoide de boca, o efeito da CrTX ainda não foi investigado. Tendo em vista os quadros de radiorresistência de indivíduos acometidos pelo câncer bucal, a investigação de estratégias terapêuticas adjuvantes é um foco interessante a ser pesquisado, visando melhorar a resposta terapêutica e a sobrevida dos pacientes. Assim, mediante a falta de estudos que evidenciem a ação sinérgica da crotoxina à radiação ionizante terapêutica do carcinoma epidermoide de boca (CEB), o presente estudo tem como objetivo principal investigar o efeito terapêutico adjuvante da CrTX à radiação ionizante em células de carcinoma epidermoide de boca.

4 PRODUTOS

4.1 Produto 1:

- Artigo científico: “Antineoplastic potential of crotoxin in different types of cancer: a systematic review”. Formatado para submissão segundo as normas do periódico “Oral Oncology”.

4.2 Produto 2:

- Artigo científico: “Isolated substance from snake venom *Crotalus durissus terrificus* potentiates the therapeutic effect of ionizing radiation on oral squamous cell carcinoma”. Formatado para submissão segundo as normas do periódico “Journal of Oral Pathology & Medicine”

4.3 Produto 3:

- *Pitch* para divulgação online dos resultados da dissertação: Foi elaborado um vídeo de curta duração para divulgação online dos resultados do estudo, direcionado à população em geral.

4.1 Produto 1

Antineoplastic potential of crotoxin in different types of cancer: a systematic review

Felipe Alberto Dantas Guimarães[1]

Renata Sousa Leite[1]

Rogério Gonçalves da Rocha[1]

Victor Hugo Dantas Guimarães[1]

Lília Fernanda Antunes[1]

André Luiz Sena Guimarães[1,2]

Lucyana Conceição Farias[1,2]

[1]Laboratory of Health Science, Postgraduate Program in Health Sciences, Universidade Estadual de Montes Claros, Montes Claros, Minas Gerais, Brazil

[2] Department of Dentistry, Universidade Estadual de Montes Claros, Minas Gerais, Brazil

*Corresponding author:

Lucyana Conceição Farias
Universidade Estadual de Montes Claros
Hospital Universitário Clemente de Faria
Laboratório de Pesquisa em Saúde
Avenida Cula Mangabeira, 562
Montes Claros, Minas Gerais, Brasil
Zip code: 39401-001
Phone: +55 38 32248327
E-mail: lucyanacfarias@gmail.com

Abstract

Cancer is one of the leading causes of death in the world; it is caused by the multiple transformations that a normal cell undergoes over time, conferring changes in the genome and exacerbated proliferative potential. Crotoxin is a neurotoxin isolated from South American rattlesnake venom, *Crotalus durissus terrificus*. It has some biological activities, which are immunomodulatory, anti-inflammatory, antimicrobial and analgesic, besides these, crotoxin has antitumor action. The aim of this study was to systematically review the literature about the potential antineoplastic of CrTX in different types of cancer. The review involved a search of in vitro and clinical studies related to the proposed aim. The study was registered in the International Prospero Platform (CRD42019137665) and followed the standards set by the Preferred Reporting Items for Systematic Reviews and Meta-analyses. Through the search for studies in the databases PubMed, Scopus, Web of Science and EBSCO, 71 articles were selected. From the analysis of studies, adopting inclusion and exclusion criteria a total of 12 articles were selected as eligibles to read the full text. The antineoplastic potential promoted by crotoxin was evidenced in this review in studies that evaluated a diversity of tumor lineages and a phase I clinical and pharmacokinetic study. Crotoxin exerted antiproliferative activity, promoted cell cycle arrest, increased apoptosis rate, up-regulated autophagy, and increased expression of caspase-3, p53, p15, caspase-3, p17, p38MAPK, LC3-II, Beclin 1, p-JNK, and H2AX. Morphological damage in cancer cells was evidenced, such as irregularity in shape, the formation of blisters and autophagic vacuoles. In phase I clinical study, crotoxin decreased pain, reduced hepatomegaly, disappeared with edema, reduced lymph node mass, reduced tumor size and allowed cessation of treatment for 6 months. Taken together our findings points that Crotoxin can be a promising therapeutic substance due to its antineoplastic role in different cancer cells. Clinical findings corroborate with in vitro studies, improving tumor progression.

Keywords: Crotoxin. Antineoplastic. Antitumoral. *Crotalus durissus terrificus*. Cancer.

Introduction

Cancer has been ranked as one of the leading causes of death in the world [1, 2]. It is characterized by the multiple transformations that a normal cell undergoes over time [3], and by disordered growth of abnormal cells and dynamic changes in the genome [4, 5].

In the development of carcinogenesis, a series of genetic and epigenetic alterations are present which, even in the absence of growth factors, confer a proliferative potential and may resist to pro-apoptotic stimuli and promote angiogenesis [6]. These changes that may occur include aneuploidy (loss or addition of chromosomes), deletions or addition of genomic material, and minor modifications in several genes that are spread throughout the genome of a neoplastic cell [7].

In most solid cancers, its clinical appearance is not clearly defined, but the carcinogenic agents that contribute to the development of cancer are well documented. These carcinogenic agents make up broad groups and can be classified as infectious (eg Human papillomavirus), chemicals (eg alcohol and tobacco), electromagnetic radiation (eg ultraviolet radiation) and immunosuppressants (eg immunosuppressive drugs) [8].

As of 2015, more than 8.7 million cancer deaths have been reported worldwide and is cited as the second leading cause of death behind cardiovascular disease alone [4, 9]. In 2018, approximately 9.6 million deaths occurred from cancer. Worldwide, one in six deaths is due to cancer, and nearly 70% of deaths occur in low-income countries [2]. The number of cases is expected to increase to 24 million by the year 2050 [10].

The most common therapeutic modalities in cancer treatment are surgery, radiotherapy, chemotherapy, immunotherapy and hormone therapy [11, 12]. Although some treatments such as chemotherapy are successful, chemotherapeutic drugs are not selective and most anticancer agents do not differentiate cancer cells from normal cells. These chemotherapy agents damage healthy tissues, induce toxicity and cause adverse side effects [13]. Relative conditional survival (SCR) is 5 to 10 years after surviving for a certain number of years [14].

Crotoxin (CrTX) is a neurotoxin isolated from the South American rattlesnake venom (*Crotalus durissus terrificus*) [15-17]. In 1938, Slotta and Fraenkel-Conrat first isolated, purified and crystallized the toxic principle of *Crotalus durissus terrificus* [18, 19]. CrTX is formed by a non-covalent complex with 2 subunits, one acidic (subunit CA) and one basic (subunit CB) [4, 20]. Subunit B is a phospholipase A2 (PLA2) formed by

a single chain of 122 amino acid residues cross-linked by 7 disulfide bonds, and CrTX corresponds to 60% of rattlesnake venom [16, 18, 21-23].

Biological activities can be attributed to CrTX, such as neurotoxicity, myotoxicity, and nephrotoxicity. Besides its toxic effects, many other studies have shown that CrTX exerts immunomodulatory, anti-inflammatory, antimicrobial and analgesic actions [17, 18, 24].

The CrTX also has antitumor activity on different cancer cell lines including leukemia, cervix, ovary, lung, colon, kidney, melanoma and brain, which have been evaluated in vitro and in vivo studies [17, 18, 24-30]. The antitumor effect of rattlesnake venom can be attributed to the CrTX due to its antiproliferative activity, which occurs through apoptotic mechanisms, triggered by mitochondrial membrane alterations, cytochrome C release, and caspase-3 activation [4, 24, 29].

Although there is a wide spectrum of medicaments, especially antineoplastic drugs, they are still unable to meet all therapeutic demands [15]. In view of this, this study systematically reviewed the literature about the antineoplastic potential of crotoxin in different types of cancer.

Methods

Resources

This systematic review was recorded in the PROSPERO platform (Registration CRD42019137665). A literature search was performed in the scientific electronic databases PubMed, Scopus, Web of Science e EBSCO to identify publications that used the crotoxin as a treatment for various types of cancer in patients or in cancer cell lines and was evaluated its antitumor effect. For more complete strategy research, the PICOS tool was used to design the systematic search strategy. Moreover, to select the studies related to purpose, we followed the standards set by the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) [31].

The data-collection occurred from June 2019 to November 2019, and we considered the published studies until November 2019 in the English language.

Search Strategy

The descriptors were selected through the Medical Subject Headings (MeSH) and common terms from literature. Furthermore, booleans AND/OR were added in the search strategy. The term combinations and descriptors were as following: [crotoxin OR crotoxin A OR crotoxin B] AND [neoplasm OR cancer OR tumor OR carcinoma] AND [antineoplastic OR antitumor OR anti-tumor OR antitumoral OR cytotoxicity OR cytotoxic OR cytostatic]. All of these terms were identified by reading the title or abstract of the articles. This procedure aimed not only to filter the results but also to cross the main terms to obtain the maximum possible studies.

Eligibility Criteria of the Studies

In order, as searching criteria, we adopted as eligible the randomized controlled trials and observational studies (cohort and case-control), and original *in vitro* studies that investigated the antineoplastic effect of crotoxin on different cancers. Animal model studies were not eligible, once the systematic reviews are registered in the PROSPERO database according to the investigated category, as animal or human. We considered the published studies until November 2019 in the English language. Duplicated papers found in the databases were excluded.

Exclusion criteria

Studies wrote in another language than English, that is not related to the purpose of this study, review article, case report, letter to the editor, conference papers, theoretical studies (bioinformatics), *in vivo* studies were excluded. In addition, studies that assessed the antineoplastic effect of other substances in the different types of cancer or other effects of crotoxin in cancer, such as analgesic actions, were not included.

Study selection

First, an inter-rater calibration was performed to select the articles. The articles included in the study were selected by two authors (FADG and RSL). The articles were identified through the electronic search in the databases, based on the reading of the title and the abstract of the articles, they were organized, reviewed for the identification of duplicates by the authors independently.

The agreement between the reviewers was based on the Kappa statistical analysis and in case of disagreement on the inclusion criteria, a third researcher (LCF) was consulted. After completing this first stage, the full text of the selected articles was read to carry out a new selection, data collection, and evaluation of studies.

Data extraction and strategy for data synthesis

Data were extracted and recorded independently, including study results and methodological steps as a systematic narrative synthesis, showing the following items: crotoxin treatment, dose and frequency of treatment, assays performed, main findings. The intervention can be the treatment with crotoxin alone or crotoxin, in addition to another cancer treatment protocol, such as chemotherapy or radiotherapy.

Quality assessment of studies

The instrument ToxRToll [32] was used to evaluate the quality criteria developed in the original *in vitro* studies. The ToxRTool provides parameters with a potential impact on the data quality of a study, where a minimum set of information is defined in each group, considered important for data reliability. To evaluate the quality of the study, 18 points were verified, including reliability, relevance, and adequacy. The spreadsheet available at the URL https://ec.europa.eu/jrc/en/scientific-tool/toxrtool-toxicological-data-reliability-assessment-tool calculates a score by categorizing the studies as follows:

15–18 (reliable unrestricted), 11 to 14 points (reliable with possible restrictions), and <11 (unreliable) are available in table 1.

Results

Inter-rater agreement

The inter-rater calibration between the two evaluators for selecting studies was assessed by Cohen's k, using the kappa statistic. The kappa value was = 0.82 ($p<0.0001$), indicating an almost perfect inter-rater agreement.

Characteristics of included studies

From the literature search, a total of 71 publications were identified, as following: PubMed (n=16), Web of Science (n=23), Scopus (n=18), EBSCO (n=14). Figure 1 represents the process of selection and eligibility of studies. After the exclusion of 46 duplicated articles in the databases, 25 of these articles were selected for reading the title and abstract. From these, 12 articles were excluded based on titles and abstracts, because they did not meet the inclusion criteria. After reading 13 full texts, 1 of the studies did not enter the review because another substance was tested, than CrTX. A Phase I study in humans was also excluded because the main aim was not to assess the antineoplastic role of CrTX, but the toxicity and pharmacokinetic profile of this substance when administered in patients with advanced cancer refractory to conventional therapy. So, a total of 11 *in vitro* articles were included in this systematic review for the result analyses [4, 15-18, 21, 24, 29, 30, 33, 34].

The quality assessment of *in vitro* studies, according to the ToxRTool revealed that 10 studies were considered "reliable without restrictions" and only one was considered reliable with possible restrictions (Table 1).

Findings on crotoxin effects in in vitro studies

In addition to the to toxic effects of CrTX, like neurotoxicity, myotoxicity, and nephrotoxicity [17, 18, 24], the studies analyzed in this systematic review demonstrated that this phospholipase also plays antitumor activity in various cancer cell lines including leukemia [21, 25], pancreas [24], esophagus [24, 33], breast [16, 34], lung [15, 17, 18,

29, 34] and brain [4, 24, 34], which were evaluated *in vitro*. The result summaries of the studies are shown in Table 2 and Figure 2.

Crotoxin promoted antiproliferative effect on several cancer cell lines, including A549 (human lung adenocarcinoma cells) [15], Eca-109 (esophageal carcinoma cells) [33], MCF-7 (human breast cancer cells) [16], K562 (myeloid leukemia cells) [21], ME-180 (parental cervical carcinoma cells) [30], SK-MES-1 (human lung squamous cell carcinoma cells) [18, 29], GH3 (benign pituitary adenoma cells) [4], SPCA-1 (lung adenocarcinoma cells) [17].

Cell viability of SPCA-1 [17], K562 [21], and MCF-7 [16] strains decreased after treatment with CrTX. Concentration and treatment period influenced the inhibition rate of MCF-7 cells and the primary rate was 28.85 ± 0.01% at 24 hours, at 48 hours the inhibitory rate was 51.61 ± 0.03 % and after 72 hours of treatment the rate was 57.04 ± 0.04% [16] and in K562 cells the inhibition rate reached 85.91% ± 1.72% at 72 hours [21].

Evaluating the cytotoxicity of CrTX in immortalized human umbilical normal endothelial cells (HUVEC) used as control cells, after receiving CrTX 50 µg/mL treatment for 72 hours, there was a 20% decrease in HUVEC cells, suggesting that CxTX has lower toxicity in normal cells [21]. Normal human keratinocyte (HaCaT) cells also received CrTX treatment and had no reduction in cell viability. Chemotherapeutic agents were used as standards in some cancers, and CrTX had high cytotoxicity in glioma, pancreatic, and cervical cell lines. CrTX exerted lower cytotoxicity in esophageal cell lines compared to the chemotherapeutic agent gemcitabine [24].

The cytotoxic role of CrTX was tested on other three cell lines, SK-LU-1 (Lung adenocarcinoma strain), Hs 578T (Breast Ductal Carcinoma Lineage) and U-87 MG (Glioblastoma Lineage) and also on control cells, NHEK-47 (human normal epidermal keratinocytes) at concentrations ranging from 2.3 to 30 µg/mL. The CrTX-treated SK-LU-1 and Hs 578T cells had a significant reduction, while U-87 MG cells were not significantly affected. The NHEK-47 cells exposed to CrTX treatment were not as affected and maintained a considerable percentage of viable cells [34].

Evaluating the CrTX effect on the different phases of the cell cycle in the A549 lineage [15], there was a significant interruption in the sub-G0/G1 phase and in the SK-MES-1 cells [29] there was a decrease in the cells in the G0/G1 phase, and an increase in S-phase cells. In cells Eca-109 [33], SK-MES-1 [18], GH3[4], SPCA-1 [17], the CrTX promoted the arrest of the cell cycle in phase G1.

The A549 [15] and SK-MES-1 [29] cell lines that received CrTX treatment increased apoptosis rate, inhibition of cell proliferation, cleaved caspase-3 expression, wild-type p53 and phospho-P38MAPK levels. The results revealed the positive regulation of autophagy after treatment with CrTX, as evidenced by high levels of LC3-II and Beclin 1 proteins, with p62 expression partially blocked by SB203580 treatment. Caspase-3 expression increased after treatment with CrTX and PCNA protein decreased in SK-MES-1 cells [29].

Gene expression of p15 and caspase-3 p17 gene in Eca-109 cells were elevated by CrTX treatment, and Bcl-2 gene expression decreased with increasing crotoxin concentration [33].

The morphological aspects of Eca-109 [33] and SK-MES-1 [29] cells were evaluated using Hoechst 33342 staining, in which untreated cells maintained regular and uniform nuclei, whereas, in cells treated with CrTX the cells suffered structure damage, making them pycnotic, some dendrites were scattered between the cells after their disruption and DNA fragmentation.

Cell morphology of GH3 cells treated with CrTX showed visible morphological changes, namely: irregularities in cell form, shrinkage and bubble formation, characteristics attributed to apoptosis, which were induced by CrTX action [4].

Autophagy vacuoles (AVs) were visualized through monodansylcadaverin (MDC) when the treatment of MCF-7 [16] and K562 [21] cells was extended for 3 hours. Some vesicles were labeled with MDC indicating the formation of autophagic vacuoles. The increase in AVs during the 12 hours of CrTX treatment occurred in MCF-7 cells [16] and after 6 hours of treatment of K562 cells [21], a steady-state of MDC accumulation in the vesicles was achieved. There was no difference in MDC labeling in vehicle-treated control cells within 0.5 to 12 hours [21].

Structural changes in MCF-7 [16] and K562 [21] cells were evaluated by transmission electron microscopy. The morphology of the untreated cells remained with normal organelles and nuclei. In K562 [21] and MCF-7 [16] cell lines treated with CrTX, typical signs of autophagy and apoptosis were observed. Through DAPI staining assay, CrTX-treated GH3 cells showed characteristics attributed to apoptosis [4].

Changes in mitochondrial potential ($\Delta\Psi$) of K562 cells [21] were detected 1.5 hours after CrTX treatment, this change lasted for 6 hours after treatment. CrTX suppressed growth cells with the highest sensitivity in EGFr overexpressing cells in ME-180R and A431 cells, suggesting a potential correlation [30].

The action of crotoxin significantly suppressed the formation of SK-MES-1 cell colonies [18, 29]. The apoptosis rate in the CrTX, Iressa, and combination treatment groups increased significantly compared to controls, and CrTX increased expression of active caspase-3 and p-JNK, but Bcl-2 protein was downregulated [18]. In SPCA-1 cells, it was possible to verify that the p-JNK protein level was up-regulated after CrTX treatment, establishing a positive relationship in CrTX activation of the JNK pathway [17]. The immunofluorescence assay also confirmed the enhanced expression of p-JNK in SPCA-1 cells after CrTX treatment, while SP600125 failed to alter the level of CrTX-induced p-JNK expression. Based on these results, they suggested that the JNK pathway is important in CrTX-induced apoptosis in SPCA-1 cells [17].

HCB 151 and SiHA cells that were not treated with CrTX had a lower percentage of early and late apoptosis than HCB 151 and had no significant effect on the SiHa cell line [24]. DiFi cells are able to express EGFr 100 times more than in HT-29 cells based on the analysis of the intrinsic activity of EGFr tyrosine kinase in cell lysates [30].

In the evaluation of cell signaling, CrTX-treated PANC-1 and HCB151 cells phosphorylated H2AX, which was overregulated, and is an important marker of DNA damage. The expression of p21, p-AKT, AKT (pan), pP42/44 and P42/44 protein did not change in any investigated cell line [24].

The antitumor action of crotoxin on GH3 cells depends on growth factor fetal bovine serum (SFB) and calcium. The CrTX in the absence of SFB had its potency is 45% lower but remains with its active antitumor effect, the action of CrTX in the GH3 cell line was reduced by 135% in the absence of calcium [4].

Discussion

Crotoxin (CrTX), a potent neurotoxin, has phospholipase A2 (PLA2) neuromuscular activity [15, 16, 30, 33]. Studies using CrTX have reported antineoplastic activity in some cancer strains [4, 15-18, 21, 29, 30, 33, 35].

In this systematic review, the CrTX promoted a decrease in cell viability of cell lines SPCA-1 [17], K562 [21] and MCF-7 [16, 17, 21], SK-LU-1 [34], Hs 578T [34] and U-87 MG [34]**.** In addition to *in vitro* results, crotoxin (CrTX) was able to reduce tumor growth on the planar surface of the hind paw of an adult rat on the 5th day of treatment, resulting in a decrease in blood vessel diameter [28].

In the cell cycle occurs various events within a cell that leads to its division and duplication. Prior to progression to the next phase of the cell cycle, several checkpoints are checked [36, 37]. Cell cycle arrest of A549 [15] cells in the sub-G0 / G1 phase and Eca-109 [33], SK-MES-1 [18], GH3 [4] and SPCA-1 [17] in phase G1 is dependent on the dose and time of treatment with CrTX, proposing the attribution of crotoxin in the growth inhibitory mechanisms of cancer cell lines.

The *TP53* tumor suppressor gene is attributed to responses to DNA damage, preventing proliferation or inducing cellular apoptosis [35]. Caspase-3 is associated with cellular sensitivity linked to various apoptotic stimuli [38]. The p38 MAPK protein is involved in the prostate, breast cancer, transformed follicular lymphoma and leukemia. Activation of p38 MAPK is linked to the epithelial-mesenchymal transition of primary tumors, acquisition of tumor cell invasion, migration and extravasation capacity [39]. The CrTX treatment positively regulated the expression of some genes such as p53, cleaved caspase-3 and phospho-P38MAPK in A549 [15], SK-MES-1 [29] cells. The CrTX acted by inhibiting cell proliferation, mediating the apoptotic response and activating autophagy [15, 29].

Crotoxin inhibited cell growth of ME-180 and A431 cells having greater excitability in EGFr overexpressing cells, implying a potential relationship [30]. The same is true of another study, in which epidermal growth factor receptor expression is closely correlated with tumor cell sensitivity to crotoxin, with the membrane receptor being associated with a malignant cell phenotype [35].

Structural changes in cells MCF-7 [16] and K562 [21] were promoted by CrTX, while morphological aspects of untreated cells remained normal. Structural disorders of cancer cells following crotoxin treatment have resulted in typical signs of autophagy and

apoptosis (loss of organelles, the formation of many giant autophagosomes, and cytoplasm vacuolization) [16, 21]. Structural changes were also evident in another study, where it was possible to identify by DAPI staining signs of apoptosis in groups treated with crotoxin, such as nucleus condensation, DNA fragmentation, and formation of apoptotic bodies in GH3 brain tumor cells[4].

Mitochondrial membrane potential analysis is a tool used for monitoring changes involved in mitochondrial physiological parameters regarding the ability of cells to generate ATP by oxidative phosphorylation [40]. Mitochondria play a central role in regulating cell death and survival. In the present review, one study found a collapse of mitochondrial membrane potential identified by loss of red fluorescence within 1.5 hours after CrTX treatment. This change reached a maximum of 6 hours after treatment with CrTX [21].

Considering that the antineoplastic effects of crotoxin are linked to growth factor fetal bovine serum (SFB), and that in the absence of calcium the CrTX has its action reduced by 135% on GH3 cells, these results show that CrTX plays an effect calcium-dependent on cancer cells [4]. Indeed, as described in another study, CrTX exerts a dependence on calcium ions for glutamate release in the cerebral cortex [41].

We have identified a dearth of clinical studies that assessed the antineoplastic effect of CrTX in cancer patients. According to the search criteria, we identified only a single human study [22], Phase I study, that was not included in this review, because the main aim was not to evaluate the antineoplastic potential of CrTX in parameters as reduction of tumor size, disease control or metastasis inhibition. It investigated the toxicity and pharmacokinetic profile of CrTX. However, this study revealed important findings related to the clinic use of CrtX, such as the maximum tolerated dose without toxic effects in patients with different types of advanced cancer and refractory to conventional therapy. Despite this study has shown tumor regression in some specific cases, it did not use intervention and control groups, as in a randomized clinical trial.

So, according to this systematic review, the CrTX can be highlighted as a potential and promising antineoplastic substance, showing antiproliferative effects in several types of cancer cells. Consistent clinical research, as randomized clinical trials, is necessary to understand whether CrTX plays its antineoplastic effects in a tumor-specific manner, and also whether it is able to control cancer progression in patients.

References

[1] Torre LA, Siegel RL, Ward EM, Jemal A. Global cancer incidence and mortality rates and trends—an update. Cancer Epidemiology and Prevention Biomarkers. 2016;25:16-27.
[2] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians. 2018;68:394-424.
[3] San Juan BP, Garcia-Leon MJ, Rangel L, Goetz JG, Chaffer CL. The Complexities of Metastasis. Cancers. 2019;11:1575.
[4] Soares M, Pujatti P, Fortes-Dias C, Antonelli L, Santos R. Crotalus durissus terrificus venom as a source of antitumoral agents. Journal of Venomous Animals and Toxins including Tropical Diseases. 2010;16:480-92.
[5] Macconaill LE, Garraway LA. Clinical implications of the cancer genome. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2010;28:5219-28.
[6] Scheel C, Onder T, Karnoub A, Weinberg RA. Adaptation versus selection: the origins of metastatic behavior. Cancer research. 2007;67:11476-80.
[7] Esteller M. Epigenetic lesions causing genetic lesions in human cancer: promoter hypermethylation of DNA repair genes. European journal of cancer. 2000;36:2294-300.
[8] Hill BT. Etiology of Cancer. Clinical Ophthalmic Oncology: Springer; 2019. p. 11-7.
[9] Fitzmaurice C, Allen C, Barber RM, Barregard L, Bhutta ZA, Brenner H, et al. Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-years for 32 Cancer Groups, 1990 to 2015: A Systematic Analysis for the Global Burden of Disease Study. JAMA oncology. 2017;3:524-48.
[10] Thun MJ, DeLancey JO, Center MM, Jemal A, Ward EM. The global burden of cancer: priorities for prevention. Carcinogenesis. 2010;31:100-10.
[11] Arruebo M, Vilaboa N, Sáez-Gutierrez B, Lambea J, Tres A, Valladares M, et al. Assessment of the evolution of cancer treatment therapies. Cancers (Basel). 2011;3:3279-330.
[12] Baskar R, Lee KA, Yeo R, Yeoh KW. Cancer and radiation therapy: current advances and future directions. International journal of medical sciences. 2012;9:193-9.
[13] Parveen S, Sahoo SK. Polymeric nanoparticles for cancer therapy. Journal of Drug Targeting. 2008;16:108-23.
[14] Tralongo P, Maso LD, Surbone A, Santoro A, Tirelli U, Sacchini V, et al. Use of the word "cured" for cancer patients—implications for patients and physicians: the Siracusa charter: Curr Oncol. 2015 Feb;22(1):e38-40. doi: 10.3747/co.22.2287.
[15] Ye B, Xie Y, Qin Z-h, Wu J-c, Han R, He J-k. Anti-tumor activity of CrTX in human lung adenocarcinoma cell line A549. Acta Pharmacologica Sinica. 2011;32:1397.
[16] YAN Ch, YANG Yp, QIN Zh, GU Zl, Reid P, LIANG Zq. Autophagy is involved in cytotoxic effects of crotoxin in human breast cancer cell line MCF-7 cells. Acta Pharmacologica Sinica. 2007;28:540-8.
[17] Wang J, Qin X, Zhang Z, Chen M, Wang Y, Gao B. Crotoxin suppresses the tumorigenic properties and enhances the antitumor activity of Iressa®(gefinitib) in human lung adenocarcinoma SPCA-1 cells. Molecular medicine reports. 2014;10:3009-14.
[18] Wang J-H, Xie Y, Wu J-C, Han R, Reid PF, Qin Z-H, et al. Crotoxin enhances the antitumor activity of gefinitib (Iressa) in SK-MES-1 human lung squamous carcinoma cells. Oncology reports. 2012;27:1341-7.
[19] Hendon R, Fraenkel-Conrat H. Biological roles of the two components of crotoxin. Proceedings of the National Academy of Sciences. 1971;68:1560-3.
[20] Freitas AP, Favoretto BC, Clissa PB, Sampaio SC, Faquim-Mauro EL. Crotoxin Isolated from Crotalus durissus terrificus Venom Modulates the Functional Activity of Dendritic Cells via Formyl Peptide Receptors. Journal of immunology research. 2018;2018:7873257.

[21] Yan C-H, Liang Z-Q, Gu Z-L, Yang Y-P, Reid P, Qin Z-H. Contributions of autophagic and apoptotic mechanisms to CrTX-induced death of K562 cells. Toxicon. 2006;47:521-30.
[22] Cura JE, Blanzaco DP, Brisson C, Cura MA, Cabrol R, Larrateguy L, et al. Phase I and pharmacokinetics study of crotoxin (cytotoxic PLA2, NSC-624244) in patients with advanced cancer. Clinical Cancer Research. 2002;8:1033-41.
[23] de Araújo Pimenta L, de Almeida MES, Bretones ML, Cirillo MC, Curi R, Sampaio SC. Crotoxin promotes macrophage reprogramming towards an antiangiogenic phenotype. Scientific Reports. 2019;9:4281.
[24] Muller SP, Silva VAO, Silvestrini AVP, de Macedo LH, Caetano GF, Reis RM, et al. Crotoxin from Crotalus durissus terrificus venom: In vitro cytotoxic activity of a heterodimeric phospholipase A2 on human cancer-derived cell lines. Toxicon. 2018;156:13-22.
[25] Corin RE, Viskatis LJ, Vidal JC, Etcheverry MA. Cytotoxicity of crotoxin on murine erythroleukemia cellsin vitro. Investigational new drugs. 1993;11:11-5.
[26] Newman RA, Vidal JC, Viskatis LJ, Johnson J, Etcheverry MA. VRCTC-310—a novel compound of purified animal toxins separates antitumor efficacy from neurotoxicity. Investigational new drugs. 1993;11:151-9.
[27] Costa LA, Miles H, Araujo CE, Gonzalez S, Villarrubia VG. Tumor regression of advanced carcinomas following intra-and/or peri-tumoral inoculation with VRCTC-310 in humans: preliminary report of two cases. Immunopharmacology and immunotoxicology. 1998;20:15-25.
[28] Brigatte P, Faiad OJ, Ferreira Nocelli RC, Landgraf RG, Palma MS, Cury Y, et al. Walker 256 tumor growth suppression by crotoxin involves formyl peptide receptors and lipoxin A4. Mediators of inflammation. 2016;2016.
[29] Han R, Liang H, Qin Z-h, Liu C-y. Crotoxin induces apoptosis and autophagy in human lung carcinoma cells in vitro via activation of the p38MAPK signaling pathway. Acta Pharmacologica Sinica. 2014;35:1323.
[30] Donato NJ, Martin CA, Perez M, Newman RA, Vidal JC, Etcheverry M. Regulation of epidermal growth factor receptor activity by crotoxin, a snake venom phospholipase A2 toxin: a novel growth inhibitory mechanism. Biochemical pharmacology. 1996;51:1535-43.
[31] Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JP, et al. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. Annals of internal medicine. 2009;151:W-65-W-94.
[32] Schneider K, Schwarz M, Burkholder I, Kopp-Schneider A, Edler L, Kinsner-Ovaskainen A, et al. “ToxRTool”, a new tool to assess the reliability of toxicological data. Toxicology letters. 2009;189:138-44.
[33] He J-k, Wu X-s, Wang Y, Han R, Qin Z-h, Xie Y. Growth inhibitory effects and molecular mechanisms of crotoxin treatment in esophageal Eca-109 cells and transplanted tumors in nude mice. Acta Pharmacologica Sinica. 2013;34:295.
[34] Rudd CJ, Viskatis LJ, Vidal JC, Etcheverry MA. In vitro comparison of cytotoxic effects of crotoxin against three human tumors and a normal human epidermal keratinocyte cell line. Investigational new drugs. 1994;12:183-4.
[35] Zhen N, Yang Q, Wu Q, Zhu X, Wang Y, Sun F, et al. A novelly synthesized phenanthroline derivative is a promising DNA-damaging anticancer agent inhibiting G1/S checkpoint transition and inducing cell apoptosis in cancer cells. Cancer Chemotherapy and Pharmacology. 2016;77:169-80.
[36] Henry CM, Hollville E, Martin SJ. Measuring apoptosis by microscopy and flow cytometry. Methods. 2013;61:90-7.
[37] Hua P, Sun M, Zhang G, Zhang Y, Song G, Liu Z, et al. Costunolide Induces Apoptosis through Generation of ROS and Activation of P53 in Human Esophageal Cancer Eca-109 Cells. Journal of biochemical and molecular toxicology. 2016;30:462-9.
[38] Lu Y, Chen G-Q. Effector caspases and leukemia. International journal of cell biology. 2011;2011.

[39] Koul HK, Pal M, Koul S. Role of p38 MAP kinase signal transduction in solid tumors. Genes & cancer. 2013;4:342-59.
[40] Perry SW, Norman JP, Barbieri J, Brown EB, Gelbard HA. Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. BioTechniques. 2011;50:98-115.
[41] da Silva Lomeo R, de Faria Gonçalves AP, da Silva CN, de Paula AT, Santos DOC, Fortes-Dias CL, et al. Crotoxin from Crotalus durissus terrificus snake venom induces the release of glutamate from cerebrocortical synaptosomes via N and P/Q calcium channels. Toxicon. 2014;85:5-16.

Figures and Tables

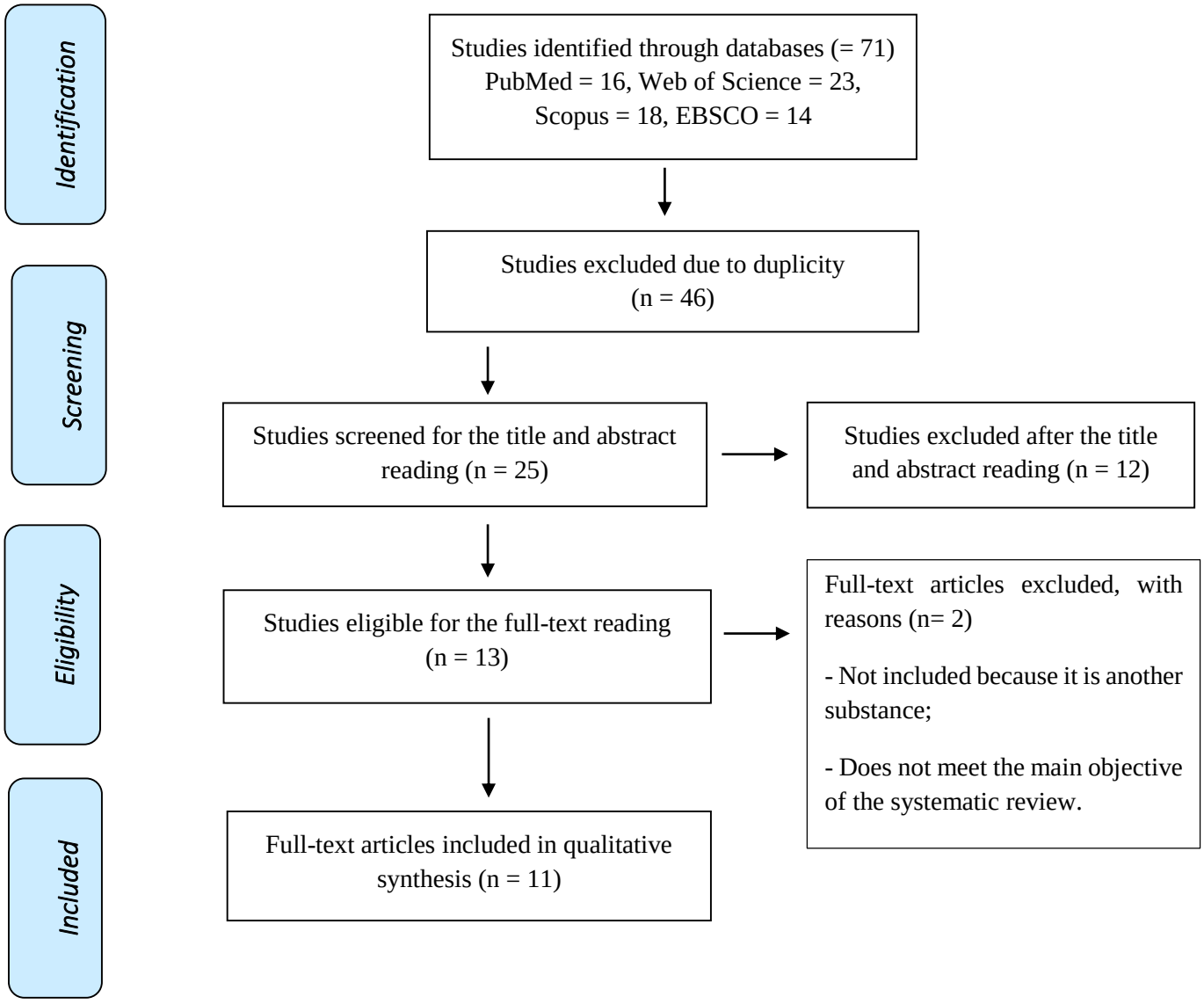

Figure 1- Flowchart of article selection about the systematic review of the antineoplastic potential of crotoxin in different types of cancer

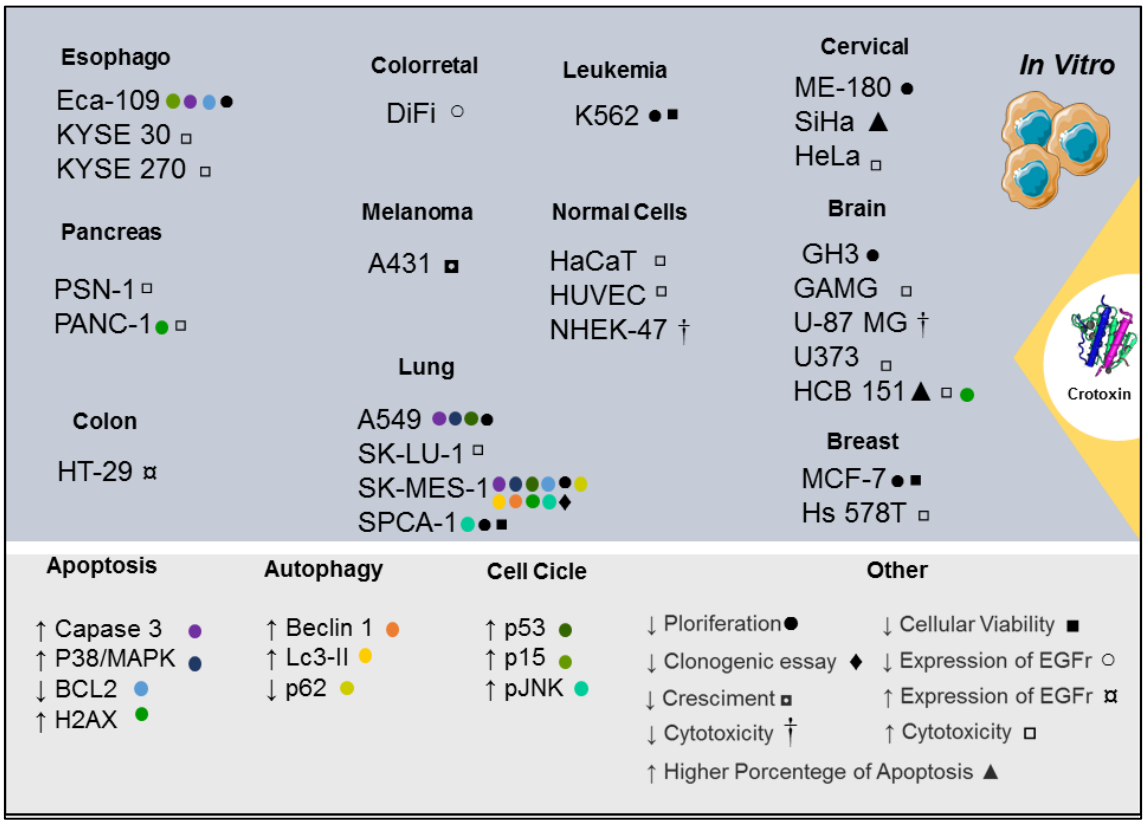

Figure 2 - Mechanisms of action of crotoxin (CrTX) and relevant aspects of in vitro and clinical trials in different types of cancer. ● Increased caspase 3; ● increase in p38 MAPK; ● decreased BCL2; ● increase in H2AX; ● decreased proliferation; ♦ decreased clonogenic formation; ■ decreased cell viability; ▫ presented cytotoxicity; § decreased cytotoxicity; ● increase in Beclin 1; ● increase in Lc3 - II; ● increased p53; ● increased p15; ● increased p-JNK; ● decreased p62; ▲ Higher porcentege of apoptosis; ◘ cresciment inibition; **Φ** decreased expression of EGFr; ¤ increased EGFr expression.;

.

Table 1. Quality assessment of *in vitro* selected articles according to the ToxRTool [32].

References	Group I: test substance identification (points: 0-4)	Group II: test system characterization (points: 0-3)	Group III: Study design description (points: 0-6)	Group IV: Study results documentation (points: 0-3)	Group V: plausibility of study design and data (points: 0-2)	Total	Reliability categorization
Ye *et al*. 2011 [15]	3	3	5	3	2	16	Reliable without restrictions
He *et al*. 2013 [33]	3	3	6	3	2	17	Reliable without restrictions
Yan *et al*. 2007 [16]	3	3	5	3	2	16	Reliable without restrictions
Yan *et al*. 2006 [21]	3	3	5	3	2	16	Reliable without restrictions
Donato *et al*. 1996 [30]	3	3	5	3	2	16	Reliable without restrictions
Wang *et al*. 2012 [18]	4	3	6	3	2	18	Reliable without restrictions
Muller *et al*. 2018 [24]	4	3	6	3	2	18	Reliable without restrictions
Soares *et al*. 2010 [4]	3	3	5	3	2	16	Reliable without restrictions
Han *et al*. 2014 [29]	4	3	6	3	2	18	Reliable without restrictions
Wang *et al*. 2014 [17]	3	3	6	3	2	17	Reliable without restrictions
Rudd *et al*. 2014 [34]	3	3	4	2	2	14	reliable with possible restrictions

Table 2. Result summary on antineoplastic effects of crotoxin in *in vitro* studies involving different cancer types

Number	*References*	*Kind of study*	*Cancer Cell line*	*Investigated assays*	*Substance used*	*Dosage and treatment frequency*	*Treatment type*	*Main effects*
1	Ye *et al.* 2011 [15]	Experimental (*in vitro* and *in vivo*)	- Cancer cell lines: A549 - Human lung adenocarcinoma cell line	Cell Viability (MTT); Flow cytometry; Apoptoses; Western blot (p53, caspase-3 and cleaved caspase-3, total P38MAPK and P38MAPK).	Crotoxin (CrTx)	25, 50, 100 and 200 µg/mL for 24 and 48 hours	Crotoxin dilution method was not reported by the author	A crotoxin exerted an antitumor effect on concentration-independent A549 cells by promoting or increasing cell arrest in the sub-G0 and G0 / G1 phases, showing the effect of crotoxin on caspase -3 application and inducing apoptosis.
2	He *et al.* 2013 [33]	Experimental (*in vitro* and *in vivo*)	- Cancer cell lines: Eca-109 Cells - Human Esophageal Carcinoma Lineage	Cell Viability (MTT); Hoechst 33342 staining of Eca-109 cells; Flow Cytometry; RT-PCR (Analysis of gene expression levels of Bcl-2, p15, caspase-3, and p17).	Crotoxin and Cisplatin as a control	25, 50 or 100 µg/mL for 24 hours and Cisplatin-treated control group (positive control): 5, 10 or 20 µg/mL for 24 hours	Crotoxin was added to the culture medium to treat cells	Eca-109 esophageal carcinoma cell growth was inhibited by the action of crotoxin. How the crotoxin-treated cells had pyknotic nuclei and clustered together, how the burst cells and their nuclear dendrites were scattered between the cells. There was an increase in diploid peak and finger cycle cell arrest in the G1 phase. Expression of p15 and caspase-3 p17 apoptosis promoter genes in crotoxin-treated cells and decrease of pro-apoptotic Bcl-2 gene with increased crotoxin.
3	Yan *et al.* 2007 [16]	Experimental (*in vitro*)	- Cancer cell lines: MCF-7 - Human Breast Cancer Cell Line	Cell Viability (MTT); Cytotoxicity Assay; Visualization of MDC-labeled vacuoles; Transmission electron microscope examination; Subcellular fractionation; Protein preparation and immunoblotting; Immunofluorescence.	Crotoxin (CrTX)	12.5, 25, 50 and 100 µg/mL and subsequently the concentration tested was 100 µg/mL for 24 and 72 hours	Crotoxin dilution method was not reported by the author	Crotoxin inhibited MCF-7 cell viability, regardless of dose and time. CrTX promoted autophagy and apoptosis formation, being a dominant mechanism in MCF-7 cell death.

4	Yan *et al.* 2006 [21]	Experimental (*in vitro*)	- Cancer cell lines: K562 - Myeloid Leukemia Cell Line - Normal cell lines: HUVEC - Immortalized human umbilical vein endothelial cell line	Cell Viability (MTT); Cytotoxicity assay; Visualization of MDC-labeled vacuoles; Transmission electron microscopic examination; Mitochondrial Potential Detection; Sub cellular fractionations; Protein preparation and immunoblotting.	Crotoxin (CrTX)	6.25, 12.5, 25 e 50 µg/mL for 12, 24, 48 and 72 hours.	Crotoxin dilution method was not reported by the author	The results shown by autophagy and apoptosis were activated during CrTX-promoted death in K562 cells. Activation of caspase-3 was reformed 12 hours after treatment with a K562 cell crotoxin that was destined to die later, suggesting autophagy triggered during initial treatment that had the effect of delaying apoptosis. Apoptosis is a major cause of cell death in the K562 cell line, while autophagy delays apoptosis and favors cell viability.
5	Donato *et al.* 1996 [30]	Experimental (*in vitro*)	- Cancer cell lines: ME-180 - Parental cervical carcinoma cell line - Cancer cell lines: A431 – Nonmalignant Melanoma Cell Line - Cancer cell lines: HT-29 - Colon carcinoma cell line - Cancer cell lines: DiFi - Carcinoma colorectal cell line	Isolation and modification of CD and CT; Epidermal growth factor and other reagents; Effect of CT on cell growth; Preparation of cell membranes; Phosphotyrosine immunoblotting of cell extracts; Immune-complex kinase assay; Studies on the influence of toxin on ^{125}1-labeled EQF binding in A431 cells	Crotoxin (CT) and Cardiotoxina	1, 10 e 100 µg/mL for 72 hours	Crotoxin dilution method was not reported by the author	According to experimental data, CrTX is involved in the decline of cell development by inhibiting the growth of EGFr overproducing cells via a cell growth regulatory pathway.
6	Wang *et al.* 2012 [18]	Experimental (*in vitro* e *in vivo*)	- Cancer cell lines: SK-MES-1 - Human lung	Cell Viability (MTT); Colony Formation Assay; Cell Cycle Analysis; Apoptosis detection by	Crotoxin (CrTX) and	12.5, 25, 50 ou 100 µg/mL) for 24,48 e 72 hours	Crotoxin dilution method was not reported by the author	CrTX increased the apoptosis rates of SK-MES-1 cells, particularly in the G1 phase, and its anti-tumor effects of CrTX were

			squamous cell carcinoma cell line	annexin-V staining; Protein preparation and immunoblotting.	Iressa and Iressa combined with CrTX			closely related to apoptosis induction and cell cycle arrest. The present study helped to understand the mechanism of CrTX-induced apoptosis in SK-MES-1 cells and allowed a new view on antitumor combinations for lung cancer therapy.
7	Muller *et al.* 2018 [24]	Experimental (in vitro)	- Cancer cell lines: KYSE 30 - Esophageal KYSE 270 - Esophageal GAMG - Glioma U373 - Glioma HCB 151 - Glioma PSN-1 - Pancreatic PANC-1 - Pancreatic HeLa - Cervical SiHa - Cervical - Normal cell lines: HaCat - immortalized keratinocytes	Cytotoxicity; cell viability assay; cell cycle; apoptosis; molecular analysis by western blot.	Crotoxin (CrTX)	0, 0.5, 1.0, 2.5, 5.0, 10, 20, 30 µg/ml for 72 hours	Crotoxin dilution method was not reported by the author	Crotoxin (CrTX) exerted cytotoxic activity against several cancer cell lines, whereas in normal cell lines, they were not affected. Pro-apoptotic effects and possible DNA damage of pancreatic (PANC-1) and glioma (HCB151) cell lines increased H2AX activity after CrTX treatment. CrTX had better cytotoxic potential than standard chemotherapeutic drugs used in clinical practice in most types of tumors (glioma, pancreatic and cervical cancer). From the results presented in this study, functionalities about knowledge about crotoxin favor the emergence of new ideas for the development of antitumor therapies.
8	Soares *et al.* 2010 [4]	Experimental (*in vitro*)	- Cancer cell lines: - Cancer cell lines: GH3 - Benign pituitary adenoma strain	Cytotoxicity Assay (MTT); morphological analysis; DAPI staining assay; DNA cell cycle analysis; Influence of calcium and growth factors on the antitumor effect of VC; Antitumor effect of major CV polypeptide: Crotoxin	Crotoxin (CrTX)	0.1 to 100 µg/mL for 24, 48, 72 e 144 hours	Crotoxin dilution method was not reported by the author	Crotoxin exerts an antitumor effect, which is eventually mediated by apoptosis, with cell cycle specificity and extracellular calcium dependence. Calcium is an important factor for poison components, including metalloproteases and phospholipases A2. Crotoxin components

				The antitumor effect: Crotoxin; Evaluation of the acute toxicity produced by antitumoral CV dose established *in vitro*				can be used to develop low toxicity cancer therapeutic
9	Han *et al.* 2014 [29]	Experimental (*in vitro*)	Cancer cell lines: SK-MES-1 - Human Lung Squamous Carcinoma Cell Line	Cell viability assay (MTT); colony formation test; cell cycle analysis and apoptosis; morphological evaluation of apoptotic cells; Western blot analysis.	Crotoxin (CrTX)	25, 50, 100 µg/mL for 24, 48 e 72 hours	Crotoxin dilution method was not reported by the author	CrTX inhibits proliferation and induced cell cycle arrest, apoptosis, and autophagy in the SK-MES-1 strain. In addition, the p38MAPK signal transduction pathway is activated by CrTX which induces apoptosis and autophagy. Through the findings on the molecular aspects based on CrTX-induced cell death in lung cancer, new possibilities may emerge with the purpose of developing therapies to assist in cancer treatment.
10	Wang *et al.* 2014 [17]	Experimental (*in vitro* e *in vivo*)	Cancer cell lines: SPCA-1 - Lung adenocarcinoma strain.	cell viability (MTT), colony formation assay, cell cycle assay and apoptosis, western blot, Immunofluorescence assays.	Crotoxin (CrTX) and Iressa and Iressa combined with CrTX	12,5, 25, 50 e 100 µg for 24 e 72 hours	Crotoxin dilution method was not reported by the author	Given the above, the present study demonstrated that CrTX induced the activation pathway via JNK and G1 phase arrest in lung cancer cells (SPCA-1), suggesting an identical mechanism in different cancer cells. All results of the present study provide information on the antitumor effect of CrTX on lung cancer and suggest that CrTX may be a potential antineoplastic agent.
11	Rudd *et al.* 1994 [34]	Experimental (*in vitro*)	Cancer cell lines: Hs 578T - Breast Ductal Carcinoma Lineage;	Cytotoxicity Assay	Crotoxin (CrTX)	0, 2.3, 3.0, 4.0, 5.3, 7.1, 9.5, 12.7, 16;9, 22.5 e 30,0 µg/ml for 24 hours	Crotoxin dilution method was not reported by the author	NHEK cells were less affected by crotoxin than tumor cells. Although the number of NHEK cells treated with CrTX was smaller than the control group, and no toxic effects

			Cancer cell lines: U-87 MG - Glioblastoma Lineage; Cancer cell lines: SK-LU-1 - Lung adenocarcinoma strain. - Normal cell lines: NHEK-47- Normal Human Epidermal Keratinocytes					were observed by microscopic examination.

*Assays and results of studies with *in vivo* approach was not considered in this study, as described in Material and Methods.

4.2 Produto 2

Crotoxin isolated from snake venom *Crotalus durissus terrificus* potentiates the therapeutic effect of ionizing radiation in oral squamous cell carcinoma: a preliminary study

Felipe Alberto Dantas Guimarães[1]
Rogério Gonçalves Da Rocha[1]
Renata Sousa Leite[1]
Eliane Macedo Sobrinho Santos[2]
Luisa Santiago[3]
Anita Mitico Tanaka Azevedo[4]
Sérgio Henrique Souza Santos[1,5]
André Luiz Sena Guimarães[1,6]
Lucyana Conceição Farias[1,6]

[1] Laboratory of Health Science, Postgraduate Program in Health Sciences, Universidade Estadual de Montes Claros, Montes Claros, Minas Gerais, Brazil
[2] Instituto Federal do Norte de Minas Gerais - Campus Araçuaí, Minas Gerais, Brazil
[3] Oncology Center, Hospital Dilson Godinho, Montes Claros, Minas Gerais, Brazil
[4] Butantan Institute, São Paulo, Brazil.
[5] Institute of Agricultural Sciences, Food Engineering College, Universidade Federal de Minas Gerais, Montes Claros, Minas Gerais, Brazil
[6] Department of Dentistry, Universidade Estadual de Montes Claros, Minas Gerais, Brazil

*Corresponding author:

Lucyana Conceição Farias
Universidade Estadual de Montes Claros
Hospital Universitário Clemente de Faria
Laboratório de Pesquisa em Saúde
Avenida Cula Mangabeira, 562
Montes Claros, Minas Gerais, Brasil
Zip code: 39401-001
Phone: +55 38 32248327
E-mail: lucyanacfarias@gmail.com

Abstract

Oral squamous cell carcinoma (OSCC) is a serious global public health problem, with 300,000 annual cases and 145,000 deaths. Radiotherapy is one of the main therapies used in the treatment of OSCC, which promotes neoplastic cell death and controls the progress of the disease. Despite advances in this therapy, the radioresistance can still be observed and leads to an unfavorable prognosis. Therefore, the development of complementary therapies that favor treatment and prolong the patient's survival is of great value. The Crotoxin (CrTX), a neurotoxin isolated from the venom of *Crotalus durissus terrificus* of South America, has been shown to have an antineoplastic effect in some types of cancer. However, it has not been investigated in OSCC. Through this study, we investigated the adjuvant potential of CrTX on the therapeutic effect of ionizing radiation in OSCC cell lines. The immortalized cell line SSC-9 was cultured and treated with 100 µg/ml CrTX and exposed to the radiation doses of 2, 4 and 6 Gy. To investigate the CrTX effects concomitantly to radiation exposure, the assays of cell proliferation, cell death, and analysis of reactive oxygen species (ROS) were performed. The CrTX was able to increase the sensitivity of SCC-9 cells to the ionizing radiation, especially at low radiation doses. This effect was associated with a decreased proliferative activity, an increase in cell mortality and high levels of ROS generation. The results of this study appoint that crotoxin enhances the therapeutic effect of ionizing radiation, increasing its therapeutic effect of radiation on OSCC cells. Additionally, lower doses of ionization can be necessary to control the neoplastic behavior of OSCC cells when associated with the CrTX treatment

Keywords: Antineoplastic Agents. Crotoxin. *Crotalus durissus terrificus*. Oral squamous cell carcinoma. Ionizing radiation.

Introduction

Cancer represents the second leading cause of death in the world. In most cases, the diagnosis is late, which can lead to high rates of morbidity[1]. It is predicted that by 2020 there will be 15 million new cancer cases and 10 million deaths [2].

Oral squamous cell carcinoma (OSCC) is the sixth most common malignant neoplasm, accounting for 2 to 4% of all cancers worldwide, representing about 90% of all oral cavity cancers [3,4]. It is a serious and growing global health problem, with an estimated 300.000 annual cases with 145.000 deaths from individuals with oral and lip cancer [3,5].

The pathogenesis of oral cancer is multifactorial, and the main etiological factors that are strongly associated with its development include primarily smoking, alcohol consumption and persistent *Human papillomavirus* infections [6,7]. Drinking and smoking are autonomously and synergistically associated with an increased risk of oral cavity cancer, and this risk may increase due to constancy of exposure [8,9]. These factors predispose the individual to a series of genetic and epigenetic events that generate a genomic inconsistency in the development and evolution of oral cancer [10].

The main modalities of treatment for OSCC are surgical resection, chemotherapy (QT), radiotherapy (RT) or combination therapy [11,12]. Treatment selection is based on consideration of disease control, functional outcomes, resource availability, and knowledge [13]. Radiotherapy (RT) provides high cure rates in many human neoplasms, especially when they are in the early stages [9,14]; nevertheless, high morbidity and mortality rates are still observed due to the advanced stage of the disease at the time of diagnosis [15]. Ionizing radiation promotes an irreparable breakdown of the DNA strand and induces apoptosis cell death by activating intracellular proteases in both normal and cancer cells [9,12]. Despite the advances made in the treatment of OSCC patients, there are still cases of low therapeutic responsiveness, either radiotherapy or chemotherapy. Thus, it is crucial to conduct research focusing on the development of complementary therapies, aiming to favor the therapeutic response and patient survival.

Venoms and toxins are promising substances for the development of new anticancer agents [16]. Crotoxin (CrTX) is the main toxin isolated from South American snake venom, *Crotalus durissus terrificus* [17]. Isolation of such a substance was initially described by Slota and Fraenkel-Sonrat (1938) [18]. The main biological effects consist of myotoxic, neurotoxic, nephrotoxic, cardiotoxic, edema inducing and anticoagulant activities [19-21]. Evidence has shown cytotoxic effects of CrTX on some types of cancer, such as leukemia, breast, lung, pancreas, and esophageal cancer [19,20,22-24].

In this context, this study aimed to investigate the adjuvant potential of CrTX on the therapeutic effect of ionizing radiation in OSCC cells.

Material and Methods

Cell culture

The human OSCC immortalized cells SCC-9 (BCRJ Cat# 0196, RRID:CVCL_1685) were cultured in Dulbecco's modified Eagle medium and Ham F-12 nutrient mixture (DMEM-F12), supplemented with 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA), antibiotics and 0.4 μg/mL hydrocortisone (Gibco, Invitrogen) and maintained at 37°C in a humidified atmosphere with 5% CO_2.

Drug Treatment

CrTX substance was produced and supplied by a researcher at Butantan Institute (São Paulo, Brazil). This was purified from the venom of the *Crotalus durissus terrificus* snake by a combination of size exclusion and anion exchange. Protein identity was confirmed by molecular weight determination by mass spectrometry (mean signals at 9500 Da and 14500 Da). The purity obtained was greater than 99%. Cells were treated with 100 μg/ml CrTX for 72 hours as standardized by the dose-response curve. Exposure of cells to ionizing radiation (IR) was performed on the day of administration of the last dose of CrTX by exposing the cells at doses of 2, 4 and 6 Gy using the Elekta Synergy linear accelerator (Atlanta, GA) to a field-source distance of 97.5 cm.

Cell proliferation assay

The assess proliferative behavior, the cells received the treatment with CrTX and were exposed to radiation doses, a density of $8.0x10^4$ was plated in 12-well plates and subjected to CrTX treatments and radiation. After treatments, cell count was analyzed by trypan blue exclusion.

Cell death test

After the experimental treatments, to analyze the ratio of dead cells/total cells, the cells were stained with ethidium bromide (EB) 100 μg/mL and acridine orange (AA) 100 μg/mL (AO and EB, Sigma, St. Louis, MO, USA). Intense EB staining (Ex360–370 filter, Em420460, DM400) indicates cell death, while intense AO (Ex460–495 filter, Em510-550, DM505) indicates living cells. To analyze the images the FSX100 microscope (Olympus, Center Valley, PA, USA) was used.

Reactive oxygen species assay

Following experimental treatments, cells were incubated with 10 μmol/L 2′7′-dichlorofluorescein diacetate (H2DCFDA; Invitrogen, CA) for 30 minutes at 37°C, washed twice with PBS buffer and analyzed immediately in a fluorescence microscope FSX100 (Olympus, Center Valley, PA, USA). Quantitation of labeled cells was performed using ImageJ software (http://rsbweb.nih.gov/ij/). Cells treated with 100 μmol/L H_2O_2 in 2% fetal bovine serum were used as internal reaction control for the formation of reactive oxygen species (ROS) [25].

Statistical analysis

Statistical analysis was performed using SPSS 22.0 software and GraphPad Prism software (version 6.0, GraphPad Software Inc., San Diego, CA, USA). Anova Two-Way test was used; probability values $p<0.05$ were considered statistically significant.

Results

Crotoxin increased the sensitivity of oral squamous carcinoma cells to the ionizing radiation, reducing proliferative activity

To evaluate whether CrTX improves the effect of the ionizing radiation on the neoplastic cell population, the assays of cell proliferation, cell death, and ROS analysis were performed.

Fig. 1A shows the proliferative behavior of CrTX-treated SCC-9 cells and the treatment-free control group after exposure to different doses of ionizing radiation. The effects of ionizing radiation reducing cell proliferation were enhanced by the action of CrTX, especially at the lowest radiation doses, 2 Gy and 4 Gy when compared to the irradiated control group.

Crotoxin enhanced cell death induced by ionizing radiation, increasing the formation of reactive oxygen species

CrTX promoted a significant increase in the number of dead cells concomitantly with exposure to ionizing radiation. These results point to a potential effect of CrTX, greatly enhancing radiation-induced cell death (Fig. 1 B). In accordance with these results, CrTX-treated cells exposed to radiation, especially at 2 Gy and 4 Gy doses, showed a significant increase in the percentage of intracellular ROS accumulation (Fig. 1 C).

Discussion

Despite its therapeutic effect in cancer treatment, ionizing radiation plays deleterious side effects to normal tissues, adjacent to neoplastic tissue, such as mucositis, xerostomia and one of the most serious effects that can affect the patient undergoing radiotherapy in head and neck region is osteoradionecrosis [26,27]. In this *in vitro* study, CrTX improved the effect of radiation, requiring lower exposure doses to control the proliferation of OSCC cells. We verify that CrTX enhances the effect of ionizing radiation, promoting a significant increase of neoplastic cell death, which may perhaps be caused by CrTX-induced apoptosis. Other studies revealed the CrTX induced apoptosis pathways, promoting increase caspase-3, cleaved caspase-3, p38MAPK, LC3-II and Beclin 1, suggesting that CRTX promoted apoptosis and autophagy in squamous cell carcinoma (SK-MES-1) and lung adenocarcinoma cells (A549) [28,29].

Although incipient, some research has investigated the antineoplastic effect of CrTX [24,29] In lung cancer, CrTX exerted a synergistic effect on gefitinib chemotherapy, widely used in lung cancer therapy [19]. There are no studies in the literature demonstrating the therapeutic adjuvant potential of CrTX on ionizing radiation in OSCC cells.

High levels of radiation-induced ROS promote oxidative stress and destabilization of neoplastic cell integrity and metabolism, inducing apoptosis mechanisms [30]. CrTX significantly exacerbated the death of the oral neoplastic cell line induced by ionizing radiation, and it was not necessary to apply high doses to obtain expressive results, especially the doses of 2 Gy and 4 Gy provided promising effects.

Taken together, our preliminary findings point the crotoxin as a potential adjuvant substance to the radiotherapy treatment on OSCC cells, highlighting a promissory effect of CrTX especially when used to at lower doses of ionizing radiation to favor the control of the neoplastic behavior and possibly to reduce the radio-induced adverse effects in head and neck region. New functional *in vivo* and clinical studies will lead to a better understanding of the action of CrTX and its pathways on the effect of radiotherapy in OSCC cells.

Ethical Approval

This research is an *in vitro* experimental study approved by the Research Ethics Committee of the State University of Montes Claros-Unimontes, under protocol nº 3.037.289.

Acknowledgments

Thanks to the Scientific Initiation Program of the State University of Montes Claros/Unimontes. This study was supported by the National Council for Scientific and Technological Development (CNPq), Higher Education Personnel Improvement Coordination (CAPES) and Minas Gerais State Research Support Foundation (FAPEMIG), Brazil.

Author Contributions

FADG, RGR, EMSS e LCF conceived and designed the experiments; RGR, FADG e RSL performed the experiments; LCF, FADG and ALSG analyzed the data; AMTA, LS, SHSS, ALSG, LCF contributed reagents/materials/analysis tools; the LCF, ALSG the FADG wrote the manuscript.

Figures

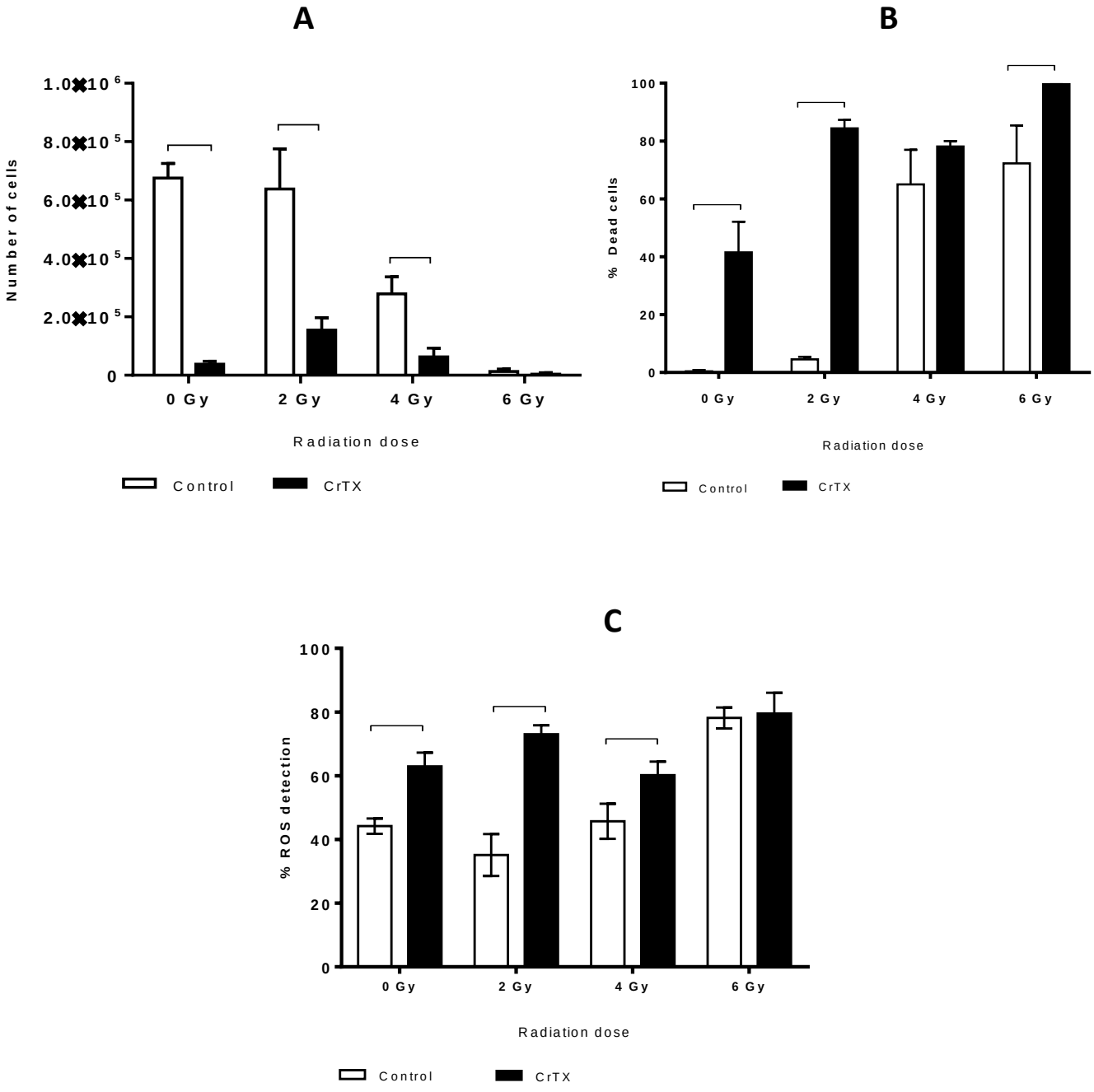

Figure 1. Effects of crotoxin on OSCC cells exposed to different doses of ionizing radiation (A) Changes in SCC9 cell proliferative behavior (B) Changes in percentage of SCC9 cell death (C) Reactive oxygen species assay in SCC9 cells. In all assays, cells were treated with 100 µg/ml crotoxin and exposed at different doses of therapeutic ionizing radiation. Anova Two-Way Test. Experimental groups: control, crotoxin treatment (CrTX). Horizontal bars indicate statistical significance: $p<0.05$.

Referências

1. Nagai H, Kim YH. Cancer prevention from the perspective of global cancer burden patterns. *Journal of thoracic disease.* 2017;9(3):448-451.
2. Hernández-Guerrero JC, Jacinto-Alemán LF, Jiménez-Farfán MD, Macario-Hernández A, Hernández-Flores F, Alcántara-Vázquez A. Prevalence trends of oral squamous cell carcinoma. Mexico City's General Hospital experience. *Medicina oral, patologia oral y cirugia bucal.* 2013;18(2):e306-311.
3. Dhanuthai K, Rojanawatsirivej S, Thosaporn W, et al. Oral cancer: A multicenter study. *Medicina oral, patologia oral y cirugia bucal.* 2018;23(1):e23-e29.
4. Markopoulos AK. Current aspects on oral squamous cell carcinoma. *The open dentistry journal.* 2012;6:126-130.
5. Ribeiro IP, Barroso L, Marques F, Melo JB, Carreira IM. Early detection and personalized treatment in oral cancer: the impact of omics approaches. *Molecular cytogenetics.* 2016;9:85.
6. Subapriya R, Thangavelu A, Mathavan B, Ramachandran CR, Nagini S. Assessment of risk factors for oral squamous cell carcinoma in Chidambaram, Southern India: a case–control study. *European Journal of Cancer Prevention.* 2007;16(3):251-256.
7. Elimairi I, Sami A, Yousef B. Oral Cancer and Potentially Malignant Disorders. In: *Histopathology-An Update.* IntechOpen; 2017.
8. Morse DE, Psoter WJ, Cleveland D, et al. Smoking and drinking in relation to oral cancer and oral epithelial dysplasia. *Cancer causes & control : CCC.* 2007;18(9):919-929.
9. Wu SY, Liu YW, Wang YK, et al. Ionizing radiation induces autophagy in human oral squamous cell carcinoma. *J BUON.* 2014;19(1):137-144.
10. Hema KN, Smitha T, Sheethal HS, Mirnalini SA. Epigenetics in oral squamous cell carcinoma. *Journal of oral and maxillofacial pathology : JOMFP.* 2017;21(2):252-259.
11. Pugazhendi SK, Thambiah L, Venkatasetty A, Thangaswamy V. Elective neck dissection versus "wait and watch" policy in tongue carcinoma. *Journal of pharmacy & bioallied sciences.* 2012;4(Suppl 2):S226-229.
12. Baker-Groberg SM, Bornstein S, Zilberman-Rudenko J, et al. Effect of ionizing radiation on the physical biology of head and neck squamous cell carcinoma cells. *Cellular and molecular bioengineering.* 2015;8(3):517-525.
13. Huang SH, O'Sullivan B. Oral cancer: Current role of radiotherapy and chemotherapy. *Medicina oral, patologia oral y cirugia bucal.* 2013;18(2):e233-240.
14. Satheeshkumar P, Chamba MS, Balan A, Sreelatha K, Bhatathiri V, Bose T. Effectiveness of triclosan in the management of radiation-induced oral mucositis: a randomized clinical trial. *Journal of cancer research and therapeutics.* 2010;6(4):466.
15. Minicucci EM, da Silva GN, Salvadori DM. Relationship between head and neck cancer therapy and some genetic endpoints. *World journal of clinical oncology.* 2014;5(2):93-102.
16. Harvey AL. Toxins and drug discovery. *Toxicon.* 2014;92:193-200.
17. de Andrade CM, Rey FM, Cintra ACO, Sampaio SV, Torqueti MR. Effects of crotoxin, a neurotoxin from Crotalus durissus terrificus snake venom, on human endothelial cells. *International journal of biological macromolecules.* 2019;134:613-621.
18. Hendon R, Fraenkel-Conrat H. Biological roles of the two components of crotoxin. *Proceedings of the National Academy of Sciences.* 1971;68(7):1560-1563.
19. Wang J, Qin X, Zhang Z, Chen M, Wang Y, Gao B. Crotoxin suppresses the tumorigenic properties and enhances the antitumor activity of Iressa®(gefinitib) in human lung adenocarcinoma SPCA-1 cells. *Molecular medicine reports.* 2014;10(6):3009-3014.
20. Muller SP, Silva VAO, Silvestrini AVP, et al. Crotoxin from Crotalus durissus terrificus venom: In vitro cytotoxic activity of a heterodimeric phospholipase A2 on human cancer-derived cell lines. *Toxicon.* 2018;156:13-22.
21. Wang J-H, Xie Y, Wu J-C, et al. Crotoxin enhances the antitumor activity of gefinitib (Iressa) in SK-MES-1 human lung squamous carcinoma cells. *Oncology reports.* 2012;27(5):1341-1347.
22. YAN Ch, YANG Yp, QIN Zh, GU Zl, Reid P, LIANG Zq. Autophagy is involved in cytotoxic effects of crotoxin in human breast cancer cell line MCF-7 cells. *Acta Pharmacologica Sinica.* 2007;28(4):540-548.
23. Corin RE, Viskatis LJ, Vidal JC, Etcheverry MA. Cytotoxicity of crotoxin on murine erythroleukemia cellsin vitro. *Investigational new drugs.* 1993;11(1):11-15.
24. He JK, Wu XS, Wang Y, Han R, Qin ZH, Xie Y. Growth inhibitory effects and molecular mechanisms of crotoxin treatment in esophageal Eca-109 cells and transplanted tumors in nude mice. *Acta Pharmacol Sin.* 2013;34(2):295-300.

25. Park WH. The effect of MAPK inhibitors and ROS modulators on cell growth and death of H2O2-treated HeLa cells. *Molecular medicine reports.* 2013;8(2):557-564.
26. Otmani N. Oral and maxillofacial side effects of radiation therapy on children. *Journal of the Canadian Dental Association.* 2007;73(3).
27. Freitas DA, Caballero AD, Pereira MM, Oliveira SKM, Silva GPE, Hernández CIV. Oral sequelae of head and neck radiotherapy. *Revista CEFAC.* 2011;13(6):1103-1108.
28. Han R, Liang H, Qin Z-h, Liu C-y. Crotoxin induces apoptosis and autophagy in human lung carcinoma cells in vitro via activation of the p38MAPK signaling pathway. *Acta Pharmacologica Sinica.* 2014;35(10):1323.
29. Ye B, Xie Y, Qin Z-h, Wu J-c, Han R, He J-k. Anti-tumor activity of CrTX in human lung adenocarcinoma cell line A549. *Acta Pharmacologica Sinica.* 2011;32(11):1397.
30. Dayal R, Singh A, Pandey A, Mishra KP. Reactive oxygen species as mediator of tumor radiosensitivity. *Journal of cancer research and therapeutics.* 2014;10(4):811.

4.3 Produto 3:

- *Pitch* para divulgação online dos resultados da dissertação: Foi elaborado um vídeo de curta duração para divulgação online dos resultados do estudo, direcionado à população em geral, cujo conteúdo pode ser acessado através do link http://www.ppgcs.unimontes.br/

5 CONSIDERAÇÕES FINAIS

A crotoxina destaca-se pelo seu potencial antineoplásico em diferentes linhagens de células neoplásicas, como evidenciado pela revisão sistemática da literatura. Além disso, a CrTX foi capaz de potencializar o efeito terapêutico da radiação ionizante no tratamento das células de carcinoma epidermoide de boca, promovendo a redução da atividade proliferativa, morte celular e altos níveis de formação de espécies reativas de oxigênio, utilizando baixas doses de radiação. A utilização de menores doses de radiação associada a estratégias terapêuticas complementares poderão minimizar os efeitos adversos provocados pelas doses elevadas de radiação ionizante, possibilitando melhor qualidade de vida e aumento da sobrevida. Novos estudos clínicos e funcionais *in vivo* poderão levar a uma melhor compreensão da ação da CrTX sobre o efeito da radioterapia, controlando o fenótipo neoplásico em indivíduos acometidos pelo carcinoma epidermoide de boca.

REFERÊNCIAS

1. Nagai H, Kim YH. Cancer prevention from the perspective of global cancer burden patterns. Journal of thoracic disease. 2017 Mar;9(3):448-51. PubMed PMID: 28449441. Pubmed Central PMCID: PMC5394024. Epub 2017/04/30. eng.
2. Pires FR, Ramos AB, Oliveira JB, Tavares AS, Luz PS, Santos TC. Oral squamous cell carcinoma: clinicopathological features from 346 cases from a single oral pathology service during an 8-year period. Journal of applied oral science : revista FOB. 2013 Sep-Oct;21(5):460-7. PubMed PMID: 24212993. Pubmed Central PMCID: PMC3881836. Epub 2013/11/12. eng.
3. Curado MP, Johnson NW, Kerr AR, Silva DRMe, Lanfranchi H, Pereira DL, et al. Oral and oropharynx cancer in South America: Incidence, mortality trends and gaps in public databases as presented to the Global Oral Cancer Forum. Translational Research in Oral Oncology. 2016;1:2057178X16653761.
4. Montero PH, Patel SG. CANCER OF THE ORAL CAVITY. Surgical oncology clinics of North America. 2015 Jul;24(3):491-508. PubMed PMID: 25979396. Pubmed Central PMCID: Pmc5018209. eng.
5. Gupta N, Gupta R, Acharya AK, Patthi B, Goud V, Reddy S, et al. Changing Trends in oral cancer - a global scenario. Nepal journal of epidemiology. 2016 Dec;6(4):613-9. PubMed PMID: 28804673. Pubmed Central PMCID: PMC5506386. Epub 2017/08/15. eng.
6. Rivera C. Essentials of oral cancer. International Journal of Clinical and Experimental Pathology. 2015;8(9):11884-94. PubMed PMID: 26617944. Pubmed Central PMCID: Pmc4637760. eng.
7. Omura K. Current status of oral cancer treatment strategies: surgical treatments for oral squamous cell carcinoma. International journal of clinical oncology. 2014;19(3):423-30.
8. Lingen MW, Kalmar JR, Karrison T, Speight PM. Critical evaluation of diagnostic aids for the detection of oral cancer. Oral oncology. 2008 Jan;44(1):10-22. PubMed PMID: 17825602. Pubmed Central PMCID: PMC2424250. Epub 2007/09/11. eng.
9. Monteiro LS, Salazar F, Pacheco J, Warnakulasuriya S. Oral cancer awareness and knowledge in the city of valongo, portugal. International journal of dentistry. 2012;2012:376838. PubMed PMID: 22919388. Pubmed Central PMCID: PMC3420131. Epub 2012/08/25. eng.
10. Núñez-González S, Delgado-Ron JA, Gault C, Simancas-Racines D. Trends and Spatial Patterns of Oral Cancer Mortality in Ecuador, 2001-2016. International journal of dentistry. 2018;2018:6086595. PubMed PMID: 30057607. Pubmed Central PMCID: PMC6051085. Epub 2018/07/31. eng.
11. Petersen PE. Oral cancer prevention and control–the approach of the World Health Organization. Oral oncology. 2009;45(4-5):454-60.
12. Ketabat F, Pundir M, Mohabatpour F, Lobanova L, Koutsopoulos S, Hadjiiski L, et al. Controlled Drug Delivery Systems for Oral Cancer Treatment-Current Status and Future Perspectives. Pharmaceutics. 2019 Jun 30;11(7). PubMed PMID: 31262096. Pubmed Central PMCID: PMC6680655. Epub 2019/07/03. eng.
13. Elkashty OA, Ashry R, Tran SD. Head and neck cancer management and cancer stem cells implication. The Saudi dental journal. 2019 Oct;31(4):395-416. PubMed PMID: 31700218. Pubmed Central PMCID: PMC6823822. Epub 2019/11/09. eng.
14. França DC, Monti LM, de Castro AL, Soubhia AM, Volpato LE, de Aguiar SM, et al. Unusual presentation of oral squamous cell carcinoma in a young woman. Sultan

Qaboos University medical journal. 2012 May;12(2):228-31. PubMed PMID: 22548144. Pubmed Central PMCID: PMC3327572. Epub 2012/05/02. eng.
15. Vigneswaran N, Williams MD. Epidemiologic trends in head and neck cancer and aids in diagnosis. Oral and maxillofacial surgery clinics of North America. 2014 May;26(2):123-41. PubMed PMID: 24794262. Pubmed Central PMCID: PMC4040236. Epub 2014/05/06. eng.
16. Al-Amad SH, Awad MA, Nimri O. Oral cancer in young Jordanians: potential association with frequency of narghile smoking. Oral surgery, oral medicine, oral pathology and oral radiology. 2014;118(5):560-5.
17. Huang SH, O'Sullivan B. Oral cancer: Current role of radiotherapy and chemotherapy. Medicina oral, patologia oral y cirugia bucal. 2013 Mar 1;18(2):e233-40. PubMed PMID: 23385513. Pubmed Central PMCID: PMC3613874. Epub 2013/02/07. eng.
18. Carneiro-Neto JN, de-Menezes JD, Moura LB, Massucato EM, de-Andrade CR. Protocols for management of oral complications of chemotherapy and/or radiotherapy for oral cancer: Systematic review and meta-analysis current. Medicina oral, patologia oral y cirugia bucal. 2017 Jan 1;22(1):e15-e23. PubMed PMID: 27918734. Pubmed Central PMCID: PMC5217492. Epub 2016/12/06. eng.
19. Manzano BR, Santaella NG, Oliveira MA, Rubira CMF, Santos P. Retrospective study of osteoradionecrosis in the jaws of patients with head and neck cancer. Journal of the Korean Association of Oral and Maxillofacial Surgeons. 2019 Feb;45(1):21-8. PubMed PMID: 30847293. Pubmed Central PMCID: PMC6400702. Epub 2019/03/09. eng.
20. Joo YH, Cho JK, Koo BS, Kwon M, Kwon SK, Kwon SY, et al. Guidelines for the Surgical Management of Oral Cancer: Korean Society of Thyroid-Head and Neck Surgery. Clinical and experimental otorhinolaryngology. 2019 May;12(2):107-44. PubMed PMID: 30703871. Pubmed Central PMCID: PMC6453784. Epub 2019/02/02. eng.
21. Ye B, Xie Y, Qin Z-h, Wu J-c, Han R, He J-k. Anti-tumor activity of CrTX in human lung adenocarcinoma cell line A549. Acta Pharmacologica Sinica. 2011;32(11):1397-401.
22. YAN Ch, YANG Yp, QIN Zh, GU Zl, Reid P, LIANG Zq. Autophagy is involved in cytotoxic effects of crotoxin in human breast cancer cell line MCF-7 cells. Acta Pharmacologica Sinica. 2007;28(4):540-8.
23. Hendon R, Fraenkel-Conrat H. Biological roles of the two components of crotoxin. Proceedings of the National Academy of Sciences. 1971;68(7):1560-3.
24. Muller SP, Silva VAO, Silvestrini AVP, de Macedo LH, Caetano GF, Reis RM, et al. Crotoxin from Crotalus durissus terrificus venom: In vitro cytotoxic activity of a heterodimeric phospholipase A2 on human cancer-derived cell lines. Toxicon : official journal of the International Society on Toxinology. 2018;156:13-22.
25. Wang J-H, Xie Y, Wu J-C, Han R, Reid PF, Qin Z-H, et al. Crotoxin enhances the antitumor activity of gefinitib (Iressa) in SK-MES-1 human lung squamous carcinoma cells. Oncology reports. 2012;27(5):1341-7.
26. Soares M, Pujatti P, Fortes-Dias C, Antonelli L, Santos R. Crotalus durissus terrificus venom as a source of antitumoral agents. Journal of Venomous Animals and Toxins including Tropical Diseases. 2010;16(3):480-92.
27. Corin RE, Viskatis LJ, Vidal JC, Etcheverry MA. Cytotoxicity of crotoxin on murine erythroleukemia cellsin vitro. Investigational new drugs. 1993;11(1):11-5.
28. Markopoulos AK. Current Aspects on Oral Squamous Cell Carcinoma. The open dentistry journal. 2012;6:126-30. PubMed PMID: 22930665. Pubmed Central PMCID: Pmc3428647. eng.

29. Bavle RM, Venugopal R, Konda P, Muniswamappa S, Makarla S. Molecular Classification of Oral Squamous Cell Carcinoma. Journal of clinical and diagnostic research : JCDR. 2016 Sep;10(9):Ze18-ze21. PubMed PMID: 27790599. Pubmed Central PMCID: Pmc5072099. Epub 2016/10/30. eng.
30. Weatherspoon DJ, Chattopadhyay A, Boroumand S, Garcia I. Oral cavity and oropharyngeal cancer incidence trends and disparities in the United States: 2000-2010. Cancer epidemiology. 2015 Aug;39(4):497-504. PubMed PMID: 25976107. Pubmed Central PMCID: PMC4532587. Epub 2015/05/16. eng.
31. Manikandan M, Deva Magendhra Rao AK, Arunkumar G, Manickavasagam M, Rajkumar KS, Rajaraman R, et al. Oral squamous cell carcinoma: microRNA expression profiling and integrative analyses for elucidation of tumourigenesis mechanism. Molecular cancer. 2016 Apr 7;15:28. PubMed PMID: 27056547. Pubmed Central PMCID: PMC4823852. Epub 2016/04/09. eng.
32. Scully C, Porter S. Oral cancer. The Western journal of medicine. 2001 May;174(5):348-51. PubMed PMID: 11342519. Pubmed Central PMCID: PMC1071397. Epub 2001/05/09. eng.
33. Xavier SD, Bussoloti Filho I, Lancellotti CLP. Prevalence of histological findings of human papillomavirus (HPV) in oral and oropharyngeal squamous cell carcinoma biopsies: preliminary study. Brazilian journal of otorhinolaryngology. 2005;71(4):510-4.
34. Ram H, Sarkar J, Kumar H, Konwar R, Bhatt ML, Mohammad S. Oral cancer: risk factors and molecular pathogenesis. Journal of maxillofacial and oral surgery. 2011 Jun;10(2):132-7. PubMed PMID: 22654364. Pubmed Central PMCID: PMC3177522. Epub 2012/06/02. eng.
35. Ribeiro ILA, Medeiros JJd, Rodrigues LV, Valença AMG, Neto L, de Andrade E. Factors associated with lip and oral cavity cancer. Revista Brasileira de Epidemiologia. 2015;18:618-29.
36. de Camargo Cancela M, Voti L, Guerra-Yi M, Chapuis F, Mazuir M, Curado MP. Oral cavity cancer in developed and in developing countries: Population-based incidence. Head & Neck: Journal for the Sciences and Specialties of the Head and Neck. 2010;32(3):357-67.
37. Dhanuthai K, Rojanawatsirivej S, Thosaporn W, Kintarak S, Subarnbhesaj A, Darling M, et al. Oral cancer: A multicenter study. Medicina oral, patologia oral y cirugia bucal. 2018 Jan 1;23(1):e23-e9. PubMed PMID: 29274153. Pubmed Central PMCID: PMC5822535 interest. Epub 2017/12/24. eng.
38. Ferlay J, Colombet M, Soerjomataram I, Mathers C, Parkin D, Piñeros M, et al. Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. International journal of cancer. 2019;144(8):1941-53.
39. Estimativa I. Incidência de câncer no Brasil [Internet]. Instituto Nacional de Câncer José Alencar Gomes da Silva. 2017. 130 p. 2018.
40. Califano J, Van Der Riet P, Westra W, Nawroz H, Clayman G, Piantadosi S, et al. Genetic progression model for head and neck cancer: implications for field cancerization. Cancer research. 1996;56(11):2488-92.
41. Seethalakshmi C. Early Detection of Oral Squamous Cell Carcinoma (OSCC) – Role of Genetics: A Literature Review. Journal of clinical and diagnostic research : JCDR. 2013 Aug;7(8):1824-6. PubMed PMID: 24086928. Pubmed Central PMCID: Pmc3782985. eng.
42. Chong V. Oral cavity cancer. Cancer imaging : the official publication of the International Cancer Imaging Society. 2005 Nov 23;5 Spec No A(Spec No A):S49-52. PubMed PMID: 16361136. Pubmed Central PMCID: PMC1665311. Epub 2005/12/20. eng.
43. Zhong L, Liu Y, Wang K, He Z, Gong Z, Zhao Z, et al. Biomarkers: paving stones on the road towards the personalized precision medicine for oral squamous cell

carcinoma. BMC cancer. 2018 Sep 21;18(1):911. PubMed PMID: 30241505. Pubmed Central PMCID: PMC6151070. Epub 2018/09/23. eng.
44. Mydlarz WK, Hennessey PT, Califano JA. Advances and Perspectives in the Molecular Diagnosis of Head and Neck Cancer. Expert opinion on medical diagnostics. 2010 Jan 1;4(1):53-65. PubMed PMID: 20161611. Pubmed Central PMCID: PMC2811380. Epub 2010/02/18. eng.
45. Rahman MS, Ingole N, Roblyer D, Stepanek V, Richards-Kortum R, Gillenwater A, et al. Evaluation of a low-cost, portable imaging system for early detection of oral cancer. Head & neck oncology. 2010 Apr 22;2:10. PubMed PMID: 20409347. Pubmed Central PMCID: PMC2867772. Epub 2010/04/23. eng.
46. Irimie AI, Ciocan C, Gulei D, Mehterov N, Atanasov AG, Dudea D, et al. Current Insights into Oral Cancer Epigenetics. International journal of molecular sciences. 2018 Feb 27;19(3). PubMed PMID: 29495520. Pubmed Central PMCID: PMC5877531. Epub 2018/03/03. eng.
47. Choi S, Myers JN. Molecular pathogenesis of oral squamous cell carcinoma: implications for therapy. Journal of dental research. 2008;87(1):14-32.
48. Chakraborty P, Karmakar T, Arora N, Mukherjee G. Immune and genomic signatures in oral (head and neck) cancer. Heliyon. 2018 Oct;4(10):e00880. PubMed PMID: 30417146. Pubmed Central PMCID: Pmc6218671. Epub 2018/11/13. eng.
49. Ahn C-H, Hong K-O, Jin B, Lee W, Jung YC, Lee H, et al. Contribution of p38 MAPK Pathway to Norcantharidin-Induced Programmed Cell Death in Human Oral Squamous Cell Carcinoma. International journal of molecular sciences. 2019;20(14):3487.
50. Jurel SK, Gupta DS, Singh RD, Singh M, Srivastava S. Genes and oral cancer. Indian journal of human genetics. 2014 Jan;20(1):4-9. PubMed PMID: 24959008. Pubmed Central PMCID: Pmc4065477. Epub 2014/06/25. eng.
51. Krishna A, Singh S, Kumar V, Pal US. Molecular concept in human oral cancer. National journal of maxillofacial surgery. 2015 Jan-Jun;6(1):9-15. PubMed PMID: 26668446. Pubmed Central PMCID: PMC4668742. Epub 2015/12/17. eng.
52. Liggett Jr WH, Sidransky D. Role of the p16 tumor suppressor gene in cancer. Journal of clinical oncology. 1998;16(3):1197-206.
53. Tan M, Myers JN, Agrawal N. Oral cavity and oropharyngeal squamous cell carcinoma genomics. Otolaryngologic clinics of North America. 2013 Aug;46(4):545-66. PubMed PMID: 23910469. Pubmed Central PMCID: Pmc3734385. Epub 2013/08/06. eng.
54. Hema KN, Smitha T, Sheethal HS, Mirnalini SA. Epigenetics in oral squamous cell carcinoma. Journal of oral and maxillofacial pathology : JOMFP. 2017 May-Aug;21(2):252-9. PubMed PMID: 28932035. Pubmed Central PMCID: Pmc5596676. Epub 2017/09/22. eng.
55. Gasche JA, Goel A. Epigenetic mechanisms in oral carcinogenesis. Future oncology (London, England). 2012 Nov;8(11):1407-25. PubMed PMID: 23148615. Pubmed Central PMCID: Pmc3569850. Epub 2012/11/15. eng.
56. Vilen ST, Salo T, Sorsa T, Nyberg P. Fluctuating roles of matrix metalloproteinase-9 in oral squamous cell carcinoma. TheScientificWorldJournal. 2013;2013:920595. PubMed PMID: 23365550. Pubmed Central PMCID: Pmc3556887. Epub 2013/02/01. eng.
57. Koontongkaew S. The tumor microenvironment contribution to development, growth, invasion and metastasis of head and neck squamous cell carcinomas. Journal of Cancer. 2013;4(1):66-83. PubMed PMID: 23386906. Pubmed Central PMCID: Pmc3564248. Epub 2013/02/07. eng.
58. Priyanka K, Sudhir KM, Reddy VCS, Kumar RK, Srinivasulu G. Impact of Alcohol Dependency on Oral Health - A Cross-sectional Comparative Study. Journal of

clinical and diagnostic research : JCDR. 2017 Jun;11(6):Zc43-zc6. PubMed PMID: 28764291. Pubmed Central PMCID: Pmc5535480. Epub 2017/08/03. eng.
59. Dong TT, Wang LJ, Liu LZ, Ma SN. Susceptibility to oral squamous cell carcinoma: correlation with variants of CYP1A1-MspI, GSTT1, GSTM1, ALDH2, EC-SOD and Lifestyle factors. Balkan journal of medical genetics : BJMG. 2016 Dec 1;19(2):61-70. PubMed PMID: 28289590. Pubmed Central PMCID: Pmc5343332. Epub 2017/03/16. eng.
60. Nishioka T, Tada H, Ibaragi S, Chen C, Sasano T. Nicotine exposure induces the proliferation of oral cancer cells through the α7 subunit of the nicotinic acetylcholine receptor. Biochemical and biophysical research communications. 2019;509(2):514-20.
61. Mishra R. Glycogen synthase kinase 3 beta: can it be a target for oral cancer. Molecular cancer. 2010 Jun 11;9:144. PubMed PMID: 20537194. Pubmed Central PMCID: Pmc2906469. Epub 2010/06/12. eng.
62. Bansal A, Singh MP, Rai B. Human papillomavirus-associated cancers: A growing global problem. International journal of applied & basic medical research. 2016 Apr-Jun;6(2):84-9. PubMed PMID: 27127735. Pubmed Central PMCID: Pmc4830161. Epub 2016/04/30. eng.
63. Mattoscio D, Casadio C, Miccolo C, Maffini F, Raimondi A, Tacchetti C, et al. Autophagy regulates UBC9 levels during viral-mediated tumorigenesis. PLoS pathogens. 2017;13(3):e1006262.
64. Feitelson MA, Arzumanyan A, Kulathinal RJ, Blain SW, Holcombe RF, Mahajna J, et al. Sustained proliferation in cancer: Mechanisms and novel therapeutic targets. Seminars in cancer biology. 2015 Dec;35 Suppl(Suppl):S25-S54. PubMed PMID: 25892662. Pubmed Central PMCID: PMC4898971. Epub 2015/04/22. eng.
65. Lam KY, Law SYK, So MKP, Fok M, Ma LT, Wong J. Prognostic implication of proliferative markers MIB-1 and PC10 in esophageal squamous cell carcinoma. Cancer: Interdisciplinary International Journal of the American Cancer Society. 1996;77(1):7-13.
66. Rozengurt E. Growth factors, cell proliferation and cancer: an overview. Molecular biology & medicine. 1983;1(1):169-81.
67. Veena V, Rajan K, Saritha V, Preethi Sara George CK, Jayasree K, Thara S. DNA replication licensing proteins for early detection of lung cancer. Asian Pacific journal of cancer prevention: APJCP. 2017;18(11):3041.
68. Ramos-Garcia P, Gil-Montoya J, Scully C, Ayén A, González-Ruiz L, Navarro-Triviño F, et al. An update on the implications of cyclin D1 in oral carcinogenesis. Oral diseases. 2017;23(7):897-912.
69. Ohnishi Y, Watanabe M, Wato M, Tanaka A, Kakudo K, Nozaki M. Cyclin D1 expression is correlated with cell differentiation and cell proliferation in oral squamous cell carcinomas. Oncology letters. 2014 Apr;7(4):1123-7. PubMed PMID: 24944679. Pubmed Central PMCID: PMC3961451. Epub 2014/06/20. eng.
70. Rajendiran S, Kpetemey M, Maji S, Gibbs LD, Dasgupta S, Mantsch R, et al. MIEN1 promotes oral cancer progression and implicates poor overall survival. Cancer biology & therapy. 2015;16(6):876-85. PubMed PMID: 25996585. Pubmed Central PMCID: Pmc4622880. Epub 2015/05/23. eng.
71. Trepat X, Chen Z, Jacobson K. Cell migration. Comprehensive Physiology. 2012;2(4):2369-92.
72. Kargahi N, Torabinia N, Razavi SM, Tahririan D, Kamani H, Nazari M. Immunohistochemically Detection of Angiogenesis in Oral Pre-Cancerous Lesions Compared with Oral Invasive Carcinomas. Asian Pacific journal of cancer prevention : APJCP. 2018 Jul 27;19(7):1805-8. PubMed PMID: 30049191. Pubmed Central PMCID: Pmc6165636. Epub 2018/07/28. eng.
73. Rich AM, Reade PC. Epithelial–mesenchymal interactions in experimental oral mucosal carcinogenesis. Journal of oral pathology & medicine. 2001;30(7):389-97.

74. Polverini PJ. Angiogenesis in health and disease: insights into basic mechanisms and therapeutic opportunities. Journal of dental education. 2002;66(8):962-75.
75. Lu P, Takai K, Weaver VM, Werb Z. Extracellular matrix degradation and remodeling in development and disease. Cold Spring Harbor perspectives in biology. 2011;3(12):a005058.
76. Folkman J, editor Role of angiogenesis in tumor growth and metastasis. Seminars in oncology; 2002: Elsevier.
77. Webb AH, Gao BT, Goldsmith ZK, Irvine AS, Saleh N, Lee RP, et al. Inhibition of MMP-2 and MMP-9 decreases cellular migration, and angiogenesis in in vitro models of retinoblastoma. BMC cancer. 2017;17(1):434.
78. Juhász A, Bárdos H, Répássy G, Ádány R. Characteristic distribution patterns of tenascin in laryngeal and hypopharyngeal cancers. The Laryngoscope. 2000;110(1):84-92.
79. Loro L, Vintermyr O, Johannessen A. Apoptosis in normal and diseased oral tissues. Oral diseases. 2005;11(5):274-87.
80. Dai W, Li Y, Zhou Q, Xu Z, Sun C, Tan X, et al. Cetuximab inhibits oral squamous cell carcinoma invasion and metastasis via degradation of epidermal growth factor receptor. Journal of Oral Pathology & Medicine. 2014;43(4):250-7.
81. Strojan P, Hutcheson KA, Eisbruch A, Beitler JJ, Langendijk JA, Lee AWM, et al. Treatment of late sequelae after radiotherapy for head and neck cancer. Cancer treatment reviews. 2017 Sep;59:79-92. PubMed PMID: 28759822. Pubmed Central PMCID: Pmc5902026. Epub 2017/08/02. eng.
82. Ishigami T, Uzawa K, Fushimi K, Saito K, Kato Y, Nakashima D, et al. Inhibition of ICAM2 induces radiosensitisation in oral squamous cell carcinoma cells. British journal of cancer. 2008;98(8):1357.
83. Baskar R, Dai J, Wenlong N, Yeo R, Yeoh KW. Biological response of cancer cells to radiation treatment. Frontiers in molecular biosciences. 2014;1:24. PubMed PMID: 25988165. Pubmed Central PMCID: Pmc4429645. Epub 2014/01/01. eng.
84. Huang A, Glick SA. Genetic susceptibility to cutaneous radiation injury. Archives of dermatological research. 2017;309(1):1-10.
85. Spyridonidou S, Yapijakis C, Nkenke E, Toyoshima T, Vylliotis A, Serefoglou Z, et al. A common 9 bp deletion in the ataxia-telangiectasia-mutated gene is not associated with oral cancer. Anticancer research. 2009;29(8):3191-3.
86. Hiro J, Inoue Y, Toiyama Y, Miki C, Kusunoki M. Mechanism of resistance to chemoradiation in p53 mutant human colon cancer. International journal of oncology. 2008;32(6):1305-10.
87. Shintani S, Mihara M, Ueyama Y, Matsumura T, Wong DT. Cyclin D1 overexpression associates with radiosensitivity in oral squamous cell carcinoma. International journal of cancer. 2001;96(3):159-65.
88. Vyas VK, Brahmbhatt K, Bhatt H, Parmar U. Therapeutic potential of snake venom in cancer therapy: current perspectives. Asian Pacific journal of tropical biomedicine. 2013 Feb;3(2):156-62. PubMed PMID: 23593597. Pubmed Central PMCID: PMC3627178. Epub 2013/04/18. eng.
89. Costa HC, Bérnils RS. Répteis do Brasil e suas Unidades Federativas: Lista de espécies. Herpetologia Brasileira. 2018;7(1):11-57.
90. Gomes C, Almeida-Santos S. Microhabitat use by species of the genera Bothrops and Crotalus (Viperidae) in semi-extensive captivity. Journal of Venomous Animals and Toxins including Tropical Diseases. 2012;18(4):393-8.
91. Lamar WW. The venomous reptiles of Latin America: Comstock Pub. Associates; 1989.
92. Hoyos MA, Almeida-Santos SM. The South-American rattlesnake Crotalus durissus: feeding ecology in the central region of Brazil. Biota Neotropica. 2016;16(3).

93. CAMILLO MA. Contribuicao ao estudo das giroxinas (enzimas semelhantes a trombina) dos venenos das serpentes brasileiras Lachesis muta muta e crotalus durissus terrificus. 1998.
94. Lourenco Jr A, Creste CFZ, de Barros LC, dos Santos LD, Pimenta DC, Barraviera B, et al. Individual venom profiling of Crotalus durissus terrificus specimens from a geographically limited region: crotamine assessment and captivity evaluation on the biological activities. Toxicon : official journal of the International Society on Toxinology. 2013;69:75-81.
95. Wang J, Qin X, Zhang Z, Chen M, Wang Y, Gao B. Crotoxin suppresses the tumorigenic properties and enhances the antitumor activity of Iressa(R) (gefinitib) in human lung adenocarcinoma SPCA1 cells. Molecular Medicine Reports. 2014 Dec;10(6):3009-14. PubMed PMID: 25310019. Epub 2014/10/14. eng.
96. He J, Wu X, Wang Y, Han R, Qin Z, Xie Y. Growth inhibitory effects and molecular mechanisms of crotoxin treatment in esophageal Eca-109 cells and transplanted tumors in nude mice. Acta Pharmacologica Sinica. 2013 Feb;34(2):295-300. PubMed PMID: 23202800. Pubmed Central PMCID: Pmc4011616. eng.
97. Bercovici D, Chudiziniski AM, Dias VdO, Esteves MI, Hiraichi E, Oishi NY, et al. A systematic fractionation of Crotalus durissus terrificus venom. Mem Inst Butantan. 1987;49(3):69-78.
98. Slotta K, Fraenkel-Conrat H. Two active proteins from rattlesnake venom. Nature. 1938;142(3587):213.
99. Aird SD, Kaiser II, Lewis RV, Kruggel WG. A complete amino acid sequence for the basic subunit of crotoxin. Archives of biochemistry and biophysics. 1986;249(2):296-300.
100. Shimizu JF, Pereira CM, Bittar C, Batista MN, Campos GRF, da Silva S, et al. Multiple effects of toxins isolated from Crotalus durissus terrificus on the hepatitis C virus life cycle. PloS one. 2017;12(11):e0187857.
101. Hendon RA, Fraenkel-Conrat H. Biological roles of the two components of crotoxin. Proceedings of the National Academy of Sciences of the United States of America. 1971 Jul;68(7):1560-3. PubMed PMID: 5283946. Pubmed Central PMCID: PMC389240. Epub 1971/07/01. eng.
102. Sampaio SC, Hyslop S, Fontes MR, Prado-Franceschi J, Zambelli VO, Magro AJ, et al. Crotoxin: novel activities for a classic β-neurotoxin. Toxicon : official journal of the International Society on Toxinology. 2010;55(6):1045-60.
103. Brigatte P, Faiad OJ, Ferreira Nocelli RC, Landgraf RG, Palma MS, Cury Y, et al. Walker 256 tumor growth suppression by crotoxin involves formyl peptide receptors and lipoxin A4. Mediators of inflammation. 2016;2016.

ANEXOS

ANEXO A - Normas para publicação no periódico *Oral Oncology*

As as normas para a submissão no periódico *Oral Oncology* estão disponíveis no link a seguir:

https://www.elsevier.com/journals/oral-oncology/1368-8375/guide-for-authors

ANEXO B - Normas para publicação no periódico *Journal of Oral Pathology & Medicine*

As as normas para a submissão no periódico *Journal of Oral Pathology & Medicine Oral Oncology* estão disponíveis no link a seguir:

https://onlinelibrary.wiley.com/page/journal/16000714/homepage/forauthors.html

ANEXO C - Aprovação da pesquisa pelo Comitê de Ética em Pesquisa/Unimontes

UNIVERSIDADE ESTADUAL DE MONTES CLAROS - UNIMONTES

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Potencial antineoplásico e efeito terapêutico adjuvante de substância isolada do veneno da serpente Crotalus durissus terrificus em células de carcinoma epidermoide de boca

Pesquisador: Lucyana Conceição Farias

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 1

CAAE: 02257618.9.0000.5146

Instituição Proponente: Universidade Estadual de Montes Claros - UNIMONTES

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.037.289

Apresentação do Projeto:

Esse estudo envolverá a condução de análises in vitro, utilizando linhagens de células imortalizadas de carcinoma epidermoide de boca- CEB (SCC-4 e SCC-9, ATCC, USA). As
linhagens serão tratadas com diferentes concentrações da crotoxina-CTX e expostas a radiação ionizante terapêutica e quimioterápico, somente e em conjunto com o tratamento com a CTX, visando avaliar o potencial antineoplásico e sinérgico da substância. O comportamento neoplásico das células, sob tratamento, será avaliado por meio dos ensaios celulares de viabilidade, morte celular e formação de colônias. Os ensaios moleculares
envolverão análise do ciclo celular, apoptose, detecção de espécies reativas de oxigênio, e expressão de genes relacionados à proliferação, morte celular e invasividade, tais como CICLINA-D1, CASP3, NFKB1 e IKB. Assim, esse estudo será norteado não somente no intuito de investigar uma possível ação antineoplásica da CTX, mas como uma ferramenta para investigação de novas abordagens terapêuticas para o CEB.

Objetivo da Pesquisa:

Objetivo Primário:

Avaliar o potencial antineoplásico e efeito terapêutico adjuvante de substância extraída do veneno

Endereço: Av.Dr Rui Braga s/n-Camp Univers Profº Darcy Rib
Bairro: Vila Mauricéia **CEP:** 39.401-089
UF: MG **Município:** MONTES CLAROS
Telefone: (38)3229-8180 **Fax:** (38)3229-8103 **E-mail:** smelocosta@gmail.com

da serpente Crotalus durissus terrificus em células de carcinoma epidermoide de boca
Objetivo Secundário:
- Avaliar a ação da crotoxina sobre os parâmetros fenotípicos proliferação, migração e morte celular em linhagens celulares de carcinoma epidermoide de boca;- Analisar o potencial sinérgico da crotoxina sobre o efeito terapêutico da radiação ionizante ou quimioterapia em células de carcinoma epidermoide de boca;- Investigar a influência da crotoxina sobre expressão de genes relacionados à proliferação celular, morte celular e invasividade em células de carcinoma epidermoide de boca.

Avaliação dos Riscos e Benefícios:
Riscos:
A pesquisa será realizada a partir do cultivo de linhagens celulares imortalizadas, adquiridas comercialmente. Os riscos são àqueles relacionados à execução dos experimentos laboratoriais, que serão minimizados com o uso de equipamentos de proteção individual e obediência às normas de biossegurança em laboratórios.
Benefícios:
Através deste estudo, poderá ser identificado o potencial antineoplásico e efeito terapêutico adjuvante de substância extraída do veneno da serpente Crotalus durissus terrificus, visando o controle do comportamento neoplásico de células de carcinoma epidermoide de boca.

Comentários e Considerações sobre a Pesquisa:
Pesquisa relevante para verificar o potencial antineoplásico e efeito terapêutico adjuvante de substância isolada do veneno da serpente Crotalus durissus terrificus em células de carcinoma epidermoide de boca

Considerações sobre os Termos de apresentação obrigatória:
Adequados.

Recomendações:
Apresentação de relatório final por meio da plataforma Brasil, em "enviar notificação".

Conclusões ou Pendências e Lista de Inadequações:
Aprovado.

Considerações Finais a critério do CEP:
O projeto respeita os preceitos éticos da pesquisa em seres humanos, sendo assim somos favoráveis à aprovação do mesmo.

UNIVERSIDADE ESTADUAL DE MONTES CLAROS - UNIMONTES

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1247187.pdf	31/10/2018 15:37:33		Aceito
Folha de Rosto	FolhaDeRostoAssinadaPDF.pdf	31/10/2018 15:17:18	Lucyana Conceição Farias	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	JustificativaAusenciaTCLE.pdf	29/10/2018 17:55:07	Lucyana Conceição Farias	Aceito
Declaração de Pesquisadores	DeclaracaoCumprimentoNormasPesquisa.pdf	29/10/2018 17:54:20	Lucyana Conceição Farias	Aceito
Projeto Detalhado / Brochura Investigador	ProjetoCompleto.pdf	29/10/2018 17:42:47	Lucyana Conceição Farias	Aceito
Orçamento	Orcamento.pdf	29/10/2018 17:39:39	Lucyana Conceição Farias	Aceito
Declaração de Instituição e Infraestrutura	DeclaracaoInfraestrutura.pdf	29/10/2018 17:37:52	Lucyana Conceição Farias	Aceito
Cronograma	Cronograma.pdf	29/10/2018 17:37:41	Lucyana Conceição Farias	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

MONTES CLAROS, 25 de Novembro de 2018

Assinado por:
SIMONE DE MELO COSTA
(Coordenador(a))

Endereço: Av.Dr Rui Braga s/n-Camp Univers Profº Darcy Rib
Bairro: Vila Mauricéia **CEP:** 39.401-089
UF: MG **Município:** MONTES CLAROS
Telefone: (38)3229-8180 **Fax:** (38)3229-8103 **E-mail:** smelocosta@gmail.com

ANEXO D - Registro da Revisão Sistemática na Plataforma PROSPERO

PROSPERO
International prospective register of systematic reviews

NHS National Institute for Health Research

UNIVERSITY of York
Centre for Reviews and Dissemination

Systematic review

1. * Review title.

Give the working title of the review, for example the one used for obtaining funding. Ideally the title should state succinctly the interventions or exposures being reviewed and the associated health or social problems. Where appropriate, the title should use the PI(E)COS structure to contain information on the Participants, Intervention (or Exposure) and Comparison groups, the Outcomes to be measured and Study designs to be included.

Antineoplastic potential of crotoxin in different types of cancer: a systematic review

2. Original language title.

For reviews in languages other than English, this field should be used to enter the title in the language of the review. This will be displayed together with the English language title.

3. * Anticipated or actual start date.

Give the date when the systematic review commenced, or is expected to commence.

02/06/2019

4. * Anticipated completion date.

Give the date by which the review is expected to be completed.

15/11/2019

5. * Stage of review at time of this submission.

Indicate the stage of progress of the review by ticking the relevant Started and Completed boxes. Additional information may be added in the free text box provided.
Please note: Reviews that have progressed beyond the point of completing data extraction at the time of initial registration are not eligible for inclusion in PROSPERO. Should evidence of incorrect status and/or completion date being supplied at the time of submission come to light, the content of the PROSPERO record will be removed leaving only the title and named contact details and a statement that inaccuracies in the stage of the review date had been identified.
This field should be updated when any amendments are made to a published record and on completion and publication of the review.

The review has not yet started: No

Review stage	Started	Completed
Preliminary searches	Yes	No
Piloting of the study selection process	No	No
Formal screening of search results against eligibility criteria	No	No
Data extraction	No	No
Risk of bias (quality) assessment	No	No
Data analysis	No	No

Provide any other relevant information about the stage of the review here (e.g. Funded proposal, protocol not yet finalised).

6. * Named contact.

The named contact acts as the guarantor for the accuracy of the information presented in the register record.

Lucyana Farias

Email salutation (e.g. "Dr Smith" or "Joanne") for correspondence:

Professor Farias

7. * Named contact email.

Give the electronic mail address of the named contact.

lucyanacfarias@gmail.com

8. Named contact address

Give the full postal address for the named contact.

Av Cula mangabeira 562, Santo Expedito

9. Named contact phone number.

Give the telephone number for the named contact, including international dialling code.

+ 55 38 32248377

10. * Organisational affiliation of the review.

Full title of the organisational affiliations for this review and website address if available. This field may be completed as 'None' if the review is not affiliated to any organisation.

Unimontes

Organisation web address:

11. Review team members and their organisational affiliations.

Give the title, first name, last name and the organisational affiliations of each member of the review team.

Affiliation refers to groups or organisations to which review team members belong.

Mr Felipe Alberto Dantas Guimarães. Postgraduate Program in Health Sciences, Universidade Estadual de Montes Claros, Minas Gerais, Brazil
Mrs Renata Sousa Leite. Postgraduate Program in Health Sciences, Universidade Estadual de Montes Claros, Minas Gerais, Brazil
Mrs Karina Marini Aguiar. Postgraduate Program in Health Sciences, Universidade Estadual de Montes Claros, Minas Gerais, Brazil
Mr Rogério Gonçalves da Rocha. Postgraduate Program in Health Sciences, Universidade Estadual de Montes Claros, Minas Gerais, Brazil
André Luiz Sena Guimarães. Department of Dentistry and Postgraduate Program in Health Sciences, Universidade Estadual de Montes Claros, Minas Gerais, Brazil
Lucyana Conceição Farias. Department of Dentistry and Postgraduate Program in Health Sciences, Universidade Estadual de Montes Claros, Minas Gerais, Brazil

12. * Funding sources/sponsors.

Give details of the individuals, organizations, groups or other legal entities who take responsibility for initiating, managing, sponsoring and/or financing the review. Include any unique identification numbers assigned to the review by the individuals or bodies listed.

This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Brazil

13. * Conflicts of interest.

List any conditions that could lead to actual or perceived undue influence on judgements concerning the main topic investigated in the review.

None

14. Collaborators.

Give the name and affiliation of any individuals or organisations who are working on the review but who are not listed as review team members.

15. * Review question.

State the question(s) to be addressed by the review, clearly and precisely. Review questions may be specific or broad. It may be appropriate to break very broad questions down into a series of related more specific questions. Questions may be framed or refined using PI(E)COS where relevant.

Does Crotoxin have an antineoplastic effect on different types of cancer?

16. * Searches.

Give details of the sources to be searched, search dates (from and to), and any restrictions (e.g. language or publication period). The full search strategy is not required, but may be supplied as a link or attachment.

Studies will be retrieved from PubMed/Medline, Web of Science, Scopus, Embase and LILACS. Use the following descriptors:

crotoxin OR crotoxin A OR crotoxin B AND neoplasm OR cancer OR tumor OR carcinoma AND antineoplastic OR antitumor OR anti-tumor OR antitumoral OR cytotoxicity OR cytotoxic OR cytostatic.

These descriptors were selected according to the Medical Subject Heading (MeSH) and common terms from literature.

17. URL to search strategy.

Give a link to the search strategy or an example of a search strategy for a specific database if available (including the keywords that will be used in the search strategies).

Alternatively, upload your search strategy to CRD in pdf format. Please note that by doing so you are consenting to the file being made publicly accessible.

Do not make this file publicly available until the review is complete

18. * Condition or domain being studied.

Give a short description of the disease, condition or healthcare domain being studied. This could include health and wellbeing outcomes.

Antineoplastic potential of crotoxin in different types of cancer.

19. * Participants/population.

Give summary criteria for the participants or populations being studied by the review. The preferred format includes details of both inclusion and exclusion criteria.

Inclusion:

1- Studies involving patients with different types of cancer or human cancer cell lines

2- Studies investigating treatments with crotoxin in different types of cancer.

Exclusion:

1- Studies that assessed the antineoplastic effect of other substances in the different types of cancer

2-Studies that assessed other effects of crotoxin in cancer, such as analgesic

actions

3- In vivo studies.

20. * Intervention(s), exposure(s).

Give full and clear descriptions or definitions of the nature of the interventions or the exposures to be reviewed.

Treatments using crotoxin without limits for dosage and frequency. The intervention might be crotoxin treatment alone or crotoxin plus other treatment protocol for cancer such as chemo- or radiotherapy.

21. * Comparator(s)/control.

Where relevant, give details of the alternatives against which the main subject/topic of the review will be compared (e.g. another intervention or a non-exposed control group). The preferred format includes details of both inclusion and exclusion criteria.

Cancer patients or human cancer cell lines without treatment with crotoxin with no limits to the dosage and frequency.

22. * Types of study to be included.

Give details of the types of study (study designs) eligible for inclusion in the review. If there are no restrictions on the types of study design eligible for inclusion, or certain study types are excluded, this should be stated. The preferred format includes details of both inclusion and exclusion criteria.

Randomized clinical trials and other original studies are included to investigate the antineoplastic effect of crotoxin in different types of cancer.

23. Context.

Give summary details of the setting and other relevant characteristics which help define the inclusion or exclusion criteria.

24. * Primary outcome(s).

Give the pre-specified primary (most important) outcomes of the review, including details of how the outcome is defined and measured and when these measurement are made, if these are part of the review inclusion criteria.

The crotoxin may exert an antineoplastic effect on different types of cancer. This outcome will be defined by reading the "results" section of the included articles, which show the antineoplastic effect of crotoxin in cancer patients and cell lines. For randomized clinical trials, an effect size analysis and/or descriptive analysis will be performed, and for in vitro studies, the antineoplastic effect on the cell lines will be evaluated through the phenotypic assays.

Timing and effect measures

Not applicable.

25. * Secondary outcome(s).

List the pre-specified secondary (additional) outcomes of the review, with a similar level of detail to that required for primary outcomes. Where there are no secondary outcomes please state ‘None’ or ‘Not applicable’ as appropriate to the review

None

Timing and effect measures

Not applicable.

26. Data extraction (selection and coding).

Give the procedure for selecting studies for the review and extracting data, including the number of researchers involved and how discrepancies will be resolved. List the data to be extracted.

First, we will perform a calibration of inter-evaluators by Kappa statistic for selection of articles. Articles will be selected independently (FADG and RSL) based on the reading and publication of the abstracts reading. The rays are reviewed from the selected abstracts. In case of disagreement between the inclusion criteria, the title, the abstract or the complete article, these will be retained for later evaluation. Disagreements regarding the inclusion criteria, a third researcher (LCF) will be consulted. Data were extracted and recorded independently, including study results and methodological steps in a standardized data collection with the following items: Crotoxin treatment with no dose limits and frequency. The intervention might be crotoxin

treatment alone or crotoxin plus other treatment protocol for cancer such as chemo- or radiotherapy.

27. * Risk of bias (quality) assessment.

State whether and how risk of bias will be assessed (including the number of researchers involved and how discrepancies will be resolved), how the quality of individual studies will be assessed, and whether and how this will influence the planned synthesis.

The risk will be assessed using the Jadad scale to classify the methodological quality of the randomized controlled trial. Original in vitro studies are very useful for a single item: the use of different cancer cell lines only for product reviews.

28. * Strategy for data synthesis.

Give the planned general approach to synthesis, e.g. whether aggregate or individual participant data will be used and whether a quantitative or narrative (descriptive) synthesis is planned. It is acceptable to state that a quantitative synthesis will be used if the included studies are sufficiently homogenous.

We will provide a systematic narrative summary of the findings from the included studies, covering the antineoplastic effect of crotoxin on cancer patients or human cancer cell lines. We will provide summaries about the crotoxin, its antineoplastic effect, dosage, frequency of treatment and intervention type, including the crotoxin treatment alone or crotoxin plus other treatment protocol for cancer such as chemo- or radiotherapy.

29. * Analysis of subgroups or subsets.

Give details of any plans for the separate presentation, exploration or analysis of different types of participants (e.g. by age, disease status, ethnicity, socioeconomic status, presence or absence or co-morbidities); different types of intervention (e.g. drug dose, presence or absence of particular components of intervention); different settings (e.g. country, acute or primary care sector, professional or family care); or different types of study (e.g. randomised or non-randomised).

None planned.

30. * Type and method of review.

Select the type of review and the review method from the lists below. Select the health area(s) of interest for your review.

Type of review

Cost effectiveness
No

Diagnostic
No

Epidemiologic
No

Individual patient data (IPD) meta-analysis
No

Intervention
No

Meta-analysis
No

Methodology
No

Narrative synthesis
Yes

Network meta-analysis
No

Pre-clinical
No

Prevention
No

Prognostic
No

Prospective meta-analysis (PMA)
No

Review of reviews
No

Service delivery
No

Synthesis of qualitative studies
No

Systematic review
Yes

Other
No

Health area of the review

Alcohol/substance misuse/abuse
No

Blood and immune system
No

Cancer
Yes

Cardiovascular
No

Care of the elderly
No

Child health
No

Complementary therapies
No

Crime and justice
No

Dental
No

Digestive system
No

Ear, nose and throat
No

Education
No

Endocrine and metabolic disorders

No

Eye disorders
No

General interest
No

Genetics
No

Health inequalities/health equity
No

Infections and infestations
No

International development
No

Mental health and behavioural conditions
No

Musculoskeletal
No

Neurological
No

Nursing
No

Obstetrics and gynaecology
No

Oral health
No

Palliative care
No

Perioperative care
No

Physiotherapy
No

Pregnancy and childbirth
No

Public health (including social determinants of health)
No

Rehabilitation
No

Respiratory disorders
No

Service delivery
No

Skin disorders
No

Social care
No

Surgery
No

Tropical Medicine
No

Urological
No

Wounds, injuries and accidents
No

Violence and abuse
No

31. Language.

Select each language individually to add it to the list below, use the bin icon to remove any added in error.
English

There is not an English language summary

32. Country.

Select the country in which the review is being carried out from the drop down list. For multi-national collaborations select all the countries involved.

Brazil

33. Other registration details.

Give the name of any organisation where the systematic review title or protocol is registered (such as with The Campbell Collaboration, or The Joanna Briggs Institute) together with any unique identification number assigned. (N.B. Registration details for Cochrane protocols will be automatically entered). If extracted data will be stored and made available through a repository such as the Systematic Review Data Repository (SRDR), details and a link should be included here. If none, leave blank.

34. Reference and/or URL for published protocol.

Give the citation and link for the published protocol, if there is one

Give the link to the published protocol.

Alternatively, upload your published protocol to CRD in pdf format. Please note that by doing so you are consenting to the file being made publicly accessible.

No I do not make this file publicly available until the review is complete

Please note that the information required in the PROSPERO registration form must be completed in full even if access to a protocol is given.

35. Dissemination plans.

Give brief details of plans for communicating essential messages from the review to the appropriate audiences.

Do you intend to publish the review on completion?

Yes

36. Keywords.

Give words or phrases that best describe the review. Separate keywords with a semicolon or new line. Keywords will help users find the review in the Register (the words do not appear in the public record but are included in searches). Be as specific and precise as possible. Avoid acronyms and abbreviations unless these are in wide use.

37. Details of any existing review of the same topic by the same authors.

Give details of earlier versions of the systematic review if an update of an existing review is being registered, including full bibliographic reference if possible.

38. * Current review status.

Review status should be updated when the review is completed and when it is published.
Please provide anticipated publication date

Review_Ongoing

39. Any additional information.

Provide any other information the review team feel is relevant to the registration of the review.

40. Details of final report/publication(s).

This field should be left empty until details of the completed review are available.

Give the link to the published review.