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JEFFERSON MUNIZ DE LIMA

LIBERAÇÃO CONTROLADA DE QUIMIOTERÁPICOS NO TRATAMENTO DE CÂNCER DE CABEÇA E PESCOÇO

Recife

2021

JEFFERSON MUNIZ DE LIMA

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Tese apresentada como requisito parcial para a obtenção do grau de Doutor pelo Programa de Pós-Graduação em Odontologia do Centro de Ciências da Saúde da Universidade Federal de Pernambuco.

Área de concentração: Clínica integrada

Orientador: Danyel Elias da Cruz Perez

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À minha família, minha mãe Valdenira, meu pai José, minhas irmãs Valéria e Thayná. Obrigado por terem me ensinado o real valor da vida;

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RESUMO

Carcinoma de células escamosas de cabeça e pescoço (HNSCC) é o sexto tipo de câncer mais prevalente no mundo, com altas taxas de recorrência locoregional e baixa taxa de sobrevida em 5 anos após o diagnóstico. O tratamento para os pacientes portadores de HNSCC é modal e frequentemente está associado a efeitos adversos significativos. Nesse trabalho, foi sintetizada e caracterizada uma formulação de quitosana (CS) e partículas de policaprolactona (PCL) carreando 5-fluorouracil (5-FU) para tratamento do HNSCC. As partículas e esponjas (EPs) preparadas foram avaliadas para tamanho, morfologia, eficiência de carreamento (EE%) e a cinética liberação de droga em modelos *in vitro*. A atividade antineoplásica das partículas e *scaffolds* contendo 5-FU foi avaliada em linhagens celulares humanas de HNSCC (CAL27 e HSC3) e modelo pré-clínico de camundongos (AT84) submetidas a ensaios de proliferação celular, motilidade, viabilidade e sobrevivência (MTT e clonogênico). As proteínas associadas com apoptose e autofagia PARP1 e LC3-II expressas em células tratadas com os materiais foram quantificadas por meio de Western Blot. As micropartículas (MP) sintetizadas exibiram tamanhos enquadrados na escala micrométrica. A EE% de 5-FU nas micropartículas foi de 38.57%. A taxa de liberação controlada da quantidade total de 5-FU nas MPs e EPs variou entre 72 e 96 horas. Nos ensaios antineoplásicos, houve redução do metabolismo, migração e formação de colônias e aumento da citotoxicidade do 5-FU nos compósitos contendo CS/PCL comparados com as amostras sem incorporação de droga ou nas amostras contendo apenas o fármaco. A expressão de proteínas evidenciou aumento da autofagia e morte celular devido ao aumento de LC3-II e aumento de PARP1 ativado, respectivamente. Em conclusão, micropartículas de PCL e esponjas de PCL/CS foram consideradas carreadoras de 5-FU para liberação controlada e com potencial terapêutico para HNSCC.

Palavras-chave**:** Neoplasias de Cabeça e Pescoço. Liberação controlada de fármacos. Materiais biocompatíveis. Quitosana. PCL.

ABSTRACT

Head and neck squamous cell carcinoma (HNSCC) is the sixth most prevalent cancer worldwide with high rates of loco-regional recurrence and lower 5-year survival. The treatment of patients with HNSCC is complex and often associated with significant side effects. In this study, we characterized a nanoformulation of chitosan solution (CS) and polycaprolactone (PCL) coating 5-fluorouracil (5-FU) in HNSCC. The prepared particles were evaluated for their particle size, particle morphology, drug entrapment efficiency (EE%) and in vitro drug release profile. The anticancer activity of 5-FU-loaded particles was assessed in HNSCC human cell lines (CAL27 and HSC3) and preclinical mouse model (AT84) utilizing cell proliferation, motility, viability and survival assays (MTT and clonogenic). The proteins LC3-II and PARP1 levels associated with apoptosis and autophagy were evaluated. The prepared microparticles (MPs) exhibited particle sizes in the range of micrometric scale. The EE% of 38.57% was obtained for PCL coating 5-FU MPs. Moreover, lowering the rate of total 5-FU in vitro release within 96h by using CS coated SPs. Furthermore, a decrease of cell metabolism, cell migration and colony formation followed by a tremendous increase in 5-FU cytotoxicity was observed by CS-decorated PCL MPs compared to all other MPs including 5-FU-free-MPs and pure 5-FU. The protein expression showed an increase of autophagy and cell death evaluated by LC3-II and PARP1 cleavage respectively. In conclusion, CS-decorated PCL MPs are a considerable 5-FU-delivery carrier with anti-neoplastic efficacy and potential therapeutic use in HNSCC.

Key words**:** Head and neck neoplasms. Drug liberation. Biocompatible materials. Chitosan. PCL.

LISTA DE SIGLAS E ABREVIATURAS

5-FU	5-fluorouracil
95% CI	95% confidence interval/intervalo de confiança
AEs	adverse events/efeitos adversos
AJCC	American Joint Committee on Cancer
ATCC	American Type Culture Collection
CEBM	Oxford Centre for Evidence Based Medicine
CRT	adjuvant radiation or chemoradiation/radioquimioterapia adjuvante
CS	chitosan/quitosana
CT	Chemotherapy/quimioterapia
CTC	Common Toxicity Criteria
DMEM	Dulbecco's Modified Eagle's
DMSO	dimethyl sulfoxide/dimetilsulfóxido
EE%	entrapment efficiency/eficiência de carreamento
FBS	fetal bovine serum/soro fetal bovino
FDA	United States Food and Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gy	Greys
h	hour/hora
HNC	Head and neck cancer/cancer de cabeça e pescoço
HNSCC	Head and neck squamous cell carcinoma/carcinoma de células escamosas de cabeça e pescoço
ICD-O	International Classification of Diseases for Oncology
ICTRP	International Clinical Trials Registry Platform
IgG:	Immunoglobulin G/imunoglobulina G
INCA	Instituto Nacional do Câncer
kD	kilodalton/quilodalton
LC3	microtubule-associated protein 1 light chain 3/cadeia leve 3 da proteína 1 associada a microtúbulos
mg	milligrams/miligramas
mL	milliliter/mililitro

MP	Microparticles/micropartículas
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide
NCC	nanoparticles carrier chemotherapy/nanoparticulas carreadoras de quimioterápicos
NCC	National Cancer Institute
NIH	National Institutes of Health NIH
nm	nanometer/nanometro
NOF	normal oral fibroblasts/fibroblastos orais normais
ºC	Celsius degree/graus Celsius
PARP1	poly-(adenosine diphosphate-ribose) polymerase/poli-(adenosine ribose-difosfato) polimerase
PBS	phosphate-buffered saline
PCL	polycaprolactone/policaprolactona
PD	progressive disease/doenca progressiva
PR	partial response/resposta parcial
Prev.	prevalence/prevalência
PVDF	polyvinylidene fluoride/fluoreto de polivinilideno
RPM	rotations per minute/rotacoes por minuto
RPMI	Roswell Park Memorial Institute Medium
RT	Radiotherapy/Radioterapia
SAE	Serious adverse effects/Efeito adverso severo
SD	stable disease/doenca estável
SEM	scanning electron microscope/microscopia eletronica de varredura
SEM	standard error/erro padrao
SFM	serum free medium/meio sem soro
SP	sponges/esponjas
TBST	tris-buffered saline and polysorbate 20/tampao tris e polisorbato 20
UADT	aerodigestive tract/trato aerodigestivo
UV	ultraviolet/ultravioleta
v/v	volume/volume
w/v	weight/volume / peso/volume
WHO	World Health Organization

µg	micrograms/microgramas
µL	microlitre/microlitro

SUMÁRIO

1 INTRODUÇÃO

O trabalho foi redigido conforme as normas para defesa de Tese do Programa de Pós-Graduação em Odontologia da Universidade Federal de Pernambuco. Os artigos científicos que o compõe foram elaborados de acordo com as normas das revistas científicas Nanomaterials e International Journal Biochemistry and Cell Biology (Anexo A e B, respectivamente).

O presente trabalho teve início em 2018 com a síntese dos biomateriais no Laboratório de Materiais e Biossistemas (LAMAB) da Universidade Federal da Paraíba e os testes biológicos foram possibilitados por meio de um estágio de doutorado sanduíche no Lady Davis Institute for Medical Research and Segal Cancer Centre (Montreal – QE) durante o ano de 2019.

O trabalho surgiu da ideia de se averiguar o emprego de um biomaterial formulado a partir dos polímeros policaprolactona (PCL) e quitosana (CS) carreando o quimioterápico 5-fluorouracil (5-FU) para o tratamento de pacientes afetados por carcinoma de células escamosas de cabeça e pescoço, baseando-se na suposição de que esse biomaterial pode ser empregado como *scaffold* para regeneração tecidual após a ressecção tumoral e liberação controlada do 5-FU para redução da recidiva local da neoplasia.

Concomitantemente, foi realizada uma revisão sistemática da literatura sobre estudos clínicos que avaliaram a eficácia e os efeitos adversos de nanoformulações de quimioterápicos no tratamento de pacientes com HNSCC.

Cânceres de cabeça e pescoço possuem tratamentos com efetividade limitada devido à heterogeneidade dessas neoplasias. O principal evento relacionado ao óbito pelo carcinoma de células escamosas é a recidiva local e locorregional. Desse modo, fazem-se necessárias terapias com biodispolibilidade de fármacos no sítio primário do tumor.

A literatura ainda é escassa sobre a associação das propriedades biológicas de blendas de CS e PCL carreando 5-FU para a liberação controlada desse fármaco. Desta forma, os objetivos dos estudos foram avaliar a atividade antineoplásica de scaffolds porosos de polímeros bioativos carreados com quimioterápicos em modelos pré-clínicos de HNSCC e revisar sistematicamente a literatura a respeito da eficácia e efeitos adversos de nanoformulações de quimioterápicos no tratamento de HNSCC.

O carcinoma células escamosas de cabeça e pescoço (HNSCC) representa um grupo de malignidades que surgem no tecido epitelial da mucosa do trato aerodigestivo superior (UADT), incluindo cavidade oral, faringe, laringe e seios paranasais (LYDIATT; O'SULLIVAN e PATEL, 2018). A categoria HNSCC inclui o sexto câncer mais prevalente em todo o mundo, com uma das maiores taxas de recorrência nos primeiros 2 anos após

a ressecção (MACDIARMID *et al.*, 2016). No Brasil, o Instituto Nacional do Câncer (INCA) estima que número de casos novos de câncer da cavidade oral, para cada ano do triênio 2020-2022, será de 11.200 casos em homens e de 4.010 em mulheres (SANTOS, 2018). Essa estimativa também evidencia que o câncer da cavidade oral ocupa a quinta posição em prevalência nacional em homens.

Os casos HNSCC apresentam baixas taxas de sobrevida em 5 anos (<50%) (REYES-GIBBY *et al.*, 2014). De acordo com variáveis anatômicas, as taxas de sobrevida global em cinco anos dos pacientes com HNSCC são de 49,0% para cavidade oral, 54,8% para orofaringe, 50,0% para hipofaringe e 63,4% para laringe (CADONI *et al.*, 2017). O prognóstico de pessoas com HNSCC dependem de vários fatores, como local comprometido, extensão e estágio da doença, fatores comportamentais e socioeconômicos (REYES-GIBBY *et al.*, 2014).

As decisões sobre terapias contra o HNSCC são baseadas em estádios clínicos e patológicos (LYDIATT; O'SULLIVAN e PATEL, 2018). A estratificação de malignidades em estadiamento é útil para auxiliar na estimativa do prognóstico clínico e no planejamento do tratamento. Conforme definido pelo American Joint Committee on Cancer (AJCC), os grupos do Manual de Estadiamento do HNSCC foram estabelecidos de acordo com critérios anatômicos, de tamanho, metástase local e à distância e, recentemente, a infecção pelo HPV foi levada em consideração (AMIN; EDGE, 2017). No entanto, na rotina clínica, as equipes profissionais negligenciam o estadiamento clínico para pequenos cânceres tratados com ressecções menos invasivas (GROOME *et al.*, 2001).

O tratamento para HNSCC em estádio inicial ou ressecável tem a cirurgia e/ou radioterapia como modalidades primárias. No entanto, o HNSCC localmente avançado exige cirurgia seguida de radioterapia adjuvante ou quimiorradioterapia (TRC). Embora haja evidências de que a quimioterapia adjuvante concomitante melhore a sobrevida global de pacientes com HNSCC ressecável, existem toxicidades substanciais associadas à quimioterapia. Para minimizar os efeitos colaterais e aumentar a eficácia, as imunoterapias surgiram para complementar os tratamentos convencionais. Entretanto, as combinações de tratamentos padrão e imunoterapias direcionadas e/ou imunológicas podem amplificar efeitos adversos típicos de tratamentos únicos (CHAN *et al.*, 2015).

No curso clínico do câncer de cabeça e pescoço, a maioria dos pacientes com HNSCC desenvolve recorrência locorregional tumoral após ressecção cirúrgica. Acontece que a mucosa circundante ainda apresenta anormalidades clinicamente não visíveis e são identificadas apenas ao microscópio como epitélio mucoso displásico. Essas anormalidades morfológicas levaram ao desenvolvimento do conceito de malignização em campo' (LEEMANS; SNIJDERS e BRAKENHOFF, 2018).

Apesar dos avanços na estratégia terapêutica, o atual tratamento padrão (TRC

ou cirurgia seguido de radioterapia adjuvante (TR) ou TRC adjuvante) deixa os sobreviventes com morbidade significativa e ao longo da vida associada a sequelas de longo prazo. Atualmente, as nanoformulações de medicamentos emergiram como uma das aplicações mais esperançosas na medicina para aumentar a atividade antineoplásica. No campo da terapia do câncer, as partículas recebem maior atenção nas investigações como portadoras de drogas devido ao aumento da biodisponibilidade no ambiente tumoral. A fim de otimizar o índice terapêutico da molécula efetora, minimizar os efeitos colaterais sistêmicos e aumentar a biodisponibilidade no local do tumor, nanoformulações de compostos antineoplásicos convencionais (NCAC) foram desenvolvidos nas últimas décadas. De acordo com ensaios clínicos aprovados ou em avaliação, a Food and Drug Administration (FDA) dos Estados Unidos classificou os nanomateriais com base no veículo de entrega: lipossômico, polimérico, limites de albumina, limites de polímeros e partículas inorgânicas (LYTTON-JEAN *et al.*, 2015). Além das drogas, essas estruturas podem ser conjugadas com grupos de direcionamento, imagem/compostos fluorescentes e agentes estabilizadores (HASSAN *et al.*, 2017).

O uso de terapias de câncer baseadas em biomateriais e liberação controlada de fármacos para o tratamento de HNSCC ainda precisam de evidências de eficácia sobre os respectivos padrões-ouros, redução de toxicidade quimioterapêutica e melhoria da qualidade de vida para justificar a translação desses materiais para a prática clínica (HASSAN *et al.*, 2017; LYTTON-JEAN e LANGER, 2015). Além disso, biomateriais baseados em biopolímeros como a quitosana (CS) podem melhorar a quimioterapia tradicional não apenas pela inibição da proliferação de células tumorais (DASS e CHOONG, 2008), mas adicionando propriedades extras como atividade antimicrobiana, estimulação da cicatrização de feridas e indução hemostática (DI MARTINO; SITTINGER e RISBUD, 2005). Portanto, como a quitosana possui atividade anticâncer inerente, isso a torna ideal para uso em encapsulamento e administração de agentes antineoplásicos tradicionais (DASS e CHOONG, 2008). Nesse contexto, o 5-Fluorouracil (5-FU) é um dos medicamentos antimetabólitos mais amplamente utilizados nos casos recorrentes de HNSCC (MACDIARMID *et al.*, 2016). Assim, a combinação que envolve o uso de CS e 5-FU parece ser promissora (ZHENG *et al.*, 2016), mas sua atividade antineoplásica contra o HNSCC permanece incerta.

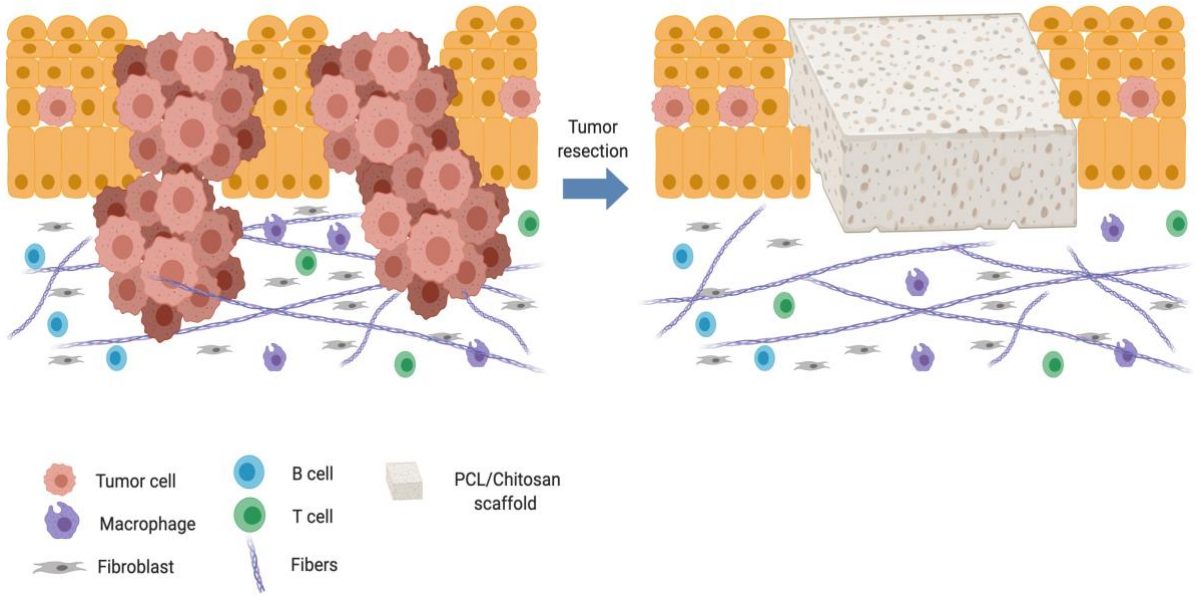
A associação da CS com outros polímeros podem agregar propriedades físicas desejáveis à engenharia tecidual. Um exemplo é a policaprolactona (PCL) que tem sido utilizado para diversos fins clínicos, dentre eles a reconstrução de tecidos: como ossos, pele, tecido nervoso, retina, entre outros (FERNANDES, 2011).

A associação entre dois polímeros é uma abordagem para o desenvolvimento de novos biomateriais exibindo combinações de propriedades que não podiam ser obtidas

por polímeros individuais. Blendas feitas de polímeros sintéticos e naturais podem absorver uma vasta gama de propriedades físico-químicas e de técnicas de processamento de polímeros sintéticos, bem como a biocompatibilidade e as interações biológicas dos polímeros naturais. Com base no que foi descrito, as respectivas características da CS e do PCL associadas resultam na formação de um biomaterial superior, onde podemos observar a presença de propriedades mutuamente complementares. É, por conseguinte, razoável esperar que suas deficiências individuais possam ser superadas a partir da formação de blendas entre eles (SARASAM e MADIHALLY, 2005).

Essa múltipla abordagem como scaffold para indução de reparo tecidual e liberação controlada de quimioterápicos e surge como hipótese especialmente formulada para reduzir a recorrência local do HNSCC após a ressecção HNSCC e fornecer condições para cicatrização tecidual (Figura 1).

Figura 1. Scaffold para liberação de fármacos como uma potencial estratégia terapêutica em medicina regenerativa. A manutenção de um epitélio circundante com potencial capacidade de transformação maligna após a remoção do tumor é um evento preocupante no HNSCC. Os scaffolds com liberação controlada e sustentada de medicamentos podem ser colocados na área cirúrgica como uma estratégia para superar a alta incidência de recidiva tumoral e recorrência locorregional. PCL: policaprolactona. A imagem foi feita usando a ferramenta BioRender.com.



Para garantir tratamentos significativamente eficazes e garantir aumento da qualidade de vida e livre de doenças para pacientes com HNSCC, é crucial identificar novos protocolos de tratamento. Neste estudo atual, nossos objetivos foram desenvolver e avaliar a atividade antineoplásica de partículas e esponjas carreando 5-FU no tratamento de HNSCC.

2 METODOLOGIAS

2.1 Artigo I

Pesquisa de literatura

Uma pesquisa sistemática da literatura foi conduzida nas bases MEDLINE (1946 até o presente), EMBASE via OVID (1980 até o presente), Cochrane Oral Health Group's Trials Register, Cochrane Central Register of Controlled Trials (CENTRAL) (The Cochrane Library, edição atual) e ClinicalTrials. gov desde o início até 10 de junho de 2020. Além disso, a Plataforma Internacional de Registro de Ensaios Clínicos (ICTRP) da Organização Mundial da Saúde (OMS) (http://apps.who.int/trialsearch/), Current Contolled trials (www.controlledtrials.com) e a Clinical Trials (www.clinicaltrials.gov) foram pesquisadas para HNC. Os resultados foram compilados por meio do software de gerenciamento bibliográfico EndNote X9 3.2 (Thomson Reuters).

A estratégia de pesquisa completa está listada no Apêndice A. Resumidamente, a pesquisa incluiu pacientes com carcinoma de células escamosas de cabeça e pescoço de acordo com a Classificação Internacional de Doenças para Oncologia (CID-O) (PERCY et al., 1990), códigos como C00 (lábio), C01-C02 (língua), C03 (goma), C04 (assoalho da boca), C05 (palato) e C06 (outras partes não especificadas da boca), C09 (amígdala), C10 (orofaringe), C11 (nasofaringe) , C12 (seio piriforme) e C13 (hipofaringe). Os resultados foram: "resposta do tumor", "sobrevida global", "sobrevida livre de doença", "sobrevida livre de progressão", "controle loco-regional", "recorrência", "efeitos adversos graves (SAE)" e "qualidade de vida".

A lista de referências relevantes foi pesquisada e os autores foram contatados a fim de identificar estudos não publicados ou em andamento. Quaisquer resumos de congressos e artigos potencialmente relevantes encontrados em suas listas de referência foram revisados e considerados para inclusão. Dois investigadores revisaram independentemente os artigos para elegibilidade. As referências recuperadas neste estudo estão listadas na Biblioteca EndNote no Material Suplementar S1.

Seleção do estudo: critérios de inclusão e exclusão

Esta pesquisa não recuperou ensaios clínicos randomizados porque apenas estudos porque apenas ensaios clínicos de braço único estão atualmente publicados. Devido à falta de evidências, apenas estudos clínicos nas Fases I ou II, incluindo mais de 50% dos pacientes com HNC, foram selecionados. Em seguida, os seguintes critérios foram usados para inclusão na meta-análise: ensaio clínico onde pelo menos o resultado foi relatado, como doença progressiva (PD), resposta parcial (PR), doença estável (SD) e eventos adversos (EAs). Os critérios de exclusão envolveram artigos em outro idioma, relatos de caso único, cartas ao

editor e revisões.

Extração de dados e avaliação da qualidade do estudo

Dois autores extraíram independentemente os dados de títulos e resumos. A leitura completa do artigo foi realizada quando os estudos preencheram os critérios de inclusão ou se as informações não eram suficientes no título ou resumo para tomar uma decisão clara. Os artigos foram classificados de acordo com os níveis de evidência do Oxford Centre for Evidence Based Medicine (CEBM) (DURIEUX et al., 2012). Discrepâncias foram identificadas e resolvidas por meio de discussão. Os dados omissos foram solicitados diretamente aos autores por e-mail. Os estudos rejeitados nesta ou nas etapas subsequentes foram registrados na tabela de características dos estudos excluídos e os motivos da exclusão anotados. As características clínicas dos estudos são apresentadas na Tabela 1. Para cada ensaio clínico, os seguintes detalhes foram extraídos e apresentados: características do estudo (primeiro autor, periódico, ano de publicação, país, centro múltiplo ou único), características do desenho do ensaio (desenho do estudo, medição do resultado, regime de terapia), população do estudo (localização do tumor primário, idade mediana, número de pacientes avaliados para eficácia e desfechos de segurança), detalhes da intervenção (tipo de intervenção, tempo, dose, modo de administração e duração, tratamentos concomitantes) resultados de eficácia (PD, PR e SD) e resultados de SAE (Tabelas 2 e 3). Um formulário padronizado e pré-piloto adaptado da Colaboração Cochrane foi usado para extrair dados dos trabalhos incluídos.

Avaliação do risco de viés

Uma vez que não foi possível encontrar ensaios clínicos randomizados relatados, todos os estudos foram classificados com alto risco de viés de acordo com o Cochrane Handbook for Systematic Reviews of Interventions versão 5.1.0 (HIGGINS et al., 2011). Esses parâmetros incluíram detalhes de randomização, ocultação de alocação, cegamento de tratamento, integridade dos dados de resultados e presença de relatórios de resultados seletivos.

Tipos de medidas de resultados

O desfecho primário foi a resposta clínica expressa como volume do tumor, bem como PD, PR e SD. Os desfechos secundários foram os efeitos adversos graves (SAE) classificados como grau III e IV de acordo com os critérios de toxicidade comuns (CTC) v2.0 (TROTTI et al., 2000).

Análise estatística

A estatística descritiva foi utilizada para resumir os dados, com média e amplitude para

variáveis contínuas e frequências e percentuais para variáveis nominais/dicotômicas. Os SAE foram calculados como o número de eventos por 100 e agrupados em modelos de efeitos aleatórios com software MetaXL (versão 5.3). Um dos estudos incluídos (HARRINGTON et al., 2001) na metanálises apresentou a análise de um evento com zero resultados ao calcular as estimativas agrupadas conforme a metodologia previamente publicada (FRIEDRICH et al., 2007). Os resultados foram considerados estatisticamente significativos para um valor de P bicaudal <0,05.

2.2 Artigo II

Infraestrutura

O desenvolvimento dos biomateriais desenvolvidos por esse trabalho foi realizado no Laboratório de Materiais e Biossistemas (LAMAB) e no Laboratório de Análise e Cultivo Celular (LACEC) da UFPB. A caracterização morfológica ocorreu no Laboratório de Materiais e Compósitos (LACOM). O perfil de liberação do fármaco e os ensaios biológicos foram efetuados no Lady Davis Institute for Medical Research e no Segal Cancer Centre.

Preparação de partículas carregadas de 5-FU

A síntese de micropartículas de PCL (MPs) foi realizada por disposição interfacial de polímeros e método de evaporação de solventes modificado a partir de Ortiz et al. (2012) (ORTIZ; PRADOS; MELGUIZO; ARIAS et al., 2012). Resumidamente, o polímero PCL foi dissolvido em acetona (2 mg/mL) sob agitação mecânica (300 RPM). A solução orgânica foi transferida gota a gota para uma solução aquosa de TWEEN® 80 a 0,2% (v/v), agitada a 1200 rpm contendo ou não 5-FU (Sigma Aldrich, F6627) a 10% da massa total de PCL. A fase orgânica foi evaporada para obter uma suspensão aquosa de MPs de PCL. A preparação da solução de CS consistiu em 2,5 g de quitosana ≥75% desacetilada de cascas de camarão (Sigma Aldrich, C3646) diluída em 500 mL de ácido acético glacial a 1% (v/v) (Química Moderna, QMA00001120301000) agitada durante pernoite à temperatura ambiente.

Para síntese das esponjas (SPs), MPs de PCL e a solução de CS foram associados nas seguintes proporções volumétricas (v/v): 100: 0; 75:25; 50:50; 0: 100. A nomenclatura correspondente a estas diluições fooram: 100CS, 100CS-5FU, 75CS, 75CS-5FU, 50CS, 50CS-5FU, 100PCL e 100PCL-5FU. Para sintetizar a amostra 100CS-5FU, foi adicionado 5-FU a 10% da massa total de quitosana na solução.

Essas soluções foram armazenadas a -20°C e colocadas em um equipamento de liofilização (marca Terroni, modelo Enterprise II) por 36h para obter as esponjas compostas.

Morfologia e tamanho de partícula

A morfologia dos MPs e SPs foi observada usando um microscópio eletrônico de varredura modelo Quanta 450 FEI (SEM) (FEITM, FEI Company). As amostras foram fixadas em suportes de alumínio e cobertos com ouro usando um sputter coater K550X Emitech. A morfologia de partículas e esponjas foi analisada por descrição visual e as medidas de tamanho de partículas foram realizadas usando o software ImageJ (National Institutes of Health, Bethesda, MD).

Eficiência de carregamento de droga

A concentração de 5-FU foi medida por absorção de luz ultravioleta (UV) no comprimento de onda de 300nm em um espectrofotômetro (Beckman, DU 800). As quantidades de 5-FU aprisionadas nas formulações foram determinadas por um método indireto (ORTIZ; PRADOS; MELGUIZO; ARIAS et al., 2012). O 5-FU livre foi determinado no sobrenadante após a filtração das MPs (tamanho da membrana: 0,1µm) para avaliar a eficiência do carregamento (%EE). Para que o método fosse preciso, considerou-se a absorvância do agente surfactante e do PCL. A carga do medicamento nas PCL MPs foi expressa em termos de eficiência de aprisionamento em 5-FU(%) (medicamento encapsulado [mg]/medicamento total nos MPs [mg] × 100).

Liberação *in vitro*

O perfil de liberação in vitro de 5-FU pelas MPs e SPs foi medido usando formulações liofilizadas pelo método da membrana de diálise (ORTIZ; PRADOS; MELGUIZO; ARIAS et al., 2012) pré-embebida em solução salina tamponada com fosfato (PBS; Corning®, 21-040-CM) por 12h antes do uso. A bolsa de diálise (corte do tubo de membrana de diálise Spectra / Por® 2, 12-14 kD; Spectrum) reteve as MPs, mas permitiu que o fármaco se difundisse no meio de dissolução. Um equivalente em peso da amostra de 20 mg dos MPs e SPs foi colocado na membrana de diálise com as duas extremidades fixadas por pinças. Após a selagem, os sacos foram colocados em um tubo Falcon de 50 mL contendo 30 mL do meio de dissolução e agitados a 200 rpm. A temperatura foi mantida a 37,0°C±0,5 °C durante as experiências de liberação do medicamento. Amostras de 1mL foram retiradas e seu teor de 5-FU analisado por

espectrofotometria UV-Vis (300nm) durante os intervalos (0,25, 0,5, 1, 2, 3, 6, 9, 12, 24, 48, 72 e 96h). Um volume igual de PBS mantido à mesma temperatura foi adicionado após a retirada da amostra para garantir condições semelhantes. As leituras de amostras sem medicamento foram subtraídas da absorbância de esponjas contendo 5-FU e os resultados foram interpolados em uma curva de concentrações padrão de 5-FU usando o software GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, EUA). Os experimentos foram realizadas em triplicatas.

Cultura de células

As linhagens celulares de câncer oral humanas CAL27 (ATCC® CRL-2095™, American Type Culture Collection) e HSC3 (JCRB 0623, uma linhagem celular de carcinoma de células escamos4as de língua humana, Instituto Nacional de Ciências da Saúde de Osaka, Japão) disponíveis comercialmente. Os fibroblastos normais (NOF) foram gentilmente fornecidos por Ricardo D. Coletta (Universidade de Campinas, Piracicaba, Brasil) (DOURADO; KORVALA; ÅSTRÖM; DE OLIVEIRA et al., 2019). Estas células foram mantidas em DMEM (Corning, 10-017-CV) suplementado com 10% de FBS (Wisent Bioproducts, 080-150) com antibióticos de penicilina e estreptomicina a 1% (Corning Inc., 30-002-CI). A linhagens de células de câncer bucal de camundongo (AT84) foi estabelecida e caracterizada por nosso grupo (Black et al., 1995) E cultivada em RPMI 1640 (Fisher Scientific, 11-875-093) suplementado com 10% de SFB e 1% de penicilina e estreptomicina. antibióticos (Corning Inc., 30-002-CI). As células foram cultivadas a 37ºC com 5% de CO2. As linhagens celulares foram testadas quanto à contaminação por micoplasma.

Ensaio clonogênico

Os efeitos crônicos de MPs e SPs no crescimento de células tumorais foram avaliados usando o ensaio de formação de colônias (FRANKEN; RODERMOND; STAP; HAVEMAN et al., 2006). As linhagens celulares CAL-27, HSC3 (600 células/poço) e AT84 (500 células/poço) foram plaqueadas em triplicatas em uma placa de 6 poços. Após 6 h de incubação, as células foram tratadas com 5 mg de MPs e SPs. Após 10 dias de incubação, as células foram lavadas com PBS e coradas com violeta de cristal a 0,05% (p/v) (Sigma Aldrich Corporation, C3886) e solução de formaldeído a 4% (v/v) (Sigma Aldrich Corporation, 8.18708) e as colônias> 100 µm de diâmetro foram contadas usando um contador de colônias (Oxford Optronix Gelcount, Inc., Milton

Park, Abingdon, Reino Unido).

Ensaio de migração celular

O ensaio de migração da cicatrização de feridas foi realizado com base na publicação de pré-visualizações (VALSTER; TRAN; NAKADA; BERENS et al., 2005). As áreas foram medidas em triplicatas e fotografadas com um microscópio invertido (sistema de imagem celular EVOS FL, Life Technologies) nas 0 e 24 horas após a incubação. A quantificação do percentual de fechamento da cicatrização de feridas foi determinada usando a macro MRI Wound Healing Tool da ImageJ (versão 1.50i, Institutos Nacionais de Saúde (NIH), Bethesda, MD).

Western Blot

As células subconfluentes foram lavadas com PBS, lisadas em tampão RIPA (Tris-HCl 50 mM, pH 7,5, NaCl 150 mM, NP40 a 1%, SDS 0,1%, EDTA 5 mM, desoxicolato de sódio 25 mM) suplementado com PMSF 1 mM, NaF 1 mM e inibidor de protease coquetel; Roche, 4693159001) por 15 min em gelo e centrifugado (14.000x g a 4 ° C por 20 min) para separar o lisado celular. A concentração celular foi medida pelo ensaio da proteína Bradford e, em seguida, adicionada com tampão de amostra SDS (Tris 17 mM, pH 6,8, glicerol a 30%, SDS a 10%, azul de bromofenol a 0,02%, azul de bromofenol a 0,02%, β-mercaptoetanol a 6%) e desnaturado por 5 min. As amostras (20 µg) foram então resolvidas através de géis de 15% de SDS-PAGE, transferidos para a membrana de fluoreto de polivinilideno (PVDF) (MerckMillipore, IPVH00010), bloqueados com TBST com 5% p / v de leite seco sem gordura, transferidos durante a noite com primeiro (Tabela 1) em diferentes diluições durante a noite a 4ºC e amplificados com anticorpos secundários conjugados com peroxidase de rábano silvestre por 1 h em temperatura ambiente e aprimorados por sistemas de detecção por quimioluminescência (Thermo Fisher Scientific, 32106 e 34095). A densitometria de bandas foi realizada utilizando ImageJ (versão 1.50i, Institutos Nacionais de Saúde (NIH), Bethesda, MD).

Tabela 1. Anticorpos utilizados neste estudo para avaliar a expressão de proteínas por western blot.

Nome	Fonte	Empresa	Diluição

PARP1	PAB coelho	Merck Millipore	1:500
LC3B	MAB de rato	Biotecnologia de Santa Cruz	1:20000
*GAPDH	Coelho	Sigma Aldrich	1:10000
*anti-rato IgG-peroxidase-conjugado	Cabra	Laboratórios Bio-Rad	1:5000
anti-coelho IgG-peroxiase-conjugado	Cabra	Laboratórios Bio-Rad	1:3000

* GAPDH: Glicealdeído 3-fosfato desidrogenase; IgG: Imunoglobulina G.

Análise estatística

Todos os dados são apresentados como média ± SEM usando o software GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, EUA). O erro alfa foi estabelecido com intervalo de confiança de 5% e 95%. As diferenças estatísticas na proliferação celular e formação de colônias foram determinadas pelo teste Anova de duas vias com o teste de comparações múltiplas de Tukey. A importância da migração celular foi analisada pelo teste T de vários alunos para amostras emparelhadas. Os dados foram considerados estatisticamente significativos se $P < 0,05$.

3 ARTIGO I

Artigo aceito para publicação no periódico Nanomaterials (Qualis A1 na área Engenharias II)

Review

Nanoparticle-Based Chemotherapy Formulations for Head and Neck Cancer: A Systematic Review and Perspectives

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Abstract: Head and neck cancer (HNC) is a complex and heterogeneous disease associated with high mortality and morbidity worldwide. Standard therapeutic management of advanced HNC, which is based on radiotherapy often combined with chemotherapy, has been hampered by severe long-term side effects. To overcome these side effects, tumor-selective nanoparticles have been exploited as a potential drug delivery system to improve HNC therapy. A combination of MEDLINE, EMBASE, Cochrane Oral Health Group's Trials Register, Cochrane Central Register of Controlled Trials (CENTRAL) and ClinicalTrials.gov from inception up to June 2020 was used for this systematic review. A total of 1747 published manuscripts were reviewed and nine relevant references were retrieved for analysis, while eight of them were eligible for meta-analysis. Based on these studies, the level of evidence about the efficacy of nanoformulation for HNC therapy on tumor response and adverse side effects (SAE) was low. Even though basic research studies have revealed a greater promise of nanomaterial to improve the outcome of cancer therapy, none of them were translated into clinical benefits for HNC patients. This systematic review summarized and discussed the recent progress in the development of targeted nanoparticle approaches for HNC management, and open-up new avenues for future perspectives.

Keywords: nanoparticles; targeted therapeutics; head and neck cancer; selective drug delivery

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1. Introduction

Head and neck cancer (HNC) is a complex multifactorial disease that originates in the epithelial layer of mucosa of the upper aerodigestive tract, including the oral cavity, pharynx and larynx showing microscopic evidence of squamous differentiation [1,2]. The main risk factors for HNC are tobacco smoke and alcohol consumption, as well as human papilloma virus (HPV) infection. Therapeutic decisions for patients with HNC are primarily based on clinical and pathological tumor stage [1]. It is estimated that 60% of the patients are diagnosed with advanced disease (stage III and IV) leading to low

survival rates. In these cases, the treatment consists of surgical ablation followed by adjuvant radiation or chemoradiation (CRT) [3,4]. Despite recent advances in these therapeutic modalities, 50–60% of the patients develop regional relapses or distant metastasis within two years [5]. Patients with recurrent and/or metastatic disease have a median survival lower than 12 months [6], in part due to the limitations of conventional treatments, in particular the severe side effects that worsen quality of life [7].

Nanotechnology based therapy approaches have attracted great interest in oncology in recent years. Nanoformulations, a class of multifunctional materials with diameters of 1-100nm, can act as carriers for drugs and targeting ligands to optimized cancer therapy. The United States Food and Drug Administration (FDA) categorized nanomaterials based on the delivery vehicle or carrier as liposomal, polymeric, albumin-bounds, polymer-bounds, and inorganic particles [8]. These materials have been explored to overcome the biological barriers to cancer treatment due to their unique features such as a large surface area allowing conjugation to biologically active molecules, structural properties (optical, electronic, catalytic and magnetic) and a long time circulation in blood compared with small molecules. Furthermore, a plethora of nanomaterials has been developed to load sufficient drugs and accurately delivery to the tumor site with excellent biocompatibility, biodistribution and biodegradation resulting in lower systemic toxicity [9]. In HNC, early studies in nanotechnology have been designed to overcome the lack of the specificity of conventional chemotherapeutic agents to target cancer cells [10]. In this systematic review, we summarized and discussed the recent progress in the development of targeted nanoparticles systems for HNC therapy opening new avenues for future opportunities of investigations in the field.

2. Materials and Methods

The systematic review and meta-analysis were performed in accordance with the protocol of interventions for the treatment of oral and oropharyngeal cancers statement and Cochrane Handbook for Systematic Reviews of Intervention. The tested hypothesis was to establish if the nanoformulation of chemotherapy drugs is able to improve HNC treatment response and prevent side effects. This study did not require ethical approval or informed consent, as the analyses were carried out based on data from previously published clinical trials.

2.1. Literature Search

A systematic literature search was conducted in MEDLINE (1946 to present), EMBASE via OVID (1980 to present), Cochrane Oral Health Group's Trials Register, Cochrane Central Register of Controlled Trials (CENTRAL) (The Cochrane Library, current issue) and ClinicalTrials.gov from inception to June 10, 2020. In addition, World Health Organization (WHO) International Clinical Trials Registry Platform (ICTRP) (http://apps.who.int/trialsearch/), Current Controlled trials (www.controlledtrials.com) and Clinical Trials (www.clinicaltrials.gov) were searched for HNC. The results were compiled using the bibliographic management software EndNote X9 3.2 (Thomson Reuters).

The complete search strategy is listed in the Appendix A. Briefly, the search included patients with squamous cell carcinoma of head and neck according to International Classification of Diseases for Oncology (ICD-O) [11] codes as C00 (lip), C01-C02 (tongue), C03 (gum), C04 (floor of mouth), C05 (palate) and C06 (other unspecified parts of mouth), C09 (tonsil), C10 (oropharynx), C11 (nasopharynx), C12 (pyriform sinus) and C13 (hypopharynx). The outcomes were: "tumor response", "overall survival", "disease free survival", "progression free survival", "locoregional control", "recurrence", "severe adverse effects (SAE)" and "quality of life".

The list of relevant references was searched, and the authors were contacted in order to identify unpublished or ongoing trials. Any potentially relevant meeting abstracts and articles found in their reference lists were reviewed and considered for inclusion. Two investigators independently reviewed the articles for eligibility. The references retrieved in this study are listed in the EndNote Library in the Supplementary Material S1.

2.2. Study Selection: Inclusion and Exclusion Criteria

This research did not retrieve randomized clinical trials because only cohort studies using single-arm clinical trials are currently published. Due to the lack of evidence, only clinical studies at Phases I or II, including more than 50% of patients with HNC, were included. Then, the following criteria were used for inclusion in the meta-analysis: clinical trial where at least one outcome was reported, such as progressive disease (PD), partial response (PR), stable disease (SD), and adverse events (AEs). The exclusion criteria involved non-English papers, single case reports, letters to editor and reviews.

2.3. Data Extraction and Study Quality Assessment

Two authors independently extracted data from titles and abstracts. The full report was retrieved when the studies met the inclusion criteria or if the information was not sufficient in the title or abstract to make a clear decision. Articles were graded using the Oxford Centre for Evidence Based Medicine (CEBM) levels of evidence [12]. Discrepancies were identified and resolved through discussion. Missing data were requested directly to the authors via e-mail. Studies rejected at this or subsequent stages were recorded in the characteristics of excluded studies table, and reasons for exclusion noted. The clinical characteristics of the studies are presented in the Table 1. For each clinical trial, the following details were extracted and presented: study characteristics (first author, journal, year of publication, country, multi or single center), trial design characteristics (study design, outcome measurement, therapy regimen), study population (primary tumor location, median age, number of patients evaluated for efficacy and safety endpoints), intervention details (type of intervention, timing, dose, mode of administration and duration, concomitant treatments) efficacy results (PD, PR and SD) and SAE outcomes (Tables 2 and 3). A standardized, pre-piloted form adapted from the Cochrane Collaboration was used to extract data from the included works. Data Extraction Form.

2.4. Risk of Bias Assessment

Once we were not able to find reported randomized clinical trials, all the studies were classified with high-risk of bias according on Cochrane Handbook for Systematic Reviews of Interventions version 5.1.0 [13] These parameters included details of sequence generation, allocation concealment, treatment blinding, completeness of outcomes data, and presence of selective outcome reporting.

2.5. Types of Outcome Measures

The primary outcome was the clinical response expressed as tumor volume, as well as PD, PR and SD. Second outcomes were the Severe Adverse Effects (SAE) classified as grade III and IV according to Common Toxicity Criteria (CTC) v2.0 [14].

2.6. Statistical Analysis

Descriptive statistics were used to summarize data, with average and range for continuous variables and frequencies and percentages for nominal/dichotomous variables. The SAE were calculated as number of events per 100 and pooled in random-effects models with MetaXL (Version 5.3). One of included studies [15] in the proportion meta-analyses presented zero total event when calculating the pooled estimates as previously advised [16]. Results were considered statistically significant for a two-tailed P value<0.05.

3. Results

3.1. Study Overview

A total of 1772 articles were identified. Following the exclusion of duplication or reports unrelated to cancer and/or nanoformulation, 1747 manuscripts were retrieved (Figure 1). An additional 1708 studies were excluded, as they were either abstracts or irrelevant studies regarding nanoformulation based on target therapy in HNC, leaving 39 studies for further full-text evaluation. From these, 30 studies were removed from the analysis because they did not match with inclusion criteria. The reasons for inclusion and exclusion after screening are listed in the Supplementary Tables S1 and S2. Thus, the

qualitative and quantitative analysis was conducted with nine clinical studies (Phases I or II) involving nanoformulations as the administration mode of antineoplastic therapies in HNC [15,17–24].

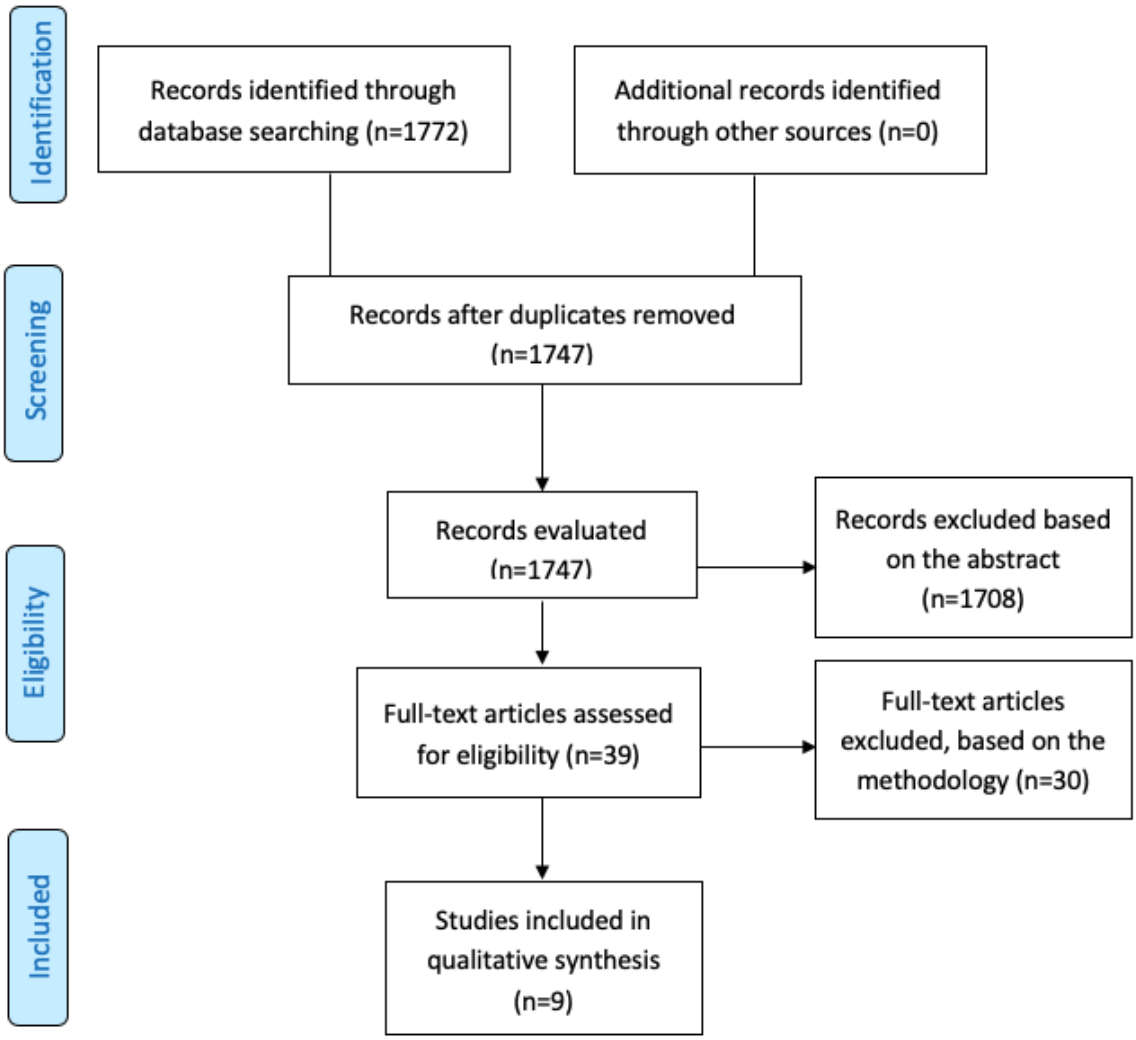

Figure 1. Study flow diagram. Following the guidelines of the Cochrane Handbook for Systematic Reviews of Intervention [25], it was performed a sensitive search in the online databases to identify the studies that examined associations between different nanoformulation and head and neck cancer (HNC) treatment. This systematic review searched for relevant studies considering publications up to June 2020. The chart diagram was reproduced from Moher, Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement; published by PLoS med., 2009 [26].

The clinical characteristics of the nine studies included in the analysis were retrieved (Table 1). The mean age of the patients was 59.23 years (range 25–87 years) and the majority of the cases were observed in males (78.16%). Most patients had previously non-treated lesions (62.1%) and non-distant metastases (89.14%) at the moment of the diagnosis. However, the majority of the cases were advanced HNC (92.56% T3 + T4) presenting metastasis in the lymph nodes (72.48%). Of the nine manuscripts exploring nanoparticles in HNC, four of them were classified at Phase I (44.4%) and five at Phase II (55.6%) non-randomized controlled clinical trials. The anatomic location was predominantly the oral cavity (n = 97, 56.7%), followed by larynx (n = 43, 25.1%), oropharynx (n = 38, 22.2%), hypopharynx (n = 28, 16.4%), maxillary sinus (n = 16, 9.4%) and other mixed sites (n = 16, 9.4%-including nasopharynx, oropharynx, paranasal sinuses, maxillary sinus, oral cavity, and hypopharynx) (Table 1).

Table 1. Clinical characteristics of the nine included clinical studies of head and neck cancer and chemotherapeutic nanoformulations.

Patient characteristics	n
Mean age	59.23 (range 25-87) years
Total patients recruited	229 (range 7-60)
Number of patients analyzed	216 (94.3%)
Gender	
Male	179 (78.17%)
Female	50 (21.83%)
Previously treated	
Yes	88 (37.9%)
No	141 (62.1%)
Tumor Size*	
T1 + T2	11 (7.43%)
T3 + T4	137 (92.57%)
Lymph nodes metastasis*	
Positive	108 (72.48%)
Negative	41 (27.52%)
Distant metastasis*	
Yes	19 (10.86%)
No	156 (89.14%)
Died of disease*	
Yes	61 (44.20%)
No	77 (55.80%)
Tumor Location	
Oral cavity	97 (41.99%)
Larynx	43 (18.62%)
Oropharynx	38 (16.45%)
Hypopharynx	28 (12.12%)
Maxilary sinus	9 (3.89%)
Others	16 (6.92%)

*Only reported data were recorded

3.2. Interventions in HNC Using Chemotherapy Nanoformulations

Nanoparticles carrying different chemotherapy drugs were identified in nine studies and the information about each intervention was compiled in the Table 2. Among the nanoparticles carrier chemotherapy (NCC) categories translated into clinical trials, the liposomes were the most common complex observed, representing 6 among 9 papers, followed by 3 papers related to albumin-bound

chemotherapeutics. Cisplatin (2/9), Doxorubicin (3/9) and Paclitaxel (4/9) were the chemotherapeutic agents used in these nine studies. The posology, the administration mode and the timing of intervention were very heterogeneous among the studies. The concentrations of NCC solutions were not possible to calculate, as most of the articles did not use a standardized system to report their concentrations.

3.3. Tumor Response and Host Toxicity

Randomized clinical trials aiming to evaluate NCC for HNC therapy were not identified in the literature. The single arms clinical trials at Phases I and II, included in the analysis, were summarized by tumor response (Table 2) and toxicity outcomes (Table 3). These studies showed small sample size (range 7–60) without control groups. It was not possible to evaluate survival outcomes with the Kaplan–Meier method and log-rank test, because these studies presented short period of follow-up (range 0.5–36 months) or the HNC patients underwent to definitive treatment after administration of NCCs.

The chemotherapies agents were heterogeneous among the articles. These nine studies used a range of different methods to assess tumor response and the adverse side effects of NCC in patients with HNC. Normally, the tumor response was evaluated in different time points in accordance with the criteria specified by World Health Organization (WHO) guidance. The treatment duration was also variable among the studies and was estimated either based on the maximum number of cycles allowed per patient, but it did not consider the progression-free survival. The most common methods used to demonstrate toxicity were the National Cancer Institute (NCI) Common Toxicity Criteria and the WHO toxicity criteria. The studies escalated NCC dose according to the chemotherapeutic drug applied until a maximum dose allowed. All these variants being evaluated thorough different criteria can introduce bias into the demonstration of the efficiency in the treatment.

Serious adverse effects (SAE - grade 3 or 4) were expressed as events per 100 (Table 3; Figure 2). The number of clinical trials included in this analysis ranged from 2 to 3 depending on the type of NCC investigated. One study was removed of the meta-analysis [24] because of the liposomal paclitaxel differs from albumin formulations presented by the other studies. For the meta-analysis, the forest plots showed non-significant and moderate heterogeneity between trials for the liposomal cisplatin ($P = 0.09$ and $i^2 = 64\%$) and paclitaxel albumin nanoparticles ($P = 0.14$ and $I^2 = 49\%$). The liposomal doxorubicin group displayed significant and substantial heterogeneity ($P = 0.001$ and $I^2 = 89\%$) (Figure 2).

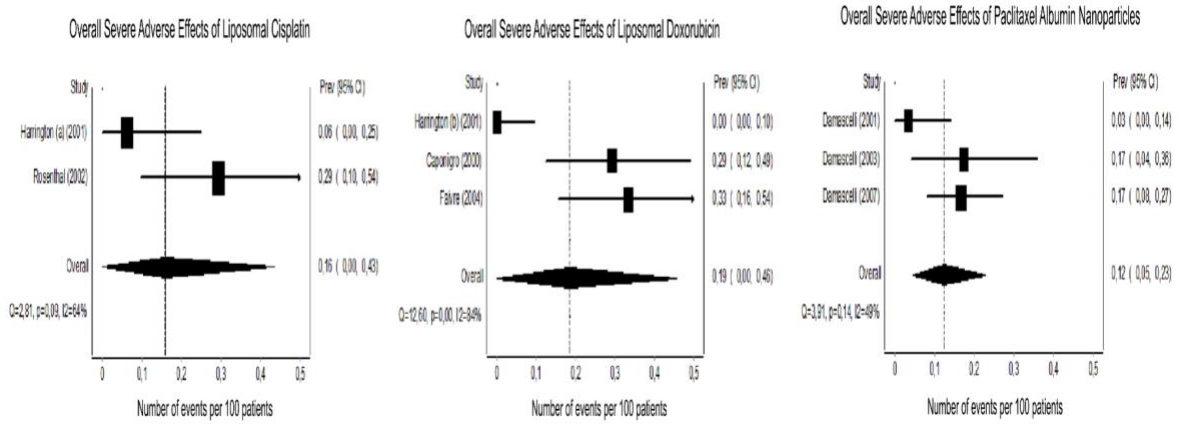

Figure 2. Forest plots showing the proportions of overall severe adverse events (SAE—grade 3 or 4). The graphs display the reference of the study with the name of the author and year of publication, the prevalence (Prev) and the 95% confidence interval (95% CI) for each of the three nanoparticles carrier chemotherapy (NCC) identified on the search. The overall rates of adverse events per 100 patients were calculated with random-effects models due to limited sample size.

Table 2. Clinical trials at Phases I and II investigating nanoformulations based on chemotherapy in patients with head and neck cancer.

Study (Year)	Phase	Number of Participants/ Number Analyzed	CT	Vehicle/Carrier	Dose	Previous Treatments	Median Follow-Up (Months) [a]/ Definitive Treatment	Tumor Response [b] (%)		
								CR	SD	PD
Harrington (a) (2001) [15]	I-II	18/16	Cisplatin	Pegylated Liposome	2 cycles of 200 mg/m^2 every 3 weeks. The last 8 patients received 260 mg/m^2	No	17/RT after the second dose	11.1	55.6	33.3
Rosenthal (2002) [17]	I	20/17	Cisplatin	Pegylated Liposome	Escalated from 20–200 mg/m^2 in six dose levels	Yes	36/Concurrent with RT	40	0	60
Harrington (b) (2001) [22]	II	20/18	Doxorubicin	Pegylated Liposome	Escalated doses starting at 10 mg/m^2 and increasing through 15 mg/m^2 to 20 mg/m2.	No	13/RT began after the last dose	57	31	13
Caponigro (2000) [18]	I	24/24	Doxorubicin	Pegylated Liposome	Initial dose of 30 mg/m^2 and subsequently escalated by 5 mg/m^2	Yes	1/Not stated	33.33	62.5	4.16
Faivre (2004) [19]	I-II	26/24	Doxorubicin	Pegylated Liposome	15 patients received a dose of 35 mg/m^2 every 3 weeks. The following 11 patients group was treated at 45 mg/m^2.	Yes	34/Not stated	17	33	50[d]
Damascelli (2001) [23]	I	31/28	Paclitaxel	Albumin nanoparticle	Starting dose of 120 mg/m^2 was increased by 30 mg/m^2 during three treatment cycles.	Yes	3-13/Not stated	75.85	17.24	6.88
Damascelli (2003) [21]	I	23/23	Paclitaxel	Albumin nanoparticle	Starting dose of 120 mg/m^2 was increased by 30 mg/m^2 at 3 subsequent levels each 4 weeks.	No	5-12/Not stated	78	13	9
Damascelli (2007) [20]	II	60/60	Paclitaxel	Albumin nanoparticle	Starting dose of 230 mg/m^2 and subsequently a reduced dose of 150 mg/m^2.	No	0.5/Surgery, CT and/or RT	75	11.67	13.33
Strieth (2014) [24]	I-II	07/05	Paclitaxel	Liposome	One group received 3 infusions of 0.55 mg/kg and another received 1.1 mg/kg.	Yes	0.75/Not stated	0	80	20

[a] Median of follow-up was recorded after the last dose. [b] Tumor response on the last follow-up was recorded. [c] Overall survival 41% and Disease-Free Survival 25%. [d] Assumed value. [e] 1 patient developed massive necrosis and was considered. Clinical response (CR), stable disease (SD), progressive disease (PD), Radiotherapy (RT) and Chemotherapy (CT).

Table 3. Severe adverse events (Grade 3 or 4) in patients receiving nanoformulations based on chemotherapy for head and neck cancer.

Study	CT and Vehicle	Number of Analyzed Patients	Main Severe Adverse Effects (n)					
			Hematological	Neutro-/Leucopenia	Gastrointestinal [b]	Mucocutaneous	Neurological	Allergy
Harrington [15]	Cisplatin (Liposome)	16	1					
Rosenthal [17]	Cisplatin (Liposome)	17	2	1	2			
Total (events per 100 patients)	..	33	3 (9)	1 (3)	2 (6)	0	0	0
Harrington [22]	Doxorubicin (Liposome)	18						
Caponigro [18]	Doxorubicin (Liposome)	24		2		5		
Faivre [19]	Doxorubicin (Liposome)	24	3	2	1			2
Total (events per 100 patients)	..	66	3 (5)	4 (6)	1 (2)	5 (8)	0	2 (4)
Damascelli [23]	Paclitaxel (Albumin nanoparticle)	29		1				
Damascelli [21]	Paclitaxel (Albumin nanoparticle)	23	2				2	
Damascelli [20]	Paclitaxel (Albumin nanoparticle)	60		4			6	
Strieth [24]	Paclitaxel (Liposome)	05						
Total (events per 100 patients)	..	117	2 (2)	5 (4)	0	0	8 (7)	0

[a] Only Severe Adverse Effects (SAE) classified as grade III and IV according to Common Toxicity Criteria (CTC) v2.0 were recorded. [b] Gastrointestinal events include diarrhoea, constipation, vomiting, nausea, anorexia, gastrointestinal perforation and gastrointestinal bleeding, bilirubin elevation, alkaline phosphatase elevation. Cells are left empty when a study did not report on an adverse event.

3.4. Nanoformulation and Chemotherapeutic Agents Used in the Clinical Trials for HNC

3.4.1. Platinum-Based Chemotherapy

The platinum agents cisplatin and carboplatin are used both as single agents and to form the backbone for most combination regimens in HNC. In this review, nanoparticles were combined with platinum-based chemotherapy in two studies. Harrington et al. [15] treated ten patients with cisplatin combined with liposomes (200 mg/m^2) using two cycles every three weeks. Because of the lack of toxicity, the last eight patients received 260mg/m^2 every three weeks. Although the drug was well tolerated and the adverse effects were minimum, the partial response was only 11.1%. The high stability of the liposome may explain the lack of efficacy, once the slow drug release kinetics reduces the cisplatin bioavailability in the body. Thus, the drug concentration fails to exceed the threshold for therapeutic effects in patients. In another study, Rosenthal et al. [17] treated 17 patients with cisplatin combined with liposomes concurrently with radiotherapy (60–72 Gy in 6–7 weeks). The dose was escalated from 20–200 mg/m^2 in six dose levels intravenously injected every two weeks. The estimated overall survival rate was 41% and disease-free survival was 25%. Among the adverse effects, liver toxicity or rash occurred in two patients. In addition, one patient showed elevated transaminases and neutropenia. Both studies observed low severe toxicities even at the highest cisplatin doses. It may be explained by the prolonged half-life of liposomal chemotherapy agents. Even though the first clinical trial [15] demonstrated a lack of efficacy, the second study [17] showed high therapeutic potential probably because of the association with radiotherapy.

3.4.2. Doxorubicin

Doxorubicin is an anthracycline drug for which the major side effect associated with its use is the cardiotoxicity. Our study identified three clinical trials that used doxorubicin combined with NCC. Harrington et al. [22] analyzed 18 patients after intravenous infusion of doxorubicin combined with liposomes. Consecutive groups of three patients received escalating doses starting at 10mg/m^2 and increasing through 15 mg/m^2 to 20 mg/m^2. The partial response to this treatment was observed in 57% of patients without severe side effects. Caponigro et al. [18] analyzed 24 patients submitted to neoadjuvant therapy with radiation and/or chemotherapy using doxorubicin combined with pegylated liposome. The compound was administered at the initial dose of 30mg/m^2 and subsequently escalated by 5 mg/m^2 per step. Partial response was 33% (95% CI: 16–55%), which is similar to the doxorubicin as a single agent. Three patients develop severe adverse effects (grade 3 and 4) with stomatitis, neutropenia, and 14 showed skin toxicity. The study conducted by Faivre et al. [19] analyzed 24 patients who received doxorubicin conjugated with pegylated liposomes by intravenous infusion at an initial dose of 35 mg/m^2, every three weeks. In the first stage of the study, 15 patients received a dose of 35 mg/m^2 every three weeks and 11 patients were treated with 45 mg/m^2 of the drug. Four patients showed complete clinical response (17%; 95% CI 0.5–32%). The time observed for disease-free survival and overall survival were 3.5 and 4.6 months, respectively. Two patients showed severe adverse effects such neutropenia, however none of them had skin, digestive, cardiac or hepatic toxicities. This study shows that the high concentration of drugs increases severe adverse effects but not necessarily improve the efficacy of the clinical response.

3.4.3. Paclitaxel

Paclitaxel (know as Taxol) is a microtubule-stabilizing drug that induces mitotic arrest, which leads to cell death. However, recent evidence demonstrates that intratumoral concentrations of single paclitaxel are too low to cause mitotic arrest and result in multipolar divisions instead. In our review, four clinical trials used paclitaxel associated with nanoparticles to increase drug efficacy. Damascelli et al. [23] evaluated 29 patients undergone to three treatment cycles and four-weeks interval using paclitaxel conjugated to albumin nanoparticles administered by percutaneous catheterization of the neck vessels. The starting dose of 120 mg/m^2 was progressively increased by 30mg/m^2 at each

subsequent level. The dose-limiting toxicity was myelosuppression. Three patients had complete clinical responses and nineteen partial responses (six previously treated patients and 13 not previously treated). In another Phase I clinical trial conducted by the same group, Damascelli et al. [21] analyzed 23 previously untreated HNC patients with paclitaxel conjugated albumin nanoparticles with the same posology. Eighteen patients (78%) had a clinical and radiologic response (complete: 26%; partial: 52%), three patients (13%) had stable disease and two cases (9%) showed disease progression. The adverse effects were hematologic (grade 3) in two patients (8.6%) and neurologic (grade 4) in two patients. These scientists in 2007 expanded the study to 60 patients in a Phase II clinical trial [20], using an initial dose of 230 mg/m^2 and subsequently a reduced dose of 150mg/m^2 of paclitaxel bounded albumin nanoparticles. Complete or partial responses were observed in 45 of 60 treated patients (75%). Seven patients (11.67%) had stable disease and eight (13.33%) showed disease progression. High-grade bone marrow depression was rare, however, the reduction in the dose eliminated this specific toxicity without losing efficacy. Strieth et al. [24] performed a Phase I/II clinical trial and analysed seven HNC patients previously exposed to surgery and/or radio-chemotherapy. They were treated with paclitaxel in a liposome formulation and, after three infusions of 0.55 mg/kg or 1.1 mg/kg, the tumor volume revealed stable disease in four cases and the disease progressed in only one patient. The applied doses in liposomal formulation are far below the doses of conventional paclitaxel usually given in clinical practice, which may also be a reason for the favourable safety profile. Mild adverse events were observed, such as fatigue, chills and hypertension. These clinical trials showed evidences that paclitaxel nanoformulation has lower systemic toxicity compared with the other clinical trials testing free formulations of paclitaxel. Unfortunately, none of these studies showed a proper control group.

3.5. Ongoing Clinical Trials

Seven clinical trials at Phases I and II evaluating the potential of innovative nanomaterials for antineoplastic drugs release are registered in the ClinicalTrials.gov (Table 4). Most of these studies used paclitaxel as chemotherapeutic and albumin as carrier. Among these trials, none of them were designed as Phase III or includes immunotherapy and/or target-therapeutic agents.

Table 4. List of ongoing clinical trials in head and neck cancer using nanoformulations (source: ClinicalTrials.gov).

Phase	Year	NCC	Identifier Number
I	2016	Cisplatin polymeric micelle	NCT02817113
I	2013	Paclitaxel albumin-nanoparticle	NCT01847326
I	2008	Paclitaxel albumin-nanoparticle	NCT00736619
II	2014	Paclitaxel albumin-nanoparticle	NCT02033538
II	2009	Paclitaxel albumin-nanoparticle	NCT00851877
II	2012	Paclitaxel albumin-nanoparticle	NCT01566435
na*	2007	Paclitaxel albumin-nanoparticle	NCT00499291

*na: not applicable.

3.6. Outcomes

Nanoformulation may have a significant impact in the future of oncology treatment due to the potential to improve efficacy while reduce the toxicities by enhancing drug stability, solubility and bioavailability. Such properties are motivating several therapeutic nanoproducts to move ahead towards clinical development in the last few years [27,28]. However, the majority of nanoformulations tested in HNC oncology have been at the preclinical stage. The clinical translation is not a reality yet, because there is still a need to show more evidences about the efficacy and safety. In this study, we systematically reviewed the published manuscripts about HNC nanotechnology-based therapies in clinical stages and all the current ongoing clinical trials registered on ClinicalTrials.gov. After carefully screening, it was not possible identify any randomized clinical trial testing nanoformulations of chemotherapeutics or the combination with immunotherapeutics agents for HNC treatment. These clinical trials used single arm (Phases I and II) to determine only the tumor response or adverse effects. It was noticed that some challenges, such as the achievement of the optimal combination of physic-chemical parameters to specifically target the tumor site and control drug release, are the key factors that are preventing the translation of nanomedicines into therapy [8,29]. However, global efforts have focused on the development of functional nanoparticles to increase the bioavailability in the tumor site. Several modifications were done along the last decades characterizing four generations of nanoformulation-mediated co-delivery of small-molecule chemotherapy (Figure 3). The first generation includes materials with passive drug-release mechanisms (e.g., coating, nanoparticles of polymers, metals or ceramics); the second generation comprises targeted and bioactive devices with active mechanism of drug-delivery; and the third and fourth generations consist of guided assembly and molecular nanoparticles (Figure 3). All the clinical studies included in this review tested nanoformulations for combination therapy belonging to the first generation of nanotechnology. These clinical studies investigated three conventional drugs (cisplatin, doxorubicin and paclitaxel) and excipients (albumin, lipids by itself or associated with polymers). The single use of these methods are approved by FDA and largely used in clinical practice, so there is no concern about the safety and toxicity of the excipients themselves [30]. However, these approved drugs are often obsolete technology when compared with the recent options under preclinical and clinical testing [31]. The most common types of nanoformulations, including the chemical composition, physical properties and target ligands that affect the biological processes involved in the drug delivery [32] to the tumor tissues identified in clinical studies of HNC are presented in the Figure 4.

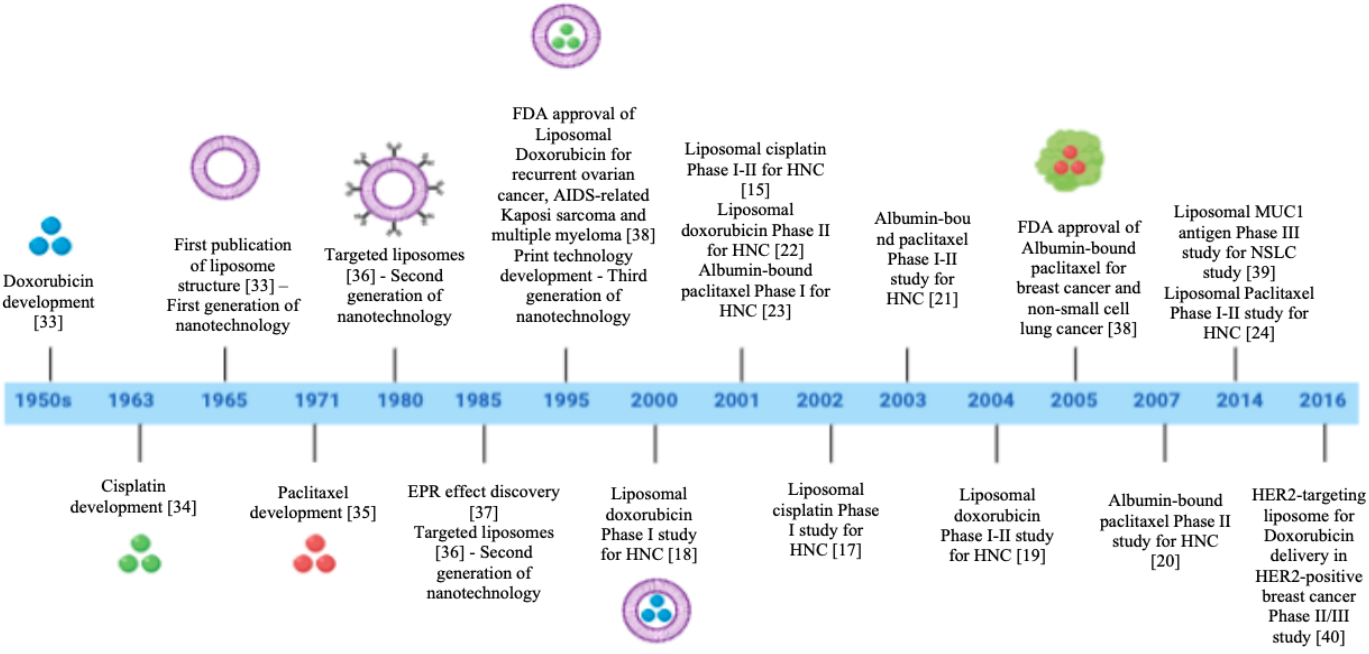

Figure 3. Timeline of development of nanoformulations identified in this study. The scheme compiles the main chemotherapeutic agents and nanostructures for cancer treatment over the last 7 decades. The year listed

in the blue line shows the moment that FDA approved the nanoformulation to the clinical practice and the intersection of the nine clinical studies analyzed in this research. NSLC: Non-Small Cell Lung Cancer; HNC: Head and Neck Cancer.

4. Remarks and Future Perspectives

This systematic review and meta-analysis reveals the recent progress in the development of targeted nanoparticle systems for HNC therapy. In HNC, chemotherapy is usually used alongside surgery and/or radiotherapy in advanced cases generating severe side effects and poor quality of life. The most common chemotherapeutic agents used are platinum-based drugs (cisplatin or carboplatin) and combinations with taxanes (e.g., docetaxel) or 5-fluorouracil. However, conventional delivery methods of chemotherapeutic agents have several limitations: Firstly, some drugs have poor solubility and low bioavailability and contain toxic solvents in their formulation. Secondly, they have a short circulation time because of their physiological instability, degradation, and clearance. Thirdly, the non-specific distribution of the drugs limits the concentration achieved in the tumor and causes harmful side effects because of their unwanted accumulation in healthy tissues. A combination of chemotherapeutic agents improved drug response for patients with advanced HNC but no effect on overall survival was observed. Therefore, advanced drug delivery systems based on nanotechnology and a tumor-targeted strategy, hold considerable potential to enhance chemotherapeutic efficacy, representing a hot topic in cancer therapy for future investigations. Even though most approaches are still in the preclinical stages, they have shown tremendous potential to fulfill the need for viable alternative cancer therapies. Further researches into higher-specificity tumor targets and more efficient NCC are needed, including complex modifications to enhance the antitumor efficacy in order to achieve the ultimate goal of personalized medicine.

The studies involving nanoformulations for HNC therapy demonstrated the difficulty and limitation to demonstrate efficacy in tumor response due to the lack of clinical studies with proper gold standard controls. Besides, the short-term follow-up and the use of co-concurrent therapies, such as radiotherapy, generate bias to determine the real impact of these strategies in the success of the treatment. However, in general, all the studies showed that nanotechnologies were not associated with increased SAE in HNC. We conclude that this topic demands future and well-designed experimental studies with proper randomized clinical trials.

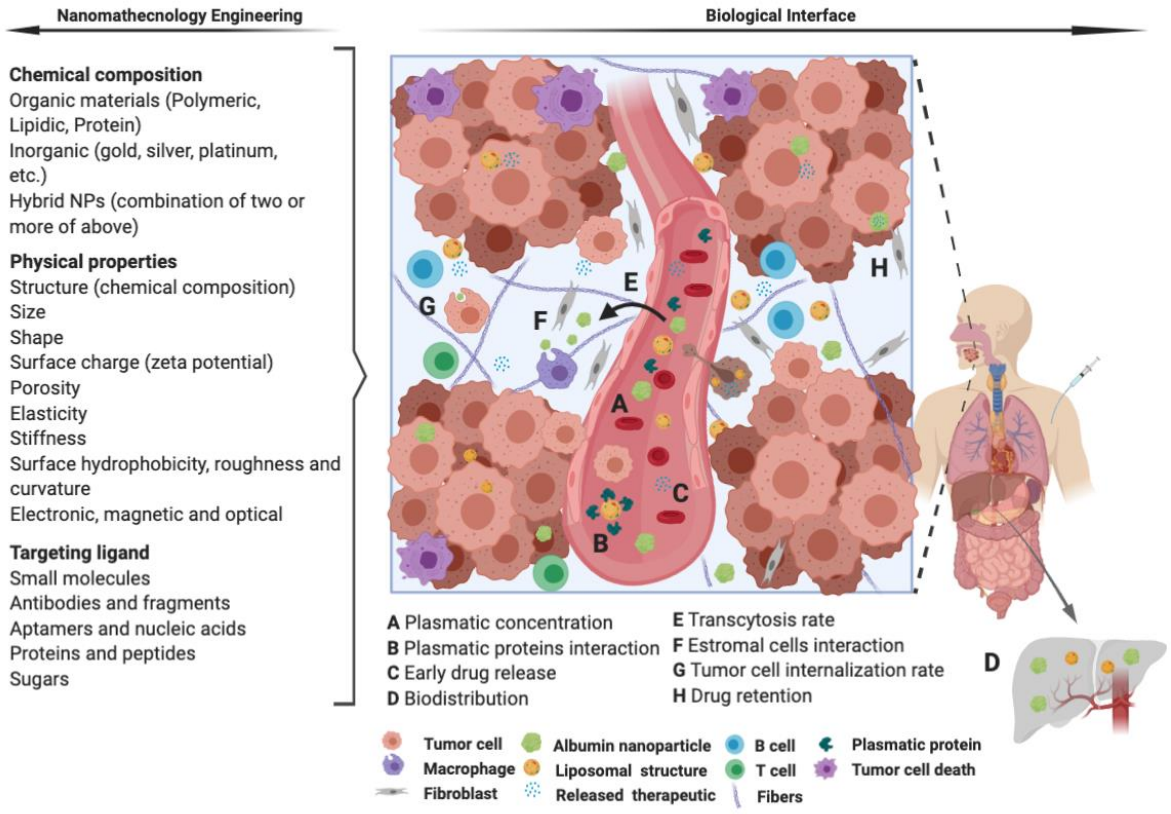

Figure 4. Representation of the most common types of nanoformulations identified in clinical studies of HNC. The nanoformulations carrying chemotherapeutics are expected perform transcytosis throughout the endothelium barrier and accumulate in tumor sites due the EPR effect and release the drug inside the cancer cells. This ideal pharmacokinetic depends on the nanotechnology engineering (chemical composition, physical properties and targeting ligand) and how this variables interacts with the biological events, e.g., plasmatic concentration depending on the administration mode (**A**), plasmatic proteins interaction (**B**), particle stability and drug release (**C** and **H**), biodistribution (**D**), transcytosis rate (**E**), stromal cells interaction (**F**) and tumor cell internalization (**G**).

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Supplementary Table S1. Characteristics of included studies after full text screening, Supplementary Table S2. Characteristics of the excluded studies after full text screening. Supplementary Material S1—Endnote Library

Author Contributions: J.M.L.: data collection, visualization and analysis; write the manuscript. P.R.B., D.E.C.P.: conceptualization, review and edition. M.H., M.A.J.: funding acquisition, review and edition. S.D.S.: conceptualization, supervision, data visualization, write, review, and edit the manuscript.

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References

1. Lydiatt, W.M.; Patel, S.G.; O'Sullivan, B.; Brandwein, M.S.; Ridge, J.A.; Migliacci, J.C.; Loomis, A.M.; Shah, J.P. Head and neck cancers—Major changes in the American Joint Committee on cancer eighth edition cancer staging manual. *CA A Cancer J. Clin.* **2017**, *67*, 122–137.

2. Groome, P.A.; Schulze, K.M.; Mackillop, W.J.; Grice, B.; Goh, C.; Cummings, B.J.; Hall, S.F.; Liu, F.F.; Payne, D.; Rothwell, D.M. A comparison of published head and neck stage groupings in carcinomas of the tonsillar region. *Cancer* **2001**, *92*, 1484–1494.
3. Marur, S.; Forastiere, A.A. Head and neck squamous cell carcinoma: Update on epidemiology, diagnosis, and treatment. *Proceedings of the Mayo Clinic Proceedings, 91,* **2016**, 386–396.
4. Chan, K.K.; Glenny, A.M.; Weldon, J.C.; Furness, S.; Worthington, H.V.; Wakeford, H. Interventions for the treatment of oral and oropharyngeal cancers: Targeted therapy and immunotherapy. *Cochrane Database Syst. Rev.* **2015**, doi:10.1002/14651858.cd010341.pub2.
5. Howlader, N.; Noone, A.; Krapcho, M. Previous Version: SEER cancer statistics review, 1975–2010. *Natl. Cancer Inst.* **2013**, *21*, 12.
6. Forastiere, A.; Koch, W.; Trotti, A.; Sidransky, D. Head and neck cancer. *N. Engl. J. Med.* **2001**, *345*, 1890–1900.
7. Carvalho, A.L.; Nishimoto, I.N.; Califano, J.A.; Kowalski, L.P. Trends in incidence and prognosis for head and neck cancer in the United States: A site-specific analysis of the SEER database. *Int. J. Cancer* **2005**, *114*, 806–816.
8. Lytton-Jean, A.K.; Kauffman, K.J.; Kaczmarek, J.C.; Langer, R. Cancer nanotherapeutics in clinical trials. *Cancer Treat. Res,* **2015**, 293–322.
9. Maeda, H. Tumor-selective delivery of macromolecular drugs via the EPR effect: Background and future prospects. *Bioconjugate Chem.* **2010**, *21*, 797–802.
10. Wang, X.; Wang, Y.; Chen, Z.G.; Shin, D.M. Advances of cancer therapy by nanotechnology. *Cancer research and treatment: official journal of Korean Cancer Association.* **2009**, 41, 1-11.
11. Percy, C.; Holten, V.V.; Muir, C.S. *International Classification of Diseases for Oncology.* 3th ed. Geneva Switzerland: World Health Organization; 2013. Available at: < https://apps.who.int/iris/bitstream/handle/10665/96612/9789241548496_eng.pdf?sequence=1> [Accessed 24 January 2020]
12. Durieux, N.; Pasleau, F.; Howick, J.; OCEBM Levels of Evidence Working Group. *Rev. Med. Liege,* **2013**, *68*, 644–649.
13. Higgins, J.P.; Altman, D.G.; Gøtzsche, P.C.; Jüni, P.; Moher, D.; Oxman, A.D.; Savović, J.; Schulz, K.F.; Weeks, L.; Sterne, J.A. The Cochrane Collaboration's tool for assessing risk of bias in randomised trials. *BMJ* **2011**, *343*, d5928.
14. Trotti, A.; Byhardt, R.; Stetz, J.; Gwede, C.; Corn, B.; Fu, K.; Gunderson, L.; McCormick, B.; Morris, M.; Rich, T. Common toxicity criteria: Version 2.0. An improved reference for grading the acute effects of cancer treatment: Impact on radiotherapy. *Int. J. Radiat. Oncol. * Biol. * Phys.* **2000**, *47*, 13–47.
15. Harrington, K.J.; Lewanski, C.R.; Northcote, A.D.; Whittaker, J.; Wellbank, H.; Vile, R.G.; Peters, A.M.; Stewart, J.S.W. Phase I-II study of pegylated liposomal cisplatin (SPI-077TM) in patients with inoperable head and neck cancer. *Ann. Oncol.* **2001**, *12*, 493–496.
16. Friedrich, J.O.; Adhikari, N.K.; Beyene, J. Inclusion of zero total event trials in meta-analyses maintains analytic consistency and incorporates all available data. *BMC Med Res. Methodol.* **2007**, *7*, 5.
17. Rosenthal, D.I.; Yom, S.S.; Liu, L.; Machtay, M.; Algazy, K.; Weber, R.S.; Weinstein, G.S.; Chalian, A.A.; Mille, L.K.; Rockwell, K., Jr., et al. A phase I study of SPI-077 (Stealth liposomal cisplatin) concurrent with radiation therapy for locally advanced head and neck cancer. *Investig. New Drugs* **2002**, *20*, 343–349.
18. Caponigro, F.; Comella, P.; Budillon, A.; Bryce, J.; Avallone, A.; De Rosa, V.; Ionna, F.; Comella, G. Phase I study of Caelyx (doxorubicin HCL, pegylated liposomal) in recurrent or metastatic head and neck cancer. *Ann. Oncol.* **2000**, *11*, 339–342.
19. Faivre, S.; Alsabe, H.; Djafari, L.; Janot, F.; Julieron, M.; Domenge, C.; Djazouli, K.; Armand, J.P.; Luboinski, B.; Raymond, E. Locoregional effects of pegylated liposomal doxorubicin (Caelyx) in irradiated area: A phase I-II study in patients with recurrent squamous cell carcinoma of the head and neck. *Eur. J. Cancer* **2004**, *40*, 1517–1521.
20. Damascelli, B.; Patelli, G.; Ticha, V.; Di Tolla, G.; Frigerio, L.F.; Garbagnati, F.; Lanocita, R.; Marchiano, A.; Spreafico, C.; Mattavelli, F., et al. Feasibility and efficacy of percutaneous transcatheter intraarterial chemotherapy with paclitaxel in albumin nanoparticles for advanced squamous-cell carcinoma of the oral cavity, oropharynx, and hypopharynx. *J. Vasc. Interv. Radiol.* **2007**, *18*, 1395–1403.
21. Damascelli, B.; Patelli, G.L.; Lanocita, R.; Di Tolla, G.; Frigerio, L.F.; Marchiano, A.; Garbagnati, F.; Spreafico, C.; Ticha, V.; Gladin, C.R., et al. A novel intraarterial chemotherapy using paclitaxel in albumin nanoparticles

to treat advanced squamous cell carcinoma of the tongue: Preliminary findings. *Am. J. Roentgenol.* **2003**, *181*, 253–260.

22. Harrington, K.J.; Lewanski, C.; Northcote, A.D.; Whittaker, J.; Peters, A.M.; Vile, R.G.; Stewart, J.S. Phase II study of pegylated liposomal doxorubicin (Caelyx) as induction chemotherapy for patients with squamous cell cancer of the head and neck. *Eur. J. Cancer* **2001**, *37*, 2015–2022.
23. Damascelli, B.; Cantuand, G.; Mattavelli, F.; Tamplenizza, P.; Bidoli, P.; Leo, E.; Dosio, F.; Cerrotta, A.M.; Tolla, G.D.; Frigerio, L.F., et al. Intraarterial chemotherapy with polyoxyethylated castor oil free paclitaxel, incorporated in albumin nanoparticles (ABI-007): Phase I study of patients with squamous cell carcinoma of the head and neck and anal canal: Preliminary evidence of clinical activity. *Cancer* **2001**, *92*, 2592–2602.
24. Strieth, S.; Dunau, C.; Michaelis, U.; Jager, L.; Gellrich, D.; Wollenberg, B.; Dellian, M. Phase I/II clinical study on safety and antivascular effects of paclitaxel encapsulated in cationic liposomes for targeted therapy in advanced head and neck cancer. *Head Neck* **2014**, *36*, 976–984.
25. Higgins, J.P.; Thomas, J.; Chandler, J.; Cumpston, M.; Li, T.; Page, M.J.; Welch, V.A. *Cochrane handbook for systematic reviews of interventions*. New Jersey: John Wiley & Sons; **2019**.
26. Moher D.; Liberati A.; Tetzlaff J.; Altman DG.; Prisma Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *Int J Surg*. **2010,** *8*, 336-41.
27. Bjornmalm, M.; Thurecht, K.J.; Michael, M.; Scott, A.M.; Caruso, F. Bridging bio–nano science and cancer nanomedicine. *ACS Nano* **2017**, *11*, 9594–9613.
28. Shi, J.; Kantoff, P.W.; Wooster, R.; Farokhzad, O.C. Cancer nanomedicine: Progress, challenges and opportunities. *Nat. Rev. Cancer* **2017**, *17*, 20–37.
29. Hassan, S.; Prakash, G.; Ozturk, A.B.; Saghazadeh, S.; Sohail, M.F.; Seo, J.; Dokmeci, M.R.; Zhang, Y.S.; Khademhosseini, A. Evolution and clinical translation of drug delivery nanomaterials. *Nano Today* **2017**, *15*, 91–106.
30. Park, K. Facing the truth about nanotechnology in drug delivery. *ACS Nano* **2013**, *7*, 7442–7447.
31. El-Sayed, I.H. Nanotechnology in head and neck cancer: The race is on. *Curr. Oncol. Rep.* **2010**, *12*, 121–128.
32. Stern, S.T.; Hall, J.B.; Lee, L.Y.; Wood, L.J.; Paciotti, G.F.; Tamarkin, L.; Long, S.E.; McNeil, S.E. Translational considerations for cancer nanomedicine. *J. Control. Release* **2010**, *146*, 164–174.
33. Bogner, J.R.; Kronawitter, U.; Rolinski, B.; Truebenbach, K.; Goebel, F.-D. Liposomal doxorubicin in the treatment of advanced AIDS-related Kaposi-sarcoma. *J. Acquir. Immune Defic. Syndr.* **1994**, 4634–68.
34. Rosenberg, B.; Van Camp, L.; Krigas, T. Inhibition of cell division in Escherichia coli by electrolysis products from a platinum electrode. *Nature* **1965**, *205*, 698–699.
35. Wani, M.C.; Taylor, H.L.; Wall, M.E.; Coggon, P.; McPhail, A.T. Plant antitumor agents. VI. Isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. *J. Am. Chem. Soc.* **1971**, *93*, 2325–2327.
36. Leserman, L.D.; Barbet, J.; Kourilsky, F.; Weinstein, J.N. Targeting to cells of fluorescent liposomes covalently coupled with monoclonal antibody or protein A. *Nature* **1980**, *288*, 602–604.
37. Matsumura, Y.; Maeda, H. A new concept for macromolecular therapeutics in cancer chemotherapy: Mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res.* **1986**, *46*, 6387–6392.
38. Smith, A. Big moment for nanotech: Oncology therapeutics poised for a leap. *OncLive* **2013**. Available at: <https://www.onclive.com/view/big-moment-for-nanotech-oncology-therapeutics-poised-for-a-leap.> [Accessed 27 may 2020].
39. Butts, C.; Socinski, M.A.; Mitchell, P.L.; Thatcher, N.; Havel, L.; Krzakowski, M.; Nawrocki, S.; Ciuleanu, T.-E.; Bosquée, L.; Trigo, J.M. Tecemotide (L-BLP25) versus placebo after chemoradiotherapy for stage III non-small-cell lung cancer (START): A randomised, double-blind, phase 3 trial. *Lancet Oncol.* **2014**, *15*, 59–68.
40. Miller, K.; Cortes, J.; Hurvitz, S.A.; Krop, I.E.; Tripathy, D.; Verma, S.; Riahi, K.; Reynolds, J.G.; Wickham, T.J.; Molnar, I. HERMIONE: A randomized Phase 2 trial of MM-302 plus trastuzumab versus chemotherapy of physician's choice plus trastuzumab in patients with previously treated, anthracycline-naïve, HER2-positive, locally advanced/metastatic breast cancer. *BMC Cancer* **2016**, *16*, 352.

4 ARTIGO II

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CHARACTERIZATION OF CHITOSAN/PCL MICROPARTICLES FOR IMPROVED DELIVERY AND ANTI-NEOPLASTIC ACTIVITY OF 5-FLUOROURACIL IN HEAD AND NECK CANCER

Running title: Anti-neoplastic activity of CS/PCL coating 5-FU in HNSCC

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ABSTRACT

Head and neck squamous cell carcinoma (HNSCC) is a prevalent cancer worldwide with high incidence of loco-regional dissemination, frequent recurrence, and lower 5-year survival rate. Current gold standard treatments for advanced disease rely primarily on radiotherapy and

chemotherapy but with limited efficacy and significant side effects. In this study, we characterized a novel 5-fluorouracil (5-FU) carrier composed of chitosan (CS) and polycaprolactone (PCL) microparticles (MPs) in HNSCC preclinical models. The designed MPs and sponges (SPs) were evaluated for their size, morphology, drug entrapment efficiency (EE%) and *in vitro* drug release profile. The anti-cancer activity of 5-FU-loaded particles was assessed in HNSCC human cell lines (CAL27 and HSC3) and in a preclinical mouse model (AT84) utilizing cell proliferation and survival, cell motility, and cell death and autophagy assays. We demonstrated that the EE% was 38.57% and 5-FU *in vitro* release was significantly reduced after 96h. Furthermore, CS-decorated PCL MPs unlike other MPs used as controls were able to promote a significant inhibition of cancer cell proliferation based on the metabolic MTT assay as well as colony formation. In addition, CS-decorated PCL MPs and SPs enhanced the efficacy of 5-FU to inhibit cancer cell migration. Last, the protein expression showed an increase of autophagy and cell death evaluated by LC3-II and PARP1 cleavage respectively. In summary, these results support the utility of CS-decorated PCL MPs and SPs as a effective 5-FU-delivery carrier for therapeutic potentiation of this drug in HNSCC.

KEYWORDS: head and neck cancer; drug delivery; biomaterial; chitosan; PCL, cancer progression.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is an epithelial malignancy in the upper aerodigestive tract (UADT), including the oral cavity, pharynx, larynx, and paranasal sinuses (1). HNSCC is a prevalent cancer worldwide with high rates of loco-regional recurrence that often occur in the first 2 years (2). The 5-year survival rates for HNSCC remain below 50% (3) (4). The treatment decision is based on clinical and pathological characteristics (1), (5). Surgery and radiotherapy are the main option for resectable HNSCC. For advanced stages, surgery combined with adjuvant radiation or chemoradiation (CRT) remain the standard treatment but these approaches often impact on lifelong morbidity associated with treatment side effects and long-term sequelae (6).

Nowadays, nanoformulations for drug delivery have emerged as a promising therapeutic strategy intended to increase the therapeutic index of drugs and prevent secondary drug side effects by delivering drugs via different mechanisms, such as solubilization, passive or active targeting and triggered slow release (7). Targeted drug delivery using microparticles (MPs)

have been shown to be advantageous for increasing the therapeutic index of many chemotherapy drugs and with reduced host toxicity due to reduced accumulation in healthy tissues. These particles have also been studied for their ability to stimulate targeted cell uptake and improve intracellular trafficking. In HNSCC, early studies in nanotechnology have been designed to overcome the lack of the specificity of conventional chemotherapeutic agents to target cancer cells.

Clinical trials approved by the Food and Drug Administration (FDA) classified drug carriers based on the delivery vehicle such as liposomal, polymeric, albumin-bounds, polymer-bounds and inorganic particles (8). In this study, we report a novel carrier for 5-Fluorouracil (5-FU), an antimetabolite drug widely used for patients with advanced tumor stages or with recurrent HNSCC but with limitations in part due to toxicity and drug resistance (9) (10). In this study, we characterized and evaluated the anti-neoplastic activity of a composite of chitosan (CS) and polycaprolactone (PCL) coating 5-fluorouracil (5-FU) in HNSCC.

MATERIALS AND METHODS

Preparation of 5-FU loaded particles

PCL microparticles (MPs) synthesis was performed by interfacial polymer disposition and solvent displacement method modified from Ortiz *et al*. (2012) (11). Briefly, PCL polymer was dissolved in acetone (2mg/mL) under mechanical stirring (300RPM). Organic solution was transferred dropwise into a 0.2% (v/v) aqueous solution of TWEEN® stirred at 1200RPM containing or not 5-FU (Sigma Aldrich, F6627) at 10% of the total PCL mass. The organic phase was evaporated to obtain an aqueous suspension of pure PCL MPs. CS solution preparation consisted in 2.5g of chitosan ≥75% deacetylated from shrimp shells (Sigma Aldrich, C3646) diluted in 500mL 1% (v/v) glacial acetic acid (Química Moderna, QMA00001120301000) stirred overnight at room temperature.

CS solution and PCL MPs were associated in the following volumetric proportions (v/v): 100:0; 75:25; 50:50; 0:100. The nomenclature corresponding these dilutions were: 100CS, 100CS-5FU, 75CS, 75CS-5FU, 50CS, 50CS-5FU, 100PCL and 100PCL-5FU. To synthetize the sample 100CS-5FU, 5-FU was added at 10% of total CS mass in the solution. These solutions were stored at -20°C and packed in a freeze-dryer equipment (Terroni brand, model Enterprise II) for 36h to obtain the composite sponges (SPs) (**Figure 1**).

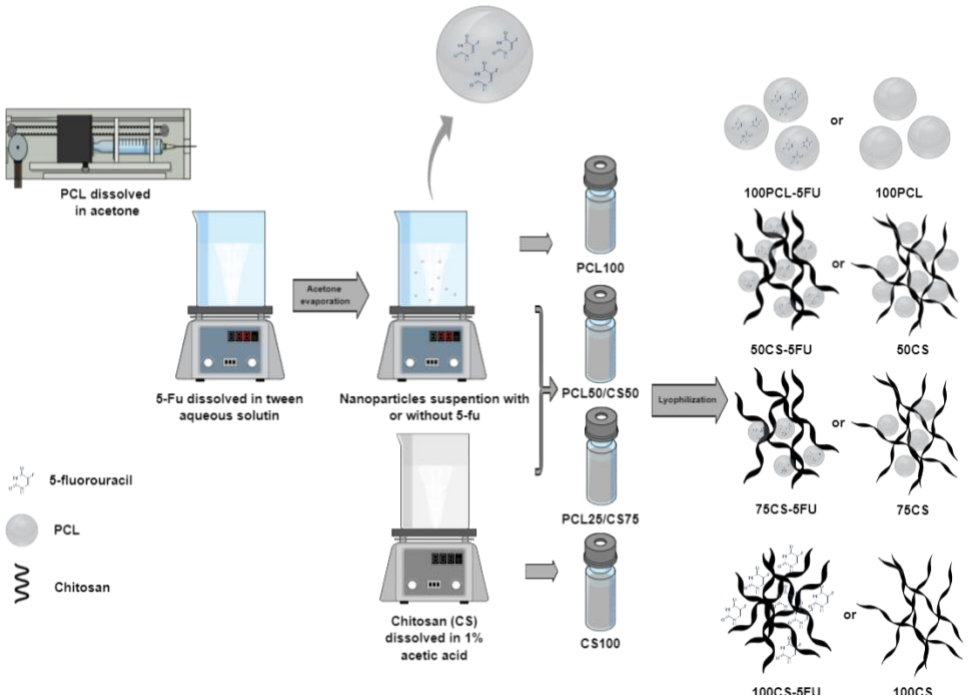

Figure 1. Preparation of 5-FU loaded particles and the composite sponges (CS): PCL particles coating 5-FU were synthesized by the interfacial polymer disposition method and associated with CS in the following volumetric proportions (v/v): 100:0; 75:25; 50:50; 0:100. PCL: polycaprolactone, 5-FU: 5-Fluorouracil, CS: chitosan. Image was done using the tool Mind the Graph (*mindthegraph.com*).

Morphology and particle size

Morphology of the MPs and SPs were observed using a model Quanta 450 FEI scanning electron microscope (SEM) (FEITM, FEI Company). Samples were fixed on aluminum stubs and sputter-coated with gold using a K550X Emitech sputter coater. The morphology of the particle and composite sponges were analysed by visual description and particle size measurements were performed using ImageJ (National Institutes of Health, Bethesda, MD) software.

Entrapment efficiency and drug loading

5-FU concentration was measured by ultraviolet (UV) absorption at the wave length of 300nm in a spectrophotometer (Beckman, DU 800). The amounts of 5-FU entrapped within the formulations were determined by an indirect method (15). The free 5-FU was determined in the supernatant upon filtration of the MPs (membrane size: 0.1μm) to evaluate the entrapment efficiency (EE%). To improve the accuracy, it was considered the absorbance of the surfactant agent and the PCL. The drug loading into PCL MPs was expressed in terms of 5-FU EE%

(encapsulated drug [mg]/total drug in the MPs [mg]×100).

In vitro drug release

In vitro release profiling of 5-FU from the MPs and SPs was measured using lyophilized formulations by the dialysis bag method (11) pre-soaked in phosphate-buffered saline (PBS; Corning®, 21-040-CM) for 12h before use. The dialysis bag (cut-off of Spectra/Por® 2, 12-14 kD dialysis membrane tubing; Spectrum) retains the MPs but allows the drug to diffuse into the medium. A weight equivalent of 20mg sample of the MPs and SPs was placed into the dialysis bag with the two ends fixed by clamps. After sealing, the bags were placed in a tube containing 30 mL of the dissolution medium and stirred at 200RPM. The temperature was maintained at 37.0°C ± 0.5°C during the drug-release experiments. Samples of 1mL were withdrawn and its 5-FU content analyzed by UV-Vis spectrophotometry (300nm) during sequential intervals (0.25, 0.5, 1, 2, 3, 6, 9, 12, 24, 48, 72, and 96h). The readings of neat samples were subtracted from the absorbance of sponges containing 5-FU and the results were interpolated in a 5-FU standard curve using GraphPad Prism 7.0 Software (GraphPad Software Inc., San Diego, USA). The experiments were done in triplicate.

Cell culture

The oral human cancer cell lines CAL27 (ATCC® CRL-2095™, American Type Culture Collection) and HSC3 (JCRB 0623, a human tongue squamous cell carcinoma cell line, Osaka National Institute of Health Sciences, Japan) were used. The normal fibroblasts (NOF) were kindly provided by Ricardo D. Coletta (University of Campinas, Piracicaba, Brazil) (12). These cells were maintained in DMEM (Corning, 10-017-CV) supplemented with 10% FBS (Wisent Bioproducts, 080-150) with 1% penicillin and streptomycin antibiotics (Corning Inc., 30-002-CI). The mouse oral cancer cell line (AT84) was establish and characterized by our group (13) and cultured in RPMI 1640 (Fisher Scientific, 11-875-093) supplemented with 10% FBS and 1% penicillin and streptomycin antibiotics (Corning Inc., 30–002-CI). Cells were cultured at 37ºC with 5% CO_2. The cell lines were tested for mycoplasma contamination.

In vitro cytotoxicity studies

The acute cytotoxicity effect of MPs and SPs was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide (MTT) assay (14). Cells were seeded at a density of $5x10^4$ cells/mL in a 96-well plate and treated with 38,5µg/well of 5-FU and 1mg/well of MPs and SPs

for 24, 48 and 74h at 37°C. Cell medium was changed to serum free medium (SFM) and 100µL of MTT solution (Sigma Aldrich Corporation) was added. Each formulation was compared with a drug-free sample as a negative control and a 5-FU solution (as a positive control). Plates were incubated at 37°C for 4h in the dark, and the purple formazan crystals were dissolved in 100µL dimethyl sulfoxide (DMSO) for 5min on orbital shaking. The color intensity was quantified by optical density on FLUOstar OPTIMA microplate reader (BMG Labtech, Ortenberg, Germany) at 560nm. Data are presented as mean absorbance *versus* medium control and represent the percentages of viable cells compared to the survival of a control group.

Clonogenic assay

The chronical cytotoxicity effects of MPs and SPs on tumor cell proliferation and survival were assessed using the colony formation assay (15). The cells lines CAL27, HSC3 (600 cells/well) and AT84 (500 cells/well) were plated in triplicates in a 6-well plate. After 6h of incubation, cells were treated with 5mg of the MPs and SPs. After 10 days of incubation, the cells washed with PBS and stained with 0.05% (w/v) crystal violet (Sigma Aldrich Corporation, C3886) and 4% formaldehyde solution (v/v) (Sigma Aldrich Corporation, 8.18708), and the colonies > 100µm in diameter were counted using a colony counter (Oxford Optronix Gelcount, Inc., Milton Park, Abingdon, UK).

Cell migration assay

Wound healing migration assay was based on methodology described earlier (16). The areas were measured in triplicates and photographed with an inverted microscope (EVOS FL cell imaging system, Life Technologies) at 0 and 24h post-incubation. Quantification of the wound healing percent closure was determined using the MRI Wound Healing Tool macro from ImageJ (version 1.50i, National Institutes of Health (NIH), Bethesda, MD).

Western blot assay

Sub-confluent cells were washed with PBS, lysed in RIPA buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, 1% NP40, 0.1% SDS, 5mM EDTA, 25 mM sodium deoxycholate) supplemented with 1mM PMSF, 1mM NaF and protease inhibitor cocktail; Roche, 4693159001) for 15 min on ice and centrifuged (14,000xG at 4°C for 20 min) to separate the cell lysate. Cell concentration was measured by the Bradford protein assay and then added with SDS sample buffer (17mM Tris, pH 6.8, 30% glycerol, 10% SDS, 0.02% bromophenol blue, 6% β-mercaptoethanol) and denatured for 5min. Samples (20µg) were then resolved through 15%

SDS-PAGE gels, transferred to polyvinylidene fluoride (PVDF) membrane (MerckMillipore, IPVH00010), blocked with TBST with 5% w/v non-fat dry milk, blotted overnight with specific first antibodies (**Table 1**) at different dilution overnight at 4ºC, and then amplified with horseradish peroxidase-conjugated secondary antibodies for 1h in room temperature and enhanced by chemiluminescence detection systems (Thermo Fisher Scientific, 32106 and 34095). The bands densitometry was performed using ImageJ (version 1.50i, National Institutes of Health (NIH), Bethesda, MD).

Table 1. Antibodies used in this study to evaluate protein expression by western blot.

Name	Source	Company	Dilution
PARP1	Rabbit pAB	Merck Millipore	1:500
LC3B	Mouse mAB	Santa Cruz Biotechnology	1:20000
*GAPDH	Rabbit	Sigma-Aldrich	1:10000
*anti-mouse IgG-peroxidase-conjugated	Goat	Bio-Rad Laboratories	1:5000
anti-rabbit IgG-peroxiase-conjugated	Goat	Bio-Rad Laboratories	1:3000

* GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; IgG: Immunoglobulin G.

Statistical analyses

All data present as mean ± SEM using the software GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, USA). The alpha error was stablished at 5% and 95% confidence interval. Statistical differences on cell proliferation and colony formation were determined by the test two-way Anova with Tukey's multiple comparisons test. The significance in cell migration was analysed by multiple student's T-Test for paired samples. Data were deemed to be statistically significant if $P < 0.05$.

RESULTS AND DISCUSSION

Morphological characterization

The interfacial PCL disposition followed by acetone displacement permitted the formation of 100PCL and 100PCL-5FU MPs with agglomeration after lyophilisation process. The irregular surface was observed in both MP with or without 5-FU (**Figure 2**). The PCL composite sponges

mixed with CS in different volume (100CS-0PCL, 75CS-25PCL and 50CS-50PCL) did not show polymer precipitation and resulted in a single-phase homogeneous solution. After lyophilisation, porous morphology of composite sponges was observed under microscopy (**Figure 2**). SEM analysis of eight samples of CS-PCL MPs (100PCL and 100PCL-5FU) and SPs (PCL50CS, PCL50CS-5FU, 75CS, 75CS-5FU, 100CS and 100CS-5FU) demonstrated different morphologies. The sponges 75CS and 100CS showed highly porous surface with channels network, which could increase the drug loading capability. The PCL addition changed the morphology and the PCL composite sponges became compact and less porous. The 50CS composite was less porous with dense surface without external particles as the others SPs. These results confirmed previous studies showing that the increased concentration of PCL increases surface homogeneity and density (11, 17). The 5-FU loading does not change the MP morphology (**Figure 2**).

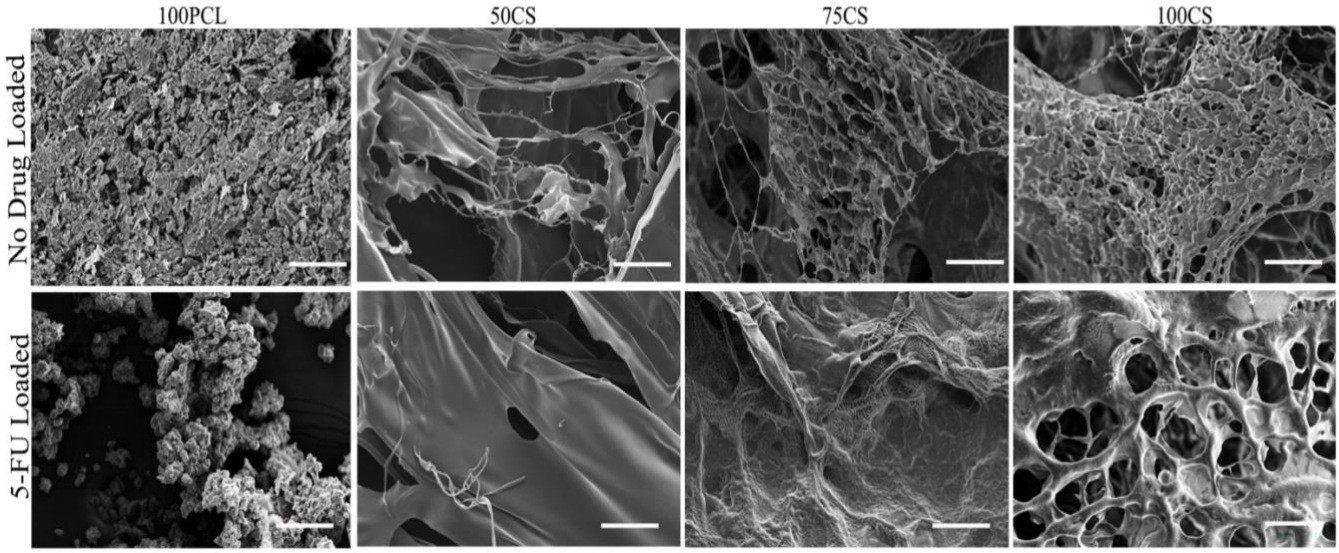

Figure 2. Scanning electron microscopy (SEM) analysis of the PCL MP (100PCL) and CS/PCL SPs (50CS, 75CS, 100CS) composite sponges loaded with or without 5-FU. The images showed an irregular aggregated microparticles and sponges with a range of asymmetric porosity. Scale bars represent 100 µm. MPs: microparticles, SPs: sponges, PCL: polycaprolactone, 5-FU: 5-Fluorouracil, CS: chitosan.

It is crucial for a scaffold-designed drug delivery and tissue engineering to have asymmetric microporous surface to facilitate exudate absorption and promote wound healing. In oncology, the asymmetry may serve as a physical barrier to reduce bacterial penetration and avoid wound dehydration (17, 18). This porosity supports cell proliferation and migration into the scaffold,

then, nutrients and metabolite wastes are exchanged with surrounding environment (18). The ideal porosity to stimulate a superior performance of the wound healing is between 60% to 90% (19) and the average of the pore size should be superior to 20μm (20). This morphological characteristic was observed in our SPs of 75CS-25PCL and 100CS. Beyond mechanical properties, the chemical composition of chitosan also add biological advantages. This compound is known to be a wound healing inductor with anti-inflammatory, hemostatic and antimicrobial properties (18, 21). These properties endorse CS-PCL composite sponges as an optimal candidate for tissue repair promotion (22).

Entrapment efficiency and drug release

The 5-FU was loaded into the composite sponges during the dripping of solubilized PCL in acetone into aqueous drug solution. 5-FU is well-known antimetabolite drug that is widely used for treatment of several cancers, including advanced and/or metastatic HNSCC. The cellular mechanism of action affects the inhibition of nucleotide synthetic enzyme thymidylate synthase and incorporation of its metabolites into nucleic acids. After entering in the cell, the 5-FU is converted in the mainly active metabolites fluorodeoxyuridine monophosphate, fluorodeoxyuridine triphosphate and fluorouridine triphosphate. These active forms disrupt RNA synthesis and lead to DNA damage (23). Ortiz *et al*. (11) suggested that 5-FU carrying by PCL MPs should use electrostatic attraction between the positively charged NH group of the 5-FU molecules (24) and the negatively charged MPs surface (11). However, the drug might be entrapped into the polymer network (11). To overcome this, the loading of 5-FU onto the MPs was evaluated by absorbance readings of the drug remaining in the aqueous solution obtained upon filtration of the MPs suspension and the entrapment efficiency was 38.57%. The 5-FU encapsulation efficiency was similar with previous studies (11) probably due to the hydrophobicity of the PCL network towards the hydrophilic properties of the drug (25). However, this is not a limitation once the non-loaded drug was entrapped into the hydrophilic bulk matrix of CS during the lyophilization (24).

Drug release from all our nanoformulations was initially slow but reached the plateau within 96h, except for CS75-PCL25 where the maximum drug release happened within 72h (**Figure 3**). The late drug release suggests that the majority of the 5-FU was entrapped in the polymeric network rather than adsorbed onto the composites surface. 5-FU release from the sample PCL100 was similar with SPs containing CS in any concentration. Although, CS polymeric matrix interfered on the drug release and the sample 100CS-5FU presented a delayed drug release rate compared to sample containing PCL only.

According to advanced HNSCC treatment regimens using 5-FU infusions (26), the drug at concentration of 1,000 mg/m^2/day must be intravenously injected during 4 days (total dose = 4,000 mg/m^2 over 96 h) and this cycle is repeated every 4 weeks. Such dose aims to guarantee a plasmatic and interstitial tumor space concentration. Coincidently, mostly of samples synthetized in this study presented a drug release profiling matching with the 4 days cycle recommended by the guideline.

The long release profile is a desired condition during the development of anti-cancer drug delivery-carriers, once the encapsulated drug will reach the maximum concentration in the tissue not immediately after surgery, but gradually during the days. Other advantage of the topical use of SPs loaded with chemotherapeutic agents is that the drug release is reducing extracellular trafficking extension and increasing the local bioavailability.

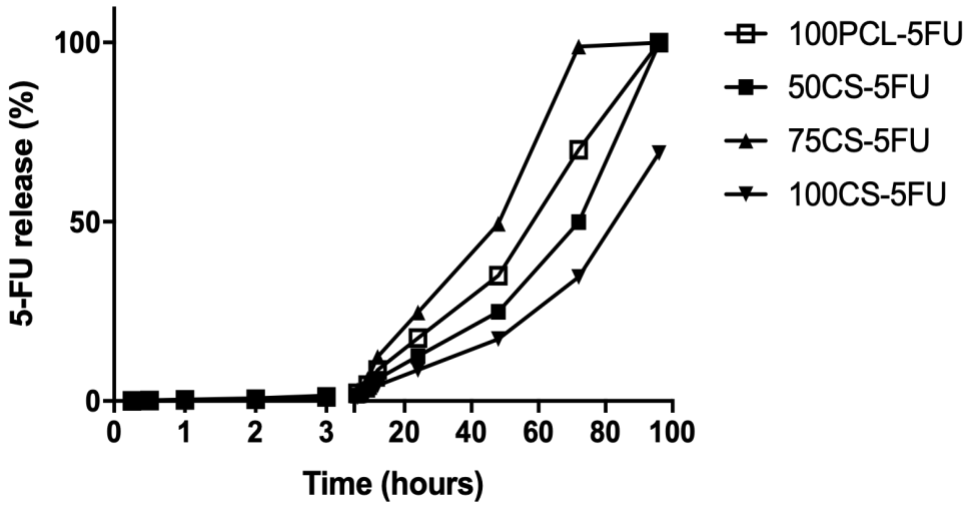

Figure 3. 5-FU kinetic release estimation. 5-FU release (%) from nanoformulations and composite sponges were quantified by spectrophotometry in different incubation times (range 0.25 – 96h). The maximum pick of drug release for CS75-PCL25 was observed after 72h of incubation. PCL: polycaprolactone, 5-FU: 5-Fluorouracil, CS: chitosan.

MPs and SPs loaded with 5-FU reduced malignant cell proliferation, colony formation, and migration

Anti-cancer activity was evaluated after incubation of MPs and SPs loaded with 5-FU in human HNSCC cell lines (CAL27 and HSC3) and oral cancer mouse cell line (AT84). Cell proliferation was evaluated comparing medium with and without 5-FU. MPs and SPs loaded with 5-FU significatively reduced metabolism in all the neoplastic cell lines (**Figure 4**). CAL 27 and HSC-3 are human cancer cell lines commonly used to in laboratory HNSCC research.

The main differences between these cell lines include non-metastatic capsular adenosquamous tumors (27, 28) formed by CAL27 cells in nude mouse models in contrast to non-capsular and highly invasive and metastatic tumors formed by HSC-3 cells (28). It means that samples carrying 5-FU were cytotoxic against human tumoral cell lines with different phenotype behavior.

The cytotoxicity against NOF, a primary normal fibroblast cell line, was measured by MTT assay. MPs and SPs with or without drug loading did not change cell metabolism and/or proliferation no more than 30% during the exposition of 72h (**Figure 4**). This criteria is considered as reference to non-cytotoxic material by International Standard ISO 10993-5 (29). Thus, the composite sponges and PCL MPs show no cytotoxicity to the normal cell line and this is an indicative that the material used in our study is specific to target tumor cell for topical drug-release purpose.

In addition, SPs with increased CS concentration showed the highest reduction in cell proliferation after incubation for 72h ($P<0.05$). The influence of composite sponges and PCL MPs on cancer cell motility showed a clear reduction in the number of cells migration after incubation with composites containing 5-FU (**Figure 5**). Cell viability was also decreased in HNSCC cells compared with cells without treatment during 10 days (**Figure 6**). These results suggest that our composites with high CS concentration have superior effects to inhibit cell growth and tumor motility. The CS anti-cancer properties were previously reported (30) and the mechanism was proposed to involve cell death. CS may trigger and amplify the signaling pathways that are engaged in cell death signals (31). Wimardhani *et al*. (2014) (32) reported that low molecular weight CS possess high cytotoxic effects and leads to cell cycle arrest and apoptosis . The selective cytotoxic effect on cancer cells promoted by CS may be explained by the positive charge on the amine group that is more attracted to cancer cell membrane compared with normal cells (33). This could facilitate bioavailability of polar drugs and their transportation through epithelial surfaces in malignant cells (33). Interestingly, the drug permeation enhancer effect of CS were also associated with anti-metastatic activity *in vitro* and *in vivo* (21). Studies involving different tumor cell lines, such as, MDA-MB-231, a human metastatic breast carcinoma cell line, showed that CS inhibited tumor cell migration due to reduction of MMP9 protein in a drug concentration dependent manner (34). Similar results were found in sarcoma in a dose and particle size dependent manner (35).

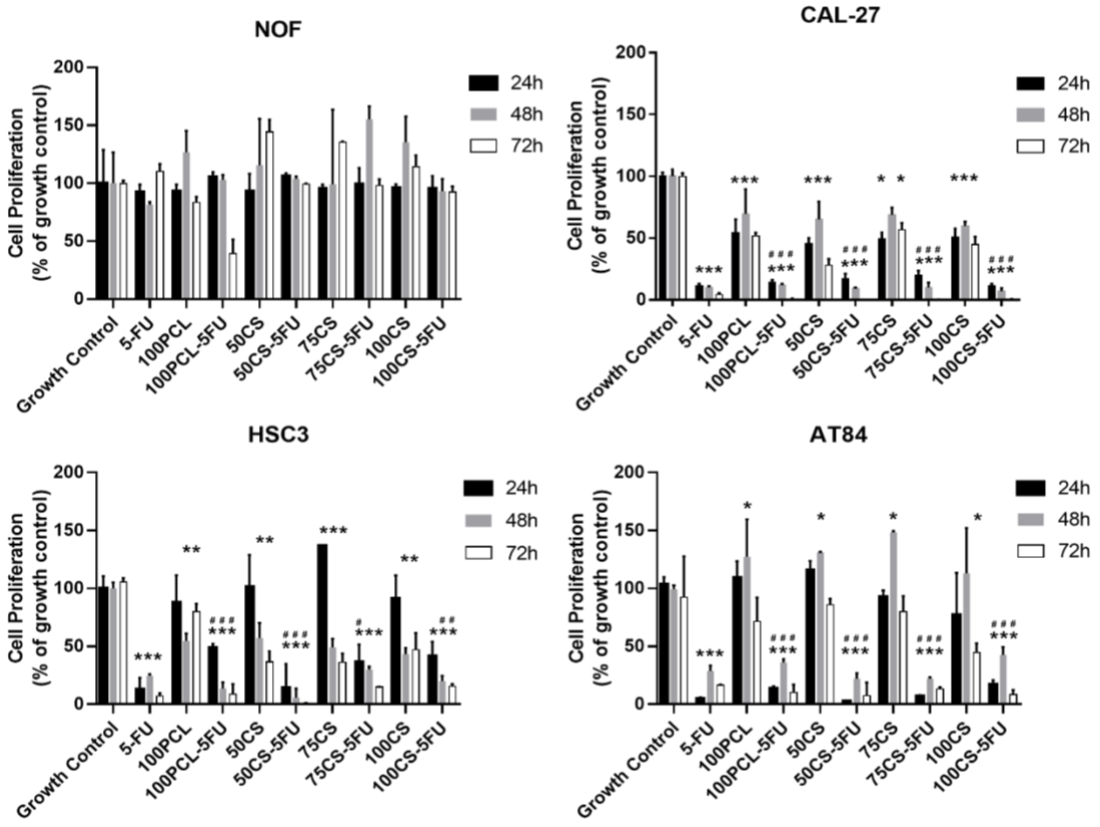

Figure 4. PCL MPs and composite sponges influence HNSCC cell proliferation. MPs and SPs loaded with 5-FU reduced metabolism of all HNSCC cell lines but not interfered in the normal oral fibroblast (NOF) proliferation. SPs with high CS concentrations reduced malignant cell lines proliferation. Data were represented as median ± 95% confidence interval of percentage of cell proliferation. *statistical difference compared with control group. #statistical differences between samples with and without 5-FU loading. NOF: normal oral fibroblasts, PCL: polycaprolactone, 5-FU: 5-Fluorouracil, CS: chitosan, MPs: microparticles and SPs: sponges.

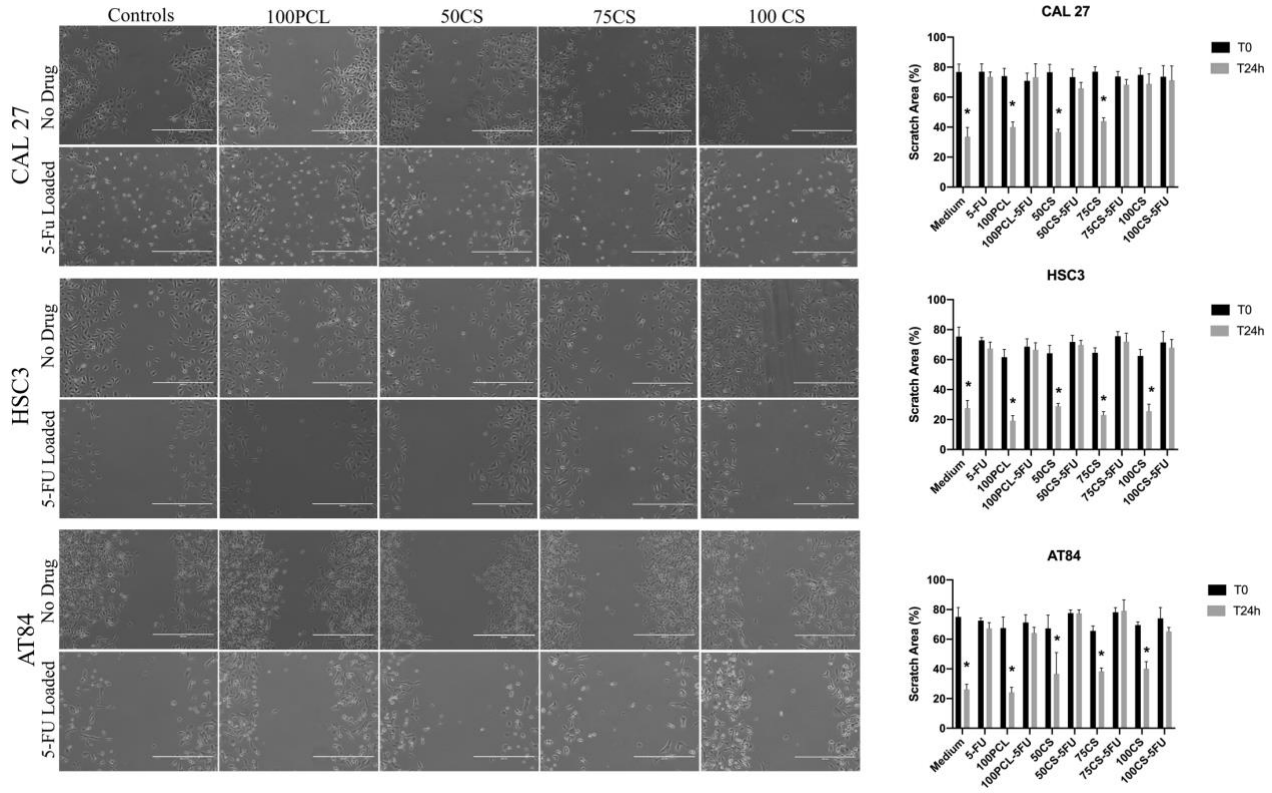

Figure 5. Cell migration assay to evaluate HNSCC treated with MPs and SPs. Micrographs and measurements (% of area) of the extension of wound healing obtained after 24h in the samples treated with MP and SP loaded or not with 5-FU compared with the control. A reduction in the number of cells filling the gap points to a decrease migration after incubation with samples containing 5-FU was clearly noted using phase-contrast microscopy. PCL: polycaprolactone, 5-FU: 5-Fluorouracil, CS: chitosan, MPs: microparticles, SPs: sponges, T0: initial analyses and T24: final analyses. *statistical difference between T0 and T24h.

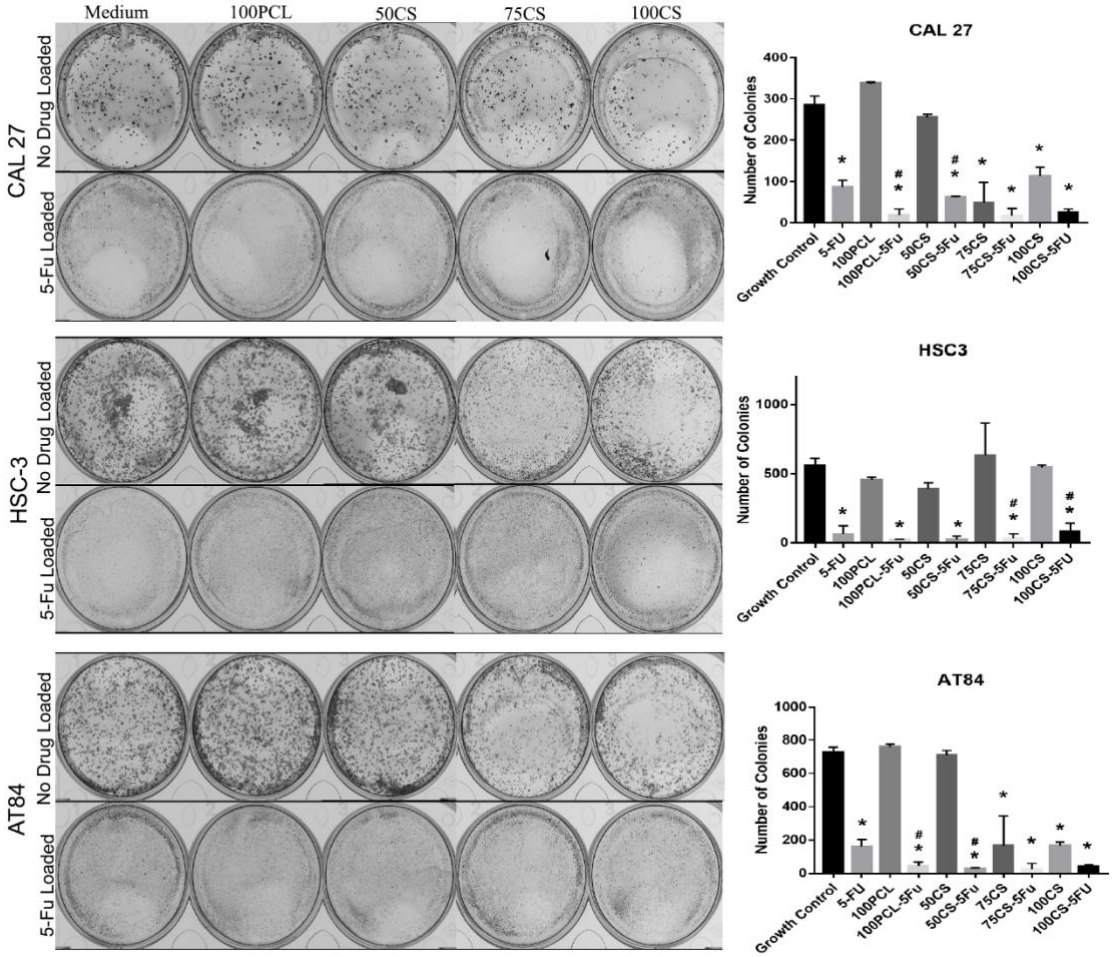

Figure 6. Viability assays after exposure of HNSCC cells to MPs and composite sponges for 10 days. The panel on the left shows representative clonogenic assays and the graphs on the right represent the respective surviving fraction after treatments. Data were represented as median ± 95% confidence interval. *statistical significance compared with the control group. #statistical significance comparing samples with and without 5-FU loading. PCL: polycaprolactone, 5-FU: 5-Fluorouracil, CS: chitosan. MPs: microparticles.

5-FU loaded MPs and SPs are able to stimulate autophagy

Cell death mechanism was evaluated by quantification of poly-(adenosine diphosphate-ribose) polymerase-1 (PARP1) cleavage in cell incubated with composite sponges and MPs during 48h. Protein expression showed an increase in the cleaved PARP1, especially in the samples containing 5-FU and the highest CS concentrations (**Figure 7)**. The PARP1 cleavage is considered a hallmark of apoptosis. Overreaction of the nuclear enzyme PARP1 mediates cell death through two mechanisms: 1: necrosis due the depletion of NAD+ and ATP observed in cases of severe DNA damage; and 2: apoptosis associated with the dissipation of the

mitochondrial inner transmembrane potential (36).

In order to determine whether composite sponges and MPs affects autophagy in HNSCC cells, we investigated formation of autophagosomes *in vitro* by detecting the conversion of microtubule-associated protein 1 light chain 3 (LC3-I) to lipidated LC3-II, a classical markers of autophagy regulation (36, 37). The protein analyses showed an increase of LC3-II conversion in cells treatment during 48h in the samples containing 5-FU and the highest CS concentrations (**Figure 7**). The LC3 lipidation can reflect the induction of a different/modified pathway such as LC3-associated phagocytosis and the noncanonical destruction pathway of the paternal mitochondria (37). The composite sponges and MPs containing 5-FU induced PARP1 expression and LC3 activation in a similar manner as the drug alone, reinforcing that the coating do not interfere with drug activity.

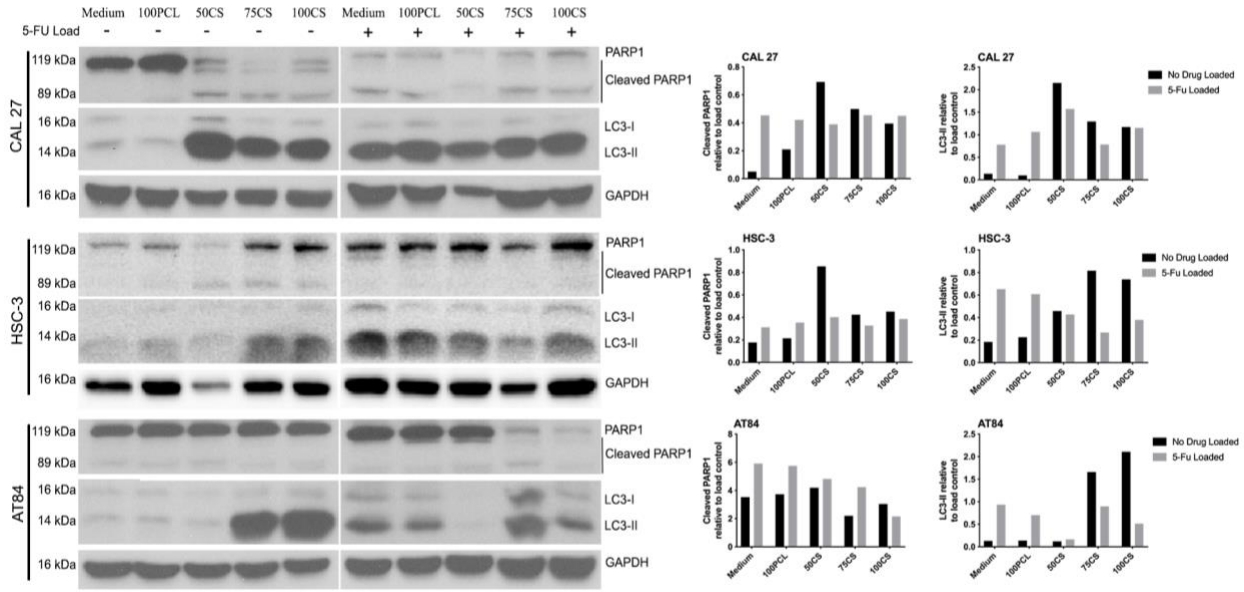

Figure 7. The MPs and composite sponges induced apoptosis and autophagy in HNSCC cells. Cells were treated with the MPs and SPs during 48h and the protein expression of PARP1, LC3B and GAPDH were analyzed. The panel shows an increased expression of PARP1 and LC3-II cleavage in the CAL27, HSC3 and AT84 cell lines incubated with samples coating or not 5-FU. This suggests increase in cell death by apoptosis and autophagy, respectively. PCL: polycaprolactone, 5-FU: 5-Fluorouracil, CS: chitosan, LC3: microtubule-associated protein 1 light chain 3, PARP1: poly-(adenosine diphosphate-ribose) polymerase-1, MPs: microparticles, SPs: sponges.

Based on our results, the potential molecular mechanisms involved in cell death in HNSCC

triggered by our synthetized composite sponges and MPs is probably through the increased autophagy and DNA damage by PARP1 hyperactivity and the lipidated LC3-II. The porous surface of our SPs offer significant benefits over others MPs such as a 3D matrix to load drugs that can also act as a niche for cell regeneration (38). We re-engineered a porous platform with 5-FU for sustainable drug release made with natural and synthetic polymers with translational potential. Once, this approach was specially designed as a strategy to reduce HNSCC early relapse and loco-regional recurrence. Aiming to archive clinical application, further investigations could provide new application of the controlled release of CT agents after HNSCC tumor resection.

CONCLUSION

The synthetized composite sponges were able to improve 5-FU efficacy in alternative HNSCC cell lines when compared to controls. The presence of PCL was not related to the drug release rate but decreased the material porosity. Thus, our results support the potential clinical utility of CS-decorated PCL MPs ans SPs as 5-FU-delivery carrier to improve therapeutic efficacy of this major drug in HNSCC.

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AUTHORS CONTRIBUTIONS

JML: data collection, visualization and analysis; write the manuscript.

LRC, ESM, PRB, DECP: conceptualization, review and edition.

KB, MH, MAJ: funding acquisition, review and edition.

SDS: conceptualization, supervision, data visualization, write, review, and edit the manuscript.

CONFLICT OF INTEREST STATEMENT: None declared.

REFERENCES

1. Lydiatt W, O'Sullivan B, Patel S. Major changes in head and neck staging for 2018. American Society of Clinical Oncology Educational Book. 2018;38:505-14.

2. MacDiarmid JA, Langova V, Bailey D, Pattison ST, Pattison SL, Christensen N, et al. Targeted Doxorubicin Delivery to Brain Tumors via Minicells: Proof of Principle Using Dogs with Spontaneously Occurring Tumors as a Model. PLoS One. 2016;11(4).
3. Reyes-Gibby CC, Anderson KO, Merriman KW, Todd KH, Shete SS, Hanna EY. Survival patterns in squamous cell carcinoma of the head and neck: pain as an independent prognostic factor for survival. The Journal of Pain. 2014;15(10):1015-22.
4. Cadoni G, Giraldi L, Petrelli L, Pandolfini M, Giuliani M, Paludetti G, et al. Prognostic factors in head and neck cancer: a 10-year retrospective analysis in a single-institution in Italy. Acta Otorhinolaryngologica Italica. 2017;37(6):458.
5. Amin MB, Edge SB. AJCC cancer staging manual: Springer; 2017.
6. Leemans CR, Snijders PJ, Brakenhoff RH. The molecular landscape of head and neck cancer. Nature Reviews Cancer. 2018;18(5):269.
7. Hua S, De Matos MB, Metselaar JM, Storm G. Current trends and challenges in the clinical translation of nanoparticulate nanomedicines: pathways for translational development and commercialization. Frontiers in pharmacology. 2018;9:790.
8. Lytton-Jean AK, Kauffman KJ, Kaczmarek JC, Langer R. Cancer nanotherapeutics in clinical trials. Nanotechnology-Based Precision Tools for the Detection and Treatment of Cancer: Springer; 2015. p. 293-322.
9. Tapazoglou E, Kish J, Ensley J, Al-Sarraf M. The activity of a single-agent 5-fluorouracil infusion in advanced and recurrent head and neck cancer. Cancer. 1986;57(6):1105-9.
10. Zheng X-F, Lian Q, Yang H, Wang X. Surface molecularly imprinted polymer of chitosan grafted poly (methyl methacrylate) for 5-fluorouracil and controlled release. Scientific reports. 2016;6:21409.
11. Ortiz R, Prados J, Melguizo C, Arias JL, Ruiz MA, Álvarez PJ, et al. 5-Fluorouracil-loaded poly (ε-caprolactone) nanoparticles combined with phage E gene therapy as a new strategy against colon cancer. International journal of nanomedicine. 2012;7:95.
12. Dourado MR, Korvala J, Åström P, De Oliveira CE, Cervigne NK, Mofatto LS, et al. Extracellular vesicles derived from cancer-associated fibroblasts induce the migration and invasion of oral squamous cell carcinoma. Journal of extracellular vesicles. 2019;8(1):1578525.
13. Hier M, Black M, Shenouda G, Sadeghi N, Karp S. A murine model for the immunotherapy of head and neck squamous cell carcinoma. The Laryngoscope. 1995;105(10):1077-80.
14. Heo DS, Park JG, Hata K, Day R, Herberman RB, Whiteside TL. Evaluation of tetrazolium-based semiautomatic colorimetric assay for measurement of human antitumor cytotoxicity. Cancer Res. 1990;50(12):3681-90.
15. Franken NA, Rodermond HM, Stap J, Haveman J, Van Bree C. Clonogenic assay of cells in vitro. Nature protocols. 2006;1(5):2315-9.
16. Valster A, Tran NL, Nakada M, Berens ME, Chan AY, Symons M. Cell migration and invasion assays. Methods. 2005;37(2):208-15.
17. Sahoo S, Sasmal A, Nanda R, Phani AR, Nayak PL. Synthesis of chitosan–polycaprolactone blend for control delivery of ofloxacin drug. Carbohydrate Polymers. 2010;79(1):106-13.
18. Miguel SP, Moreira AF, Correia IJ. Chitosan based-asymmetric membranes for wound healing: A review. International journal of biological macromolecules. 2019;127:460-75.
19. Freyman T, Yannas I, Gibson L. Cellular materials as porous scaffolds for tissue engineering. Progress in Materials science. 2001;46(3-4):273-82.
20. Lee JW, Han SS, Zo SM, Choi SM. Cellulose/poly-(m-phenylene isophthalamide) porous film as a tissue-engineered skin bioconstruct. Results in Physics. 2018;9:113-20.
21. Yogeshkumar NG, GuravAtul S, Adhikrao VY, editors. Chitosan and Its Applications: A Review of Literature2013.
22. Wang D, Zhang N, Meng G, He J, Wu F. The effect of form of carboxymethyl-chitosan dressings on biological properties in wound healing. Colloids and Surfaces B: Biointerfaces. 2020;194:111191.
23. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. Nature reviews cancer. 2003;3(5):330-8.

24. Ahmadi F, Oveisi Z, Samani SM, Amoozgar Z. Chitosan based hydrogels: characteristics and pharmaceutical applications. Res Pharm Sci. 2015;10(1):1-16.
25. Cameron RE, Kamvari-Moghaddam A. Synthetic bioresorbable polymers. 2008. p. 43-66.
26. Ho C. BC Cancer Protocol Summary for Treatment of Advanced Squamous Cell Carcinoma of the Head and Neck Cancer Using Fluorouracil and Platinum.
27. Jiang L, Ji N, Zhou Y, Li J, Liu X, Wang Z, et al. CAL 27 is an oral adenosquamous carcinoma cell line. Oral oncology. 2009;45(11):e204-e7.
28. Rodriguez AE. Characterization of CAL 27 and HSC-3 cell lines. DPAGT1 gene expression and association with oral squamous cell carcinoma genesis and metastasis 2016.
29. Wallin RF, Arscott E. A practical guide to ISO 10993-5: Cytotoxicity. Medical Device and Diagnostic Industry. 1998;20:96-8.
30. Adhikari HS, Yadav PN. Anticancer activity of chitosan, chitosan derivatives, and their mechanism of action. International journal of biomaterials. 2018;2018.
31. Park JK, Chung MJ, Choi HN, Park YI. Effects of the molecular weight and the degree of deacetylation of chitosan oligosaccharides on antitumor activity. Int J Mol Sci. 2011;12(1):266-77.
32. Wimardhani YS, Suniarti DF, Freisleben HJ, Wanandi SI, Siregar NC, Ikeda MA. Chitosan exerts anticancer activity through induction of apoptosis and cell cycle arrest in oral cancer cells. J Oral Sci. 2014;56(2):119-26.
33. Zhang J, Xia W, Liu P, Cheng Q, Tahirou T, Gu W, et al. Chitosan modification and pharmaceutical/biomedical applications. Mar Drugs. 2010;8(7):1962-87.
34. Nam KS, Shon YH. Suppression of metastasis of human breast cancer cells by chitosan oligosaccharides. J Microbiol Biotechnol. 2009;19(6):629-33.
35. Qi L, Xu Z. In vivo antitumor activity of chitosan nanoparticles. Bioorg Med Chem Lett. 2006;16(16):4243-5.
36. Tang D, Kang R, Berghe TV, Vandenabeele P, Kroemer G. The molecular machinery of regulated cell death. Cell Research. 2019;29(5):347-64.
37. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Autophagy. 2016;12(1):1-222.
38. Rambhia KJ, Ma PX. Controlled drug release for tissue engineering. Journal of controlled release : official journal of the Controlled Release Society. 2015;219:119-28.

5 CONCLUSÃO

A presença de PCL não alterou a taxa de liberação do medicamento, mas diminuiu a porosidade do material. As esponjas sintetizadas foram capazes de reduzir o metabolismo celular, a migração celular e a formação de colônias das linhagens celulares HNSCC quando comparadas ao controle de crescimento. Portanto, a amostra 100CS-5FU provou eficácia e eficiência quando comparada às amostras contendo PCL. Em síntese, este estudo descreveu um biomaterial com atividade antineoplásica *in vitro* com potencial translacional como terapia complementar após a ressecção do tumor, seguida ou não da modalidade tradicional de quimioterapia e/ou radioterapia para HNSCC. Para testar esta hipótese, ainda é necessário examinar as esponjas no modelo de recorrência local HNSCC *in vivo*.

A atual literatura sobre nanoformulações de quimioterápicos apresenta dificuldade em mostrar eficácia melhorada na resposta do tumor devido à falta de estudos clínicos com os respectivos controles de padrões ouro, mas as nanotecnologias identificadas têm desempenhado de uma maneira geral um fator importante na redução da toxicidade quimioterápica. Essas observações sugerem que a nanomedicina do câncer como terapia de HNSCC ainda permanece com um status promissor e deve atingir a maturidade antes do impacto translacional total. Embora ainda seja necessário estudos com desenhos apropriados para entender a toxicidade e a resposta tumoral aos NCCs em comparação com tratamentos tradicionais.

REFERÊNCIAS

ADHIKARI, H. S.; YADAV, P. N. Anticancer activity of chitosan, chitosan derivatives, and their mechanism of action. **International journal of biomaterials**, 2018, 2018.

AHMADI, F.; OVEISI, Z.; SAMANI, S. M.; AMOOZGAR, Z. Chitosan based hydrogels: characteristics and pharmaceutical applications. **Research in pharmaceutical sciences**, 10, n. 1, p. 1-16, Jan-Feb 2015.

AMIN, M. B.; EDGE, S. B. **AJCC cancer staging manual**. Springer, 2017. 3319406175.
Bjornmalm, M.; Thurecht, K.J.; Michael, M.; Scott, A.M.; Caruso, F. Bridging bio–nano science and cancer nanomedicine. *ACS nano* **2017**, *11*, 9594-9613.

Bogner, J.R.; Kronawitter, U.; Rolinski, B.; Truebenbach, K.; Goebel, F.-D. Liposomal doxorubicin in the treatment of advanced AIDS-related Kaposi-sarcoma. *Journal of acquired immune deficiency syndromes* **1994**, 463-468.

Butts, C.; Socinski, M.A.; Mitchell, P.L.; Thatcher, N.; Havel, L.; Krzakowski, M.; Nawrocki, S.; Ciuleanu, T.-E.; Bosquée, L.; Trigo, J.M. Tecemotide (L-BLP25) versus placebo after chemoradiotherapy for stage III non-small-cell lung cancer (START): a randomised, double-blind, phase 3 trial. *The lancet oncology* **2014**, *15*, 59-68.

CADONI, G.; GIRALDI, L.; PETRELLI, L.; PANDOLFINI, M. *et al.* Prognostic factors in head and neck cancer: a 10-year retrospective analysis in a single-institution in Italy. **Acta Otorhinolaryngologica Italica**, 37, n. 6, p. 458, 2017.

CAMERON, R. E.; KAMVARI-MOGHADDAM, A. Synthetic bioresorbable polymers. *In*, 2008. p. 43-66.

Caponigro, F.; Comella, P.; Budillon, A.; Bryce, J.; Avallone, A.; De Rosa, V.; Ionna, F.; Comella, G. Phase I study of Caelyx (doxorubicin HCL, pegylated liposomal) in recurrent or metastatic head and neck cancer. *Annals of Oncology* **2000**, *11*, 339-342.

Carvalho, A.L.; Nishimoto, I.N.; Califano, J.A.; Kowalski, L.P. Trends in incidence and prognosis for head and neck cancer in the United States: a site-specific analysis of the SEER database. *International journal of cancer* **2005**, *114*, 806-816.

CHAN, K. K.; GLENNY, A. M.; WELDON, J. C.; FURNESS, S. *et al.* Interventions for the treatment of oral and oropharyngeal cancers: targeted therapy and immunotherapy. **Cochrane Database of Systematic Reviews**, n. 12, 2015.

Chan, K.K.W.; Glenny, A.M.; Furness, S.; Worthington, H.V. Interventions for the treatment of oral and oropharyngeal cancers: targeted therapy and immunotherapy. *Cochrane Database of Systematic Reviews* **2013**, 10.1002/14651858.CD010341, doi:10.1002/14651858.CD010341.

Damascelli, B.; Cantuand, G.; Mattavelli, F.; Tamplenizza, P.; Bidoli, P.; Leo, E.; Dosio, F.; Cerrotta, A.M.; Tolla, G.D.; Frigerio, L.F., et al. Intraarterial chemotherapy with polyoxyethylated castor oil free paclitaxel, incorporated in albumin nanoparticles (ABI-007): Phase I study of patients with squamous cell carcinoma of the head and neck and anal canal: Preliminary evidence of clinical activity. *Cancer* **2001**, *92*, 2592-2602.

Damascelli, B.; Patelli, G.; Ticha, V.; Di Tolla, G.; Frigerio, L.F.; Garbagnati, F.; Lanocita, R.; Marchiano, A.; Spreafico, C.; Mattavelli, F., et al. Feasibility and efficacy of percutaneous transcatheter intraarterial chemotherapy with paclitaxel in albumin nanoparticles for advanced squamous-cell carcinoma of the oral cavity, oropharynx, and hypopharynx. *Journal of Vascular & Interventional Radiology* **2007**, *18*, 1395-1403.

Damascelli, B.; Patelli, G.L.; Lanocita, R.; Di Tolla, G.; Frigerio, L.F.; Marchiano, A.; Garbagnati, F.; Spreafico, C.; Ticha, V.; Gladin, C.R., et al. A novel intraarterial chemotherapy using paclitaxel in albumin nanoparticles to treat advanced squamous cell carcinoma of the tongue: preliminary findings. *AJR. American Journal of Roentgenology* **2003**, *181*, 253-260.

DASS, C. R.; CHOONG, P. F. The use of chitosan formulations in cancer therapy. **Journal of microencapsulation**, 25, n. 4, p. 275-279, 2008.

DI MARTINO, A.; SITTINGER, M.; RISBUD, M. V. Chitosan: a versatile biopolymer for orthopaedic tissue-engineering. **Biomaterials**, 26, n. 30, p. 5983-5990, 2005.

DOURADO, M. R.; KORVALA, J.; ÅSTRÖM, P.; DE OLIVEIRA, C. E. *et al.* Extracellular vesicles derived from cancer-associated fibroblasts induce the migration and invasion of oral squamous cell carcinoma. **Journal of extracellular vesicles**, 8, n. 1, p. 1578525, 2019.

Durieux, N.; Pasleau, F.; Howick, J.; Group, O.L.o.E.W. Oxford Centre for Evidence-Based Medicine 2011 Levels of Evidence-Traduction française. **2012**.

El-Sayed, I.H. Nanotechnology in head and neck cancer: the race is on. *Current oncology reports* **2010**, *12*, 121-128.

Faivre, S.; Alsabe, H.; Djafari, L.; Janot, F.; Julieron, M.; Domenge, C.; Djazouli, K.; Armand, J.P.; Luboinski, B.; Raymond, E. Locoregional effects of pegylated liposomal doxorubicin (Caelyx) in irradiated area: A phase I-II study in patients with recurrent squamous cell carcinoma of the head and neck. *Eur. J. **Cancer*** **2004**, *40*, 1517-1521.

FERNANDES, M. d. S. **Membranas de policaprolactona e quitosano para aplicação estomatológica**. 2011. -, Faculdade de Ciências e Tecnologia.

Forastiere, A.; Koch, W.; Trotti, A.; Sidransky, D. Head and neck cancer. *New England Journal of Medicine* **2001**, *345*, 1890-1900.

FRANKEN, N. A.; RODERMOND, H. M.; STAP, J.; HAVEMAN, J. *et al.* Clonogenic assay of cells in vitro. **Nature protocols**, 1, n. 5, p. 2315-2319, 2006.

FREYMAN, T.; YANNAS, I.; GIBSON, L. Cellular materials as porous scaffolds for tissue engineering. **Progress in Materials science**, 46, n. 3-4, p. 273-282, 2001.

Friedrich, J.O.; Adhikari, N.K.; Beyene, J. Inclusion of zero total event trials in meta-analyses maintains analytic consistency and incorporates all available data. **BMC medical research methodology 2007**, *7*, 5.

GROOME, P. A.; SCHULZE, K. M.; MACKILLOP, W. J.; GRICE, B. *et al.* A comparison of published head and neck stage groupings in carcinomas of the tonsillar region. **Cancer**, 92, n. 6, p. 1484-1494, 2001.
Groome, P.A.; Schulze, K.M.; Mackillop, W.J.; Grice, B.; Goh, C.; Cummings, B.J.; Hall, S.F.; Liu, F.F.; Payne, D.; Rothwell, D.M. A comparison of published head and neck stage groupings in carcinomas of the tonsillar region. *Cancer* **2001**, *92*, 1484-1494.

Harrington, K.J.; Lewanski, C.; Northcote, A.D.; Whittaker, J.; Peters, A.M.; Vile, R.G.; Stewart, J.S. Phase II study of pegylated liposomal doxorubicin (Caelyx) as induction chemotherapy for

patients with squamous cell cancer of the head and neck. *European Journal of Cancer* **2001**, *37*, 2015-2022.

Harrington, K.J.; Lewanski, C.R.; Northcote, A.D.; Whittaker, J.; Wellbank, H.; Vile, R.G.; Peters, A.M.; Stewart, J.S.W. Phase I-II study of pegylated liposomal cisplatin (SPI-077TM) in patients with inoperable head and neck cancer. *Annals of Oncology* **2001**, *12*, 493-496.

HASSAN, S.; PRAKASH, G.; OZTURK, A. B.; SAGHAZADEH, S. *et al.* Evolution and clinical translation of drug delivery nanomaterials. **Nano Today**, 15, p. 91-106, 2017.

HEO, D. S.; PARK, J. G.; HATA, K.; DAY, R. *et al.* Evaluation of tetrazolium-based semiautomatic colorimetric assay for measurement of human antitumor cytotoxicity. **Cancer Res**, 50, n. 12, p. 3681-3690, Jun 15 1990.

HIER, M.; BLACK, M.; SHENOUDA, G.; SADEGHI, N. *et al.* A murine model for the immunotherapy of head and neck squamous cell carcinoma. **The Laryngoscope**, 105, n. 10, p. 1077-1080, 1995.

Higgins, J.P.; Altman, D.G.; Gøtzsche, P.C.; Jüni, P.; Moher, D.; Oxman, A.D.; Savović, J.; Schulz, K.F.; Weeks, L.; Sterne, J.A. The Cochrane Collaboration's tool for assessing risk of bias in randomised trials. *Bmj* **2011**, *343*, d5928.

Howlader, N.; Noone, A.; Krapcho, M. Previous Version: SEER cancer statistics review, 1975-2010. National Cancer Institute, **2013**.

HUA, S.; DE MATOS, M. B.; METSELAAR, J. M.; STORM, G. Current trends and challenges in the clinical translation of nanoparticulate nanomedicines: pathways for translational development and commercialization. **Frontiers in pharmacology**, 9, p. 790, 2018.

KLIONSKY, D. J.; ABDELMOHSEN, K.; ABE, A.; ABEDIN, M. J. *et al.* Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). **Autophagy**, 12, n. 1, p. 1-222, 2016.

LEE, J. W.; HAN, S. S.; ZO, S. M.; CHOI, S. M. Cellulose/poly-(m-phenylene isophthalamide) porous film as a tissue-engineered skin bioconstruct. **Results in Physics**, 9, p. 113-120, 2018.

LEEMANS, C. R.; SNIJDERS, P. J.; BRAKENHOFF, R. H. The molecular landscape of head and neck cancer. **Nature Reviews Cancer**, 18, n. 5, p. 269, 2018.

Leserman, L.D.; Barbet, J.; Kourilsky, F.; Weinstein, J.N. Targeting to cells of fluorescent liposomes covalently coupled with monoclonal antibody or protein A. *Nature* **1980**, *288*, 602-604.

LONGLEY, D. B.; HARKIN, D. P.; JOHNSTON, P. G. 5-fluorouracil: mechanisms of action and clinical strategies. **Nature reviews cancer**, 3, n. 5, p. 330-338, 2003.

LYDIATT, W.; O'SULLIVAN, B.; PATEL, S. Major changes in head and neck staging for 2018. **American Society of Clinical Oncology Educational Book**, 38, p. 505-514, 2018.

LYTTON-JEAN, A. K.; KAUFFMAN, K. J.; KACZMAREK, J. C.; LANGER, R. Cancer nanotherapeutics in clinical trials. *In*: **Nanotechnology-Based Precision Tools for the Detection and Treatment of Cancer**: Springer, 2015. p. 293-322.

MACDIARMID, J. A.; LANGOVA, V.; BAILEY, D.; PATTISON, S. T. *et al.* Targeted doxorubicin delivery to brain tumors via minicells: proof of principle using dogs with spontaneously occurring tumors as a model. **PloS one**, 11, n. 4, p. e0151832, 2016.

Maeda, H. Tumor-selective delivery of macromolecular drugs via the EPR effect: background and future prospects. *Bioconjugate chemistry* **2010**, *21*, 797-802.

Marur, S.; Forastiere, A.A. Head and neck squamous cell carcinoma: update on epidemiology, diagnosis, and treatment. *In Proceedings of Mayo Clinic Proceedings* **2016,** 386-396.

Matsumura, Y.; Maeda, H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer research* **1986**, *46*, 6387-6392.

MIGUEL, S. P.; MOREIRA, A. F.; CORREIA, I. J. Chitosan based-asymmetric membranes for wound healing: A review. **International journal of biological macromolecules**, 127, p. 460-475, 2019.

Miller, K.; Cortes, J.; Hurvitz, S.A.; Krop, I.E.; Tripathy, D.; Verma, S.; Riahi, K.; Reynolds, J.G.;

Wickham, T.J.; Molnar, I. HERMIONE: a randomized Phase 2 trial of MM-302 plus trastuzumab versus chemotherapy of physician's choice plus trastuzumab in patients with previously treated, anthracycline-naïve, HER2-positive, locally advanced/metastatic breast cancer. *BMC cancer* **2016**, *16*, 1-11.

Moher D.; Liberati A.; Tetzlaff J.; Altman DG.; Prisma Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *Int J Surg.* **2010,** *8,* 336-41.

NAM, K. S.; SHON, Y. H. Suppression of metastasis of human breast cancer cells by chitosan oligosaccharides. **J Microbiol Biotechnol**, 19, n. 6, p. 629-633, Jun 2009.

ORTIZ, R.; PRADOS, J.; MELGUIZO, C.; ARIAS, J. L. *et al.* 5-Fluorouracil-loaded poly (ε-caprolactone) nanoparticles combined with phage E gene therapy as a new strategy against colon cancer. **International journal of nanomedicine**, 7, p. 95, 2012. PARK, J. K.; CHUNG,

M. J.; CHOI, H. N.; PARK, Y. I. Effects of the molecular weight and the degree of deacetylation of chitosan oligosaccharides on antitumor activity. **Int J Mol Sci**, 12, n. 1, p. 266-277, Jan 6 2011.

Park, K. Facing the truth about nanotechnology in drug delivery. *ACS nano* **2013**, *7*, 7442-7447.

Percy, C.; Holten, V.v.; Muir, C.S.; Organization, W.H. International classification of diseases for oncology. **1990**.

QI, L.; XU, Z. In vivo antitumor activity of chitosan nanoparticles. **Bioorg Med Chem Lett**, 16, n. 16, p. 4243-4245, Aug 15 2006.

RAMBHIA, K. J.; MA, P. X. Controlled drug release for tissue engineering. **Journal of controlled release : official journal of the Controlled Release Society**, 219, p. 119-128, 2015.

REYES-GIBBY, C. C.; ANDERSON, K. O.; MERRIMAN, K. W.; TODD, K. H. *et al.* Survival patterns in squamous cell carcinoma of the head and neck: pain as an independent prognostic factor for survival. **The Journal of Pain**, 15, n. 10, p. 1015-1022, 2014.

Rosenberg, B.; Van Camp, L.; Krigas, T. Inhibition of cell division in Escherichia coli by electrolysis products from a platinum electrode. *Nature* **1965**, *205*, 698-699.

Rosenthal, D.I.; Yom, S.S.; Liu, L.; Machtay, M.; Algazy, K.; Weber, R.S.; Weinstein, G.S.;

Chalian, A.A.; Mille, L.K.; Rockwell, K., Jr., et al. A phase I study of SPI-077 (Stealth liposomal cisplatin) concurrent with radiation therapy for locally advanced head and neck cancer. *Investigational New Drugs* **2002**, *20*, 343-349.

SAHOO, S.; SASMAL, A.; NANDA, R.; PHANI, A. R. *et al.* Synthesis of chitosan–polycaprolactone blend for control delivery of ofloxacin drug. **Carbohydrate Polymers**, 79, n. 1, p. 106-113, 2010/01/05/ 2010.

SANTOS, M.O. Estimativa 2018: incidência de câncer no Brasil. *Revista brasileira de cancerologia* **2018,** 64, 119-20.

SARASAM, A.; MADIHALLY, S. V. Characterization of chitosan–polycaprolactone blends for tissue engineering applications. **Biomaterials**, 26, n. 27, p. 5500-5508, 2005.

Shi, J.; Kantoff, P.W.; Wooster, R.; Farokhzad, O.C. Cancer nanomedicine: progress, challenges and opportunities. *Nature Reviews Cancer* **2017**, *17*, 20-37.

Smith, A. Big moment for nanotech: oncology therapeutics poised for a leap. *OncLive* **2013**.

Stern, S.T.; Hall, J.B.; Lee, L.Y.; Wood, L.J.; Paciotti, G.F.; Tamarkin, L.; Long, S.E.; McNeil, S.E. Translational considerations for cancer nanomedicine. *Journal of Controlled Release* **2010**, *146*, 164-174.

Strieth, S.; Dunau, C.; Michaelis, U.; Jager, L.; Gellrich, D.; Wollenberg, B.; Dellian, M. Phase I/II clinical study on safety and antivascular effects of paclitaxel encapsulated in cationic liposomes for targeted therapy in advanced head and neck cancer. *Head Neck* **2014**, *36*, 976-984.

TANG, D.; KANG, R.; BERGHE, T. V.; VANDENABEELE, P. *et al.* The molecular machinery of regulated cell death. **Cell Research**, 29, n. 5, p. 347-364, 2019/05/01 2019.

TAPAZOGLOU, E.; KISH, J.; ENSLEY, J.; AL-SARRAF, M. The activity of a single-agent 5-fluorouracil infusion in advanced and recurrent head and neck cancer. **Cancer**, 57, n. 6, p. 1105-1109, 1986.

Trotti, A.; Byhardt, R.; Stetz, J.; Gwede, C.; Corn, B.; Fu, K.; Gunderson, L.; McCormick, B.; Morris, M.; Rich, T. Common toxicity criteria: version 2.0. an improved reference for grading the acute effects of cancer treatment: impact on radiotherapy. *International Journal of Radiation Oncology* Biology* Physics* **2000**, *47*, 13-47.

VALSTER, A.; TRAN, N. L.; NAKADA, M.; BERENS, M. E. *et al.* Cell migration and invasion assays. **Methods**, 37, n. 2, p. 208-215, 2005.

WANG, D.; ZHANG, N.; MENG, G.; HE, J. *et al.* The effect of form of carboxymethyl-chitosan dressings on biological properties in wound healing. **Colloids and Surfaces B: Biointerfaces**, 194, p. 111191, 2020/10/01/ 2020.

Wani, M.C.; Taylor, H.L.; Wall, M.E.; Coggon, P.; McPhail, A.T. Plant antitumor agents. VI. Isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. *Journal of the American Chemical Society* **1971**, *93*, 2325-2327.

WIMARDHANI, Y. S.; SUNIARTI, D. F.; FREISLEBEN, H. J.; WANANDI, S. I. *et al.* Chitosan exerts anticancer activity through induction of apoptosis and cell cycle arrest in oral cancer cells. **J Oral Sci**, 56, n. 2, p. 119-126, Jun 2014.

YOGESHKUMAR, N. G.; GURAVATUL, S.; ADHIKRAO, V. Y., 2013, **Chitosan and Its Applications: A Review of Literature**.

ZHANG, J.; XIA, W.; LIU, P.; CHENG, Q. *et al.* Chitosan modification and pharmaceutical/biomedical applications. **Marine drugs**, 8, n. 7, p. 1962-1987, 2010.

ZHENG, X.-F.; LIAN, Q.; YANG, H.; WANG, X. Surface molecularly imprinted polymer of chitosan grafted poly (methyl methacrylate) for 5-fluorouracil and controlled release. **Scientific reports**, 6, p. 21409, 2016.