UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA

PALOMA SANTOS DE CAMPOS

ANÁLISE DO PAPEL DA CURCUMINA SOBRE A MIGRAÇÃO DE LINHAGENS CELULARES

Porto Alegre
2016

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Trabalho de Conclusão de Curso apresentado ao Curso de Graduação em Odontologia da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do título de Cirurgiã-Dentista.

Orientador: Prof. Dr. Marcelo Lazzaron Lamers

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RESUMO

CAMPOS, Paloma Santos. **Análise do papel da curcumina sobre a migração de linhagens celulares**. 2016. 33 f. Trabalho de Conclusão de Curso (Graduação em Odontologia) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2016.

O processo de invasão tecidual e metástase é um dos principais fatores do insucesso clínico no tratamento de tumores malignos e ocorre principalmente devido ao comportamento migratório desenvolvido pelas células tumorais. Nesse contexto, os produtos naturais têm sido estudados quanto aos seus efeitos sobre várias propriedades fisiológicas das células, na tentativa de descobrir novas drogas que modulem o comportamento migratório. A curcumina, um polifenol lipofílico amarelado oriundo da *Curcuma longa* possui propriedades antibacterianas, antioxidantes e anti-tumorais. O objetivo deste estudo foi avaliar o efeito da curcumina na migração de fibroblastos (3T3-NIH) e de carcinoma espinocelular oral (SCC25) e CAL27). Inicialmente, foi analisado os efeitos da curcumina sobre a atividade proliferativa através da quantificação de conteúdo de DNA. Observou-se que a curcumina em baixas concentrações (2uM) diminuiu em 50% a proliferação celular ($n=4$, $p<0,05$) das linhagens com perfil mesenquimal. Após, as linhagens foram tratadas com curcumina (2 uM, 5 uM e 10 uM), plaqueadas em condições promotoras de migração (fibronectina 2ug/ml) e submetidos a ensaio de time lapse (20 h). Utilizando o software ImageJ, cada célula migratória foi acompanhada e os dados foram analisados quanto à velocidade de migração e à direcionalidade. Observou-se uma inibição da velocidade de migração em 50% para 3T3-NIH ($n=5$, $p<0,05$) e em 40% para SCC 25 ($n=4$, $p<0,05$). Adicionalmente, foi observado a diminuição da persistência de migração celular a partir de 2 uM, com a diminuição da direcionalidade de migração. Para analisar o perfil de adesão célula-célula, foi realizado ensaio de esferas, no qual as células são plaqueadas em uma superfície não aderente (1,5% de agarose) e expostas ou não ao tratamento com curcumina (5 µM, 10 µM, 20 µM, 50 µM e 200µM). Foi observado que curcumina induz desagregação das esferas de forma dose dependente. Esses resultados sugerem que a curcumina é capaz de modular a migração e a proliferação celular em duas linhagens celulares, indicando um possível uso em futuras terapias.

Palavras-chave: Carcinogênese. Invasão. Metástase. *Curcuma longa*. Câncer bucal

ABSTRACT

CAMPOS, Paloma Santos. **Analysis of the role of Curcuma longa on cell migration.** 2016. 33 p. Final Paper. (Graduation in Dentistry) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2016.

Cell invasion and metastasis is a major factor in the clinical failure in the treatment of malignant tumors and is mainly due to migratory behavior developed by tumor cells. In this context, natural products have been studied for their effects on various physiological properties of the cells in an attempt to discover new drugs that modulate the migratory behavior. The curcumin, a yellow lipophilic polyphenol derived from *Curcuma longa* has antibacterial, antioxidant and anti-tumor properties. The aim of this study was to evaluate the effect of curcumin on the migration of fibroblasts (3T3-NIH) and squamous cell carcinoma (SCC25,CAL27). Initially, we examined the effects of curcumin on the proliferative activity by measuring DNA content. It was observed that curcumin at low concentrations (2μM) decreased by 50% the cell proliferation ($n = 4$, $p < 0.05$) in cell lines with mesenchymal characteristics. Then, cell were treated for 24h with low doses of curcumin (2 μM, 5 μM and 10 μM), plated in conditions that promote migration (2μg fibronectin/ml) and subjected to time-lapse test (20 h). Using ImageJ software, each migratory cell was tracked and data were analyzed regarding migration speed and directionality. It was observed a migration inhibition rate of 50% for NIH-3T3 cells ($n = 5$, $p < 0.05$) and 40% for SCC25 ($n = 4$, $p < 0.05$). Additionally, 2 μM of curcumin decreased cell migration persistence due to impairment of migration directionality. To analyze the effects of curcumin on cell-cell adhesion, it was performed espheroids by platting Cal27 and SCC25 cells on a non-stick surface (1.5% agarose), in the presence/absence of curcumin (5 μM, 10 μM, 20 μM, 50 μM and 200μM). It was observed that curcumin induces breakdown of the spheres of dose-dependent manner. These results suggest that curcumin can modulate migration and cell proliferation in both cell lines, indicating a possible future use in therapy.

Keywords: Carcinogenesis, Invasion, Metastasis, *Curcuma longa*, Oral cancer

SUMÁRIO

1 INTRODUÇÃO

1.1 NEOPLASIAS MALIGNAS

A Organização Mundial da Saúde (OMS) em 2014 apontou que são diagnosticados 14 milhões de novos casos de câncer por ano e que até o ano de 2035 este número deve aumentar para 24 milhões de diagnósticos/ano devido ao aumento da expectativa de vida das populações. A OMS ainda atenta para a necessidade de realizar o diagnóstico precoce além de combater os fatores de risco (tabagismo, etilismo e obesidade, por exemplo).

O câncer é um problema de saúde pública, sendo que no Brasil há cerca de 600 mil novos doentes por ano dos quais 60% tem seu diagnóstico já em estágio avançado (INSTITUTO NACIONAL DE CÂNCER JOSÉ ALENCAR GOMES DA SILVA, 2016). Em relação ao carcinoma espinocelular oral, a última estimativa mundial apontou que ocorreram cerca de 300 mil novos casos e 145 mil óbitos em 2012 (GLOBOCAN, 2012). Desses, cerca de 80% ocorreram em países em desenvolvimento. No Brasil, foram estimados 11.280 novos casos de câncer bucal em homens – sendo o quinto tipo de câncer mais comum – e 4.010 em mulheres para o ano de 2014 (INSTITUTO NACIONAL DE CÂNCER JOSÉ ALENCAR GOMES DA SILVA, 2015). Sendo assim, para o ano de 2016 a previsão é de 596 mil novos casos de câncer para todo o país e de 15.490 novos casos para câncer de cavidade oral (INSTITUTO NACIONAL DE CÂNCER JOSÉ ALENCAR GOMES DA SILVA, 2016). Ou seja, havendo um aumento de 1,3% de casos em dois anos o que sugere uma tendência de elevação para os próximos anos.

O foco terapêutico da maioria das neoplasias malignas é o tratamento cirúrgico e/ou radioterapia e/ou quimioterapia. Tratamentos cirúrgicos são, normalmente, bastante invasivos, nos quais ocorre grande perda de tecido, levando, na grande parte dos casos, a uma elevada morbidade e diminuição da qualidade de vida do paciente. Já a radioterapia e a quimioterapia são procedimentos que possuem muitos efeitos adversos ao paciente, uma vez que não conseguem diferenciar o tecido maligno do tecido sadio (HOSKIN, 2008). Devido à complexidade da doença e ao seu caráter invasivo o prognóstico para os pacientes acometidos por câncer de boca não é favorável e não vem melhorando ao longo dos anos, pois a taxa de sobrevida em cinco anos segue por volta de 50% (AGGARWAL et al., 2009). Os estudos vêm se aprimorando e buscando uma alternativa terapêutica, no entanto o foco das pesquisas se mantém na proliferação celular.

1.2 PROCESSO DE TUMORIGÊNESE

O desenvolvimento do câncer, processo denominado de tumorigênese, se inicia a partir de um grupo de células que sofreram mutações genéticas, principalmente nos genes que controlam a proliferação e a apoptose celular, levando a um crescimento descontrolado. Conforme o tumor progride, inúmeras alterações ocorrem nas diferentes células presentes no local e na própria matriz extracelular (MEC), propiciando que a lesão se desenvolva cada vez mais, sendo a metástase o estágio mais avançado desse evento (TSANTOULIS, 2007). Além desse descontrole na proliferação, as células tumorais alteram seu metabolismo, passando a apresentar um comportamento totalmente diferente dos outros tecidos do organismo (ZHANG, 2013). Hanahan e Weinberg (2011) descrevem as seguintes alterações que as células tumorais são capazes de promover para o seu desenvolvimento: 1- manutenção da sinalização para proliferação, 2- escape dos supressores de crescimento, 3- fuga da destruição pelo sistema imune, 4- imortalidade replicativa, 5- promoção de inflamação crônica, 6- indução de angiogênese e linfangiogênese, 7- mutação e instabilidade genômica, 8- resistência à morte celular, 9- desregulação do sistema energético, e 10- ativação da invasão tecidual e metástase (HANAHAN; WEINBERG, 2011). A partir deste estudo observamos a grande complexidade desta doença e podemos trabalhar na busca de uma terapia mais abrangente para tentar controlar os diferentes mecanismos usados pelas células tumorais para progredir e sobreviver.

1.3 MECANISMO DE INVASÃO TUMORAL

A invasão tecidual e o desenvolvimento de potencial metastático de tumores malignos é a maior causa de insucessos clínicos em termos de terapia e prognóstico. As invasões celulares, que podem ser coletivas ou individuais, são caracterizadas por alterações moleculares importantes, tais como modificação da adesão entre as células e da adesão à matriz extracelular, facilitando o processo de invasão (FRIEDL P., 2004; SAHAI E.; 2005, GUARINO M., 2007, ETIENNE-MANNEVILLE S., 2008, PAINTER K.J.,2010). Contudo, as células migratórias de um tumor não são coordenadas, estão randomicamente orientadas e se dividem em grupos de confusa organização ou se isolam, levando à alteração da estrutura tecidual. Por exemplo, no tecido epitelial ocorre a ruptura da lâmina basal e invasão do tumor em direção ao tecido adjacente. Dentro deste processo invasivo, ocorre a mudança de uma migração coletiva para uma migração individual, sendo este um dos passos da transição epitélio-mesênquima (EMT) e está relacionada a progressão do tumor. Assim sendo, a

invasão tumoral facilita a emergência das metástases, espalhando as células do câncer para outras partes do corpo e contribuindo para a formação de tumores (FRIEDL P., 2004; SAHAI, 2005; GUARINO, 2007; ETIENNE-MANNEVILLE, 2008).

A migração celular é um processo essencial para o desenvolvimento e também pode contribuir para patologias importantes (HORWITZ; WEBB; ZHANG, 2004). A migração celular é um fenômeno fisiológico que permite que diversos eventos ocorram como, por exemplo, a cicatrização de feridas. No entanto, em algumas doenças como inflamação crônica e câncer esse processo é alterado causando graves injúrias ao paciente, ou seja, torna-se um processo patológico. Quando a migração é iniciada, ocorre a polimerização de actina na porção frontal da célula (leading edge), promovendo a formação de grandes projeções de membrana (lamelipódios) e o estabelecimento de novas adesões ao substrato. A adesão celular é um processo dinâmico e ocorre a partir da interação de proteínas transmembrana (integrinas) com proteínas da MEC resultando na liberação de moléculas sinalizadoras intracelulares bem como no recrutamento de proteínas moduladoras da ligação entre integrinas e proteínas do citoesqueleto. Cada etapa da migração celular é regulada por GTPases de baixo peso molecular pertencente à família Rho (de Ras-homology), que desempenham um papel fundamental nesse processo. Estas proteínas ciclam entre um estado inativo (ligadas a GDP) e um estado ativo (ligadas a GTP). A ativação das Rho GTPases, principalmente RhoA (envolvida, principalmente, na formação de fibras de estresse) e Rac1 (envolvida na formação de lamelípodios e complexos focais), estão associados a uma maior capacidade de invasão e migração de diferentes tumores, e a expressão elevada dessas proteínas tem sido associada a um pior prognóstico dos pacientes (ZHU Y., 2013;GEST C., 2013, LIU SY,2004; PATEL V,2007; LAI SY, 2008; CHANG KW 2013;).

Caderinas e integrinas são as maiores classes de receptores de superfície que mediam, respectivamente, a adesão célula-célula e adesão célula-matriz (HYNES; HUGHES, 2002). Embora os receptores de adesão e os ligantes da MEC terem sido estudados como potenciais alvos terapêuticos, diferenças na dinâmica de adesão e maturação pode também podem desempenhar um papel na doença e, portanto, oferecerem alvos de intervenção e diagnóstico (PARSONS, 2011). O papel das integrinas é regular a migração e a invasão celular através da interação com componentes da matriz extracelular e a transdução de sinais intracelulares relacionados à migração celular, sendo a sua expressão associada a pior prognóstico dos pacientes. Vários trabalhos têm sido realizados com antagonistas de integrinas que mostraram inibir o crescimento do tumor, através da inibição das integrinas

produzidas por células tumorais e por células associadas ao tumor que levavam a diversificados efeitos celulares (DESGROSELLIER; CHERESH, 2010). Desta forma, mostra-se a aplicabilidade do conhecimento de migração celular que influencia no tratamento do câncer.

1.3 COMPONENTES NATURAIS ANTITUMORAIS

Quimioprevenção foi descrita como o uso de produtos químicos naturais ou sintéticos que permitem a supressão, inversão ou retardamento da carcinogênese (KELLOFF et al., 1994). Os produtos quimioprotetores apresentam poucos efeitos colaterais e baixa toxicidade. A maioria dos agentes quimiopreventivos conhecidos até hoje são extratos de plantas que se subdividem em duas classes: agentes de bloqueio, os quais inibem a etapa de inicialização por prevenção da ativação cancerígena e agentes de supressão, os quais inibem a proliferação de células malignas durante a promoção e progressão das etapas da carcinogênese (DUVOIX et al., 2004). Neste contexto, componentes naturais são interessantes como adjuvantes na terapia anti-tumoral. Na busca por compostos que se mostrem efetivos surge a curcumina que vem sendo estudada há vários séculos como um agente promissor.

1.4 CURCUMINA

Os produtos naturais têm sido estudados quanto aos seus efeitos sobre várias propriedades fisiológicas das células, na tentativa de descobrir novas drogas para o tratamento de diferentes doenças. A curcumina, um polifenol lipofílico amarelado oriundo da *Curcuma longa* possui propriedades antibacterianas, antioxidantes e anti-tumorais (KUNNUMAKKARA et al.,2008). A curcumina (diferuloylmethane) é um composto ativo muito utilizado na India e tem sido relatado na literatura por ter propriedades antitumorais em muitos tipos de câncer, incluindo câncer oral (LI et al., 2002; RAVINDRAN et al., 2009; SARKAR et al., 2010). Foi isolada pela primeira vez em 1815, sendo a forma cristalina obtida em 1870 (KUNNUMAKKARA et al, 2008). A curcumina desempenha um papel crítico na proliferação celular, crescimento, sobrevivência, apoptose, migração, invasão, angiogênese e metástase (ANAND et al., 2008; HATCHER et al., 2008; STRIMPAKOS E SHARMA, 2008). Algumas moléculas importantes pertencentes ao microambiente tumoral podem atuar como promotoras de tumor e alguns estudam mostram a atuação da curucmina sobre elas. Por exemplo, em linhagens celulares malignas de pulmão, próstata e melanoma tem demonstrado

que a curcumina é capaz de inibir a migração dessas células, através da produção de VEGF (fator de crescimento de endotélio vascular), MMPs -2 e -9 (metaloproteínases de matrix), IL-6 (interleucina) e vias de regulação gênica como os fatores de transcrição STAT-3 e NF-κB e as vias de sinalização PI3K, ERK 1/2, o que, demonstra o potencial antimetastático dessa molécula (KUNNUMAKKARA et al., 2008). Foi identificado o efeito inibitório da curcumina na invasão e migração de células de câncer de pulmão, onde observou-se a redução na capacidade de metástases nas células tratadas com curcumina devido à inibição da Rac1 (CHEN Q.Y. et al., 2014). Também em linhagem de câncer de pulmão foi mostrado que a curcumina bloqueia migração e invasão celular através da via de sinalização JAK/STAT 3 (YANG et al, 2012). Outro estudo mostra que a curcumina bloqueia migração e invasão de células da retina de ratos através da inibição da expressão de mRNA de MMP-2 e -7, Rho A, Rho kinases- ROCK-1 (ROCK-1) e FAK em células N18 (LIN, 2010).

Além disso, verificou-se que a curcumina inibe o crescimento, proliferação, invasão e migração de células de câncer oral, demonstrando ser capaz de suprimir o crescimento de tumores em modelos xenográficos derivados de câncer de cabeça e pescoço em modelo animal (Gao et al., 2012). Outros estudos, como o de Shin et al. (2010), relatou que a curcumina inibiu a motilidade das células de CCEO humano através da supressão da ativação de NF -kB. Também há relatos na literatura mostrando que a curcumina é capaz de atuar sobre os processos de invasão e migração celular, diminuindo transição epitélio-mesênquima, expressão de Cdc42 e metaloproteinases (CHEN Q.Y., 2012; TSANG R.K.,2012; SUN X.D.,2013; KIM H.J.,2013). Além desses, um estudo realizado com linhagem celular de câncer de trato aerodigestivo superior mostrou que a curcumina é capaz de ativar simultaneamente as vias supressoras de tumor p53 / p73 e inibir as pró -apoptóticas p - AKT e Bcl - 2, induzindo assim a apoptose de forma independe de p53 (RUHUL AMIN A.R.M. et al, 2015). Em linhagem de CAL-27 foi demonstrado na literatura que a curcumina diminiu proliferação e invasão através da inibição do crescimento celular, indução de apoptose, diminuição da expressão de Notch-1 e inibição da ativação de NF-kB (Liao et al., 2011).

A curcumina é uma molécula que suprime múltiplas vias de sinalização inibindo a proliferação celular, invasão, metástase (KUNNUMAKKARA et al., 2008). Esta característica multi-target, ou seja, atua interferindo em diversas vias de sinalização, pode trazer vantagens em relação às drogas convencionais, cuja atuação geralmente afeta apenas uma via de sinalização. Tendo em vista os trabalhos já realizados que demostram a influencia

da curcumina no câncer, a hipótese deste trabalho é que baixas doses de curcumina são capazes de alterar a migração celular em linhagens de câncer de cavidade oral.

2 OBJETIVOS

2.1 OBJETIVO GERAL

O objetivo deste trabalho é avaliar o papel da curcumina no perfil migratório de linhagens celulares tumorais (SCC 25, CAL 27) e não tumorais de fibroblastos (NIH-3T3).

2.2 OBJETIVOS ESPECÍFICOS

- avaliar a proliferação celular com e sem tratamento com diferentes concentrações de curcumina.

- através de ensaios de migração (SCC25, CAL27), observar o comportamento migratório das diferentes linhagens celulares quanto à direcionalidade, velocidade e persistência do movimento com ou sem tratamento prévio com curcumina.

- avaliar a coesão celular através do ensaio de esferoides com e sem tratamento.

3 ARTIGO CIENTÍFICO

O desenvolvimento do trabalho está apresentado na forma de artigo científico de periódico em inglês, o qual será submetido para publicação no periódico Molecular Nutrition & Food Research.

Analysis of the role of Curcuma longa on cell migration

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Abstract

Cell invasion and metastasis is a major factor in the clinical failure in the treatment of malignant tumors and is mainly due to migratory behavior developed by tumor cells. In this context, natural products have been studied for their effects on various physiological properties of the cells in an attempt to discover new drugs that modulate the migratory behavior. As the curcumin, a yellow lipophilic polyphenol derived from *Curcuma longa* that antibacterial, antioxidant and anti-tumor properties. The aim of this study was to evaluate the effect of curcumin on the migration of fibroblasts (3T3-NIH) and squamous cell carcinoma (SCC25CAL27). Initially, we examined the effects of curcumin on the proliferative activity by measuring DNA content. It was observed that curcumin at low concentrations (2μM) decreased by 50% the cell proliferation ($n = 4$, $p < 0.05$) in cell lines with mesenchymal characteristics. Then, cell were treated for 24h with low doses of curcumin (2 μM, 5 μM and 10 μM), plated in conditions that promote migration (2μg fibronectin/ml) and subjected to time-lapse test (20 h). Using ImageJ software, each migratory cell was tracked and cell migration speed and directionality were analyzed. It was observed a migration inhibition rate of 50% for NIH-3T3 cells ($n = 5$, $p < 0.05$) and 40% for SCC25 ($n = 4$, $p < 0.05$). Additionally, 2 μM of curcumin decreased cell migration persistence due to impairment of migration directionality. To analyze the effects of curcumin on cell-cell adhesion, it was performed espheroids by platting Cal27 and SCC25 cells on a non-stick surface (1.5% agarose), in the presence/absence of curcumin (5 μM, 10 μM, 20 μM, 50 μM and 200μM). It was observed that curcumin induces breakdown of the spheres of dose-dependent manner. These results suggest that curcumin can modulate migration and cell proliferation in both cell lines, indicating a possible future use in therapy.

Keywords: Carcinogenesis, Invasion, Metastasis, *Curcuma longa*, Oral Cancer

Introduction

Cancer is a public health problem and 20 million people worldwide are affected. In Brazil, there are 596.000 new patients with cancer in 2016, where 15.490 new cases are at the oral cavity, and 60 % have the diagnosis at an advanced stage (NATIONAL INSTITUTE OF CANCER JOSÉ ALENCAR GOMES DA SILVA, 2016). The therapeutic focus of most malignant tumors is surgical and / or radiotherapy and / or chemotherapy. Surgical treatments

are usually quite invasive, in which there is great loss of tissue, leading in most cases, a high morbidity and decrease the quality of life of the patients. Both radiotherapy and chemotherapy are procedures that have many adverse effects to the patient, since it does not differentiate malignant tissue from healthy tissue (1). Because of the complexity and its invasive phenotype of the disease, prognosis for patients suffering from oral cancer is not favorable with a five-year survival rate around 50% (2) which is not improving over the years. Studies have been performed in order to create alternative therapies that affect not only the tumor proliferation, but also other tumor properties such as invasion. In this context, natural components are interesting adjuvants in anti-tumor therapy and curcumin has been studied for several years as a promising agent. However, the mechanism by which curcumin affects tumor behavior, especially on tumor invasion, is still unclear.

Cell migration is an essential process for development and physiological events but may also contribute to major pathologies including cancer invasion (3). To migrate, cells must acquire a spatial asymmetry enabling them to translate intracellular generated forces into cell body translocation. One manifestation of this asymmetry is a polarized morphology, i.e., a clear distinction between cell front and rear. At the cell front, there is the extension of active membrane processes, including both lamellipodia and filopodia, the assemble of a multi-protein complex related to cell adhesion and the activation of signaling pathways (4,5,6,7,8). Tumor cells might migrate as a collective or as single cells. During collective migration, it is observed a supracellular process, where the coordinated behaviour of a group of cells improves the migratory capacities of each individual cell to induce a movement that is faster and more directed (9). During single cell migration, individual cells detach from tumor islands and this behavior is related to a more aggressive phenotype especially at the tumor front (9). In view of the importance of cell migration to tumor progression many drugs that act to regulate this phenomena.

Natural products have been extensively examined because of its antioxidant, anti-inflammatory and anticancer activities with few side effects. For example, half a century ago, curcumin and its family of compounds has been reported by their antibacterial effects, beyond antioxidant and anticancer properties (11,12,13,14). Curcumin is a yellowish lipophilic polyphenol from *Curcuma longa* (turmeric) and its mechanism of action involves the transcriptional regulation of several growth factors, cytokines, protein kinases and enzymes (14). Curcumin has demonstrated an anti-proliferative effect in many cancers. Studies of curcumin in cell lines from head and neck cancer have demonstrated a decrease in the growth

and survival of tumor cells (15,16). However, there are few evidences related how curcumin affects cell migration.

The aim of this study was to evaluate the effect of curcumin *in vitro* in different cell lines, fibroblast (3T3-NIH), a highly invasive oral squamous cell carcinoma (SCC 25) and low invasive oral squamous cell carcinoma (CAL 27). It was observed that curcumin decreased the migration speed and directionality in a dose-dependent way, as well as impair the cell-cell adhesion.

Materials and Methods

Reagents and cell culture

It was used a highly (SCC25 – CRL-1628) and a low invasive (CAL27 – CRL-2095) human OSCC cell line as well as highly migratory mouse fibroblasts (3T3 – NIH) obtained from American Type Culture Collection, ATCC, Manassas, VA. Fibroblasts and Cal27 cells were maintained in Dulbecco's modified Eagle's medium (DMEM - Gibco) with low and high glucose, while SCC25 were cultivated in DMEM/DMEM F12 media. All media was supplemented with 10% fetal bovine serum (Gibco), 1% penicillin and streptomycin (Gibco). For SCC25 cell, it was added hydrocortisone (400ng/ml). For imaging experiments, it was added HEPES (25mM) to the media. Curcumin and DMSO (Sigma) were obtained from Sigma (St Louis, MO). Curcumin was dissolved in DMSO to make 20 mg/mL stock solution.

Cell proliferation assay

Cells were cultured in 96-well plates for 12 hours. Curcumin was used in different concentrations (5, 10, 20, 30, 40 and 50 μM). After 24 hours, cell proliferation was analyzed by CyQUANT® NF Cell Proliferation Assay Kit (Invitrogen) according to the manufacturer's instructions.

Spheroid assay

Three-dimension culture model resembles avascular tumor and reestablish morphological, functional and mass transport properties of the corresponding tissue in vivo. We tested the effect of increasing doses of curcumin on cancer spheroids (17). Cells were plated in a non-adherent agarose-coated 96-well plate (3x104 cells/well) with an immediate or a late (24h) curcumin treatment (10, 20, 50, 200 μM). After 24, 48, and 72 hours, pictures were taken from spheroids with charge coupled device camera Axiocam mrn, Zeiss,

Göttingen, Germany) attached to an inverted microscope (Axio Observer Z1, Zeiss, Göttingen, Germany) using AxioVision Software (Zeiss, Göttingen, Germany).

Microscopy and image processing

Cells were plated in a 6-well plate ($1x10^5$cells/well) and then treated with curcumin (2 and 5μM) for 24 hours. After the treatment, cells were detached with trypsin-0.25% EDTA, washed and plated on fibronectin-coated glass-bottomed dishes (2μg/ml) in media with or without curcumin (2 and 5μM) for 3 h in incubator (37°C, 5% CO2). For analysis of the migration properties, phase microscopy time-lapse images were captured for a period of 20 h at 10 min intervals (migration speed and spatial trajectory (ST) with a charge coupled device camera (Axiocam mrn, Zeiss, Göttingen, Germany) attached to an inverted microscope (Axio Observer Z1, Zeiss, Göttingen, Germany) using AxioVision Software (Zeiss, Göttingen, Germany). The values for the assessment of migration speed and ST were obtained using Image J (National Institute of Health, MA, USA) software, and the data were processed as previously described (18). For ST analysis, a polar plot graph was constructed, which represents the spatial trajectory developed by each migratory cell, where the X and Y coordinates of each cell trajectory were normalized to start at a virtual (X = 0 and Y = 0) position.

Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences version 17.0 (SPSS Inc, Chicago, IL, USA). Data were presented as number, percentage, mean and standard deviation. To compare means between groups was applied t-test and analysis of variance (ANOVA) followed by Tukey's post-test. The level of statistical significance was 5% ($p < 0.05$).

Results

Curcumin reduces cell proliferation in a dose-dependent manner

In order to analyze the effects of curcumin on cell proliferation, NIH-3T3 fibroblasts, SCC25 OSCC and CAL-27 (Figure 1) were incubated with different doses of curcumin 2, 5, 10, 20, 30, 40 and 50 μM for 24h and submitted to proliferation assay. It was observed that curcumin decreased cell proliferation of NIH-3T3 fibroblasts in a dose dependent manner after 24 hours of treatment with cells showing a 30% decrease in

proliferation with a dose between 2 and 5 µM. For the low differentiated and highly invasive OSCC cell line (SCC 25), curcumin induced a 50% decrease in cell proliferation at 2 µM, The highly-differentiated and low invasive epithelial–derived tumor cell line (CAL27) showed a 30% decrease in cell proliferation only at 10 µM, showing that the line is more resistant to curcumin. These results indicate that curcumin has a more pronounced effect in cells that show a mesenchymal phenotype.

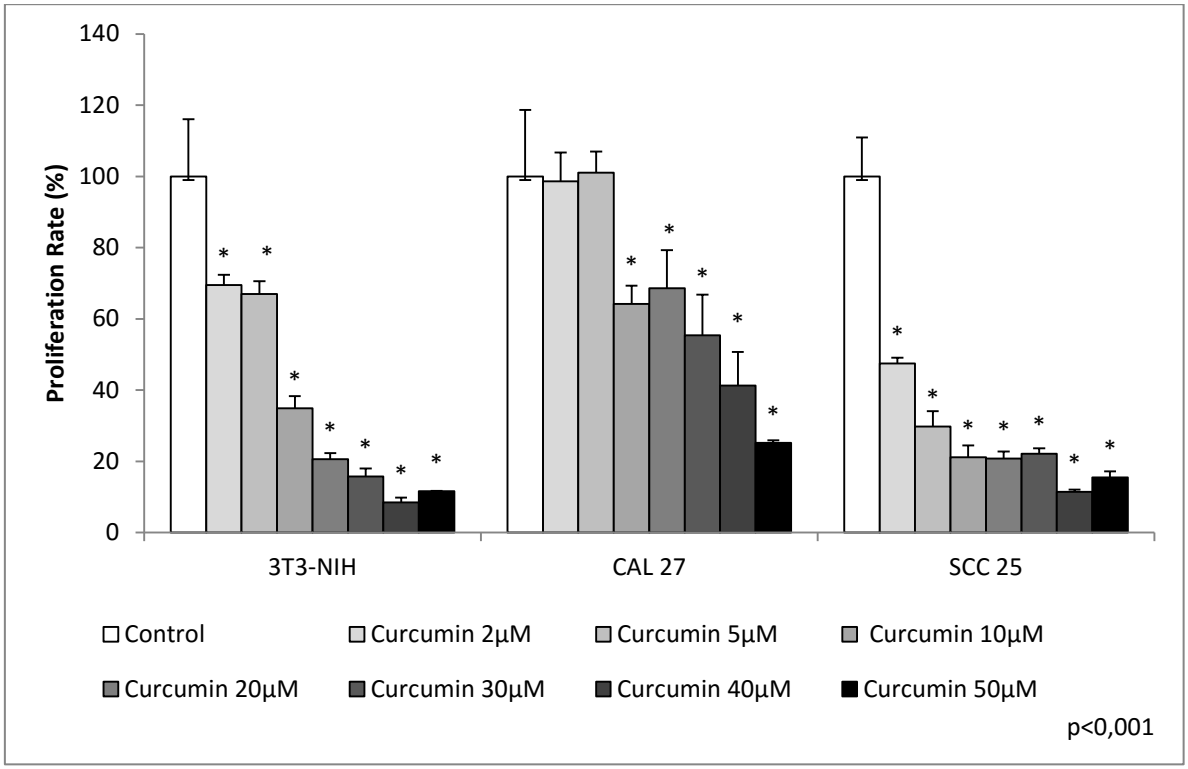


FIGURE 1: Curcumin has a more pronounced effect on cell proliferation in low differentiated cells. Fibroblasts (NIH-3T3), highly-differentiated (CAL27) and low differentiated (SCC25) Oral Squamous Cell Carcinoma derived cell lines were treated for 24h with different doses of curcumin (2, 5, 10, 20, 30, 40 and 50 µM) and submitted to proliferation assay. ANOVA, $p < 0.001$ and $n = 3$.

Curcumin reduces cell migration speed and modifies directionality

Since metastasis is a very important process for the progression of cancer and it is characterized by an increase in cell migration, we analyze the effect of curcumin in the migratory properties of highly migratory cell lines. Both, NIH-3T3 and SCC25, were treated with low doses of curcumin (2µM, 5µM) for 24h, plated under migration promoting conditions and imaged for 20h with a time interval of 10min in the presence/absence of curcumin. Using the time–lapse movies, individual migratory cells were tracked and it was

analyzed the data corresponding to migration speed and directionality (Figure 2). The OSCC (SCC25) and fibroblast (NIH-3T3) cell lines showed a decrease in cell migration (20% and 40% respectively) when treated with curcumin (2μM) that did not change over time of treatment (figure A and B and Supplementary Material 1). Moreover, the analysis of cell directionality demonstrated that, besides small changes in migration speed, an increase on curcumin concentration and/or on total time of administration led to decrease in directionality in tumor cells, resulting in non-productive migration.

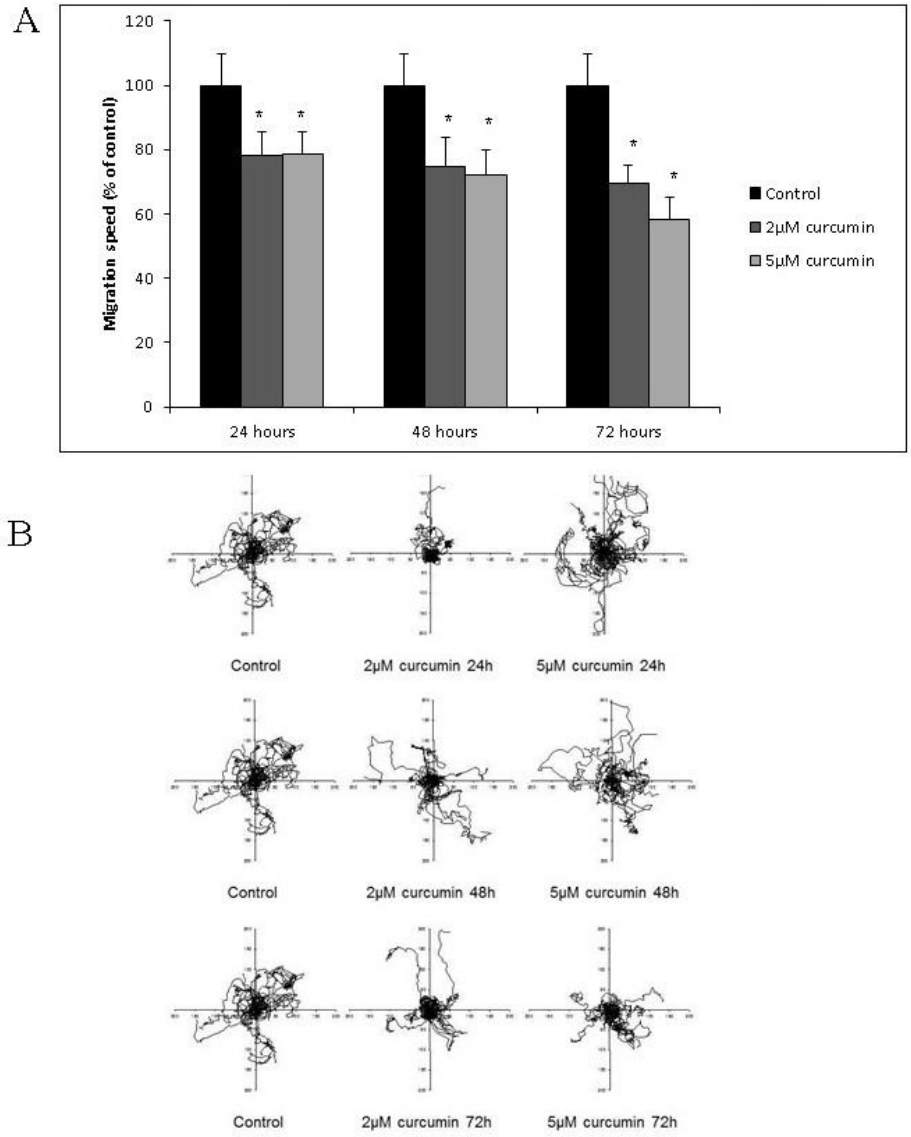


FIGURE 2: Curcumin induces decreased cell migration and modifies directionality of cell migration. From the analysis of cell migration, highly migratory OSCC cells (SCC25) were plated in migration-promoting conditions and time-lapse movies (20h) were performed. After the tracking of individual migratory cells, it was performed the ratio of

the total distance traveled by the time (A). It was observed that 24h of treatment with low doses of curcumin (2 and 5 µM) decreased (20%) migration velocity, which did not change with an increase in the time of pre-treatment (48 or 72h). For the analysis of directionality (B), the spatial trajectory of each migratory cell (individual lines) was normalized to start at a virtual X=0 and Y=0. It was observed that longer treatments with curcumin induced impairment on migration trajectory. ANOVA, n = 3 independent experiments, * = $p < 0.001$).

Curcumin modifies cellular cohesion in the spheroid

Multicellular spheroids are probably the most classical approach for 3D culturing. Studies to relate drug resistance phenomena in tumor tissue to these in cultured spheroids have shown impressive similarities and it has been widely used for drug basic screening (13,14,15,16,17). We analyzed the effects of curcumin in spheroids from SCC25 and CAL 27 cells. The highly invasive OSCC cell line (SCC25) formed a less homogeneous spheroid, while the low invasive OSCC cell line (CAL27) formed a cohesive sphere, indicating changes on cell-cell adhesion properties. It was observed that, for both cell lines, curcumin (5uM) induced a loss of cohesion between cells after 24h/48h/72h of spheroid formation (Figure 3) and this effect was more evident with an increase in curcumin concentration. These results indicate that low doses of curcumin are able to modify cell-cell adhesion of both low and highly invasive OSCC cell lines, leading to a decrease of spheroids integrity.

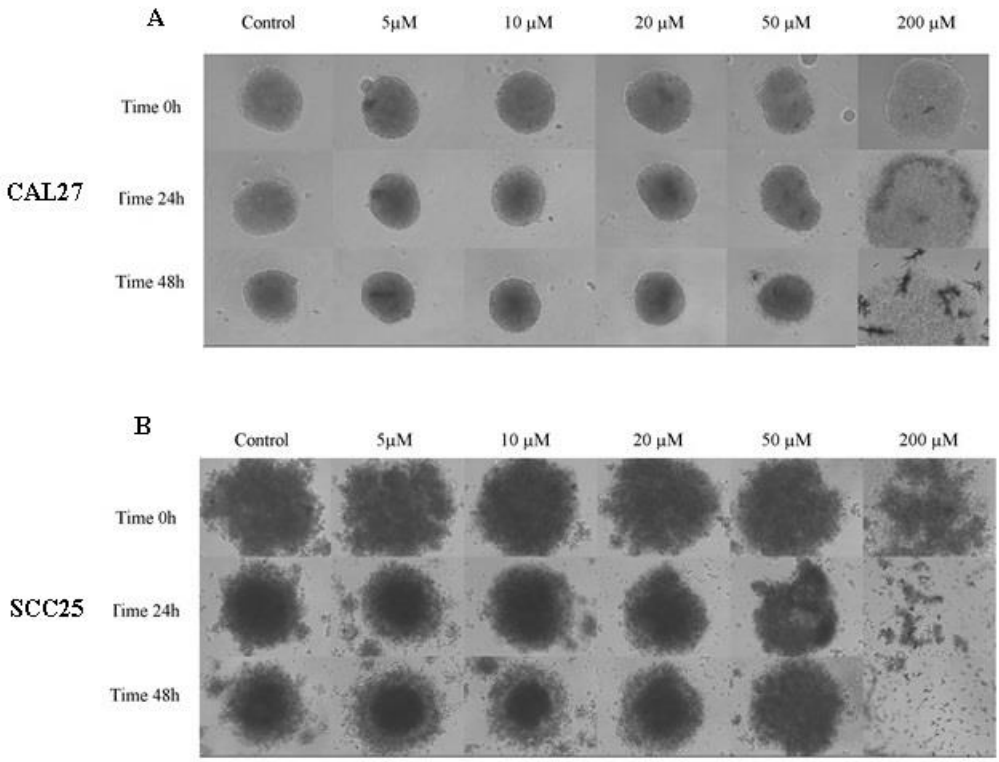


FIGURE 3: Curcumin impairs the cell-cell adhesion: assay spheroids with CAL27 (A) and SCC25 (B), after treatment with curcumin (5,10, 20, 50 and 200 μM) there was a loss of cohesion between the cells after 24h dose-dependent means.

Discussion

Cancer cells show distinctive and complementary capabilities that enable tumor growth and metastatic dissemination (19). Among these capabilities, tumor invasion and metastasis stand out as one of the biggest problem related to cancer. Regarding metastasis, it is necessary to consider two main factors: intrinsic cell migration properties and tumor microenvironment. For instance, Ramos et al 2016 suggest that the invasive behavior of OSCC is influenced by extracellular matrix composition, since fibronectin induced a fast single cell migration phenotype in high invasive and low E-cadherin expressing OSCC cells, while in low invasive and high E-cadherin OSCC cells, fibronectin produces a collective, non-directional migration (20). This evidence indicates that complimentary strategies for OSCC treatment might contemplate the regulation of migration pattern of heterogeneous tumor cell population as well as its microenvironment.

Curcumin has antioxidant, antiinflammatory and anticancer properties, showing anti-proliferative effect in many types of cancers. The effects of Curcumin (Curcuma root extract, also known as turmeric) result from its ability to inhibit tumor growth and metastasis (21,22,23,24). Curcumin is a drug known for its multi-target action that modulates different signaling pathways to inhibit tumor survival (14). The activity of curcumin reported against leukemia and lymphoma, gastrointestinal cancers, breast cancer, ovarian cancer, head and neck squamous cell carcinoma, lung cancer, melanoma reflects its ability to affect multiple targets (25,26,27,28). Moreover, it has been found that curcumin inhibits the oral cancer cell growth (29,30,31). However, the studies show variable drug concentration and in most of them the dosages are usually higher which turns out to be cytotoxic to the patients.

We observed that low doses of curcumin already decreased cell proliferation in highly invasive OSCC cells. The cell cycle is a fundamental biological process in the human body, but disruption of this process is an important point in tumors. Curcumin inhibits multiple levels within transcriptional network to restrict cell proliferation. It induces p53-dependent apoptosis in various tumor types, although both p53-dependent and –independent G2/M phase arrest by curcumin has been observed in colorectal cancer cells (32, 33, 34, 35, 36, 37, 38,39). Curcumin promotes caspase-3-mediated cleavage of β-catenin, decreases β-catenin/Tcf-Lef transactivation capacity for c-Myc and cyclin D1 (40). Curcumin can inhibit the NFkB pathways at multiple levels (41, 42), significantly inhibiting the growth of head and neck squamous cell carcinoma (HNSCC) xenograft tumors in nude mice. Inhibition of nuclear and cytoplasmic IkB-b kinase (IKKb) in the xenograft tumors decreased NF-kB activity (43). It is possible that the decrease growth of tumor observed in study is due by inhibition of cytoplasmic and nuclear IKKβ resulting in inhibition of NFκB activity.

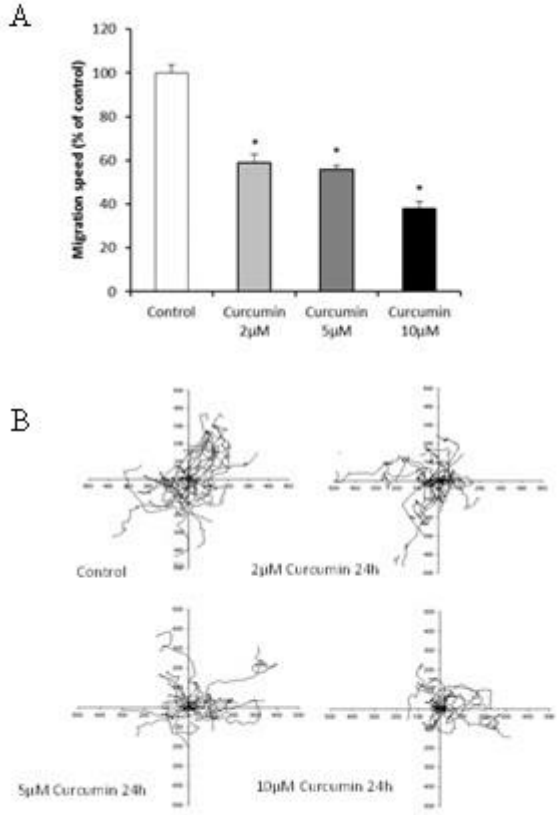
In this study we have shown that low doses of curcumin also impaired migration properties of oral cancer cells. Used for the assay time lapse cell lines with highly migratory profile (SCC25 and 3T3-NIH), since the cell line CAL27 was not used due to its non-migratory profile in control conditions under fibronectin surface. Cells with more mesenchymal phenotype and a consequent high level of invasiveness, such as SCC25, were more affected by curcumin treatment. Migration speed and directionality behavior affected at low doses of curcumim exposure. Our results show that directionality modifies of curcumin, the persistence of the movement that we can correlate with inhibition / decrease of proteins responsible for cell migration. Most of the events observed during cell migration are regulated by low molecular weight GTPases belonging to the Rho family (Ras-homology), where RhoA

is involved in the formation of stress fibers and Rac1 coordinates the formation of lamelipodia and focal complexes. Changes in the expression/activity of Rac1 and RhoA are associated with increased invasiveness and migration of different tumor, which correlates with a worse prognosis of patients (44,45,46,47,48,49) It has already demonstrated that curcumin inhibits Rac1 pathway in lung cancer cells and curcumin inhibited the expression of mRNA of Rho A in N18 cells (hybrid mouse -rat retinal ganglion cells) (50,51) and, therefore, curcumin may have a significant role in reducing in cancer cells migration and proliferation . Additionaly to this here we demonstrated that low doses of curcumin already impairs the migratory behavior of OSCC cells in 2D microenviroment.

When 3D analyses was performed, we performed a test with tumor spheroids, which demonstrated that curcumin has an important role in cell-cell adhesion of the spheroid. Observed that SCC25 spheroids looser than CAL27 3D structures, which is probably due to the low levels of E-cadherin and indicates a more invasive phenotype. On the other hand, the CAL27 spheroids form a more cohesive spheroid indicating a low level of invasion. We demonstrated that curcumin led to a dissociation of cells from spheroids, which may indicate an important therapeutic potential by increasing permeability of tumor islands suggesting that curcumin may be administered as an adjuvant for traditional antitumor therapy. To our knowledge, this is the first evidence of curcumin as a modulator of cell-cell adhesion.

Our data show that curcumin reduces cell proliferation (in 2D) in oral cancer cells, and significantly reduce migration and cell invasion. Also, a three-dimensional assay showed that curcumin modifies the cell cohesion in spheroids formed by SCC25 and CAL27, indicating that the drug can be used as an adjuvant in antitumor therapies to make the tumor more permeable to other drugs for example. Curcumin is a natural compound very promising in the search for stagnation or regression of the disease, but the search for more information about this drug mechanism of action is still necessary.

Supplementary material:



SUPPLEMENTARY FIGURE 1: Curcumin induces decreased cell migration and modifies directionality of cell migration. 3T3-NIH cells were treated 24 hours with curcumin, plated on fibronectin 2μg/ml and imaged using time-lapse analysis. (A) Curcumin reduces cell migration speed in a dose-dependent manner (40%). (B) Each line in the plot represents one cell spatial trajectory. In control and in treatment with 2μM of curcumin, cells reached longer distances when compared to treatment with 5 and 10μM. ANOVA, n = 3 independent experiments, * = $p < 0.001$).

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4 CONCLUSÃO

Os dados do trabalho apresentado mostram que a curcumina reduz a proliferação celular e a velocidade de migração celular de forma significativa, além de alterar a direcionalidade e modificar a agregação das células em saio de esferas. Portanto, com baixas doses da droga é possível observar efeitos na migração celular, já em relação a proliferação as doses que produzem efeito são mais altas, assim como no ensaio de esferoides. Desta forma, a curcumina apresenta efeitos em diferentes tipos de células neoplásicas e pode atuar como possível alvo terapêutico podendo atuar no controle de metástases oriundas do tumor.

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