Ellagic Acid Cytotoxic and Metalloproteinases Inhibitory Effects Against Oral Squamous Cell Carcinoma: An in Vitro Study

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Abstract

Objectives

This study aimed to evaluate the *in vitro* antitumoral potential of different concentrations of EA against two OSCC cell lines with distinct tissue invasiveness profiles.

Material and methods

Normal keratinocytes (NOK) and OSCC´s cells CAL-27 and SCC-9 were treated with concentrations of EA varying from 5 to 662 μM during 24, 48 or 72h. After each time of treatment, cells were submitted to viability analysis using MTT and the secretion of metalloproteinases (MMP-2 and MMP-9) and tissue metalloproteinases inhibitors (TIMP-1 and TIMP-2) were performed by Enzyme-Linked Immunoassay (ELISA). Data were submitted to ANOVA, followed by Bonferroni´s test, considering 5% as significance level.

Results

EA was cytotoxic to OSCC cells in all exposure times, rarely affecting normal cell viability, except for concentrations higher than 82 μM and after 72h treatment. For OSCC cells, EA decreased MMPs and increased TIMPs´s expression without effect on those enzymes for normal cell lines during all times of exposure. Conclusion

EA is a promising therapeutic adjuvant to treat oral cancer, however, further *in vivo* studies are required to clinically validate its potential.

Clinical Relevance

The *in vitro* anticancer properties showed by Ellagic acid, a phenolic compound that could easily be accessed by oral cancer patients, provides data to base future clinical studies intended to develop a safe topical oral anticancer product.

1. Introduction

Cancer arrests a continuous growth globally, that requires a physical, emotional and financial strain on individuals, families, communities and health systems and that get worst for low and middle-income countries, where a great amount of cancer patients have no access to quality diagnosis and treatment [1]. The most common types of cancer include breast, lung and colorectal, responsible for 6.4 million from 19.2 million cases in the world, in 2020, whilst lip and oral cavity cancer affected 0.3 million of cancer population, being responsible for 0.1 million deaths [2].

Oral squamous cell carcinoma (OSCC) is the most common histological type of cancer of the oral cavity, and has an important and well-established pattern of dissemination to cervical lymph nodes and local

tissue invasiveness, that leads a patient survival rate from 50–60% [3]. Besides more than 80% of the OSCC patients in its initial stage can be completely recovered from the disease, 70% of the OSCC progressive patients cannot achieve that positive result with the available therapies [4].

Tumor invasiveness is a dynamic process resulting from a complex interaction between host tissues and the tumoral microenvironment, in which a great number of extracellular enzymes act [5, 6]. Matrix metalloproteinases (MMPs) belong to the group of extracellular matrix degrading enzymes, acting on intracellular matrix proteins as collagen, proteoglycan, elastin, fibronectin, and regulating cellular growth, differentiation and angiogenesis potential [7, 8]. The imbalance of these enzymes and their specific inhibitors, the tissue inhibitors of matrix metalloproteinases (TIMPs), have been linked with pathological destruction in cancer [9]. Specifically, an increasing in MMP-2 and MMP-9 and decreasing in TIMP-1 and TIMP-2 levels showed to be significantly higher in OSCC patients. Furthermore, MMPs were related to the differentiation, stage and infiltration of the OSCC [6].

The type of treatment recommend to the OSCC depends basically on where the tumor is, how far the cancer has spread and patient's characteristics, varying from surgical excision of the tumor, lymph nodes removal, chemotherapy and radiation therapy, which may cause profound damage to patients health, facial esthetics and function [10]. Therefore, there is an increased need for novel and more efficient therapeutic strategies against the disease [11, 12]. In order to improve cancer prevention and therapy, many studies have focused therapeutic benefits of secondary metabolites from plant origin with chemopreventive actions as polyphenolic substances and, among them, ellagic acid (EA) has been extensively studied [13–15].

EA is a naturally occurring phenolic constituent contained in grapes, nuts, strawberries, black currents, raspberries, green tea and pomegranates [16]. Studies have reported a potent preventive and therapeutic effects for EA against different types of cancers such as colon, breast, prostate, skin esophageal cancers, and osteogenic sarcoma [17–21], however there is no study reporting EA activities against OSCC cells such as SCC-9 and CAL-27.

Therefore, herein we report EA effects on viability and secretion of MMP-2, MMP-9, TIMP-1 and TIMP-2 in the OSCC cells with different invasiveness and malignancy profiles (SCC9 and CAL27).

2. Material And Methods

2.1 Cell culture

Two commercial human tongue oral squamous carcinoma cell (OSCC) line representing different levels of aggressiveness, were used in this study, as follows: CAL 27 (CLR-2095) and SCC-9 (CLR-1629), both from ATCC (American Type Culture Collection, VA, USA). As control, an immortalized normal oral keratinocytes NOK were used (NOK-SI, Castilho et al., 2010). The cells were maintained in Dulbecco's modified Eagle's medium /Ham´s F12 (DMEM/Ham´s F12; Gibco®) supplemented with 10% fetal bovine

serum (FBS; Gibco®), 50 µg/mL vancomycin, 50 µg/mL gentamicin and 0,4 µg/mL hydrocortisone (Sigma®). The cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C.

2.2 Ellagic acid (EA)

Ellagic acid (≥ 95% - HPLC – powder, Sigma-Aldrich. ST Louis. MO-USA, #E2250) was dissolved in dimethylsulfoxide (DMSO, Sigma, at a final concentration of 0.1%) and was diluted in PBS to prepare the required concentrations (5; 20; 41; 82; 165; 331 and 662 µM). The control group was treated with 0.1% (v/v) dimethyl sulfoxide (DMSO), serving as the vehicle.

2.3 Cell Viability Tests (MTT)

Cells at an initial concentration of 110 cells/mm^2 were cultured in 96-well plates, in triplicate, for all the experimental groups. After 24 h, 48 h or 72 h of treatment, viable cells were determined using MTT assay according to manufacturer's recommendation. Briefly, cells were washed with PBS (200 µL/well, pH 7.4), stained with MTT solution (5 mg/mL, Sigma®; DMEM) 10 µL/well, incubated at 37°C during 3 h. After, sodium dodecyl sulfate (SDS) solution (10%, 100 µL/well) and chloridric acid (0,01 M) was added to solubilize the stain and absorbance was read using a microplate reader at a wavelength of 590 nm.

2.4 Enzyme-Linked Immunoassay (ELISA)

Tested concentrations of ellagic acid that maintained cells viability above 50% at 3 different times (24, 48 and 72 h) were chosen for enzyme-linked immunoassay tests. Concentrations of MMP-2, MMP-9, TIMP-1, and TIMP-2 were measured using commercially available ELISA kits (eBioscience, San Diego, CA, USA). The ELISA were carried out as per manufacturer's instructions. ELISA values were measured in triplicate for each sample in order to ensure accuracy.

For quantification of MMPs and TIMPs, cell culture supernatant was immediately collected after viability assays and separated by centrifugation at 5,000 g for 15 min at 4°C. Samples were stored at − 80°C for further processing. Total MMP-2, MMP9, TIMP-1, and TIMP-2 was quantified in picograms per mL (pg/mL). The results were calculated using the standard curves created in each assay. The ELISA assays were performed in a blind fashion in triplicate.

2.5 Statistical Analysis

The data were first analyzed for normality, using the Shapiro–Wilk test. One-way analysis of variance (ANOVA) with post hoc Bonferroni test was applied to all assays performed, at a significance level of 5%. The results were expressed as the mean ± standard deviation.

3. Results

3.1 Cell Viability

Elagic acid (EA) did not decrease normal keratinocytes (NOK) viability after 24 h or 48 h treatment ($p > 0.05$). However, after 72 h of EA treatment, cells showed a significant decrease in viability when exposed

to concentrations higher than 25 µg/mL (Fig. 1A).

For CAL 27, a significant cell viability decrease was observed after 24 h of EA treatment with concentrations up to 25 µg/mL ($p < 0.05$); whilst after 48 h or 72 h a significant decrease in cell viability occurred after treating cells with concentrations higher than 12.5 µg/mL of EA ($p < 0.05$; Fig. 1B).

A significant viability decreasing for SCC-9 was observed after treating cells with different EA concentrations for different times of exposure (24, 48 or 72 h); except with 2 µg/mL of EA concentration after 72 h, that showed 82.8% (± 14.0) of cell viability (Fig. 1C).

3.2 MMPs and TIMPs secretion

Considering outcomes obtained from control cells (NOK) at the viability assay, the lower concentrations of EA (5; 10; 20; 41 µM) were chosen for ELISA immunoassays (Figs. 2 and 3).

A significant decrease in MMP-2 and MMP-9 secretion was observed after treating OSCC with EA ($p < 0.05$), whilst no difference occurred for normal keratinocytes treated with EA ($p > 0.05$) (Figs. 2A and 2B).

For TIMPs, no difference was observed of TIMP-1 secretion on NOK cells after 24 and 48 h ($p > 0.05$); however, after 72 h there was a significant increasing at all EA concentrations in a dose-dependent manner ($p < 0.05$. Figure 3A). For TIMP-2 expression, a significant increase was observed after 48 h at 5 µM EA treatment when compared to control ($p < 0.05$), with no difference among 10, 20 and 41 µM concentrations of EA ($p > 0.05$. Figure 3B). Additionally, no difference was found in TIMP-2 expression when cells were treated after 24 or 72h ($p < 0.05$).

The expression of TIMP-1 was not statistically significant among different times of exposure or EA concentrations for CAL-27 cell line ($p > 0.05$. Figure 3A). EA induced an increase of TIMP-2 expression after 48 and 72h treatment, when compared to control cells ($p < 0.05$. Figure 3B), with no significant difference among EA concentrations ($p > 0.05$. Figure 3B).

TIMP-1 expression was significantly increased, in a concentration-dependent manner, after 72h EA treatment in SCC-9 cell line ($p < 0.05$. Figure 3A), however, no difference was observed among control and treated cells after 24 or 48h of EA treatment ($p > 0.05$. Figure 3A). For TIMP-2, a higher secretion was observed after 48 or 72 h EA treatment, when compared to control cells ($p < 0.05$. Figure 3B).

Figure 1

Cell viability assay after 24, 48 and 72 h of treatment of different doses of ellagic acid (EA).

Figure 2

Quantification of MMP-2 and MMP-9 by means of Elisa, after 24, 48 and 72 h of treatment of different doses of ellagic acid (EA).

Figure 3

Quantification of TIMP-1 and TIMP-2 by means of Elisa, after 24, 48 and 72 h of treatment of different doses of ellagic acid (EA).

4. Discussion

Oral squamous cell carcinoma (OSCC) corresponds to more than 90% of all oral cancers [22, 23] and mainly risk factors associated with are tobacco or betel quid and the regular drinking of alcoholic beverages [24, 25]. As to the anatomical site of the tumor, lower lip shows an occurrence about 30% of OSCC [26–28], however other studies revealed a major prevalence in tongue and cheek [29–32]. Patients with cancer in the lip region had presented better survival rates [33] possibly due to its easy accessibility and early detection [34] whilst those located in the oropharynx had the worst rates [35]. Oropharyngeal cancer is strongly associated with cervical metastases, with incidence of 50 to 70%, [36] due to its greater tumor dissemination, besides being located in regions difficult to visualize and diagnose, contributing negatively to patient survival [37]. Due to the necessity to improve or develop new cancer therapies, searching for new anticancer agents from plant sources is an actual and promising approach [38, 39].

Ellagic acid (EA), a naturally occurring phenolic constituent from some natural resources [16], has *in vitro* and *in vivo* cancer chemoprotective properties [40] and may induce mainly antiproliferative and pro-apoptotic activities demonstrating antitumoral activities [41, 42]. Herein, we verified EA actions for OSCC tongue cell lines compared to a non-tumoral keratinocyte lineage.

EA did not decrease cell viability of normal keratinocytes after 24 and 48 h (for all EA concentrations) and after 72h treatment with concentrations up to 82 μM. The results corroborates with studies that revealed no cytotoxic effect of EA for normal human keratinocytes HaCaT (in concentrations up to 75 μM) [43] or for normal human fibroblasts (concentrations up to 100 μM) after 24 h of treatment [15] which reinforces that EA is safe for a normal cell lineage. In this study, decreasing in cell viability at 72 h may be due to a possible viability induction by EA at higher concentrations (50, 100 and 200 μg/mL) ongoing at 24 h, which may initiate cell death after 72 h hypothetically due to medium depletion [44].

Regarding cancer cells, in this study two OSCC cells with different invasiveness phenotypes were tested: SCC-9, with a more aggressive profile, and CAL-27 [45]. Therefore, a significant decrease in cell viability was observed for both OSCC cells treated with EA at three different times, however more expressive EA effects were obtained for SCC-9 compared to CAL-27 and NOK. Whilst at 24 h the decrease in CAL-27 viability was achieved with EA concentrations above 25 μg/mL, for SCC-9 cells, EA activity decreased cells's viability for all concentrations and times of exposure, mainly. That could represent that the highest SCC-9 cell growth rate turns the cell more susceptibility to EA effects [46].

The viability outcomes for cancer cells obtained in this study are in accordance with literature reports. In fact, antiproliferative in vitro effects of EA at concentrations from 1-100 μM were reported for colon, breast, prostate, skin, esophageal, osteogenic, pancreatic, ovarian and nasopharyngeal cancers [17–21, 40, 47–49]. Until now, no study has reported EA effects in OSCC cells from tongue.

Besides cell's viability, other situations can occur in a tumoral microenvironment for the malignant cancer progression [50] during tissue invasiveness process, as the degradation of normal cells' basal membrane by enzymes like metalloproteinases (MMPs)[51]. Specifically, MMP-2 and MMP-9 have the ability to degrade type IV collagen, a major component of basement membrane, and therefore, are considered important substances indicative of growth, invasiveness and metastasis of oral cancer [52, 53]. Imbalances in the activities of MMPs and tissue inhibitors of matrix metalloproteinases (TIMPs) have been linked with pathological destruction in cancer, as TIMPs are responsible for blocking MMPs [9, 54]. Studies revealed that MMP-2, MMP-9, TIMP-1 and TIMP-2 may be extensively found at protein plasma as well tissue in OSCC patients, that represents a significant clinical usefulness [52–54].

In the present study, because of more than 50% decrease in cell viability was observed after 25, 50, 100 and 200 µg/mL EA supplementation, specially at 72h, the evaluation of MMP and TIMP expression was conducted with samples treated with EA concentrations of 5, 10, 20 and 41 µM.

Herein, all EA concentrations used decreased MMP-2 and MMP-9 levels for both OSCC cell lines, without any MMPs effect on normal keratinocytes. EA activity in MMP-2 and/or MMP-9 concentrations has been reported by few *in vivo* and corroborative studies. Huang et al. [55] described EA inhibited the activity and secretion of MMP-2 in vascular endothelial cells, whilst Devipriya et al. [56] verified coadministration of EA with alcohol decreased MMP-2, MMP-9 and TIMP-2 expression in hepatic tissue. The present study observed EA was able to increase TIMP-1 and TIMP-2 secretion notably at NOK cells.

Apparently, EA has the ability to inhibit both MMP-2 and MMP-9 initially, then, the substance increased the expression of their own cellular inhibitors as TIMP-1 and TIMP-2. EA is a potent phenolic compound presenting important antioxidant and anticarcinogenic properties. Considering different mechanisms of action described for EA's anticarcinogenic effect, it can be hypothesized that the inhibition of xenobiotic metabolizing enzymes property could be acting against MMPs [57]. Furthermore, it is well known that EA is able to increase glutathione S- transferase enzyme (GST) [58], that endogenously regulates oxidative status of the cell acting as a free radical's neutralizing agent, and GST is related to the inhibition of MMPs activities [59–61]. Both described mechanisms could be inducing MMP's decrease.

Other mechanisms from EA anticarcinogenic activities involves the modulation of the metabolism of environmental toxin [62], preventing direct binding of that carcinogens to the DNA [58] and inhibiting DNA topoisomerases which are involved in carcinogenesis [63]. EA's protective effects to cell's DNA hypothetically could allow a gene expression increase towards regulatory enzymes of the carcinogenic process.

Considering all EA obtained outcomes, it can be assumed the cytotoxic and antiproliferative activities of the substance against OSCC rarely affect normal cell viability. Furthermore, all EA concentrations tested under 12.5 µg/mL, *in vitro*, were capable of decrease MMPs and increase TIMPs. Therefore, as concentrations equal or above 12.5 µg/mL showed cytotoxic effects against OSCC and concentrations higher than 25 µg/mL of EA were toxic to normal keratinocytes after 72 h treatment, it could be proposed an optimal concentration of EA against OSCC cells ranging from 12.5 µg/mL to 25 µg/mL, for *in vitro*

tests. In conclusion, EA is a promising therapeutic adjuvant to treat oral cancer, however, further preclinical and clinical studies are required to validate its potential.

Declarations

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Conflicts of interest

None.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Code availability

Not applicable.

Authors´ contribution

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Figures

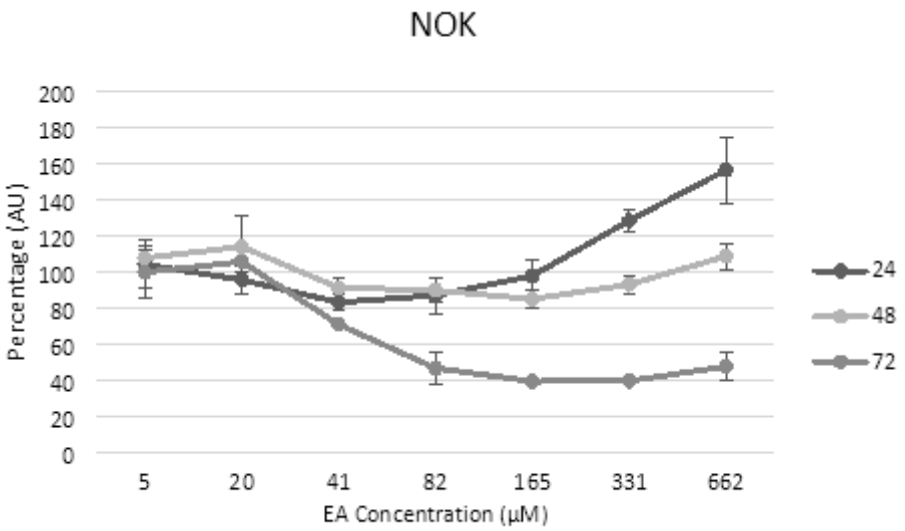

Figure 1

Cell viability assay after 24, 48 and 72 h of treatment of different doses of ellagic acid (EA).

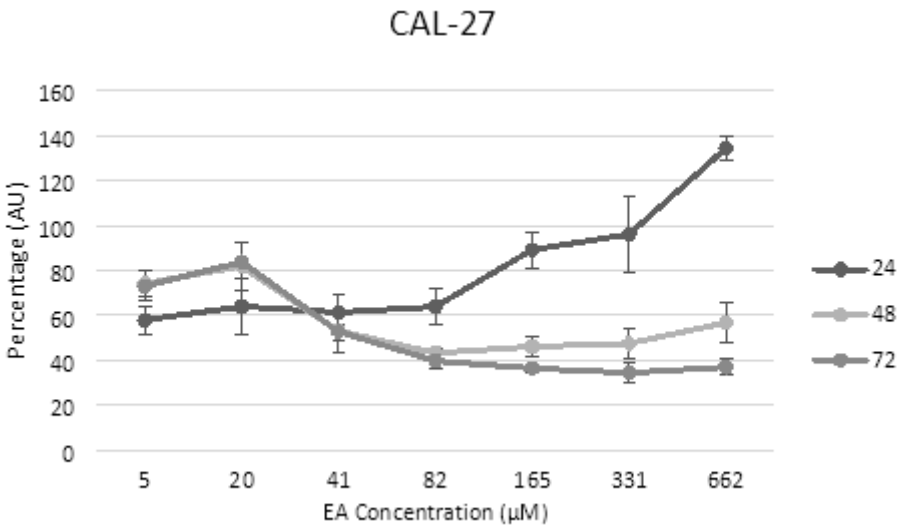

Figure 2

Quantification of MMP-2 and MMP-9 by means of Elisa, after 24, 48 and 72 h of treatment of different doses of ellagic acid (EA).

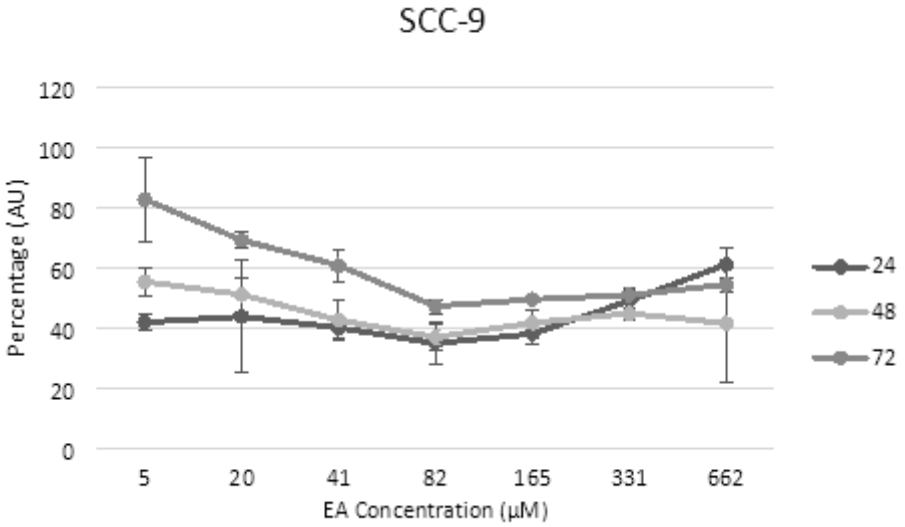

Figure 3

Quantification of TIMP-1 and TIMP-2 by means of Elisa, after 24, 48 and 72 h of treatment of different doses of ellagic acid (EA).