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EXPLORING THE ROLE OF THE KRAS PROTEIN IN THE MOLECULAR LANDSCAPE OF ORAL SQUAMOUS CELL CARCINOMA

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Cuprins

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is an aggressive tumor characterized by recurrence and therapeutic resistance, requiring selective strategies that affect tumor cells without causing toxicity to normal tissues. OSCC progression is associated with redox imbalance, the interplay between autophagy and apoptosis, and the activation of oncogenic pathways including KRAS/PI3K/AKT/mTOR and RAF/MEK/ERK.

The study is based on the hypothesis that apigenin, curcumin, and cordycepin differentially modulate the ROS–autophagy–apoptosis axis in OSCC cells compared to normal epithelial cells, through mechanisms associated with KRAS.

The objectives included the evaluation of selective cytotoxicity, investigation of oxidative stress, autophagy, apoptosis, and KRAS expression, as well as the analysis of compound combinations. The research employed a multiparametric in vitro approach on OECM-1 and HGEpiC cells, including viability assays, cytotoxicity testing, oxidative stress assessment, analyses of autophagy/apoptosis, and real-time morphological monitoring.

Overall, the study highlights the therapeutic potential of natural compounds in targeting molecular vulnerabilities of OSCC.

I. General part: Current state of knowledge

Chapter 1. OSCC

1.1 Characterization of OSCC

Data on the relationship between OSCC and KRAS have been previously published [40].

1.1.1 Mutations

OSCC develops through the accumulation of mutations in tumor suppressor genes (*TP53, CDKN2A*) and proto-oncogenes (*EGFR, PIK3CA*) [2,3]. The main risk factors include smoking, alcohol consumption, HPV infection, and exposure to genotoxic substances [3,5].

1.1.2 Phenotype

Clinically, OSCC manifests as ulcerative or exophytic lesions with an infiltrative pattern**.** These characteristics reflect underlying genetic alterations and influence treatment response [3].

1.1.3 Aggressiveness

OSCC has an aggressive behavior characterized by lymph node metastasis and local recurrence [2]. Resistance to conventional therapies justifies the development of targeted treatments aimed at specific molecular mechanisms [2,3].

1.1.4 Frequency

OSCC has a high global incidence, being more common in South and Southeast Asia, Africa, and Eastern Europe, predominantly in males [4]. The geographical distribution is influenced by tobacco and alcohol consumption [5]. In 2019, approximately 370,000–380,000 new cases and up to 180,000 deaths were reported worldwide [4].

1.2 The Role of KRAS in OSCC

Although KRAS gene mutations are more frequently reported in other types of cancer, such as pancreatic, colorectal, or lung cancers, RAS family–dependent signaling represents a central component of oncogenic networks involved in carcinogenesis, including OSCC [14,41].

1.2.1 Molecular Functions of KRAS

The *KRAS* proto-oncogene, a member of the RAS gene family, encodes a GTPase protein (KRAS) involved in the regulation of fundamental cellular processes such as growth, proliferation, and survival **[**41].

1.2.2 Prevalence of KRAS Mutations in OSCC

RAS mutations, particularly KRAS, are frequent in many types of tumors; however, they occur rarely in OSCC [51]. Nevertheless, activation of the MAPK pathway can support cellular proliferation and survival [18]. Available studies report low-frequency KRAS mutations, predominantly G12D and G12V, with variability influenced by geographic and ethnic factors as well as detection methods.

1.2.3 KRAS Protein and OSCC Development

The KRAS protein plays an important role in the pathogenesis of OSCC. Inhibition of ICMT affects the anchoring and activity of KRAS at the cell membrane, reducing cellular proliferation and migration and inducing apoptosis in TSCC [60]. Additionally, regulation of KRAS expression by miR-181d and miR-181a inhibits proliferation and promotes apoptosis in OSCC cells, supporting its role as a tumor promoter [61,62].

1.2.4 KRAS Expression Levels in OSCC

KRAS expression may be associated with invasion, metastasis, and aggressiveness in OSCC, suggesting a potential prognostic role [14,63,64]. Its involvement in the MAPK and PI3K/Akt/mTOR pathways indicates possible relevance for targeted therapies and treatment response. However, current evidence is limited, and further studies on larger cohorts are required for validation [14,63,64].

1.3. KRAS-Associated Signaling Pathways

1.3.1 KRAS and MAPK Signaling in OSCC

Overall, KRAS activates the MAPK pathway, promoting carcinogenesis through cellular proliferation, survival, and invasion. Thus, inhibitors of the Ras/Raf/MEK/ERK pathway represent promising therapeutic strategies in OSCC [46].

1.3.2 KRAS and Apoptosis in OSCC

In OSCC, the RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways are frequently activated, promoting cellular proliferation and resistance to apoptosis [79,80]. KRAS contributes to this process by activating cell survival signals, and inhibition of these pathways can induce apoptosis and reduce tumor progression [20].

1.3.3 KRAS and Autophagy in OSCC

In OSCC, autophagy regulates proliferation and apoptosis, playing a dual role in tumor progression [87–89]. KRAS can inhibit autophagy through the PI3K/AKT/mTOR pathway or activate it via MAPK, depending on the cellular context [91]. In KRAS-dependent tumors, autophagy may promote both cell survival and cell death, making KRAS and autophagy promising therapeutic targets [91–93].

Chapter 2. Bioactive Compounds with Antitumor Potential

2.1. Curcumin

2.1.1 Origin

Curcumin, the main polyphenol derived from *Curcuma longa* (turmeric), is traditionally used as both a food ingredient and a medicinal agent and is studied for its antioxidant, anti-inflammatory, and anticancer effects [28].

2.1.2 Chemical Structure

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), also known as diferuloylmethane, has the molecular formula $C_{21}H_{20}O_6$ and a symmetric structure composed of two phenolic rings connected by a central heptadiene chain.

2.1.3 Mechanism of Action and Biological Effects

Curcumin has anticancer and pro-apoptotic effects, modulating pathways involved in inflammation and proliferation, such as NF-κB, Wnt/β-catenin, and EGFR [95].

2.1.4 Bioavailability

Curcumin has low bioavailability due to poor solubility and rapid metabolism; however, nano-formulations can improve its stability and tumor delivery [96].

2.2. Apigenin

2.2.1 Origin

Apigenin is a natural flavonoid found in many plants, including chamomile, parsley, and celery, as well as in various fruits and vegetables [98].

2.2.2 Chemical Structure

Apigenin (4′,5,7-trihydroxyflavone), with the formula $C_{15}H_{10}O_5$, is a flavonoid composed of two benzene rings linked by a heterocyclic pyrone ring [98].

2.2.3 Mechanism of Action and Biological Effects

Apigenin exhibits antioxidant, anti-inflammatory, and antitumor activity [23].

2.2.4 Bioavailability

The therapeutic use of apigenin is limited by its low bioavailability, caused by poor solubility, limited intestinal absorption, and rapid metabolism [23].

2.3. Cordycepin

2.3.1 Origin

Cordycepin is a major bioactive compound derived from *Cordyceps* fungi, responsible for their pharmacological effects and traditionally used in Chinese medicine [33].

2.3.2 Chemical Structure

Cordycepin (3′-deoxyadenosine) is an adenosine analog characterized by the absence of a hydroxyl group at the 3′ position of the ribose [33].

2.3.3 Mechanism of Action and Biological Effects

The mechanisms of cordycepin include interference with RNA and protein synthesis, modulation of the AMPK and PI3K/AKT/mTOR pathways involved in proliferation and apoptosis, as well as antioxidant and anti-inflammatory effects, contributing to its anticancer activity [33–35,106,107].

2.3.4 Bioavailability

Cordycepin has low bioavailability due to limited absorption and rapid metabolism; however, nano-formulations can improve its stability and absorption [33,107].

II. Personal Contributions

Chapter 3: Hypothesis and General Objectives

3.1. Working Hypothesis

Curcumin, apigenin, and cordycepin exert selective cytotoxic effects on oral squamous cell carcinoma cells (OECM-1) compared to normal gingival epithelial cells (HGEpiC), through modulation of oxidative stress, autophagy, apoptosis, and KRAS protein expression.

Main objective of the study: To evaluate the comparative cytotoxic effects of curcumin, apigenin, and cordycepin on HGEpiC and OECM-1 cells, and to identify the underlying molecular mechanisms involved, with a focus on autophagy, apoptosis, and KRAS protein.

3.2. Research Objectives

1. Investigation of the cytotoxic effects of curcumin, apigenin, and cordycepin on HGEpiC and OECM-1 cell lines, in order to assess their antitumor selectivity.
2. Analysis of the autophagy process in OSCC under the influence of apigenin, to highlight its involvement in oral cancer progression.
3. Evaluation of oxidative stress induced by curcumin, apigenin, and cordycepin in OSCC, in order to identify oxidative vulnerabilities of tumor cells and the associated therapeutic potential.
4. Evaluation of apoptosis induced by curcumin, apigenin, and cordycepin in OSCC, to characterize the molecular mechanisms involved in their antitumor effects.
5. Investigation of the role of KRAS protein in OSCC progression and its influence on tumor cell response to treatment with cordycepin and curcumin.
6. Evaluation of the effects of the curcumin–cordycepin combination on OSCC, in order to identify a potential synergistic antitumor effect.
7. Comparative evaluation of the antitumor potential of curcumin, apigenin, and cordycepin in OSCC, in order to identify the compound with the highest cytotoxic selectivity toward tumor cells and protective effect on normal oral epithelial cells.

Chapter 4. General Methodology of the Research

4.1. Biocompounds

The biocompounds curcumin (≥90%, CAS: 458-37-7), apigenin (≥98%, CAS: 520-36-5, Cayman Chemical), and cordycepin (≥98%, CAS: 73-03-0, Cayman Chemical) were used. They were dissolved in DMSO, UV-sterilized (1 h), and diluted in culture medium to final concentrations of 150 μM and 50 μM.

4.2. Cell Culture

The HGEpiC cell line (Innoprot, Bizkaia, Spain; P10864) was cultured in supplemented Epithelial Cell Medium-Plus at 37 °C and 5% CO_2, and seeded in 96-well plates pre-coated with poly-L-lysine at 4×10^4 cells/well. The OECM-1 cell line (Sigma-Aldrich; SCC180) was cultured in RPMI-1640 supplemented with 5% FBS and 1% antibiotics at 37 °C and 5% CO_2, and seeded at the same density (4×10^4 cells/well) in 96-well plates without pre-coating.

4.3. Real-Time Cell Monitoring (HoloMonitor® M4)

Morphological changes, cell migration, and cell proliferation were evaluated using the HoloMonitor® M4 digital holographic microscope (PHI AB, Sweden). Cells were treated with apigenin, curcumin, cordycepin, and the curcumin–cordycepin combination, with untreated cells serving as control. Time-lapse images were acquired every 30 minutes for 48 hours and analyzed using HoloMonitor® App Suite 4.0.1.546, without fluorescent labeling. The system was maintained at 37 °C and 5% CO_2.

4.4. Mitochondrial Metabolic Activity (MTT Assay)

Mitochondrial activity was assessed using the MTT assay (Biotium, Cat. No. 30006) after 24 and 48 h of treatment with apigenin, curcumin, cordycepin, and the curcumin–cordycepin combination. Formazan was solubilized with isopropanol, and absorbance was measured at 570/630 nm using a FLUOstar Omega (BMG Labtech).

4.5. Lactate Dehydrogenase (LDH) Assay

LDH release was used as a marker of membrane integrity and cytotoxicity, using the LDH Cytotoxicity Assay Kit (Merck; Cat. No. MAK529A). Cells were treated with cordycepin, curcumin, and their combination for 24 and 48 h. LDH activity was measured at 500 nm.

4.6. Griess Test (NO Measurement)

For the evaluation of nitric oxide (NO) production, the Nitric Oxide Assay Kit (Merck; Cat. No. MAK454) was used, based on the Griess reaction for the detection of nitrites (NO_2^-). Cells were treated with cordycepin, curcumin, and their combination for 24 and 48 h, after which the medium was incubated with Griess reagent (1:2) for 60 min at 37 °C in the dark. Absorbance was measured at 540 nm.

4.7. Reactive Oxygen Species (ROS) Assay

Intracellular H_2O_2 levels were determined using the ROS-Glo™ H_2O_2 Assay (Promega; Cat. No. G8820) after treatment with apigenin, curcumin, cordycepin, and their combination. After 24 and 48 h, the reagent was added and incubated for 6 h at 37 °C, and luminescence was measured using a MyGlo® Reagent Reader (Promega), with the signal being proportional to H_2O_2 levels.

4.8. Reduced Glutathione (GSH) Assay

GSH was detected using BioTracker™ 625 Red GSH (Sigma-Aldrich; Cat. No. SCT036) according to the manufacturer's protocol; after 24 and 48 h of apigenin treatment, cells were

incubated for 30 min at 37 °C in the dark, and fluorescence was measured using an Optika IM-3LD4D microscope and FLUOstar Omega.

4.9. Intracellular ATP Measurement

Intracellular ATP content was measured using the CellTiter-Glo® 2.0 assay (Promega; Cat. No. G9241), following the manufacturer's protocol; after 24 and 48 h of apigenin treatment, reagent was added and incubated for 10 min in the dark, and luminescence was read on a MyGlo® Reagent Reader as RLU proportional to ATP levels.

4.10. Autophagy Assessment

Autophagic vacuoles were labeled using the Autophagy Assay Kit (Merck; Cat. No. MAK138) after 24 h of treatment with apigenin. Cells were incubated for 30 min with Autophagosome Detection Reagent at 37 °C in the dark, then washed with PBS. Fluorescence (Ex/Em = 480/530 nm) was captured using an Optika microscope and analyzed with ImageJ, with signal intensity reflecting autophagic activity.

4.11. Caspase-3/7 Activity Assay

For apoptosis detection, the Caspase-3/7 Green Detection Reagent (Merck; Cat. No. SCT100) was used, which detects caspase-3/7 activity via green fluorescence (Ex/Em ~488/530 nm). Cells were treated with apigenin, curcumin, cordycepin, and their combination for 24 and 48 h, then incubated for 30 min with the reagent (5 μM) at room temperature in the dark. Fluorescence was captured using an Optika microscope and quantified with a FLUOstar Omega.

4.12. Multiplex Apoptosis Assay

After 48 h of treatment, cells were lysed using Cell Signaling Lysis Buffer (Promega) with protease inhibitors, and lysates were stored at −80 °C for Luminex analysis. Early apoptosis-related proteins (p-AKT, p-BCL-2, p-p53, p-JNK, caspases 8/9) were measured using the MILLIPLEX® MAP 7-Plex kit (Merck Millipore). Total protein concentration was determined by Bradford assay (Sigma-Aldrich), and samples were normalized to 0.7 mg/mL. Analysis was performed on a Luminex® 200 platform based on mean fluorescence intensity (MFI).

4.13. RAS Isoform Analysis (KRAS, HRAS, NRAS)

After 48 h of treatment, cells were lysed and supernatants collected and stored at −80 °C for Luminex analysis. Proteins (Total Ras, RasG12D, RasG12V, pCRAF, pMEK1) were quantified using the Ras-Raf Oncoprotein Panel 6-Plex (Merck Millipore). Protein concentration was determined by Bradford assay (Sigma-Aldrich) and samples were standardized to 0.7 mg/mL. Analysis was performed on a Luminex® 200 platform based on mean fluorescence intensity (MFI).

4.14. Statistical Analysis

Statistical analyses were performed on raw data. Normality and homogeneity were assessed using Shapiro–Wilk and Levene tests. Depending on distribution, one-way/two-way ANOVA or Welch ANOVA with Tukey HSD or Games–Howell post-hoc tests was used, or Kruskal–Wallis with Dunn's test for non-parametric data. Correlations were analyzed using Pearson's test ($p < 0.05$). Mann–Whitney U test was applied for two-group comparisons (HoloMonitor® M4). Data are presented as mean ± SD ($n = 3$), as percentages vs. control, and plotted using Microsoft Excel.

Chapter 5. Comparative Evaluation of Curcumin Effects in OSCC and Normal Oral Cells (Study 1)

5.1. Introduction

Working hypothesis: curcumin exerts a differential effect on OECM-1 cells compared to HGEpiC cells, through modulation of oxidative stress, induction of apoptosis, and involvement of specific molecular pathways.

5.2. Materials and Methods

See Chapter 4.

5.3. Results

The findings regarding the effects of curcumin on normal oral epithelial HGEpiC cells and tumor OECM-1 cells have been previously published [108].

5.3.1. **Differential Cytotoxicity of Curcumin**

Curcumin reduced metabolic activity in both cell lines in a dose- and time-dependent manner, with the strongest effect observed at 150 µM, particularly after 48 h. These changes were accompanied by increased LDH release, indicating impaired cell membrane integrity and correlating with decreased viability. Nitric oxide levels showed a slight, non-significant increase in HGEpiC cells and were not significantly altered in OECM-1 cells. In contrast, ROS levels were significantly increased in both cell lines, with a much more pronounced effect in OECM-1 cells, indicating enhanced oxidative stress induced by the treatment.

5.3.2. **Caspase-3/7 Activity**

Curcumin increased caspase-3/7 activity in both cell lines, indicating the induction of apoptosis. In HGEpiC cells, the effect was more pronounced at 150 µM (24–48 h), whereas in OECM-1 cells a significant increase occurred earlier, at 24 h.

5.3.3. **Digital Holographic Microscopy: Cell Volume and Morphology**

In OECM-1 cells, curcumin progressively reduced confluence and proliferation at 50 and 150 µM, accompanied by decreased cell surface area and viability. Confluence was significantly reduced at 24 and 48 h, with no evidence of cell division under the treatment conditions.

5.3.4. Single-Cell Tracking: Cellular Shrinkage

Cu150 induced progressive cellular contraction in OECM-1 cells and a significant reduction in cell size, with decreases in height, length, volume, and surface area at 24 and 48 h, as well as a shift of the population toward smaller cells ($p < 0.001$).

5.3.5. Motility and Migration Inhibition

Curcumin treatment had differential effects on OECM-1 cell motility: cordycepin did not significantly influence cell displacement, whereas curcumin (Cu50, Cu150) and the combinations markedly reduced cellular motility and migration. Cells treated with Cu150 showed an almost complete absence of movement, observed rather as a consequence of cellular contraction rather than true migration, with significant differences compared to the control ($p < 0.001$).

5.3.6. Apoptosis-Related Proteins

In HGEpiC cells, curcumin increased caspase-8 at 50 µM, while at 150 µM it reduced caspase-8/9 levels as well as phosphorylated AKT and JNK proteins, correlating with decreased metabolic activity. In OECM-1 cells, apoptotic markers were no longer detectable at 150 µM

after 48 h, suggesting an earlier activation of apoptosis, whereas at 50 μM no significant differences were observed compared to the control.

5.3.7. Ras–Raf Signaling Panel

Basal levels of Ras–Raf proteins were similar between HGEpiC and OECM-1 cells. Curcumin reduced the activation of this pathway in a dose-dependent manner, particularly at 150 μM, with decreases in total Ras and MEK1 in HGEpiC cells and in RasG12D in OECM-1 cells, as well as reductions in pBRAF/pCRAF in both cell lines. Overall, it predominantly inhibited downstream signaling of the Ras–Raf pathway.

5.4. Discussion

The study shows that curcumin exerts dose- and time-dependent cytotoxic effects on HGEpiC and OECM-1 cells, with a stronger impact on tumor cells. It reduces mitochondrial metabolic activity, increases LDH release, and induces marked oxidative stress through ROS accumulation, correlated with caspase-3/7 activation and induction of apoptosis. In parallel, it also affects cell migration, proliferation, and morphology, particularly in OECM-1 cells, where cellular contraction, inhibition of division, and loss of motility are observed.

At the molecular level, curcumin inhibits RAS/RAF/MEK/ERK signaling, reducing Ras activation and downstream phosphorylation of proteins (BRAF, CRAF, MEK1), thereby contributing to the suppression of cell proliferation and survival. Although basal RAS protein levels do not differ between the two cell lines, treatment leads to a clear inhibition of the MAPK cascade.

Oxidative stress plays a central role in its mechanism of action, with ROS having a dual function: at moderate levels they support cellular adaptation, whereas at high levels they induce cell death. Tumor cells attempt to counteract this stress through antioxidant mechanisms and autophagy, but curcumin disrupts these adaptive responses.

Overall, the observed effects result from the interaction between increased ROS production, inhibition of survival pathways (RAS/RAF/MEK/ERK and PI3K/AKT/mTOR), activation of apoptosis, and modulation of autophagy processes, suggesting a potential antitumor mechanism of curcumin in OSCC.

5.5. Conclusions

In HGEpiC cells, ROS levels indicated a milder response, suggesting a lower redox vulnerability compared to OECM-1 cells. In OECM-1 cells, curcumin induced morphological changes, reduced motility, and activated apoptosis, associated with increased ROS production and caspase-3/7 activation. Ras–Raf pathway analysis suggests that the cytotoxic effects of curcumin involve inhibition of the RAS/RAF/MEK/ERK pathway, supporting its potential to modulate proliferative signaling and induce cell death in OSCC.

Chapter 6. Comparative Analysis of Apigenin Effects in OSCC and Normal Oral Cells (Study 2)

6.1. Introduction

Working hypothesis: apigenin exerts a differential effect on OECM-1 cells compared to HGEpiC cells through modulation of oxidative stress, induction of apoptosis and autophagy, involving specific molecular pathways.

6.2. Materials and Methods

See Chapter 4.

6.3. Results

The findings regarding the effects of apigenin on normal oral epithelial HGEpiC cells and tumor OECM-1 cells have been previously published [131].

6.3.1. Proliferation, Metabolic Activity, and Energy Status

Apigenin does not inhibit proliferation in HGEpiC cells, which maintain their organization and even show a slight increase in metabolic activity, whereas OECM-1 cells remain proliferative but exhibit a dose-dependent reduction in confluence and metabolic activity. Specifically, apigenin exerts minimal effects on normal cells but induces significant decreases in viability and metabolism in OECM-1 tumor cells, particularly after 48 h. ATP levels initially decrease in both cell lines at 24 h but subsequently increase at 48 h, especially in OECM-1 cells, suggesting metabolic adaptation and a possible shift toward anaerobic glycolysis. Overall, apigenin shows a selective effect, with limited impact on normal cells and inhibitory metabolic effects on tumor cells.

6.3.2. Oxidative Stress and Antioxidant Defense

Apigenin differentially influences redox balance depending on the cell type. In HGEpiC cells, GSH levels increase over time, suggesting maintained or slightly enhanced antioxidant capacity, while ROS levels show a modest increase, particularly at 48 h, without major toxic effects. In OECM-1 cells, GSH initially decreases at 24 h, followed by a partial recovery at 48 h, indicating activation of adaptive antioxidant mechanisms. Regarding oxidative stress, apigenin induces a significant increase in ROS in OECM-1 tumor cells, much more pronounced than in normal cells, with a peak at 24 h and partial reduction at 48 h, suggesting an adaptive response in surviving cells. Overall, apigenin induces a more pronounced redox imbalance in tumor cells, associated with oxidative stress induction and activation of compensatory antioxidant mechanisms, while normal cells remain relatively protected.

6.3.3. Modulation of Autophagy

Apigenin induces opposite autophagic responses in normal and tumor cells. In HGEpiC cells, treatment significantly increases autophagy, suggesting activation of a protective cellular mechanism positively correlated with concentration. In OECM-1 cells, apigenin reduces autophagy in a dose-dependent manner, indicating inhibition of this process, with a negative correlation between concentration and autophagic activity. Overall, autophagy is stimulated in normal cells and suppressed in tumor cells, suggesting a marked differential cellular response to apigenin.

6.3.4. Pro-survival vs Pro-apoptotic Signaling

Multiplex analysis revealed distinct apoptotic responses between the two cell types following apigenin treatment. In HGEpiC cells, apigenin significantly reduced survival signals

(AKT, BCL-2) and increased apoptotic markers (p53, caspase-8, and caspase-9), suggesting activation of regulated apoptotic pathways. In contrast, OECM-1 cells exhibited a predominantly anti-apoptotic profile, with increased AKT and BCL-2 levels, decreased JNK, and limited or absent caspase activation at higher concentrations, indicating a compromised apoptotic response and possible resistance to cell death.

Overall, the results demonstrate a clear differential effect of apigenin: HGEpiC cells maintain viability through adaptive responses (relatively stable proliferation, redox regulation via GSH, and moderate activation of apoptosis and autophagy), whereas OECM-1 cells are characterized by pronounced oxidative stress, dysregulation of redox homeostasis, inhibition of autophagy, impaired energy metabolism, and an incomplete apoptotic response. These differences suggest a selective vulnerability of OECM-1 tumor cells to treatment-induced redox imbalance and the ability of normal cells to activate coordinated protective mechanisms.

6.3.5. Late Apoptosis

Apigenin induces caspase-3/7 activation, indicating the onset of late apoptosis in both cell types. In HGEpiC cells, activation is moderate but significant at 48 h, suggesting a controlled, dose- and time-dependent apoptotic response. In OECM-1 cells, apoptosis is initially more pronounced at 24 h but decreases thereafter at 48 h, indicating incomplete apoptotic execution or survival of a resistant subpopulation. Overall, apigenin induces more stable apoptosis in normal cells and a transient or limited apoptotic response in tumor cells.

6.4. Discussion

This study highlights clear differences between normal HGEpiC cells and tumor OECM-1 cells in their response to apigenin, particularly at the redox, autophagic, and apoptotic levels. In HGEpiC cells, apigenin induces an adaptive response, with moderate increases in ROS, maintenance of redox balance through GSH, activation of autophagy as a protective mechanism, and limited apoptosis, without significantly affecting cell viability.

In contrast, in OECM-1 cells, treatment leads to pronounced oxidative stress, mitochondrial dysfunction, and disturbances in energy metabolism, with an initial decrease in ATP followed by metabolic adaptation toward glycolysis. At the same time, autophagy is inhibited, and the apoptotic response is incomplete, accompanied by increased anti-apoptotic signaling and dysregulation of the AKT/BCL-2/JNK axis.

Overall, apigenin acts as a cell type–dependent modulator, inducing homeostasis and protective mechanisms in normal cells, but redox imbalance and functional vulnerability in tumor cells, supporting its potential as an adjuvant agent in OSCC.

6.5. Conclusions

Apigenin exhibits cell type–dependent effects: in HGEpiC cells, it maintains redox homeostasis and viability, with balanced adaptive responses between autophagy and apoptosis, whereas in OECM-1 cells it induces pronounced oxidative stress, reduces autophagy, and reveals tumor vulnerability associated with altered JNK signaling. The results support the potential of the redox, autophagy, and JNK axes as therapeutic targets in OSCC. Study limitations include its exclusively in vitro design and incomplete assessment of late apoptosis, mainly based on caspase-3/7 analysis. Further in vivo validation and additional studies on the interaction between autophagy and apoptosis (e.g., AKT/mTOR signaling) are required.

Chapter 7. Comparative Analysis of Cordycepin Effects in OSCC and Normal Oral Cells (Study 3)

7.1. Introduction

Working hypothesis: cordycepin exerts a differential effect on OECM-1 cells compared to HGEpiC cells through modulation of oxidative stress, induction of apoptosis, and involvement of specific molecular pathways.

7.2. Materials and Methods

See Chapter 4.

7.3. Results

The findings regarding the effects of cordycepin on normal oral epithelial HGEpiC cells and tumor OECM-1 cells have been previously published [108].

7.3.1. Differential Cytotoxicity of Cordycepin

Cordycepin reduced metabolic activity in both cell lines in a dose- and time-dependent manner, without significant changes in LDH and NO levels. In contrast, it induced an increase in ROS in both cell types, which was much more pronounced in OECM-1 cells, where oxidative stress was markedly elevated, suggesting higher sensitivity of tumor cells compared to normal cells.

7.3.2. Caspase-3/7 Activity

Fluorescence microscopy images and fluorescence intensity quantification showed an increase in fluorescence intensity compared to control, directly proportional to caspase-3/7 activity, the executioner proteins of apoptosis, following cordycepin treatment in both HGEpiC and OECM-1 cells.

7.3.3. **Digital Holographic Microscopy: Cell Division and Apoptosis**

Cordycepin did not completely inhibit proliferation in OECM-1 cells at low and medium doses, where a progressive increase in cell density similar to the control was observed. In contrast, at the highest concentration (Co150), confluence remained stable and significantly reduced compared to the other groups, although signs of cell division were still present. At the same time, apoptotic-like morphological changes occurred, suggesting a cytostatic and partially cytotoxic effect at high cordycepin concentration.

7.3.4. **Early Apoptosis-Related Proteins**

Cordycepin induced differential apoptotic responses between the two cell types: in HGEpiC cells, activation of the intrinsic apoptotic pathway was observed through a significant increase in caspase-9, whereas in OECM-1 cells an increased expression of BCL-2 was detected, suggesting an anti-apoptotic response and possible resistance to cell death.

7.3.5. **Ras–Raf Signaling Panel**

Cordycepin induced a downward trend in Ras–Raf pathway proteins, but without statistically significant differences compared to control in either cell line.

7.4. Discussion

Cordycepin did not induce a relevant inflammatory response, as nitric oxide levels remained unchanged; however, it increased oxidative stress, more pronounced in OECM-1 cells than in HGEpiC, suggesting greater redox vulnerability of tumor cells. Biologically, the main effect was cytostatic, with maintenance or limited reduction of proliferation and only discrete

morphological/apoptotic changes, without significant activation of caspase-3/7, indicating the absence of a clear terminal apoptotic response.

At the molecular level, partial activation of the intrinsic apoptotic pathway was observed (increased caspase-9 in HGEpiC), alongside increased BCL-2 in OECM-1 cells, suggesting an anti-apoptotic profile in tumor cells that limits apoptotic execution. In addition, the RAS/RAF/MEK pathway remained largely stable, with minor, non-significant variations.

Overall, cordycepin acts predominantly as a cytostatic agent, inducing oxidative stress and early apoptotic signaling, but without full activation of programmed cell death, with its main effect being proliferation control rather than strong cytotoxicity.

7.5. Conclusions

In HGEpiC cells, the response to cordycepin was milder, characterized by minimal ROS increases compared to OECM-1. In OECM-1 cells, cordycepin exerted mainly cytostatic effects, with relatively stable cell density and no significant increase in caspase-3/7 activity.

Chapter 8. Comparative Analysis of Curcumin–Cordycepin Combination Effects (Study 4)

8.1. Introduction

Working hypothesis: the combination of curcumin and cordycepin exerts a differential effect on OECM-1 cells compared to HGEpiC cells through modulation of oxidative stress, induction of apoptosis, and involvement of specific molecular pathways.

8.2. Materials and Methods

See Chapter 4.

8.3. Results

The findings regarding the effects of the curcumin–cordycepin combination on normal oral epithelial HGEpiC cells and tumor OECM-1 cells have been previously published [108].

8.3.1. **Differential Cytotoxicity of the Combination**

The curcumin–cordycepin combination induced a dose- and time-dependent cytotoxic effect in both cell lines, with a marked decrease in MTT metabolic activity, much more pronounced in OECM-1 cells, especially at CC150, where viability was almost completely suppressed. These changes were supported by increased LDH levels, indicating membrane damage, more evident at higher doses and in tumor cells. NO levels remained essentially unchanged, suggesting the absence of a major inflammatory response. In contrast, ROS levels increased significantly in both cell lines, but extremely pronounced in OECM-1 cells, where values reached thousands to over ten thousand at CC150, indicating severe oxidative stress as the main mechanism of selective cytotoxicity toward tumor cells.

8.3.2. **Caspase-3/7 Activity**

The combined treatment induced caspase-3/7 activation in both cell lines, with a more pronounced effect at CC150 and at 48 h, indicating apoptosis induction in response to treatment-induced stress. In HGEpiC cells, caspase activation was dose- and time-dependent, with significant increases at both 24 h (CC50 and CC150) and 48 h (CC150), suggesting a progressive apoptotic response. In OECM-1 cells, activation was more limited, with significant increases only at CC150 and no marked time-dependent amplification, indicating a more restricted apoptotic response in tumor cells.

8.3.3. **Digital Holographic Microscopy: Antitumor Effects**

Treatment with CC50 and CC150 induced significant morphological and functional changes in OECM-1 cells, particularly at CC150, where reduced cell size, complete inhibition of division, and loss of motility were observed. These effects were supported by 2D images and a progressive decrease in cell confluence over time, indicating strong inhibition of proliferation. The reduction in cell-occupied area was significant at 24 h and 48 h compared to baseline, confirming the pronounced antiproliferative effect of the combination, especially at the higher concentration.

8.3.4. Early Apoptosis-Related Proteins

In HGEpiC cells, the curcumin–cordycepin combination generally reduced survival proteins and induced modest activation of extrinsic apoptosis (↑ caspase-8 at CC50), without major changes in BCL-2. In OECM-1 cells, effects were more complex and dose-dependent: at CC50 mixed pro-survival and pro-apoptotic signals were observed, while at CC150 increased

caspase-8, phosphorylated BCL-2, and phosphorylated JNK were detected, together with decreased phosphorylated p53, suggesting a major imbalance in cellular signaling.

8.3.5. Ras–Raf Signaling Panel

The Ras–Raf analysis indicates that this combined treatment reduces the activity of this signaling pathway. In HGEpiC cells, significant decreases were observed in total Ras, RasG12D, and MEK1 at CC150, whereas in OECM-1 cells the effect was more limited, being significant only for MEK1 at the same concentration. Overall, the results suggest a stronger inhibition of Ras–Raf signaling in normal cells compared to tumor cells.

8.4. Discussion

The curcumin–cordycepin combination exerted strong dose-dependent cytotoxic effects, especially at CC150, where it drastically reduced metabolic activity and mitochondrial function in both cell lines, with more pronounced effects in OECM-1 cells. These changes were accompanied by increased LDH, indicating membrane damage, as well as morphological alterations, inhibition of migration, and proliferation arrest, suggesting a pronounced curcumin-driven antitumor effect.

At the molecular level, the combination activated apoptosis through both extrinsic and intrinsic pathways, with different responses between normal and tumor cells: HGEpiC cells predominantly exhibited protective mechanisms, while OECM-1 cells showed mixed pro- and anti-apoptotic signaling (AKT, BCL-2, JNK), indicating stress adaptation and partial resistance to cell death. Nevertheless, increased caspase-3/7 activity in both lines confirms the onset of terminal apoptosis.

Ras–Raf analysis revealed general inhibition of proliferative signaling (total Ras and MEK1), without affecting mutant RasG12D and RasG12V forms, suggesting an effect on downstream signaling rather than oncogenic mutations. Overall, the curcumin–cordycepin combination induces oxidative stress, inhibits proliferation, and activates apoptosis, with stronger effects in OECM-1 tumor cells, supporting its therapeutic potential in OSCC.

8.5. Conclusions

The curcumin–cordycepin combination reflected the morphological and functional changes induced by curcumin, with cordycepin coexisting without counteracting its effects. In OECM-1 cells, the treatment led to loss of motility, reduced cell size, and apoptosis activation, most likely driven by ROS accumulation. Overall, these results suggest that both curcumin and cordycepin contribute to increased ROS production and modulation of Ras–Raf pathway proteins, supporting further investigation in in vivo models as a potential therapy for OSCC.

Chapter 9. Conclusions and Personal Contributions

9.1 Conclusions

This thesis investigated the effects of curcumin, apigenin, cordycepin, and the curcumin–cordycepin combination on normal HGEpiC cells and tumor OECM-1 cells, focusing on the role of oxidative stress, autophagy, apoptosis, and the Ras–Raf–MEK signaling pathway in OSCC. Curcumin induced the strongest cytotoxic effect in tumor cells by increasing ROS levels, activating apoptosis, and inhibiting proliferative signaling. Apigenin showed context-dependent effects, activating autophagy and maintaining homeostasis in normal cells, while inducing oxidative stress and autophagy inhibition in tumor cells. Cordycepin acted predominantly as a cytostatic agent, limiting proliferation without marked apoptosis. The curcumin–cordycepin combination amplified oxidative stress and apoptotic effects, especially in OECM-1 cells, through inhibition of the Ras–Raf–MEK pathways. Overall, the results highlight the redox and signaling vulnerabilities of OSCC cells and the potential of natural compounds as selective adjuvant therapies.

9.2 Personal Contributions

This thesis provides an integrated contribution to oral oncology by comparatively evaluating the effects of curcumin, apigenin, and cordycepin on tumor (OECM-1) and normal (HGEpiC) oral epithelial cells. The main novelty lies in the simultaneous analysis of oxidative stress, autophagy, apoptosis, and oncogenic signaling pathways (including KRAS) within a biologically relevant comparative model.

Methodologically, the study combines viability assays (MTT, LDH), redox stress markers (ROS, NO, GSH, ATP), multiplex apoptosis analysis, autophagy assessment, and real-time imaging (HoloMonitor M4), providing a dynamic and multiparametric characterization of cellular responses.

The results highlight differential compound effects: curcumin exhibits the strongest cytotoxicity via oxidative stress and Ras–Raf inhibition; apigenin modulates redox balance and autophagy in a cell type–dependent manner; cordycepin is predominantly cytostatic; and the curcumin–cordycepin combination enhances oxidative stress and antiproliferative effects in tumor cells.

Overall, the thesis demonstrates that these natural compounds can exploit redox and signaling vulnerabilities in OSCC, suggesting their potential as adjuvant therapeutic agents and a foundation for combined treatment strategies in oral cancer, while emphasizing the need for validation in more complex models.

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1. **Voicu Bălașea B**, Stan MS, Dinescu M, Imre M, Rădulescu R, Cernega A, Musteanu M, Ripszky A, Pituru SM. Differential effects of apigenin on normal and squamous oral epithelial cells reveal redox–autophagy signaling vulnerabilities in OSCC. Int J Mol Sci. 2026;27:2091, FI -4,9/2026, Q1, capitolul VI, pag. 63-88

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