



Article

Differential Effects of Apigenin on Normal and Squamous Oral Epithelial Cells Reveal Redox–Autophagy Signaling Vulnerabilities in OSCC

Bianca Voicu Balasea ¹, Miruna-Silvia Stan ², Miruna Dinescu ³, Marina Imre ³, Radu Radulescu ⁴, Ana Cernega ⁵, Monica Musteanu ^{6,*}, Alexandra Ripszky ^{1,4,*,†} and Silviu Mirel Pituru ^{1,5,†}

¹ The Interdisciplinary Center for Dental Research and Development, “Carol Davila” University of Medicine and Pharmacy, 19-21 Jean Louis Calderon, 030167 Bucharest, Romania; bianca.voicu-balasea@drd.umfcd.ro (B.V.B.); silviu.pituru@umfcd.ro (S.M.P.)

² Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Bucharest, 91–95 Splaiul Independentei, 050095 Bucharest, Romania; miruna.stan@bio.unibuc.ro

³ Department of Prosthodontics, Faculty of Dentistry, “Carol Davila”; University of Medicine and Pharmacy, 37 Dionisie Lupu Street, District 2, 020021 Bucharest, Romania; miruna.dinescu@drd.umfcd.ro (M.D.); marina.imre@umfcd.ro (M.I.)

⁴ Department of Biochemistry, Faculty of Dental Medicine, “Carol Davila”; University of Medicine and Pharmacy, 17-23 Plevnei Street, 020021 Bucharest, Romania; radu.radulescu@umfcd.ro

⁵ Department of Organization, Professional Legislation and Management of the Dental Office, Faculty of Dental Medicine, “Carol Davila” University of Medicine and Pharmacy, 17-23 Plevnei Street, 020021 Bucharest, Romania; ana.cernega@umfcd.ro

⁶ Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of Complutense of Madrid, 28040 Madrid, Spain

* Correspondence: mmustean@ucm.es (M.M.); alexandra.ripszky@umfcd.ro (A.R.)

† These authors contributed equally to this work.

Abstract

The aim of this study was to compare the responses of normal human gingival epithelial cells (HGEpiC) and oral squamous cell carcinoma cells (OECM-1) to apigenin, a natural flavonoid, focusing on redox balance, autophagy, and apoptosis. This study is among the first to directly compare apigenin-induced responses in normal and cancerous oral epithelial cells. Cells were exposed to apigenin for 24 or 48 h, with untreated cells as controls. Mitochondrial activity, ATP, ROS (H₂O₂), and GSH were measured. Proliferation and morphology were monitored using HoloMonitor[®] M4. Autophagy was assessed by fluorescent vacuole labeling, and apoptosis-related proteins (p-AKT, p-BCL-2, p-p53, p-JNK, caspase-8/9) by Luminox assay. Late apoptosis was evaluated by caspase-3/7 activity. Apigenin elicited a differential response: in HGEpiC cells, it was non-cytotoxic and increased metabolic activity, induced a moderate ROS increase, and activated autophagy as a pro-survival mechanism; in contrast, OECM-1 cells exhibited a significant reduction in metabolic activity, a marked ATP decrease at 24 h, and a pronounced ROS increase. These alterations were associated with reduced autophagy and decreased p-JNK signaling. These findings indicate that apigenin exerted no harmful effects on HGEpiC cells, while inducing redox imbalance in OECM-1 cells, highlighting a context-dependent cellular response.

Keywords: apigenin; oral squamous cell carcinoma; gingival epithelial cells; ROS; autophagy



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1. Introduction

Oral squamous cell carcinoma (OSCC) is the most common type of oral cancer, accounting for over 90% of all cases of head and neck squamous cell carcinoma (HNSCC) [1]. Mutagenic factors in the oral cavity include smoking, alcohol consumption, HPV infection, and genotoxicity of some modern dental materials, including those used in 3D printing technologies [2]. OSCC is among the most aggressive HNSCC types due to metastasis, recurrence, and resistance to apoptosis. The 5-year survival rate after surgery, radiotherapy, and targeted therapies remains approximately 60% [3]. These challenges require an alternative approach that emphasizes molecular signaling pathways focusing on oxidative stress, autophagy, and apoptosis, and developing treatment methods that distinguish between healthy and cancerous cells.

Reactive oxygen species (ROS) are key mediators linking oxidative stress, autophagy, and apoptosis. In normal cells, moderate levels of ROS act as regulatory signals that maintain cellular homeostasis. In malignant cells, ROS are chronically elevated, promoting proliferation and resistance to apoptosis. Tumor cells utilize ROS as signaling molecules [4–6]. Although elevated levels of ROS support tumor cell survival through the PI3K-AKT-mTOR axis, excessive oxidative stress can trigger autophagy or apoptosis [5–8]. Oxidative stress can activate JNK, which phosphorylates BCL-2, facilitating Beclin-1 release and autophagy initiation. If stress persists, intrinsic apoptosis is triggered through activation of caspase-9 and effector caspases 3/7 [9–11].

Apigenin (4',5,7-trihydroxyflavone) is a natural flavonoid found in chamomile, parsley, and celery, with anti-inflammatory, antioxidant, and antitumor properties via inhibition of PI3K/AKT/mTOR, NF- κ B, and MAPK cascades [12–14]. It induces apoptosis and inhibits proliferation in several epithelial cancers, such as hepatic, pancreatic, colorectal, pulmonary, breast, prostate, and skin cancers [12–14]. In oral tumor cells (SCC-25), apigenin reduces viability, causes cell-cycle arrest, and apoptosis [15], while in normal skin keratinocytes (HaCaT), it shows antioxidant/photoprotective effects [16]. However, its impact on redox status and autophagy remains insufficiently explored, presenting a critical knowledge gap.

Previous studies have examined apigenin in normal oral cell types such as periodontal ligament cells (hPDL), demonstrating anti-inflammatory effects via HO-1 induction [17]. Similarly, investigations using SCC-25 tumor cells compared with HaCaT (a non-oral line) provided limited relevance to oral tissue physiology [15]. Although previous studies have targeted the effect of apigenin in oral carcinoma cell lines, such as SCC-25, SCC-9, and CAL-27 [15,18,19], to date, no research has directly compared the effects of apigenin on HGEpiC versus OECM-1 cells, leaving a gap in understanding its differential redox, autophagic, and apoptotic effects in OSCC. HGEpiC cells are isolated from healthy gingival epithelium [20], whereas OECM-1 cells originate from malignant gingival epithelial tissue and represent a well-established in vitro model of OSCC [21]. These two cell lines were selected because they share a common gingival epithelial origin, allowing a direct comparison between normal and malignant phenotypes.

The novelty of our study lies in providing the first integrative analysis of ROS, ATP, autophagy, and apoptotic pathways in HGEpiC versus OECM-1 cells, combining real-time monitoring of cell morphology using HoloMonitor[®] M4 with biochemical and molecular assays.

Accordingly, this study aims to comparatively evaluate the effects of apigenin on HGEpiC and OECM-1 cells, with a focus on potential differential modulation of the redox–autophagy–apoptosis triad and elucidating molecular vulnerabilities specific to OSCC cells. If behavioral differences between HGEpiC and OECM-1 cells are observed, they could provide a key starting point for understanding the molecular mechanisms underlying chemotherapy resistance in OSCC.

2. Results

2.1. Impact on Proliferation, Metabolic Activity, and Energy Status

Phase-contrast holographic images provided information on the proliferation and morphology of HGEpiC and OECM-1 cells following exposure to apigenin. HGEpiC cells maintained their characteristic honeycomb organization, and the cultures showed a larger density and structure at 48 h, both in the control and after apigenin treatments (50 and 150 μ M), indicating sustained growth (Figure 1a). OECM-1 cells showed proliferative activity in the control, characterized by numerous dimensions (in mitotic and protrusive activity) at all monitored time intervals (0, 24, 48 h), indicating rapid division. Proliferation also continued in OECM-1 cells after apigenin treatment (Figure 1b).

The HoloMonitor[®] M4 plots support the morphological observations. In HGEpiC cells, proliferation occurred in both control and treated groups, with slightly higher cell confluence observed after apigenin treatment compared with the control at 48 h (Figure 1c). One-way ANOVA revealed a significant main effect of concentration on HGEpiC cell confluence at 24 h ($F(2,6) = 5.438$, $p = 0.045$), with lower confluence in the 50 μ M group compared with the control ($p = 0.046$). Time-dependent increases in confluence were observed in the 150 μ M group ($F(2,6) = 6.125$, $p = 0.036$) and in the control group (Welch ANOVA: $F(2,2.819) = 71.95$, $p = 0.004$). Post hoc Tukey test showed significant increases at 48 h versus 0 h in the control and 150 μ M groups. In OECM-1 cells, proliferation was also observed in both the control and treated groups, with a slightly lower cell confluence following treatment with 150 μ M apigenin compared to the control (Figure 1d). Two-way ANOVA revealed a significant main effect of concentration on OECM-1 cell confluence ($F(2,18) = 3.924$, $p = 0.039$) and a strong main effect of time ($F(2,18) = 40.919$, $p < 0.001$), with no significant concentration $\times$ time interaction ($F(4,18) = 0.056$, $p = 0.994$), indicating a consistent concentration effect across time points. Post hoc Tukey test showed significantly lower confluence in the 150 μ M group compared with control ($p = 0.032$), while no differences were observed between 50 μ M and control. One-way ANOVA confirmed a significant time-dependent increase in confluence within all groups (control: $F(2,6) = 7.628$, $p = 0.022$; 50 μ M: $F(2,6) = 11.98$, $p = 0.008$; 150 μ M: $F(2,6) = 55.02$, $p = 0.001$). Post hoc Tukey test showed significant increases at 48 h versus 0 h in all conditions.

Following the MTT test (Figure 1e), in HGEpiC cells, mitochondrial metabolic activity significantly increased only at the highest concentration of apigenin, by 20% compared to the control after 24 h of exposure. One-way ANOVA indicated a significant effect of concentration ($F(2,6) = 6.489$, $p = 0.032$). Post hoc Tukey test indicated that 150 μ M significantly increased metabolic activity compared with the control at 24 h ($p = 0.028$), while no significant differences were observed between treated groups and control at 48 h. Two-way ANOVA indicated a time-dependent effect on cell metabolic activity (ANOVA $F(1,8) = 28.204$, $p < 0.001$), while the effects of concentration ($p = 0.978$) and the concentration $\times$ time interaction ($p = 0.322$) were not significant. Post hoc Tukey test indicated that only in the control group did metabolic activity increase significantly from 24 h to 48 h ($p = 0.009$), suggesting that this increase over time reflects a normal physiological response.

In contrast, OECM-1 cells showed significant reductions in mitochondrial metabolic activity at both doses and time points. After 24 h, a decrease of approximately 19% compared to the control was observed at 50 μ M. After 48 h, a more pronounced decrease of 23% (50 μ M) and 31% (150 μ M) was observed. One-way ANOVA indicated a significant effect of concentration at 24 h ($F(2,6) = 6.602$, $p = 0.031$). Post hoc Tukey test indicated that 50 μ M significantly reduced metabolic activity compared with the control ($p = 0.029$). Two-way ANOVA indicated a significant effect of concentration ($F(2,12) = 21.24$, $p < 0.001$) and time ($F(1,12) = 18.27$, $p = 0.001$), as well as a significant concentration $\times$ time interaction ($F(2,12) = 4.445$, $p = .036$). Post hoc Tukey test indicated that at 48 h, both 50 μ M ($p = 0.007$)

and 150 μM ($p < 0.001$) significantly reduced metabolic activity compared with the control. In the control group, metabolic activity increased from 24 h to 48 h ($p = 0.009$).

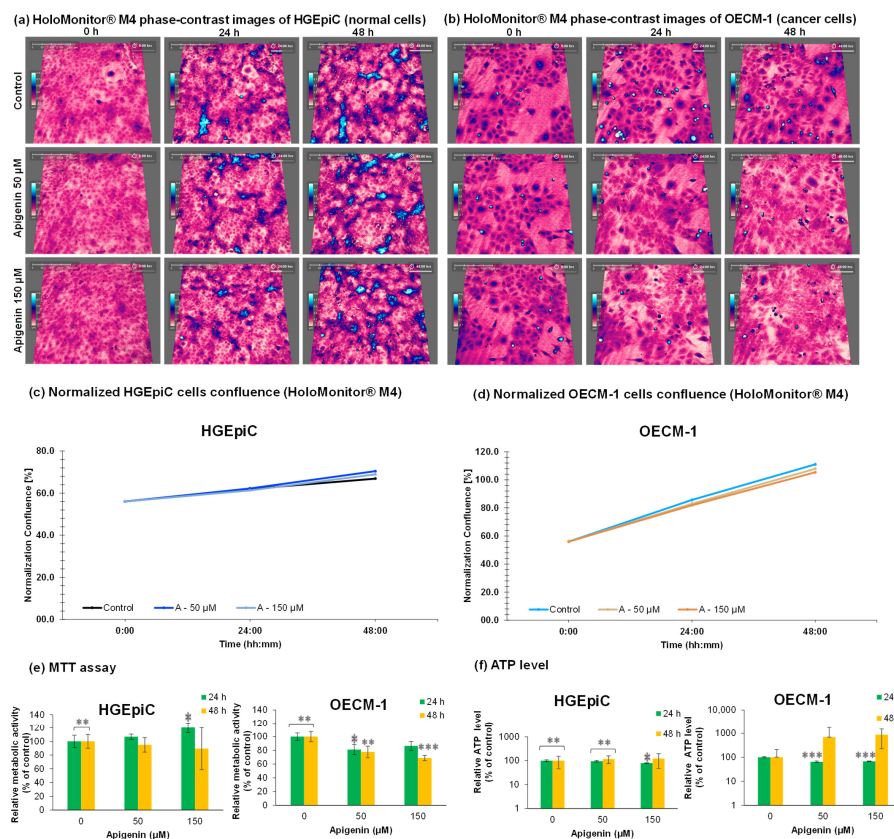


Figure 1. Comparative analysis of the effect of apigenin treatment (50 μM and 150 μM) on HGEpiC and OECM-1 cells in terms of proliferation, metabolic activity, and ATP production after 24 and 48 h. (a) Representative holographic phase contrast images of HGEpiC cells at 0, 24, and 48 h following exposure to apigenin. Controls represent untreated cells; (b) Representative holographic phase contrast images of OECM-1 cells at 0, 24, and 48 h following exposure to apigenin. Controls represent untreated cells; (c) Representative plot showing confluence of HGEpiC cells at 0, 24, and 48 h following exposure to apigenin. Controls represent untreated cells. Data are presented as mean $\pm$ SD ($n = 3$). One-way ANOVA followed by Tukey post hoc test; 50 μM vs. control * $p < 0.05$ (24 h); 0 h vs. 48 h * $p < 0.05$ (150 μM); Welch ANOVA followed by Games–Howell post hoc test; 0 h vs. 48 h ** $p < 0.01$ (control). (d) Representative plot showing confluence of OECM-1 cells at 0, 24, and 48 h following exposure to apigenin. Controls represent untreated cells. Data are presented as mean $\pm$ SD ($n = 3$). Two-way ANOVA followed by Tukey post hoc test; 150 μM vs. control * $p < 0.05$; One-way ANOVA followed by Tukey post hoc test; 0 h vs. 48 h (* $p < 0.05$ control; ** $p < 0.01$ 50 μM ; *** $p < 0.001$ 150 μM); (e) Quantification of the metabolic mitochondrial activity of HGEpiC vs. OECM-1 cells by MTT assay after 24 and 48 h of exposure to apigenin. Controls, symbolized as 0 on the graph, represent untreated cells. Data are presented as mean $\pm$ SD ($n = 3$). HGEpiC: One-way ANOVA; 150 μM vs. control * $p < 0.05$ (24 h); Two-way ANOVA followed by Tukey post hoc test 24 h vs. 48 h ** $p < 0.01$ (control). OECM-1: One-way ANOVA; 50 μM vs. control * $p < 0.05$ (24 h); Two-way ANOVA followed by Tukey post hoc test; 50 μM vs. control ** $p < 0.01$ (48 h); 150 μM vs. control *** $p < 0.001$ (48 h); control 24 h vs. control 48 h ** $p < 0.01$. (f) Quantification of the total ATP level in HGEpiC vs. OECM-1 cells after 24 and 48 h of exposure to apigenin. Controls, symbolized as 0 on the graph, represent untreated cells. Data are presented as mean $\pm$ SD ($n = 3$). HGEpiC: One-way ANOVA followed by Tukey post hoc test; 150 μM vs. control * $p < 0.05$ (24 h); Two-way ANOVA followed by Tukey post hoc test; 24 h vs. 48 h (control) ** $p < 0.01$; 24 h vs. 48 h (50 μM) * $p < 0.05$. OECM-1: One-way ANOVA followed by Tukey post hoc test; 50 μM vs. control *** $p < 0.001$ (24 h); 150 μM vs. control *** $p < 0.001$ (24 h). The Y-axis is plotted on a $\log_{10}$ scale to allow visualization of large increases.

Regarding the total ATP level in HGEpiC cells, at 24 h, treatment with 150 μ M apigenin led to a significant 20% decrease in ATP compared with the control, while at 48 h, ATP levels increased by 22% relative to the control (Figure 1f). One-way ANOVA indicated a significant effect of concentration at 24 h ($F(2,6) = 8.758$, $p = 0.017$). Post hoc Tukey test indicated that 150 μ M significantly reduced ATP level compared with the control ($p = 0.017$), while no significant differences were observed between treated groups and the control at 48 h.

Two-way ANOVA revealed a significant effect of time on ATP levels ($F(1,12) = 35.982$, $p < 0.001$), while the effects of concentration ($p = 0.757$) and the concentration $\times$ time interaction ($p = 0.343$) were not significant. Post hoc Tukey test indicated that ATP decreased significantly from 24 h to 48 h in the control group ($p = 0.008$) and in the 50 μ M group ($p = 0.032$), but not in the 150 μ M group. Thus, absolute ATP levels are higher at 24 h than at 48 h. However, when compared with the control at the same time point, ATP in treated cells is lower than that of the control at 24 h and higher than that of the control at 48 h.

In contrast, the ATP level in OECM-1 cells compared to the control decreased significantly at 24 h, followed by a marked increase at 48 h. Therefore, at 50 and 150 μ M apigenin, a decrease of 35% and 33%, respectively, was observed after 24 h, while after 48 h, a 5.8-fold and 7.7-fold increase, respectively, was observed, suggesting metabolic recovery (Figure 1f). One-way ANOVA indicated a significant effect of concentration ($F(2,6) = 48.97$, $p < 0.001$). Post hoc Tukey tests indicated that at 24 h, both 50 μ M and 150 μ M significantly reduced viability compared with the control ($p < 0.001$). At 48 h, no significant differences were observed between treated groups and the control, nor between time points.

Despite the reduction in mitochondrial dehydrogenase activity (MTT assay results), intracellular ATP levels increased approximately 5–8-fold at 48 h, suggesting bioenergetic compensation through a metabolic shift toward anaerobic glycolysis for ATP production under oxidative stress.

2.2. Oxidative Stress and Antioxidant Defense

Fluorescent GSH labeling showed an increase in fluorescence intensity at 24 and 48 h in HGEpiC compared to the control, consistent with an increase in GSH level and antioxidant defense (Figure 2a,c). Welch ANOVA indicated a significant effect of time on GSH ($F(1,2.851) = 47.02$, $p = 0.007$). Post hoc Games–Howell indicated that only in the control group, GSH increased significantly from 24 h to 48 h ($p = 0.007$).

In contrast, in OECM-1 cells, fluorescence intensity decreased at 24 h compared with the control, indicating a reduction in GSH levels, whereas at 48 h fluorescence increased, indicating partial recovery of antioxidant capacity (Figure 2b,c). One-way ANOVA revealed a significant increase in GSH levels from 24 h to 48 h in the control group ($F(1,4) = 82.66$, $p < 0.001$) and in the 50 μ M group ($F(1,4) = 101.3$, $p < 0.001$). In the 150 μ M group, Welch's ANOVA confirmed a significant increase in GSH over time ($F(1,2.08) = 60.95$, $p = 0.014$). Post hoc tests (Tukey or Games–Howell as appropriate) showed that GSH increased significantly from 24 h to 48 h in all groups.

Specifically, when compared to control, ROS (H_2O_2) levels in HGEpiC increased mainly at 48 h following apigenin exposure, reaching a statistically significant increase of 83% at 50 μ M and approximately 40% at 150 μ M (Figure 2d). One-way ANOVA revealed a significant effect of treatment on ROS levels at 48 h ($F(2,6) = 6.689$, $p = 0.03$). Post hoc Tukey analysis showed a significant increase in ROS levels in the 50 μ M group compared with the control ($p = 0.025$). At 24 h, no treatment concentration differed significantly from the control.

Further one-way ANOVA comparisons showed that ROS levels in the control group were significantly higher at 24 h than at 48 h ($F(1,4) = 17.85$, $p = 0.013$). Similarly, ROS levels

in the 150 μM group were significantly higher at 24 h compared with 48 h ($F(1,4) = 8.491$, $p = 0.044$). The decrease in absolute ROS levels observed at 48 h occurred in both control and treated groups and therefore cannot be attributed to apigenin treatment.

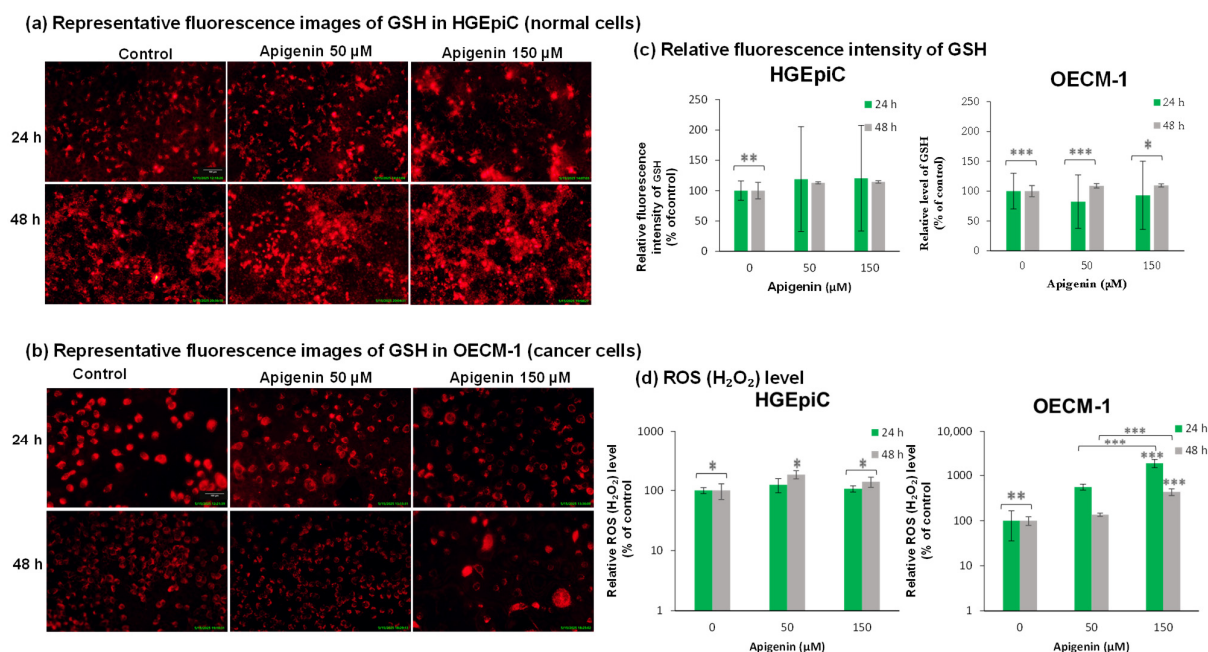


Figure 2. Differential redox responses to apigenin treatment (50 μM and 150 μM) in HGEpiC cells and OECM-1 cells after 24 and 48 h. Fluorescence microscopy images of the glutathione (GSH) activity in: (a) HGEpiC and (b) OECM-1 cells. Controls represent untreated cells. Scale bar: 100 μm (applies to all images); (c) Quantification of relative GSH fluorescence intensity using FLUOstar Omega microplate reader. Controls, symbolized as 0 on the graph, represent untreated cells. Data are presented as mean $\pm$ SD ($n = 3$). HGEpiC: Welch's ANOVA followed by Games–Howell post hoc test; 24 h vs. 48 h $** p < 0.01$ (control). OECM-1: One-way ANOVA followed by Tukey post hoc test; 24 h vs. 48 h $*** p < 0.001$ (control); 24 h vs. 48 h $*** p < 0.001$ (50 μM); Welch's ANOVA followed by Games–Howell post hoc test; 24 h vs. 48 h $* p < 0.05$ (150 μM) (d) Quantification of ROS (H_2O_2) relative luminescence intensity. Controls, symbolized as 0 on the graph, represent untreated cells. Data are presented as mean $\pm$ SD ($n = 3$). HGEpiC: One-way ANOVA followed by Tukey post hoc test; 50 μM vs. control $* p < 0.05$ (at 48 h); 24 h vs. 48 h $* p < 0.05$ (control); 24 h vs. 48 h $* p < 0.05$ (150 μM). OECM-1: Two-way ANOVA followed by Tukey post hoc test; 150 μM vs. control $*** p < 0.001$ (at 24 h and at 48 h); 150 μM vs. 50 μM $*** p < 0.001$ (at 24 h and at 48 h); One-way ANOVA; 24 h vs. 48 h $** p < 0.01$ (control). The Y-axis is plotted on a log₁₀ scale to allow visualization of large increases.

Surprisingly, apigenin induced a time-dependent response in terms of oxidative stress in OECM-1 cells. Although ROS levels increased approximately 17-fold at 24 h and approximately 3.3-fold at 48 h compared to control, they partially recovered over time. At 24 h, H_2O_2 treatment significantly increased ROS levels, while at 48 h, ROS levels decreased relative to 24 h, suggesting a partial restoration of antioxidant mechanisms in surviving cells (Figure 2d). Two-way ANOVA revealed a significant main effect of concentration on ROS ($F(2,12) = 97.34$, $p < 0.001$), whereas the effect of time (24 h vs. 48 h) was not statistically significant ($F(1,12) = 4.27$, $p = 0.061$), and the interaction between time and concentration was also not significant ($F(2,12) = 0.67$, $p = 0.531$). Post hoc Tukey HSD tests showed that 150 μM significantly increased ROS compared with control at 24 h and 48 h (all $p < 0.001$). Also, 150 μM significantly increased ROS compared with 50 μM at 24 h and 48 h (all $p < 0.001$). No significant differences were observed between control and 50 μM at either time point, nor between the same concentrations across 24 h and 48 h (all $p > 0.05$).

Further one-way ANOVA comparisons demonstrated that ROS levels in the control group were significantly lower at 24 h than at 48 h ($F(1,4) = 28.52$, $p = 0.006$).

2.3. Autophagy Modulation

HGEpiC and OECM-1 cells can be observed in both controls and apigenin treatments using phase contrast holographic imaging (Figure 3a) after 24 h. HGEpiC cells retain the characteristic honeycomb-like organization in both the control and treatment. On the other hand, in the case of OECM-1 cells, in the control, a much higher number of dividing cells (round and prominent) is observed; in contrast, after apigenin treatment (50 and 150 μM), the number of dividing cells is visibly reduced.

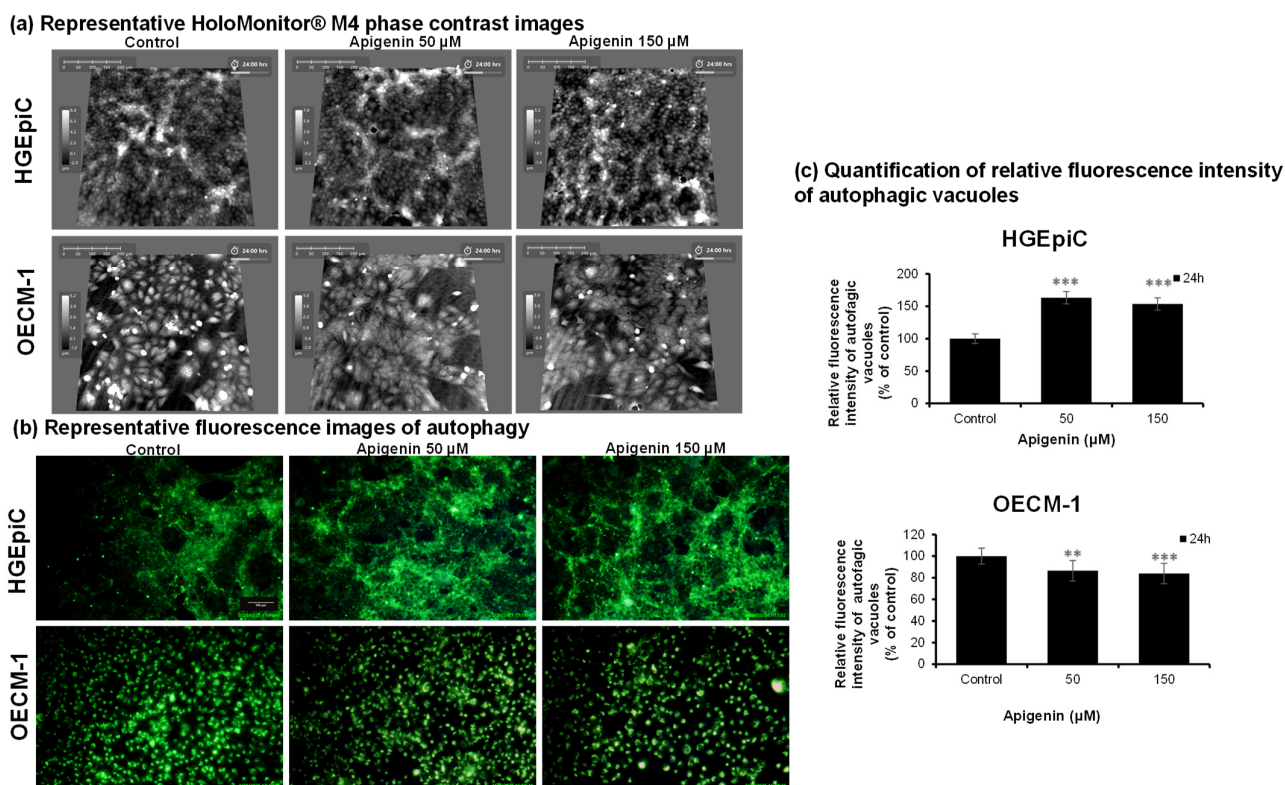


Figure 3. Differential autophagy responses to apigenin treatment (50 μM and 150 μM) in HGEpiC and OECM-1 cells after 24 h. **(a)** Representative holographic phase contrast images of HGEpiC and OECM-1 cells. Controls represent untreated cells; **(b)** Fluorescence microscopy representative images of autophagic vacuoles in HGEpiC and OECM-1 cells. Scale bar: 100 μm (applies to all images); **(c)** Quantification of relative fluorescence intensity of autophagic vacuoles using ImageJ software (Version 1.54p). Controls represent untreated cells. Data are presented as mean $\pm$ SD ($n = 10$ OECM-1; $n = 8$ HGEpiC). HGEpiC: one-way ANOVA followed by Tukey post hoc test; 50 μM *** $p < 0.001$ vs. control; 150 μM *** $p < 0.001$ vs. control. OECM-1: one-way ANOVA followed by Tukey post hoc test; 50 μM ** $p < 0.01$ vs. control; 150 μM *** $p < 0.001$ vs. control.

In HGEpiC cells, fluorescence intensity of the autophagic vacuoles significantly increased by 63% at 50 μM and by 54% at 150 μM , compared to the control, supporting the activation of autophagy as a protective mechanism (Figure 3b,c). One-way ANOVA showed a significant effect of concentration on autophagy ($F(2,21) = 29.33$, $p < 0.001$). Tukey post hoc tests indicated significant increases at 50 μM ($t(21) = 7.106$, $p < 0.001$) and 150 μM ($t(21) = 6.027$, $p < 0.001$) compared with the control, with no difference between concentrations ($p = 0.537$). Pearson correlation revealed a significant positive association between concentration and autophagy ($r = 0.563$, $p = 0.004$).

Conversely, OECM-1 cells showed a significant decrease in the fluorescence intensity of autophagic vacuoles by 14% and by 16% at 50 μ M and 150 μ M, respectively, compared with the control, indicating inhibition of autophagy (Figure 3b,c). One-way ANOVA showed a significant effect of concentration on autophagy ($F(2,27) = 9.636, p < 0.001$). Tukey post hoc tests indicated significant reductions at 50 μ M ($t(27) = -3.433, p = 0.005$) and 150 μ M ($t(27) = -4.086, p < 0.001$) compared with control, with no difference between concentrations ($p = 0.792$). Pearson correlation revealed a significant negative association between concentration and autophagy ($r = -0.545, p = 0.002$).

2.4. Pro-Survival vs. Pro-Apoptotic Signaling

Multiplex analysis of the parameters of early apoptosis in the phosphorylated or active state performed with the MILLIPLEX® Early Apoptosis 7-Plex kit (Figure 4) provides discrepant data between the molecular pathways and mechanisms of apoptosis and survival activated in the case of HGEpiC cells vs. OECM-1 cells after 48 h of exposure to 50 and 150 μ M apigenin.

In HGEpiC cells, apigenin treatment decreased p-AKT and p-BCL-2 levels compared to the control, but induced an increment of the p53 level and both extrinsic (caspase-8) and intrinsic (caspase-9) apoptotic responses. One-way ANOVA showed a significant effect of concentration ($F(2,6) = 12.32, p = 0.008$). Post hoc Tukey test indicated that the AKT decreased at 50 μ M compared with control ($p = 0.007$) and increased at 150 μ M compared with 50 μ M ($p = 0.034$), with no significant difference between 150 μ M and control. Pearson correlation showed no significant linear association ($r = -0.095, p = 0.808$).

On the other hand, in OECM-1 cells, apigenin increased the level of p-AKT and p-BCL-2, decreased p-JNK level, and exhibited only partial caspase-8 and caspase-9 activation at 50 μ M, and a lack of response at 150 μ M, consistent with impaired apoptotic signaling at higher doses. Welch's ANOVA showed a significant effect of concentration on JNK levels ($F(2,3.09) = 16.56, p = 0.022$). Post hoc Games–Howell analysis indicated that 150 μ M caused a significant decrease in JNK levels compared with the control ($p = 0.03$), whereas no difference was observed between 50 μ M and the control.

Together, these results indicate that apigenin exerts differential effects on HGEpiC compared with OECM-1 cells. HGEpiC cells maintained proliferation and mitochondrial metabolic activity over time, with only moderate changes in ATP levels, while OECM-1 cells continued to proliferate but exhibited reduced metabolic activity and a transient ATP decrease at 24 h, followed by a marked increase at 48 h. Regarding redox status, HGEpiC cells showed moderate ROS increases accompanied by enhanced GSH-mediated antioxidant defense, whereas OECM-1 cells displayed pronounced ROS accumulation and a transient reduction in GSH at 24 h; at 48 h, ROS levels remained elevated compared with control. Autophagy was activated in HGEpiC cells, whereas it was inhibited in OECM-1 cells. Finally, HGEpiC cells showed decreased p-AKT and modest caspase-3/7 activity, whereas OECM-1 cells exhibited limited apoptotic responses, associated with increased anti-apoptotic signaling (elevated p-AKT and p-BCL-2) and reduced p-JNK levels. Overall, these findings highlight the selective vulnerability of OECM-1 cells to apigenin-induced oxidative stress and suggest that HGEpiC cells preserve homeostasis through coordinated redox, autophagy, and apoptotic responses.

2.5. Late Apoptosis

Regarding late apoptosis in Figure 5, the effector caspase-3/7 was fluorescently labeled. Both HGEpiC (Figure 5a) and OECM-1 (Figure 5b) cells showed detectable caspase-3/7 activation after apigenin exposure. In HGEpiC, caspase-3/7 fluorescence increased slightly but significantly at 48 h, indicating late apoptosis (Figure 5c). Two-way ANOVA showed

a significant effect of concentration on caspase activity ($F(2,12) = 4.022$, $p = 0.046$) and a significant time $\times$ concentration interaction ($F(2,12) = 7.547$, $p = 0.008$), while time alone had no effect. Tukey post hoc analysis indicated that at 48 h, both 50 μM ($p = 0.048$) and 150 μM ($p = 0.006$) significantly increased caspase 3/7 activity compared to control, with no significant differences between the two concentrations. In the control group, caspase activity was significantly higher at 24 h than at 48 h ($p = 0.027$).

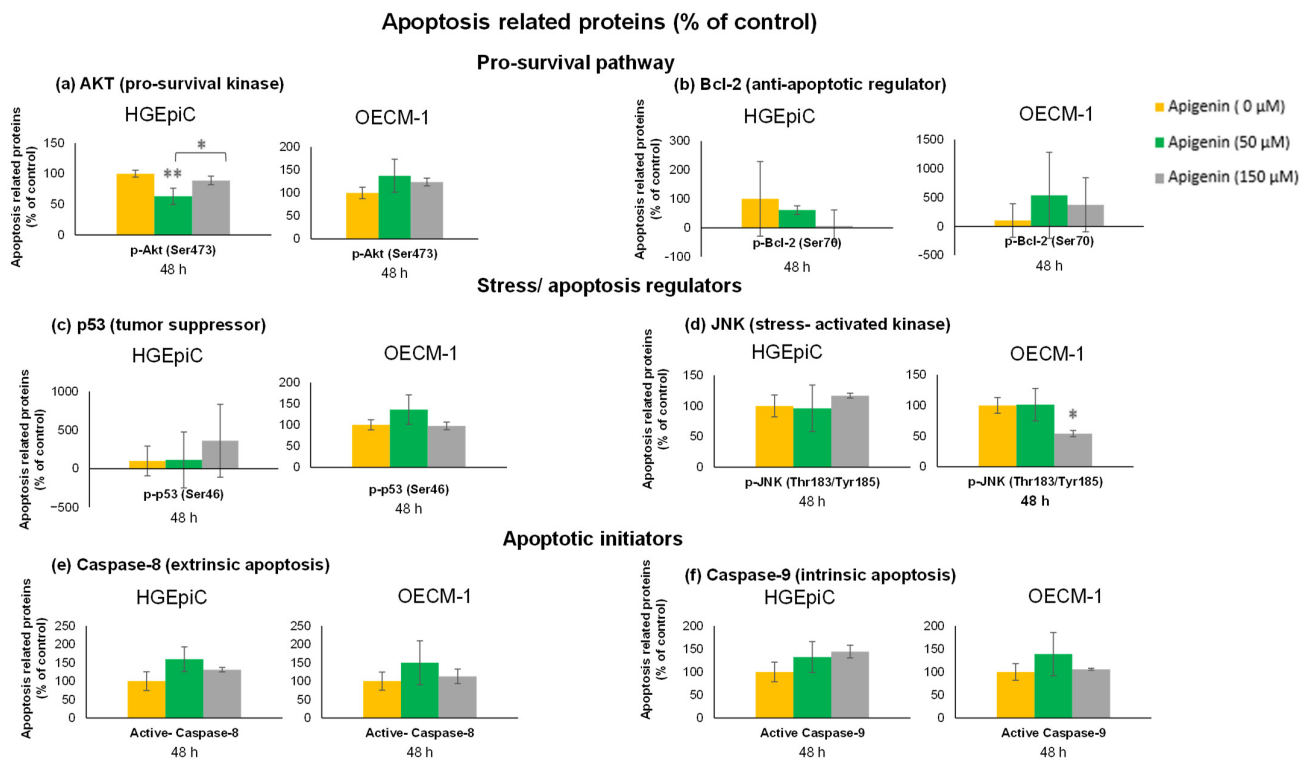


Figure 4. Relative level of phosphorylated or active apoptosis-related proteins quantified after 48 h of HGEpIC cells and OECM-1 cells exposure to apigenin treatment (50 μM and 150 μM). **(a)** Phospho-AKT (Ser473). Apigenin significantly decreased AKT phosphorylation in HGEpIC, whereas in OECM-1 cells, AKT activity showed a slight, non-significant increase (ns), indicating compensatory pro-survival signaling activation. HGEpIC: one-way ANOVA followed by Tukey post hoc test; 50 μM vs. control ** $p < 0.01$; 150 μM vs. 50 μM * $p < 0.05$; **(b)** Phospho-BCL-2 (Ser70). In HGEpIC cells, phosphorylated BCL-2 showed a mean decrease (−39% at 50 μM ; −93% at 150 μM), although variability rendered the 150 μM comparison non-significant; this trend is concordant with reduced p-AKT and supports attenuation of anti-apoptotic signaling. In contrast, in OECM-1 cells, anti-apoptotic protein levels increased by 436% > control 50 μM , 271% > control non-significant, representing tumor cell adaptation to apigenin-induced stress; **(c)** Phospho-p53 (Ser46). In HGEpIC the p-p53 level increased (ns) at 150 μM apigenin. In OECM-1 cells, no change was significant; **(d)** Phospho-JNK (Thr183/Tyr185). HGEpIC cells exhibited a slight, nonsignificant increase in JNK activation, while JNK phosphorylation was significantly inhibited in OECM-1 cells, suggesting suppression of stress-induced apoptotic pathways. OECM-1: Welch's ANOVA 150 μM vs. control * $p < 0.05$; **(e)** Active caspase-8. In HGEpIC cells, apigenin treatment increased caspase-8 activity by 59% at 50 μM (ns) and by 31% at 150 μM (ns) compared to the control. In OECM-1 tumor cells, caspase-8 activity increased by ~50% at 50 μM (ns) and by 12% at 150 μM (ns), indicating a mild, dose-dependent activation of the extrinsic apoptotic pathway; **(f)** Active caspase-9. In HGEpIC, caspase-9 activity increased by 32% at 50 μM (ns) and by 44% at 150 μM (ns), consistent with activation of the intrinsic (mitochondrial) apoptotic pathway. In OECM-1 cells, caspase-9 activity showed a 39% increase at 50 μM (ns), but no significant change at 150 μM , suggesting partial activation of the intrinsic pathway at lower concentrations and resistance at higher doses. Controls represent untreated cells. Data are presented as mean $\pm$ SD ($n = 3$).

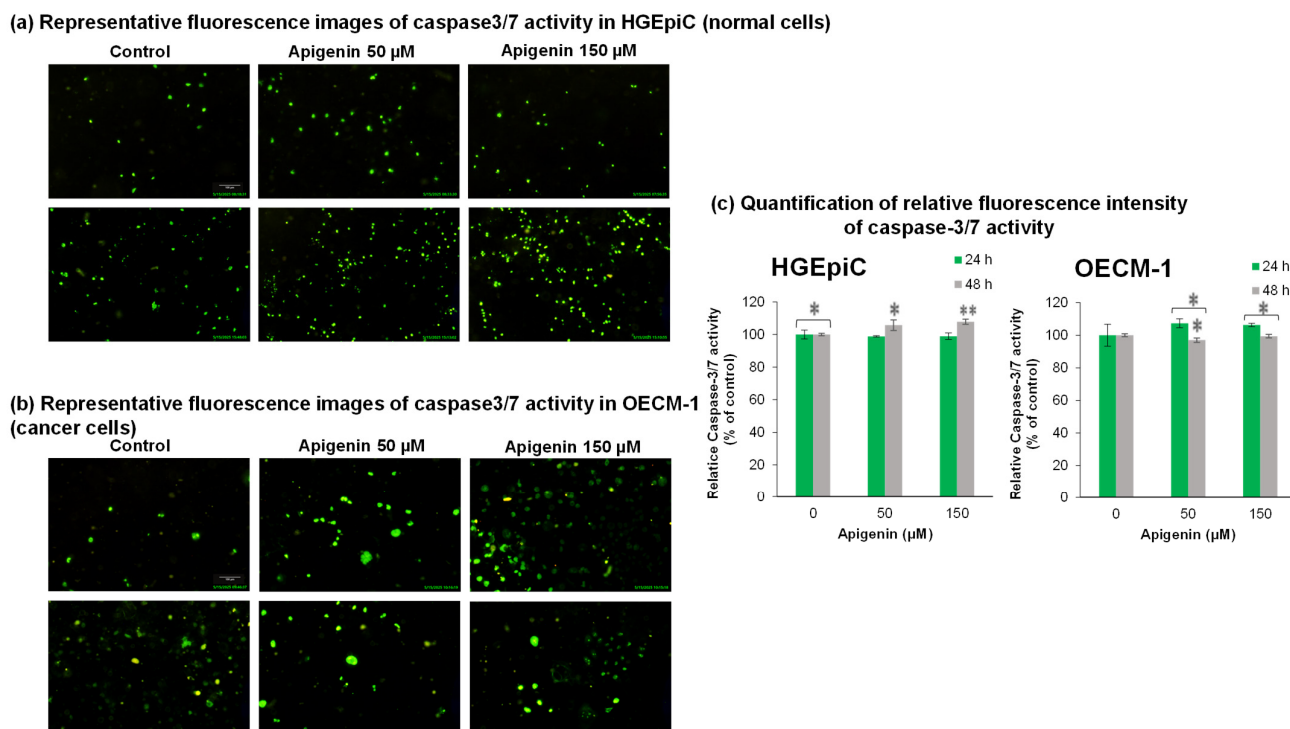


Figure 5. Late apoptosis in HGEpiC and OECM-1 cells after 24 and 48 h of exposure to apigenin treatment (50 μ M and 150 μ M). Fluorescence microscopy representative images of caspase-3/7 activity in: (a) HGEpiC, and (b) OECM-1 cells. Controls represent untreated cells. Scale bar: 100 μ m (applies to all images); (c) Quantification of relative fluorescence intensity of caspase-3/7 activity using FLUOstar Omega microplate reader. Controls, symbolized as 0 on the graph, represent untreated cells. Data are presented as mean $\pm$ SD ($n = 3$). HGEpiC: Two-way ANOVA followed by Tukey post hoc test; 50 μ M vs. control * $p < 0.05$ (48 h); 150 μ M vs. control ** $p < 0.01$ (48 h); 24 h vs. 48 h (Control)* $p < 0.05$. OECM-1: One-way ANOVA; 24 h vs. 48 h (50 μ M)* $p < 0.05$; 24 h vs. 48 h (150 μ M)* $p < 0.05$; 50 μ M vs. control * $p < 0.05$ (48 h).

In OECM-1, caspase-3/7 activation was visible at both concentrations, but overall weaker and attenuated by 48 h, suggesting a limited apoptotic execution in surviving tumor cells (Figure 5c). Caspase-3/7 activity was significantly higher at 24 h compared with 48 h in cells treated with 150 μ M ($F(1,4) = 11.2$, $p = 0.029$). A similar time-dependent decrease was observed in the 50 μ M group, with caspase-3/7 activity being significantly higher at 24 h than at 48 h ($F(1,4) = 14.05$, $p = 0.02$). At 48 h, one-way ANOVA revealed a significant effect of treatment on caspase-3/7 activity ($F(2,6) = 6.276$, $p = 0.034$), with post hoc analysis indicating significantly lower activity in the 50 μ M group compared with the control ($p = 0.037$).

3. Discussion

Our study is the first to compare the effects of apigenin on redox balance, autophagy, and apoptosis in normal (HGEpiC) versus malignant (OECM-1) gingival epithelium. We selected apigenin because it is a flavonoid known for its antioxidant and pro-apoptotic properties. However, its redox-related mechanisms and differential effects in OSCC remain insufficiently investigated [22,23], which justifies its exploration as a potential adjuvant agent in OSCC therapy. Given the involvement of apigenin in the JAK/STAT, PI3K/Akt/mTOR, MAPK/ERK, NF- κ B, and Wnt/ β -catenin pathways in multiple cancer types [24], the aim of our study was to assess the effects of apigenin on OECM-1 cells by specifically examining redox homeostasis and the AKT/BCL-2/JNK signaling axis in relation to autophagy and apoptosis, in parallel with HGEpiC cells. To our knowledge, few

studies have examined apigenin's effects on signaling pathways and autophagy specifically in OSCC cells, highlighting the novelty of our approach.

To summarize the differential effects observed, a holistic schematic is presented in Figure 6. Apigenin treatment in HGEpiC cells induced a moderate increase in ROS compared to OECM-1 cells, accompanied by activation of autophagy as a pro-survival adaptive mechanism and the induction of partial apoptosis. In contrast, in OECM-1 cells, apigenin exposure resulted in a pronounced elevation of ROS levels, suppression of autophagic activity, and resistance to apoptosis, effects that are likely associated with the downregulation of phosphorylated JNK (p-JNK).

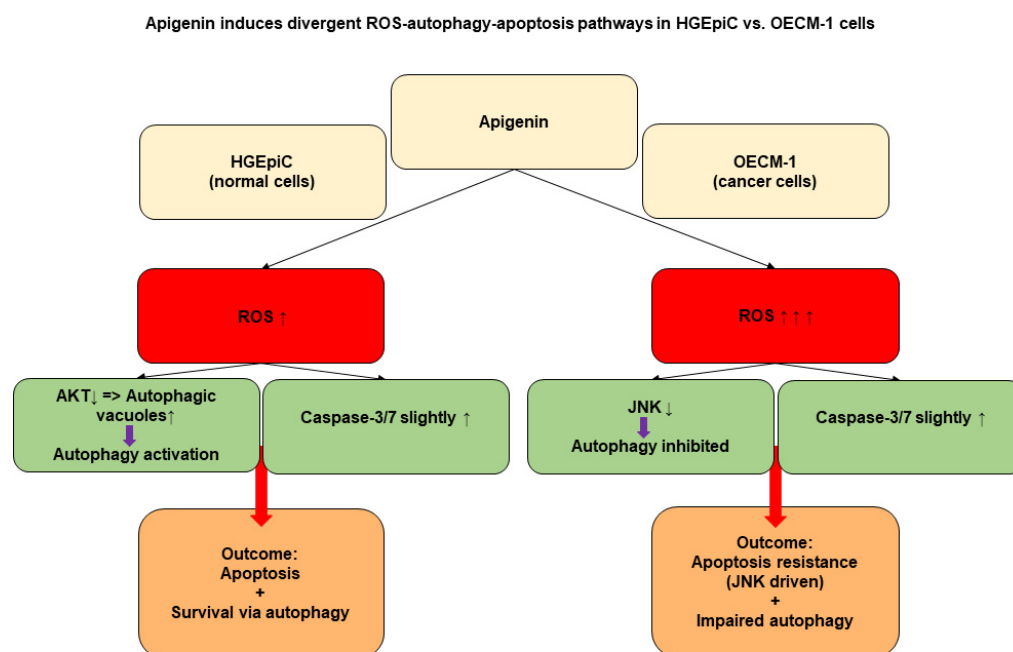


Figure 6. The holistic model encompasses intracellular aspects of apigenin on HGEpiC versus OECM-1 cells in our study.

Our results suggest that apigenin exerts distinct effects on HGEpiC versus OECM-1 cells.

MTT assays showed that apigenin did not exert cytotoxic effects on HGEpiC cells over time; in fact, it promoted a gradual increase in cell viability ($p < 0.05$). In addition, real-time monitoring using the HoloMonitor[®] M4 revealed that HGEpiC maintained its characteristic epithelial architecture. These findings are supported by existing literature, where apigenin is classified as having a low cytotoxic profile [25,26].

In contrast, post hoc tests confirmed that both concentrations of apigenin significantly reduced the metabolic activity of OECM-1 cells compared with the control, with a more pronounced effect at 48 h ($p < 0.001$). This was accompanied by visibly altered morphology in HoloMonitor[®] M4 images and reduced three-dimensional expansion (Figure 1b), consistent with the literature describing that OECM-1 cells must modify their phenotypic characteristics to adapt to the microenvironment, proliferate, and evade cell death in the presence of apigenin [27–29].

In HGEpiC cells, the significant increase in MTT at 24 h versus control (by 20%) indicates proliferation, whereas the 20% decrease in ATP is likely explained by the energy consumption required for activating antioxidant and autophagic mechanisms. Conversely, in OECM-1 cells, mitochondrial activity and ATP levels decreased significantly at 24 h compared with control, consistent with apigenin-induced mitochondrial dysfunction and ATP depletion reported in other malignancies [30]. Interestingly, in our results after 48 h, ATP

levels in OECM-1 cells increased markedly despite the persistent reduction in mitochondrial dehydrogenase activity. This suggests a metabolic shift from oxidative phosphorylation to anaerobic glycolysis, serving as a bioenergetic compensation mechanism under conditions of mitochondrial stress; indeed, the literature reports that cancer cells produce ATP through anaerobic glycolysis under stress conditions such as hypoxia or oxidative stress, thereby sustaining survival despite mitochondrial dysfunction [31]. Through this adaptive response, OECM-1 cells likely maintain ATP production to support survival, highlighting the plasticity of cancer cell metabolism in response to apigenin-induced stress.

Glutathione (GSH), the main intracellular thiol, plays a central role in detoxifying H_2O_2 via glutathione peroxidase GPx, with regeneration through glutathione reductase maintaining redox homeostasis [32,33]. In OECM-1 cells, the significant increase in GSH at 48 h compared with 24 h might suggest an adaptive cellular response to elevated ROS. Regarding oxidative stress, ROS- H_2O_2 levels in HGEpiC cells increased moderately (83% at 48 h), yet still much lower than the increases of 873% and 331% observed in OECM-1 cells exposed to 150 μM apigenin at 24 h and 48 h, respectively. Pearson correlation confirmed a strong dose-dependent association ($r = 0.946$, $p < 0.001$) for OECM-1 cells. These results align with data from Lee et al., who also reported apigenin-induced ROS accumulation [30], further supporting a cancer cell-specific oxidative response. Our findings are consistent with the concept that cancer cells can tolerate higher ROS levels by upregulating antioxidant proteins, supporting survival and resistance to apoptosis [34]. While cancer cells generally have higher ROS than normal cells, they counteract potential damage through antioxidant genes and metabolic adaptations, making the GSH synthesis pathway an attractive therapeutic target [35]. Consequently, targeting ROS regulation represents a promising therapeutic strategy, given the dysregulated redox balance characteristic of cancer cells. Overall, these findings support the potential use of apigenin for redox priming strategies in OSCC therapy, whereby controlled oxidative stress sensitizes tumor cells prior to chemotherapy or radiotherapy [6,36].

Another key aspect in the cancer cell's defense mechanisms is represented by the modulation of autophagy. Autophagy is a catabolic process through which aggregated proteins and damaged organelles are transported to lysosomes for degradation [37,38]. Basally, autophagy maintains cellular homeostasis and normal growth. Under pathological conditions, it is not always clear whether autophagic alterations act protectively or contribute to damage [39,40]. Autophagy has also been classified as a type II programmed cell death mechanism [37,41].

In HGEpiC cells, autophagy increased as a pro-survival mechanism, correlating with MTT results. In contrast, in OECM-1 cells, autophagy decreased along with reduced GSH and increased ROS at 24 h compared with the control. Contrary to our results, Enrico et al. showed in several tumor lines (HeLa, HepG2, H1299, MCF-7) that GSH depletion promotes autophagy activation [32]. In OECM-1 cells, although GSH decreased following apigenin treatment, autophagy was not activated, suggesting a protective mechanism through which tumor cells inhibit autophagy. Thus, our data clearly suggest that OECM-1 cells did not use autophagy as a survival mechanism, consistent with the reduced MTT results. In contrast to our observations, several studies on malignant cell lines have shown that apigenin-induced autophagy can be pro-survival, and its inhibition can enhance apigenin toxicity through apoptosis induction [12,42–44].

The paradox of autophagy in cancer remains unresolved. Autophagy can either protect or suppress tumor cells depending on context and stage. In early stages, autophagy inhibition may be beneficial, whereas in advanced stages, its induction may promote oncogenesis or senescence. Therapeutic strategies must therefore be adapted to the tumor's molecular profile and autophagic status [45].

At the molecular level (Figure 4), apigenin reduced AKT phosphorylation in HGEpiC cells, promoting the formation of autophagic vacuoles. The decrease in AKT at 50 μ M confirms enhanced autophagy, consistent with its known anti-autophagic role [12,44]. In OECM-1 cells, p-JNK levels were significantly decreased (post hoc analysis indicated that 150 μ M caused a significant decrease compared with both 50 μ M and control). Jun-Mo Moon et al. demonstrated in colorectal cancer that JNK is interconnected with both autophagy and apoptosis, with increased p-JNK expression stimulating both processes in vitro and in vivo [46]. Other studies highlight that JNK inhibition represents a resistance and protective mechanism in oxidative-stress conditions in cancer cells [47,48]. Therefore, in OECM-1 cells, the inhibition of autophagy and partial activation of apoptosis following apigenin treatment can be explained by reduced p-JNK, as an adaptive mechanism of tumor cells. Thus, our results confirm the importance and therapeutic potential of JNK in OSCC treatment, consistent with the existing literature.

In HGEpiC cells, caspase-3/7 activity increased slightly compared with the control at 50 and 150 μ M after 48 h ($p < 0.05$), whereas in OECM-1 cells, caspase-3/7 also increased slightly compared with the control after 24 h ($p > 0.05$) (Figure 5). These data suggest that the OECM-1 cells respond to apigenin by reducing the level of autophagy, probably to avoid the pro-death functions of autophagy [45], most likely via JNK dephosphorylation.

In summary, our findings suggested that apigenin exerts differential effects on HGEpiC versus OECM-1 cells, modulating redox balance, autophagy, and apoptosis in a cell-type-dependent manner. These results highlight the potential of apigenin as an adjuvant in OSCC therapy and provide a strong rationale for further mechanistic and in vivo investigations. This study was conducted in vitro on two cell lines; further in vivo studies and exploration of additional OSCC models are warranted to validate these findings.

Raw data of all measured assays, showing mean $\pm$ SD values for HGEpiC and OECM-1 cells, are presented in Table 1. Values represent results from confluence, MTT, ATP, GSH, ROS, autophagy, AKT, BCL-2, p53, JNK, Caspase-3/7, Caspase-8, and Caspase-9 measurements at the indicated time points (h).

Table 1. Raw data presentation, including mean values and standard deviation (SD) for all assays, for the graphs generated.

	Time (h)	Mean values	SD
Confluence	0	82.5	2.2
	24	91.2	2.2
	48	98.4	0.4
A50 HGEpiC	0	67.5	13.2
	24	75.0	9.5
	48	84.9	0.5
A150 HGEpiC	0	71.7	7.0
	24	78.4	5.0
	48	88.1	5.2
Control OECM-1	0	33.5	5.3
	24	51.2	11.9
	48	66.3	12.3
A50 OECM-1	0	38.8	7.0
	24	57.5	13.0
	48	74.7	5.0

Table 1. *Cont.*

	Time (h)	Mean values	SD
A150 OECM-1	0	42.3	3.2
	24	61.8	5.6
	48	79.5	3.8
MTT			
Control HGEpiC	24	0.79	8.88
	48	1.13	10.11
A50 HGEpiC	24	0.85	3.82
	48	1.07	10.6
A150 HGEpiC	24	0.96	7.42
	48	1.01	30.66
Control OECM-1	24	0.55	5.36
	48	0.70	7.31
A50 OECM-1	24	0.44	7.50
	48	0.54	8.54
A150 OECM-1	24	0.47	6.52
	48	0.48	4.03
ATP			
Control HGEpiC	24	50,083	8.20
	48	19,428	54.45
A50 HGEpiC	24	47,638.33	7.02
	48	22,721	42.33
A150 HGEpiC	24	39,751.33	1.68
	48	23,861	75.69
Control OECM-1	24	23,542	6.65
	48	4190	110.20
A50 OECM-1	24	15,301.33	4.61
	48	28,647.67	1069.22
A150 OECM-1	24	15,964.33	1.99
	48	36,456	643.68
GSH			
Control HGEpiC	24	89,681.33	15.78
	48	220,801.7	13.56
A50 HGEpiC	24	106,644.3	86.32
	48	249,292.7	1.78
A150 HGEpiC	24	107,971.7	87.08
	48	252,421	2.08
Control OECM-1	24	71,154	29.82
	48	229,531.3	9.34
A50 OECM-1	24	58,599	44.63
	48	249,788.3	3.74
A150 OECM-1	24	66,128.33	57.18
	48	251,366	2.51
ROS			
Control HGEpiC	24	799	11.47
	48	424.66	28.97

Table 1. *Cont.*

	Time (h)	Mean values	SD
A50 HGEpiC	24	990.33	32.57
	48	781	28.35
A150 HGEpiC	24	853.33	13.02
	48	593	26.96
Control OECM-1	24	75.33	64.49
	48	359.33	21.77
A50 OECM-1	24	419.33	83.51
	48	485	11.11
A150 OECM-1	24	1411.66	385.66
	48	1549.66	72.85
Autophagy			
Control HGEpiC	24	47.14	19.86
A50 HGEpiC	24	76.92	18.31
A150 HGEpiC	24	72.39	14.77
Control OECM-1	24	118.09	7.34
A50 OECM-1	24	102.16	9.42
A150 OECM-1	24	99.12	9.42
AKT			
Control HGEpiC	48	1900.66	5.72
A50 HGEpiC	48	1201	13.33
A150 HGEpic	48	1691	7.08
Control OECM-1	48	1922.66	12.39
A50 OECM-1	48	2642	36.23
A150 OECM-1	48	2380.33	8.36
BCL-2			
Control HGEpiC	48	−5.16	128.87
A50 HGEpiC	48	−3.16	14.78
A150 HGEpic	48	−0.33	55.87
Control OECM-1	48	2.33	290.93
A50 OECM-1	48	8.66	742.30
A150 OECM-1	48	12.5	465.71
p53			
Control HGEpiC	48	2.66	192.43
A50 HGEpiC	48	3	361.63
A150 HGEpic	48	9.66	471.86
Control OECM-1	48	767.5	11.91
A50 OECM-1	48	1044.83	34.67
A150 OECM-1	48	750.83	9.16
JNK			
Control HGEpiC	48	276.41	17.83
A50 HGEpiC	48	265.91	37.95
A150 HGEpic	48	322.91	4.02
Control OECM-1	48	262.58	12.86
A50 OECM-1	48	265.75	26.5
A150 OECM-1	48	141.75	5.03
Caspase-8			
Control HGEpiC	48	33.25	25.65
A50 HGEpiC	48	53.08	33.83
A150 HGEpic	48	43.58	6.26
Control OECM-1	48	137.41	24.7
A50 OECM-1	48	206.08	59.68
A150 OECM-1	48	155.25	19.62

Table 1. *Cont.*

	Time (h)	Mean values	SD
Caspase 9			
Control HGEpiC	48	49.83	21.19
A50 HGEpiC	48	66	33.39
A150 HGEpic	48	72	13.9
Control OECM-1	48	428	18.11
A50 OECM-1	48	594.33	47.1
A150 OECM-1	48	453	2.37
Caspase 3/7			
Control HGEpiC	24	144,472.7	2.73
	48	135,789.3	0.72
A50 HGEpiC	24	142,837	0.4
	48	143,681.3	3.23
A150 HGEpic	24	142,956.7	1.99
	48	146,452.3	1.54
Control OECM-1	24	151,655	6.74
	48	157,517.7	0.87
A50 OECM-1	24	162,821.3	2.74
	48	152,735.3	1.32
A150 OECM-1	24	161,250.7	1.04
	48	156,677.7	1.11

4. Materials and Methods

Figure 7 summarizes the parameters and intracellular events monitored following exposure of the two cell lines to apigenin for 24 and 48 h. The experimental workflow focused on oxidative stress, mitochondrial function, autophagy, apoptosis, and morphology (Figure 1a), quantifying ROS, GSH, ATP, MTT, autophagic vacuoles, apoptosis-related proteins (p-JNK, p-BCL-2, active caspase-8/9), and monitoring live-cell morphology (Figure 1b), respectively. Early apoptosis markers were quantified using multiplex testing with Luminex 200 technology, while live-cell monitoring was performed with HoloMonitor® M4.

4.1. Cell Culture

Two cell lines were used in this study: HGEpiC and OECM-1 cells.

The HGEpiC cell line was purchased from Innoprot (Bizkaia, Spain; Cat. No. P10864). The cells were cultured in Epithelial Cell Medium-Plus (Innoprot, Bizkaia, Spain; Cat. No. P60106-PLUS), completely supplemented with fetal bovine serum, supplement EpiCGS, and penicillin/streptomycin (P/S) solution 100×, FBS Innoprot, in a humidified environment at 37 °C and 5% CO₂. Before seeding, the flasks were pre-treated with poly-L-lysine to improve cell adhesion, according to the manufacturer's instructions. For the experiments, the cells were seeded in 96-well plates pre-treated with poly-L-lysine at a density of 4×10^4 cells/well. Cells were maintained overnight in a humidified incubator at 37 °C and 5% CO₂ to promote cell adhesion before the experiments.

The OECM-1 was purchased from Sigma-Aldrich (St. Louis, MO, USA; Cat. No. SCC180). Cells were cultured in RPMI-1640 medium (VWR, Radnor, PA, USA; Cat. No. 392-0426) supplemented with 5% fetal bovine serum and 1% antibiotics, in a humidified environment at 37 °C and 5% CO₂, without pre-treatment of the culture vessel surface with poly-L-lysine. For the experiments, OECM-1 cells were seeded at the same density as normal cells, namely 4×10^4 cells/well in 96-well plates, and maintained overnight at 37 °C and 5% CO₂ to promote cell adhesion before the experiments.

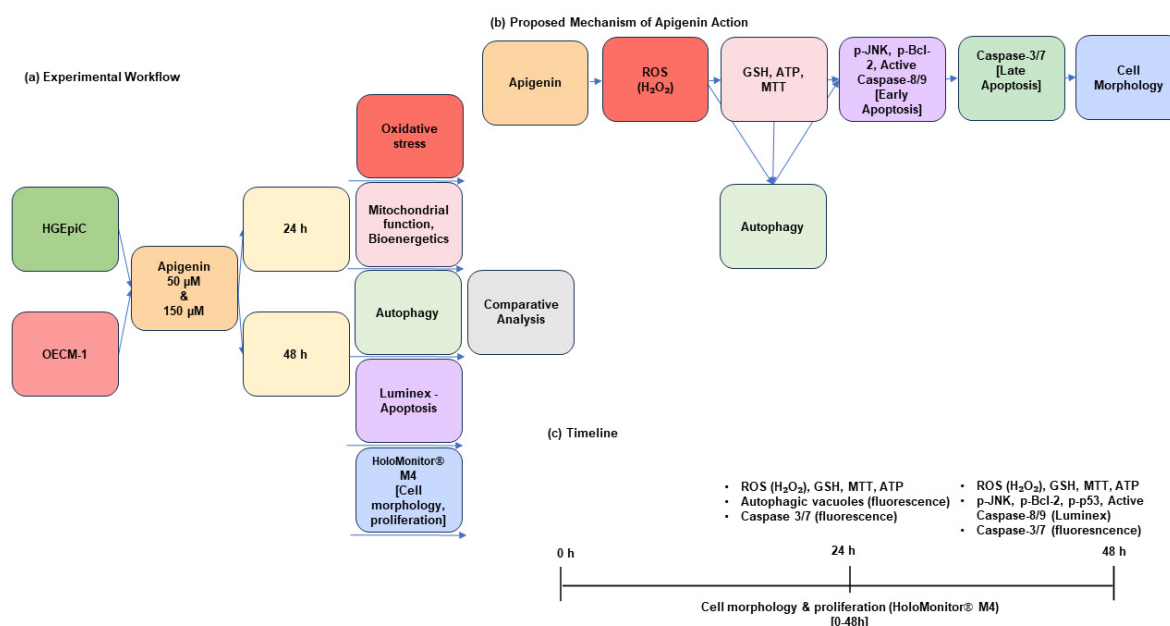


Figure 7. Experimental workflow and proposed mechanism of apigenin action; (a) Experimental workflow illustrating the comparative analysis of HGEpiC and OECM-1 cells exposed to the bio-compound apigenin in terms of oxidative stress, mitochondrial function, autophagy, and apoptosis. Proposed mechanistic model of apigenin action through ROS, autophagy, and apoptosis modulation; (b) Schematic mechanistic model of the intracellular action of apigenin on autophagy and apoptosis via ROS; (c) Timeline of the study, including apigenin treatments and analyses performed.

Although the two cell lines started from an equivalent cell seeding density, considering the faster division cycle of HGEpiC (for example, doubling time 24–30 h reported for primary human gingival keratinocytes, a biologically comparable epithelial cell population [49]), unlike OECM-1 (doubling time 30–38 h, according to the supplier's data (Merck/Sigma-Aldrich, Cat. SCC180 [21])), we expect that, over time, a difference in the number of HGEpiC vs. OECM-1 cells will appear, including in the control. Moreover, this statement is supported by the pre-treatment with a special substrate (poly-L-lysine) and the addition of extra serum in the case of HGEpiC, conditions that favor proliferation.

4.2. Biocompound

Apigenin obtained from Cayman Chemical ($\geq 98\%$ purity; CAS No. 520-36-5) was solubilized in dimethyl sulfoxide (DMSO). The stock solutions were sterilized by exposure to UV radiation for 1 h. Prior to use, stock solutions were diluted in complete, cell-type-specific culture medium to obtain final concentrations of 150 μM and 50 μM , with a final DMSO content of $\leq 0.5\%$. The concentrations of apigenin (50 μM and 150 μM) were selected based on preliminary viability testing and previously published studies [15]. Higher concentrations were not used to avoid non-specific cytotoxic effects on normal gingival epithelial cells (HGEpiC) and to remain within biologically relevant ranges.

4.3. Live-Cell Imaging—HoloMonitor® M4

Morphological changes, cell migration, and proliferation were monitored using the HoloMonitor® M4 digital holographic microscope (Phase Holographic Imaging PHI AB, Lund, Sweden). Cells were seeded in 96-well plates and exposed to apigenin, with unexposed cells used as controls. A HoloLid™ (PHI AB, Göteborg, Sweden) was placed over the plate to improve image quality and reduce evaporation. Time-lapse images were captured every 30 min for 48 h and analyzed using the HoloMonitor® App Suite version

4.0.1.546 (PHI AB, Göteborg, Sweden) in a label-free approach. The system was maintained in a humidified incubator at 37 °C and 5% CO₂.

4.4. Mitochondrial Metabolic Activity

Mitochondrial metabolic activity was assessed using the MTT reagent from Biotium (Fremont, CA, USA; Cat No. 30006), after 24 and 48 h of incubation of normal and tumor cells with apigenin (50 µM and 150 µM). Following incubation, 10 µL of MTT reagent and 100 µL of culture medium were added per well. Solubilization of formazan crystals was performed with isopropanol, and the absorbance was measured at 570 and 630 nm using a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany).

4.5. Determination of ATP Levels

Intracellular ATP content was determined using the CellTiter-Glo[®] 2.0 Assay (Promega, Madison, WI, USA; Cat. No. G9241), according to the manufacturer's instructions. After 24 and 48 h of treatment with apigenin, an equal volume of reagent was added to the culture medium and incubated for 10 min at room temperature, protected from light. Luminescence was measured using the MyGlo[®] Reagent Reader (Promega, Madison, WI, USA), and results were expressed as relative luminescence units (RLU), corresponding to intracellular ATP concentration.

4.6. Reactive Oxygen Species (ROS)

The intracellular level of hydrogen peroxide (H₂O₂), the main reactive oxygen species, was determined using the ROS-Glo[™] H₂O₂ Assay Kit (Promega, Madison, WI, USA; Cat. No. G8820) after treatment with apigenin in the two oral cell lines: normal and tumoral. After 24 and 48 h of exposure to apigenin, the ROS-Glo[™] reagent was added to the culture medium and incubated for 6 h at 37 °C. Luminescence was recorded using the MyGlo[®] Reagent Reader (Promega), with signal intensity proportional to the amount of H₂O₂ in the samples.

4.7. Glutathione (GSH)

Reduced glutathione (GSH), the main intracellular antioxidant, was fluorescently labeled using the BioTracker[™] 625 Red GSH Dye (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. SCT036), according to the manufacturer's protocol. After 24 and 48 h of exposure to apigenin, cells were incubated with the fluorescent dye for 30 min at 37 °C in the dark. Fluorescence images were captured using an Optika IM-3LD4D fluorescence microscope (Optika, Ponteranica, Italy), and fluorescence intensity was quantified using the FLUOstar Omega microplate reader (BMG Labtech, NC, USA).

4.8. Autophagy Activation

Fluorescent labeling of autophagic vacuoles (autophagosomes and autophagolysosomes) was performed using the Autophagy Assay Kit (Merck, Darmstadt, Germany; Cat. No. MAK138) after 24 h of exposure of HGEpiC and OECM-1 cells to 50 and 150 µM apigenin. Images were acquired using an IM-3LD4D fluorescence microscope (Optika, Ponteranica, Italy), with the excitation/emission wavelength filter suitable for the kit ~488/530 nm. Fluorescence intensity was quantified using the FLUOstar Omega microplate reader (BMG Labtech, NC, USA) and correlated with the degree of apoptosis activation.

4.9. Multiplex Assay (Early Apoptosis Markers)

Cell lysates were collected after 48 h of exposure of normal and tumor cells to apigenin (50 µM and 150 µM), using Cell Signaling Lysis Buffer supplemented with Protease Inhibitor

Cocktail (Promega, Madison, WI, USA; Cat. No. G6521). The filtered lysates were stored at -80°C in 1.5 mL Eppendorf tubes for subsequent analyses.

Total Protein Kit, Micro (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. TP0100), based on the Bradford method, was used to determine the total protein concentration. Subsequently, the samples were diluted in Milliplex Assay Buffer to a final concentration of 0.7 mg/mL and analyzed with the MILLIPLEX[®] MAP 7-Plex Early Apoptosis Magnetic Bead kit (Merck Millipore), which quantifies p-AKT, p-BCL-2, p-p53, p-JNK, and active caspase-8 and -9. Mean fluorescence intensity (MFI) was measured using Luminex 200 technology.

4.10. Caspase-3/7 Activity (Fluorescence)

Late apoptosis, mediated by caspases-3 and -7, was assessed using the Caspase-3/7 Green Detection Reagent Kit (Merck, Darmstadt, Germany; Cat. No. SCT100) after 24 h and 48 h of exposure of both cell lines (HGEpiC and OECM-1) to apigenin. Cells were incubated with the caspase-specific reagent at a final concentration of 5 μM for 30 min at room temperature in the dark.

Images were acquired using an IM-3LD4D fluorescence microscope (Optika, Ponteranica, Italy), with the excitation/emission wavelength filter suitable for the kit $\sim 488/530$ nm. Fluorescence intensity was quantified using the FLUOstar Omega microplate reader (BMG Labtech, Ponteranica, Italy).

4.11. Statistical Analysis

All statistical analyses were performed on raw data. Data normality and homogeneity of variances were assessed using the Shapiro–Wilk and Levene’s tests, respectively. For normally distributed variables, group comparisons were performed using one-way or two-way ANOVA, or Welch’s ANOVA when variances were unequal, followed by Tukey post hoc test where appropriate. Correlations were analyzed using Pearson’s correlation, with statistical significance set at $p < 0.05$.

Results are presented as mean $\pm$ SD from triplicate measurements ($n = 3$). Graphs were generated using Microsoft Excel and show data normalized to 100% of the control (untreated cells).

5. Conclusions

In conclusion, the present study suggests that apigenin elicits cell type-specific and context-dependent responses in HGEpiC and OECM-1 cells. Apigenin preserves cell viability, redox homeostasis, and the balance between autophagy and apoptosis in HGEpiC cells. In contrast, in OECM-1 cells, apigenin induces pronounced redox imbalance and reduces autophagic activity, highlighting a selective vulnerability of the malignant phenotype, accompanied by modulation of JNK signaling. These observations support the potential relevance of redox, autophagy, and JNK signaling as therapeutic entry points in OSCC.

Several limitations of the present study should be acknowledged. The experimental design was restricted to in vitro models, and late apoptosis was assessed mainly through executioner caspase-3/7 activity, without additional multiparametric validation. Therefore, further validation using in vivo models and additional late apoptosis markers is warranted. Future studies should also explore the interplay between autophagy and apoptosis through pathways such as AKT/mTOR.

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Abbreviations

The following abbreviations are used in this manuscript:

AKT	Protein kinase B
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BCL-2	B-cell lymphoma 2
DMSO	Dimethyl sulfoxide
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
GSH	Glutathione (reduced form)
H ₂ O ₂	Hydrogen peroxide
HaCaT	Human adult low calcium high temperature keratinocytes
HGEpiC	Human gingival epithelial cells
HNSCC	Head and neck squamous cell carcinoma
HO-1	Heme oxygenase-1
HPV	Human papillomavirus
hPDL	Human periodontal ligament cells
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
mTOR	Mechanistic target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa B
OECM-1	Oral squamous cell carcinoma cell line
OSCC	Oral squamous cell carcinoma
PBS	Phosphate-buffered saline
PI3K	Phosphoinositide 3-kinase
p-AKT	Phosphorylated AKT
p-BCL-2	Phosphorylated BCL-2
p-JNK	Phosphorylated JNK
p-p53	Phosphorylated p53
RLU	Relative luminescence units
ROS	Reactive oxygen species
SCC	Squamous cell carcinoma
SD	Standard deviation

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