Acta Odontologica Scandinavica. 2014; 72: 481–488

informa healthcare

ORIGINAL ARTICLE

Effects of resveratrol and irradiation upon oral squamous cell carcinoma cells

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Abstract

Objective. To evaluate the effects of resveratrol and irradiation on oral squamous cell carcinoma (OSCC). ***Materials and methods.*** Resveratrol was administered at doses of 5, 10, 25, 50 and 100 μM to PE/CA-PJ15 (OSCC) cultures irradiated with different doses (1, 2.5 and 5 Gy). Effects upon cell viability, apoptosis and migration were evaluated after 24, 48 and 72 h incubation. ***Results.*** After 72 h of incubation, the 100 μM dose of resveratrol induced the greatest decrease in cell viability at all irradiation doses. After 24, 48 and 72 h of incubation, 100 μM of resveratrol induced the greatest cell apoptosis at all irradiation doses. The greatest alterations in the distribution of the G_0–G_1, G_2–M and S phases of the cell cycle were recorded with 50 and 100 μM of resveratrol; after 24, 48 and 72 h of incubation, both these doses resulted in an increase in the S phase, at the expense of the G_0–G_1 and G_2–M phases. ***Conclusions.*** Resveratrol increases cytotoxic activity in the PE/CA-PJ15 cell line and reduces cell migration capacity, while the combination of resveratrol and irradiation exerts a synergic effect.

Key Words: *resveratrol, squamous cell carcinoma, irradiation, in vitro* cell line

Introduction

Head and neck cancer (HNC) accounts for 5% of all malignancies and ~100 000 new cases are diagnosed each year in Europe alone [1]. Oral malignancy is one of the most common causes of cancer death worldwide, ranking 12th in order of frequency among all malignancies. Oral squamous cell carcinoma (OSCC) is the most common head and neck cancer and has a poor prognosis closely related to frequent lymph node metastasis and local invasion. Although there have been advances in the diagnosis and treatment of OSCC, the mortality rate remains close to 50% [2].

Multiple factors are implicated in the etiology of oral cancer, including genetic, environmental, viral, social and behavioral factors [1]; of these, diet and nutrition have attracted growing interest for the prevention of oral malignancy [3,4]. Given the need to develop an oral cancer-specific anti-cancer drug, traditional natural products have become a focus of research because they suffer few side-effects and have powerful anti-oxidative, anti-inflammatory and cancer-preventing properties [5].

Resveratrol (trans-3,4′,5-trihydroxystilbene) is a naturally occurring polyphenol. Its synthesis in plants can be induced by microbial infections, UV light and exposure to ozone [6]. Resveratrol is present in fruits, vegetables and beverages (including wine) that are part of the human diet [7,8]. In recent years, numerous studies have associated resveratrol with different pharmacological activities, including anti-carcinogenic, antioxidant, anti-inflammatory, anti-diabetic and anti-asthmatic effects, and other beneficial properties in cases of pancreatitis and osteoarthritis [9–11].

Regarding the anti-carcinogenic potential of this compound, a number of studies have reported that resveratrol inhibits the proliferation of certain types of cancer cells such as myeloid leukemia, colon carcinoma, hepatocarcinoma and head and neck malignancies [7,12–14].

Although the precise chemopreventive mechanisms of resveratrol remain unclear, they may include: modulation of the activity of carcinogen-metabolizing enzymes, scavenging of free radicals, the inhibition of cell proliferation, the suppression of cyclo-oxygenase activity, induction of apoptosis and the inhibition of angiogenesis [15,16]. In this setting, resveratrol

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(Received 13 August 2013; accepted 8 November 2013)

ISSN 0001-6357 print/ISSN 1502-3850 online © 2014 Informa Healthcare
DOI: 10.3109/00016357.2013.865787

interacts with certain molecules directly related to carcinogenesis and cancer metastasis [17]. Specifically, resveratrol inhibits STAT3 (signal transducer and activator of transcription 3) and NF-κB (nuclear factor-kappaB-dependent transcription), Bcl-xL expression (B-cell lymphomal-xL) and hypoxia-induced expression of HIF-1 alpha (hypoxia-inducible factor 1-alpha) and VEGF (vascular endothelial growth factor) [18–20] and activates p53 and TRAIL (TNF-related apoptosis-inducing ligand) expression.

Research into plant extracts and phytochemicals as modifiers of irradiation effects represents a new field; this involves experimenting with radiosensitization in combination with different chemotherapeutic drugs (cisplatin, 5-fluorouracil, taxol) either prior to irradiation or as concomitant treatment for patients with head and neck malignancies [21]. As a result, another phenolic compound—curcumin—has been shown to be a potent radio-sensitizer of OSCC [22].

Radiation is known to exert a more effective cytotoxic effect upon cells that are in the G2 and mitotic phases than on cells in other phases of the cell cycle. Resveratrol has a capacity to produce increased cell apoptosis and increased cell cycle arrest in the S-phase in other types of malignancy such as prostate cancer [23] or cervical cancer [24]. So, it is reasonable to hypothesize that cell pre-treatment with resveratrol could result in radiosensitization as a secondary effect to specific cell cycle inhibition.

Hence, the aim of the present study was to evaluate the effect of resveratrol and irradiation upon OSCC.

Materials and methods

Cell line

The present study used the PE/CA-PJ15 human oral squamous carcinoma cell line (European Collection of Cell Cultures) cultured in Iscove's modified Dulbecco's modified Eagle medium (IMDM) supplemented with 10% fetal calf serum (FCS), 1% penicillin and 1% streptomycin (full medium) at 37°C, in an atmosphere of 95% oxygen and 5% CO_2. The medium (IMDM) also contained 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and dimethylsulfoxide (DMSO).

Drug preparation

Resveratrol (trans-3,4′,5-trihydroxystilbene) (Figure 1) (Sigma-Aldrich Chemistry, S.A., Madrid, Spain) was dissolved in 0.5% DMSO, with 1 mg/ml of resveratrol used as stock solution. The working solutions were diluted with sterile distilled water. Different doses were created with 5, 10, 25, 50 and 100 μM of resveratrol. All manipulations of resveratrol were performed under subdued light.

Irradiation

Irradiation of the cells was performed using a linear accelerator (Yxlon Smart, Krautkrämer-Forster Española, S.A., Madrid, Spain). The machine was calibrated for the field size of interest using small ionization chambers and thermoluminescence dosimetry. The cells were irradiated in 96-microwell plates, administering single irradiation doses of 1, 2.5 and 5 Gy.

Cell viability test (MTT assay). The study used the technique described by Carmichael et al. (1987a, b, adapted to the study's culture conditions) for the quantification of cell viability [25]. The cells were cultured at a density of 5×10^3 cells per well in 96-microwell plates. Fifteen minutes after plate irradiation (1, 2.5 and 5 Gy), resveratrol was added at different concentrations (5, 10, 25, 50 and 100 μM).

At different time-points after the start of treatment (24, 48 and 72 h), the medium was eliminated and the cells were incubated with MTT (Sigma-Aldrich Chemistry, S.A.) (1 mg/ml) for 4 h, after which the non-metabolized MTT was discarded and 100 μl of DMSO was added to each well. Absorbance in each well was measured with an enzyme-linked immunosorbent assay (ELISA), using a plate spectrophotometer (Multiskan MCC/340P, Labsystems, Helsinki, Finland) at a reading wavelength of 570 nm and a reference wavelength of 690 nm. This evaluation was performed in triplicate.

Apoptosis: histone-associated-DNA-fragments, enzyme-linked immunosorbent assay (ELISA). The ELISA kit was used to detect apoptosis in cells treated with resveratrol and irradiation, following the manufacturer's instructions. Cells were seeded in 96-well plates at a density of 1×10^4 cells per well for 24 h. Fifteen minutes after plate irradiation (1, 2.5 and 5 Gy), the medium containing different concentrations of resveratrol (5, 10, 25, 50 and 100 μM) was added. After 24, 48 or 72 h, cytoplasm was transferred to the 96-well plate, coated with streptavidin and incubated with biotinylated histone antibody and peroxidase-tagged mouse anti-human DNA for 2 h at room temperature. The absorbance at 405 nm was measured with EXL-800 type enzyme-linked immunosorbent apparatus (Bio-Tek, Shanghai, China). The evaluation was performed in triplicate.

Flow cytometry and cell cycle analysis

The cell line was cultured at an adequate density in 6-well plates (PE/CA-PJ15: 200,000 cells), allowing the cells to grow for 24 h. Fifteen minutes after plate irradiation (1, 2.5 and 5 Gy), the medium containing different concentrations of resveratrol was added (5, 10, 25, 50 and 100 μM). After 24, 48 and 72 h of

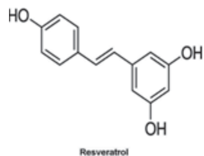

Figure 1. Chemical composition of resveratrol.

treatment with resveratrol, the cells exhibiting a confluence of <80% were collected by trypsinization and centrifuged at 214 g for 10 min. The cells were re-suspended in 200 µl of phosphate buffered saline (PBS) and fixed in ethanol (70% in PBS) at 4°C for 30 min. Then, centrifugation was repeated at 214 g for 10 min and the cells were re-suspended in 800 µl of PBS. Lastly, incubation was carried out at 37°C for 30 min with 100 µl of propidium iodide (end concentration 40 µg/ml) and 100 µl of ribonuclease (Rnase) (end concentration 100 µg/ml).

The DNA content of the stained cells was studied using a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA, USA), with a 488 nm argon laser for forward scatter (FSC) and side scatter (SSC) data collection and for registering the propidium iodide fluorescence intensity (PLA-2) of 20 000 events. Data were processed using CELLQUEST software and the MODFIT program (Becton Dickinson). This technique calculated the fluorescence intensity of the fluorochrome bound to the DNA (propidium iodide) and differentiated the cells in different cell cycle phases: G_0–G_1, G_2–M or S. This evaluation was performed in triplicate.

Migration (scratch wound healing). Scratch wounds were generated in confluent monolayers of cells using a sterile 200 µl pipette tip. Suspended cells were washed away with PBS and, after 15 min plate irradiation (1, 2.5 and 5 Gy), the culture medium was changed and different concentrations of resveratrol added (5, 10, 25, 50 and 100 µM).

Migration into the wound space was photographed (using an inverted microscope equipped with a digital camera) at the moment of wounding and at time intervals of 4 and 8 h post-wounding. The relative distances between edges of the injured monolayer were obtained via pixel counts at a minimum of 10 sites of the wound, using MIP-4 image software (CID, Barcelona, Spain) and applying the formula: migration distance = initial distance of free-of-cells space – distance of free-of-cells space at 4 or

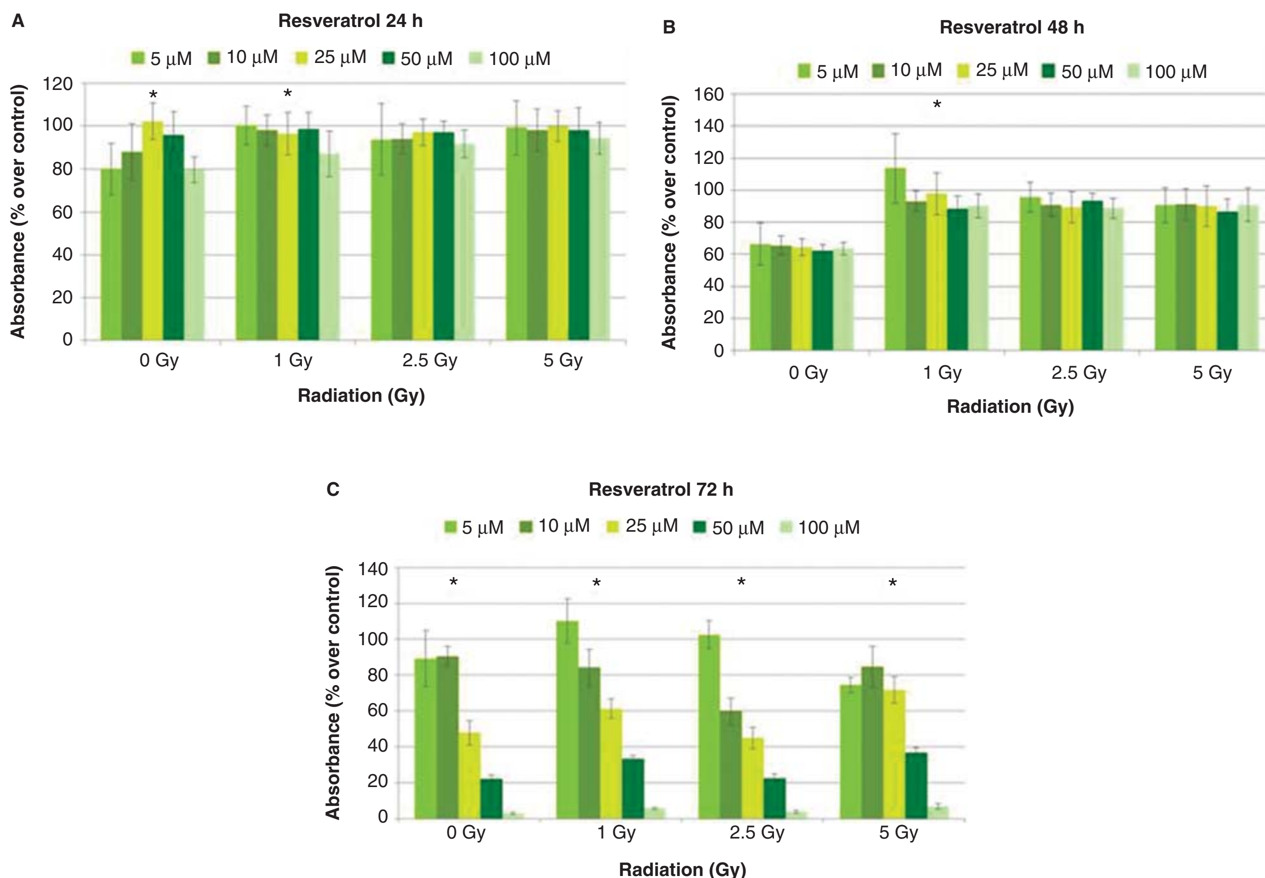

Figure 2. Effects of resveratrol and irradiation upon PE/CA-PJ15 cell viability, (A) at 24-h of incubation, (B) at 48-h of incubation and (C) at 72-h of incubation (A: 0 Gy, $p < 0.001$; 1 Gy, $p = 0.016$; 2.5 Gy, $p = 0.622$; 5 Gy, $p = 0.725$. B: 0 Gy, $p = 0.704$; 1 Gy, $p < 0.001$; 2.5 Gy, $p = 0.268$; 5 Gy, $p = 0.881$. C: 0 Gy, $p < 0.001$; 1 Gy, $p < 0.001$; 2.5 Gy, $p < 0.001$; 5 Gy, $p < 0.001$).

8 h (Valster et al., 2005). This evaluation was performed in triplicate.

Statistical analysis

Data were analyzed using the SPSS version 12.0 statistical package (SPSS Inc., Chicago, IL). A descriptive study was made of each variable. The associations between different quantitative variables were studied using one-way analysis of variance (ANOVA) for more than two samples, verifying in each case that variances were homogeneous. Statistical significance was established at $p < 0.05$.

Results

Effects of resveratrol and irradiation upon PE/CA-PJ15 cell viability

Twenty-four hours of incubation. Resveratrol at a concentration of 100 μM induced the greatest decrease in cell viability at all the irradiation doses administered, with statistically significant differences at 0 and 1 Gy ($p < 0.001$ and $p = 0.016$, respectively) (Figure 2A).

Forty-eight hours of incubation. Resveratrol at a concentration of 50 μM induced the greatest decrease in cell viability at 0 and 1 Gy, with statistically significant differences at 1 Gy ($p < 0.001$). At a concentration of 100 μM, resveratrol induced the greatest decrease in cell viability at 2.5 and 5 Gy, although no statistically significant differences were observed ($p = 0.268$ and $p = 0.881$, respectively) (Figure 2B).

Seventy-two hours of incubation. The greatest decrease in cell viability was observed after 72 h incubation, at all resveratrol concentrations and all irradiation doses; at this time-point, resveratrol induced the greatest decrease in cell viability at a concentration of 100 μM, at all the irradiation doses used, with statistically significant differences at 0 ($p < 0.001$), 1 ($p < 0.001$), 2.5 ($p < 0.001$) and 5 Gy ($p < 0.001$) (Figure 2C).

Effects of resveratrol and irradiation upon PE/CA-PJ15 cell apoptosis

After 24, 48 and 72 h incubation, resveratrol at a concentration of 100 μM induced the greatest cell

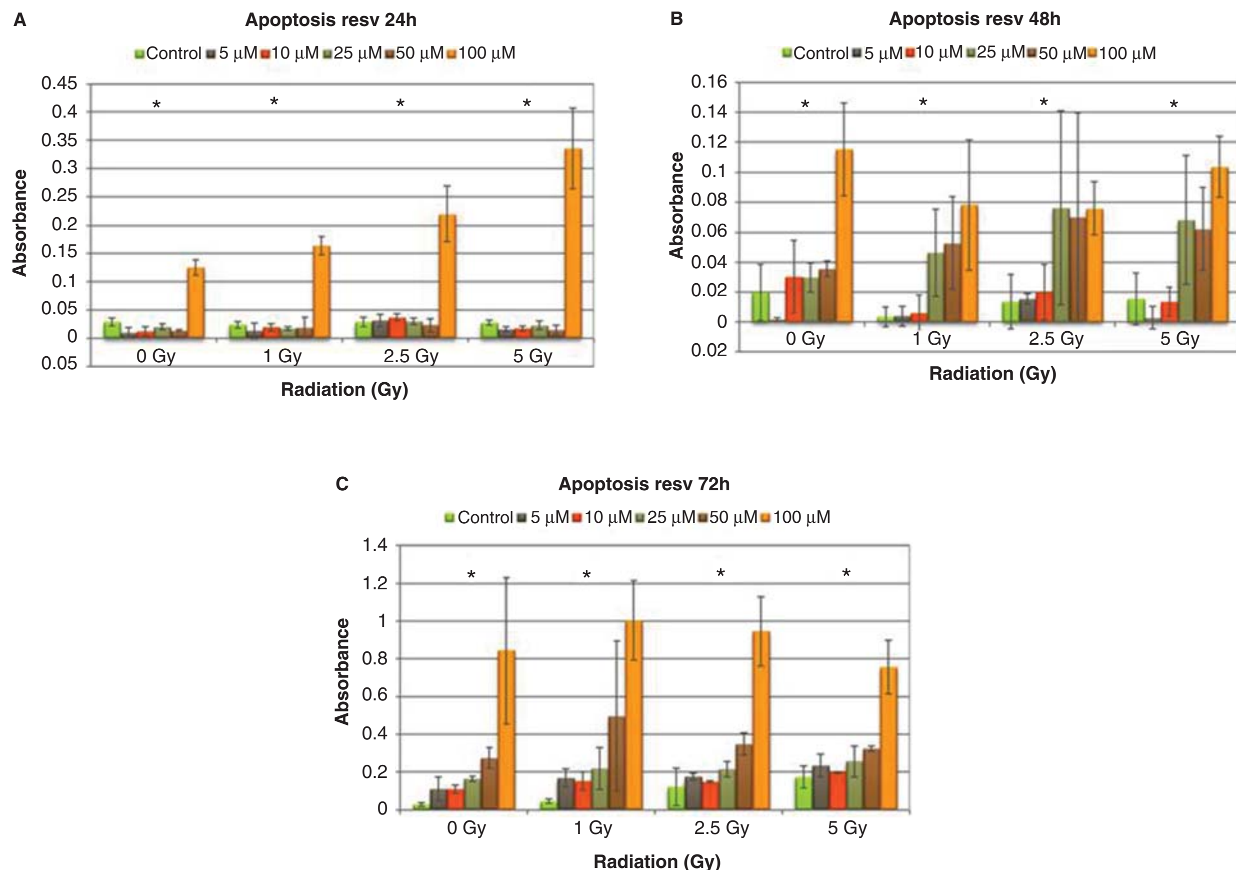

Figure 3. Effects of resveratrol and irradiation upon PE/CA-PJ15 cell apoptosis, (A) at 24-h of incubation, (B) at 48-h of incubation and (C) at 72-h of incubation (A: 0 Gy, $p < 0.001$; 1 Gy, $p < 0.001$; 2.5 Gy, $p < 0.001$; 5 Gy, $p < 0.001$. B: 0 Gy, $p < 0.001$; 1 Gy, $p = 0.014$; 2.5 Gy, $p = 0.038$; 5 Gy, $p = 0.001$. C: 0 Gy, $p = 0.001$; 1 Gy, $p = 0.001$; 2.5 Gy, $p < 0.001$; 5 Gy, $p < 0.001$).

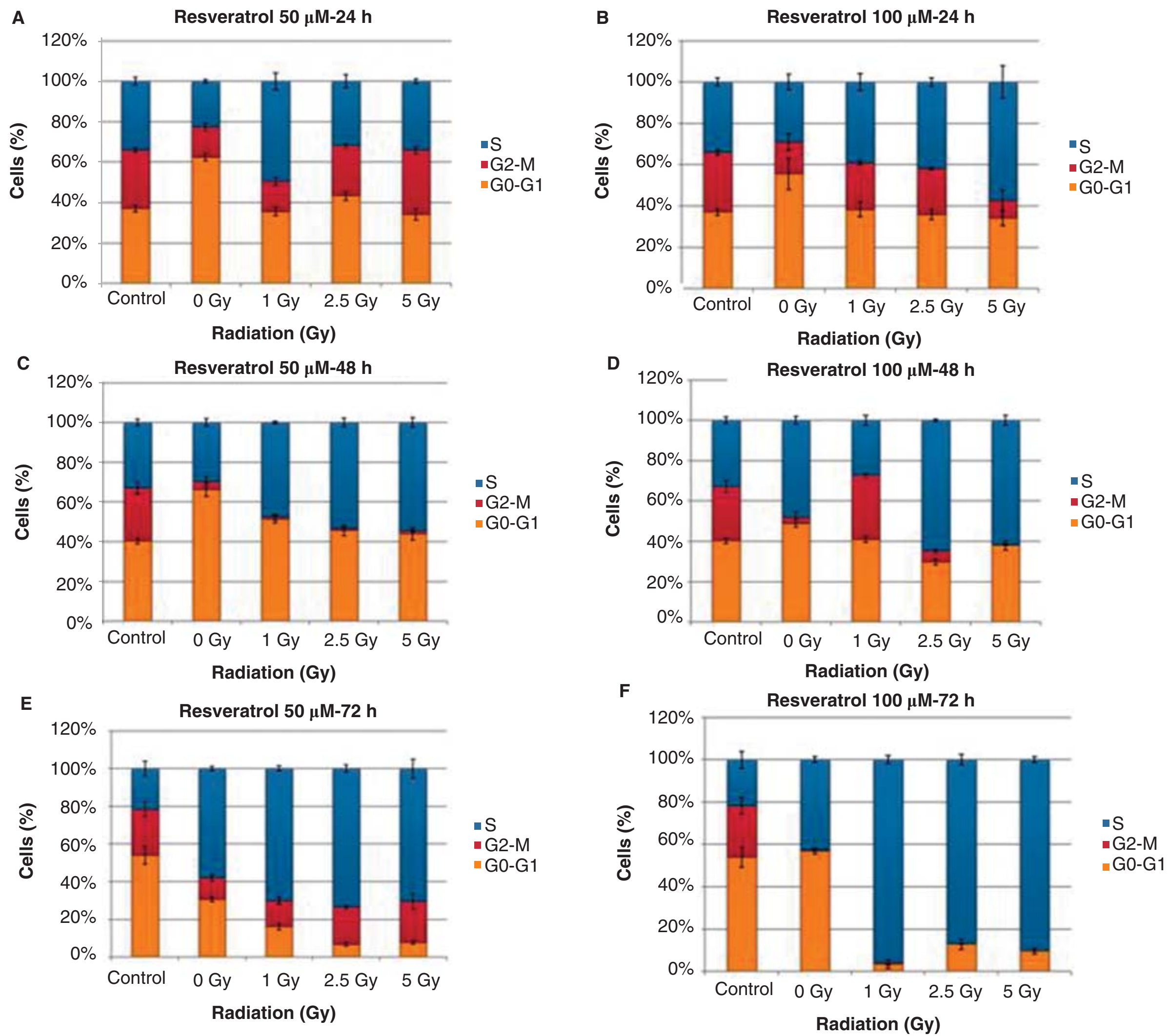

Figure 4. Effects of resveratrol and irradiation upon PE/CA-PJ15 cell cycle, (A and B) at 24-h of incubation, (C and D) at 48-h of incubation and (E and F) at 72-h of incubation (A: G_0–G_1, $p < 0.001$; G_2–M, $p < 0.001$; S, $p < 0.001$. B: G_0–G_1, $p = 0.001$; G_2–M, $p < 0.001$; S, $p < 0.001$. C: G_0–G_1, $p < 0.001$; G_2–M, $p < 0.001$; S, $p < 0.001$. D: G_0–G_1, $p < 0.001$; G_2–M, $p < 0.001$; S, $p < 0.001$. E: G_0–G_1, $p < 0.001$; G_2–M, $p = 0.001$; S, $p < 0.001$. F: G_0–G_1, $p < 0.001$; G_2–M, $p < 0.001$; S, $p < 0.001$).

apoptosis at all irradiation doses, with statistically significant differences (Figures 3A–C).

Effects of resveratrol and irradiation upon PE/CA-PJ15 cell cycle

After 24 h, the untreated cells were distributed almost equally among the three cell cycle phases. However, after 48–72 h, an increase in the G_0–G_1 phase was observed at the expense of the S-phase.

The greatest alterations in the distribution of the G_0–G_1, G_2–M and S phases of the cell cycle were recorded with resveratrol at 50 μM and 100 μM. After 24, 48 and 72 h of incubation, an increase was observed in the S-phase with both resveratrol concentrations, at the expense of the G_0–G_1 and G_2–M phases, with statistically significant differences at all three incubation periods (p-values shown in Figures 4A–F).

Effects of resveratrol and irradiation upon PE/CA-PJ15 cell migration

The 25 μM and 100 μM concentrations of resveratrol produced the greatest reduction in cell migration capacity. Statistically significant differences were observed with 25 μM and 5 Gy at 4 h post-wounding ($p < 0.001$) and with 100 μM and 1, 2.5 and 5 Gy at 8 h post-wounding ($p = 0.001$, $p = 0.006$ and $p < 0.001$, respectively) (Figures 5A–D).

Discussion

Dietary flavonoids have been shown to have beneficial biological effects upon different cell types, including anti-inflammatory, antioxidant, anti-proliferative and pro-apoptotic effects [26,27]. Numerous cell and animal studies have suggested that resveratrol may

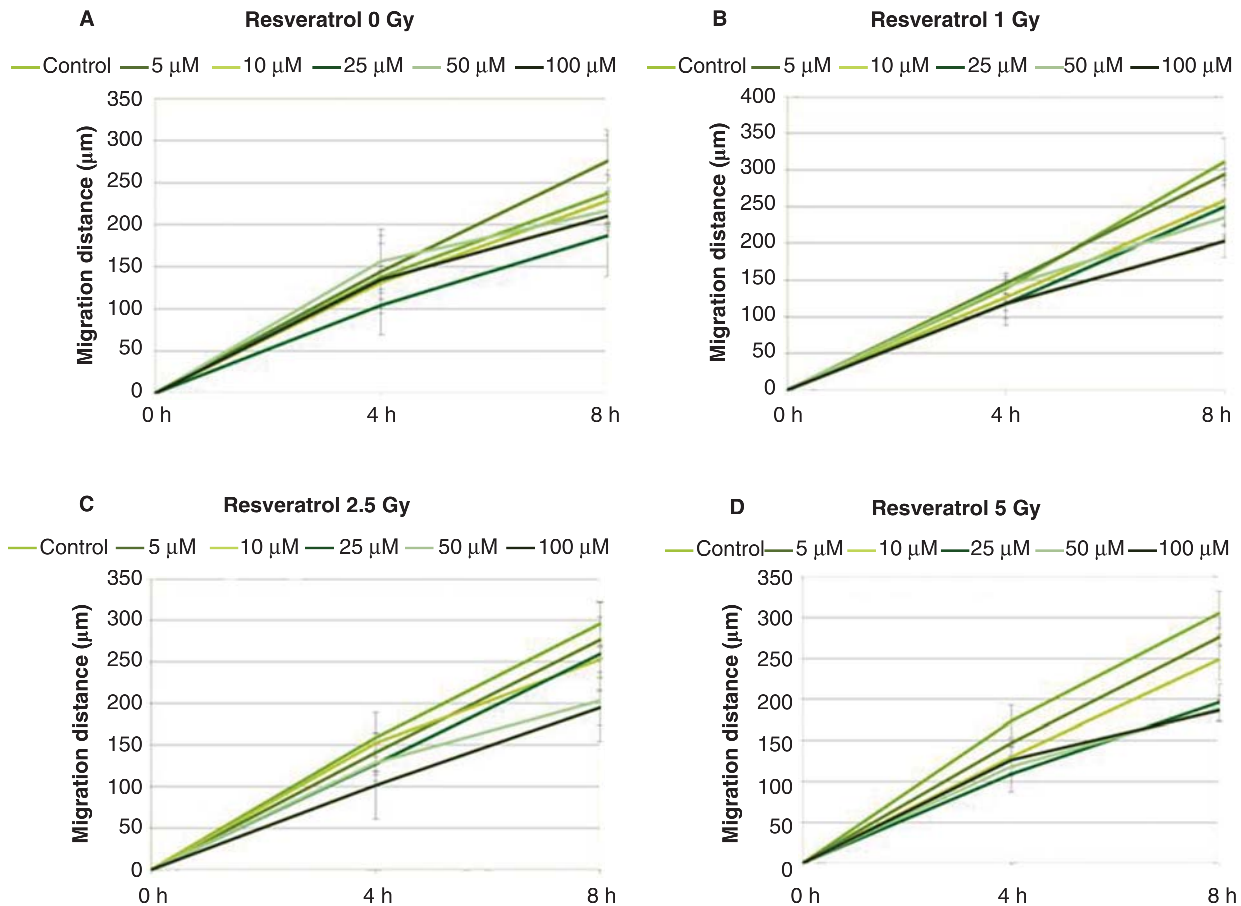

Figure 5. Effects of resveratrol and irradiation upon PE/CA-PJ15 cell migration (A: T4, $p = 0.386$; T8, $p = 0.104$. B: T4, $p = 0.246$; T8, $p = 0.001$. C: T4, $p = 0.082$; T8, $p = 0.006$. D: T4, $p < 0.001$; T8, $p < 0.001$).

be associated with potential therapeutic or preventive effects on a variety of malignancies. Data from carcinogenetic studies involving experimental models have shown that resveratrol is an attractive candidate for chemoprevention clinical trials. In addition to its anti-carcinogenic activity, resveratrol has a number of cardioprotective, anti-aging and other biological activities that suggest its possible usefulness in the prevention of cardiovascular and other chronic diseases [15]. *In vitro* and *in vivo* results in cancer chemoprevention studies demonstrate that resveratrol can inhibit carcinogenesis at several organ sites [28]. Resveratrol confers significant protection against cancer induction in the rat and mouse mammary gland [29], rat and mouse colon, mouse skin neutrophil collagenase (mNC) [6] and OSCC [14]; however, the compound appears to be inactive in chemoprevention in the mouse lung [30].

In the present study, resveratrol exerted a cytotoxic effect upon PE/CA-PJ15 tumor cells. The magnitude of this effect was directly proportional to the drug concentration used, the greatest reduction in cell viability and increase in apoptosis being achieved with doses of 50 µM and 100 µM. Elattar and Virgi [14] used resveratrol doses of 10–100 µM in application to OSCC cells (SCC-25), finding that cell viability decreased as the resveratrol concentration increased, the greatest reduction in cell viability corresponding to the 100 µM dose. Hu et al. [6] also observed the same cytotoxic effects upon head and neck cancer, which increased as resveratrol concentrations strengthened.

The present study recorded increases in apoptotic activity after applying resveratrol to the OSCC cells. While this chemopreventive agent induces apoptosis in different cancer cell lines [13,14], it does not appear to induce apoptosis in healthy cells and tissues [20]. Resveratrol seems to promote cell cancer apoptosis either by blocking anti-apoptotic protein expression or by inhibiting the transduction of different signals.

Regarding the effects of resveratrol and irradiation upon the PE/CA-PJ15 cell cycle, the greatest alterations to the distribution of the G_0–G_1, G_2–M and S phases of the cell cycle were produced at resveratrol doses of 50 µM and 100 µM. After 24, 48 and 72 h of incubation, both resveratrol concentrations induced an increase in the S-phase at the expense of the G_0–G_1 and G_2–M phases. Rubiolo et al. [7] found that, in application to rat primary hepatocytes, resveratrol induced cell arrest in the G_0–G_1 phase but not in the S-phase. It would appear that resveratrol has the

capacity to alter the distribution of the G_0–G_1, G_2–M and S phases in tumor cells and that this alteration is dose-dependent.

In relation to the effects of resveratrol and irradiation upon PE/CA-PJ15 cell migration, the present results suggest an increased reduction in migration capacity at resveratrol concentrations of 25 μM and 100 μM, at all irradiation doses. Data obtained by Salado et al. [8] showed that resveratrol inhibited IL-18 secretion from tumor-activated hepatic tissue and, subsequently, the effects of IL-18 on both host and cancer cells. This in turn prevented inflammation-dependent metastasis in the pro-metastatic microenvironment created by tumor-induced IL-18 in the liver. Previous studies have already reported anti-metastatic effects of resveratrol in various rodent and human tumors [18–20]. However, the specific sites where resveratrol might be effective for therapeutic intervention in metastatic processes remain unclear.

Radiotherapy plays an important role in cancer management, helping to secure local tumor control after surgery in patients with early stage malignancies. However, radiotherapy alone will not suppress tumors, which recur and become radioresistant. The factors conditioning radioresistance in patients with recurring malignancies remain unclear. Many studies have reported that antioxidants such as vitamin E, curcumin, β-carotene, lycopene or selenium, in addition to radiotherapy, have beneficial effects in the management of different types of cancer (head and neck squamous cell carcinoma, prostate cancer, etc.). The present results suggest a synergic effect against OSCC cells when radiotherapy is combined with resveratrol treatment. A review published by Reagan-Shaw et al. [26] reported the usefulness of resveratrol for preventing damage caused by UVB radiation and its ability to improve the radiosensitivity of tumor cell lines.

Conclusions

After 72 h of incubation, resveratrol at a dose of 100 μM induced the greatest decrease in cell viability, at all the irradiation doses administered. This was also true at the 24- and 48-h time-points. The greatest alterations in the distribution of the G_0–G_1, G_2–M and S phases of the cell cycle were registered at resveratrol concentrations of 50 μM and 100 μM; after 24, 48 and 72 h of incubation, both resveratrol concentrations induced an increase in the S phase at the expense of the G_0–G_1 and G_2–M phases. Lastly, the maximum reduction in cell migration capacity was recorded with the 25 μM and 100 μM doses of resveratrol.

Resveratrol increases cytotoxic activity in the PE/CA-PJ15 tumor cell line and reduces cell migratory capacity, while the combination of resveratrol with irradiation exerts a synergic effect.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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