Differential Effects of Resveratrol on HECa10 and ARPE-19 Cells

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Abstract: Age-related macular degeneration (AMD) and diabetic retinopathy are the leading cause of blindness in developed countries. Pathological angiogenesis has a causal role in these eye diseases. Resveratrol (RSV), a plant-derived polyphenol, has anti-proliferative and anti-angiogenic properties that could improve its management. Here, the effects of various concentrations of RSV (1, 5, 10, 50, 100 µM) were compared in two types of cell lines: HECa10 (endothelial cell line) and ARPE-19 (retinal pigment epithelial cell line). We assayed the impact on proliferation rate, viability, cell cycle progression, and secretion of selected proangiogenic factors VEGF and bFGF. We show that lower concentrations of RSV (1, 5, 10 µM) had no effect on proliferation, viability or cell cycle progression in HECa10 cells. However, higher concentrations (50, 100 µM) significantly enhanced the reduction in the cell number and stimulated apoptosis. In ARPE-19 cells, lower concentrations of RSV increased the rate of proliferation, while higher concentrations had no effect on proliferation and viability. Both ARPE-19 and HECa10 cell lines were affected to different degrees in the secretion of proangiogenic cytokines: reducing VEGF and enhancing bFGF secretion. These results suggest that RSV may be useful in the prevention or treatment of pathological angiogenesis in eye disorders.

Keywords: resveratrol; endothelial cells; retinal pigment epithelial cells; proliferation; cell cycle; cell death; VEGF; bFGF

1. Introduction

Resveratrol (3,4',5-trihydroxystilbene-RSV) is a biologically active compound from the phytoalexin family. This phenolic substance exists in isomeric cis- and trans-form, wherein trans-form is more stable in the biological systems [1]. RSV is synthesised by many plant species, such as grape wine (Vitis vinifera), berries, peanuts, and medicinal plants [2]. It is also present in plant-derived products, including wine, which is the common source of RSV in the Western European diet [3]. This compound is well characterised in terms of its antioxidant properties as a scavenger of the reactive oxygen species (ROS) [4]. RSV has various medical applications. The main biological role of RSV is associated with its positive effect on the cardiovascular system. RSV reduces inflammation and apoptosis in vascular endothelium, increases the flexibility of the aorta, decreases the level of cholesterol and...
triglycerides in the blood, protects most organs (kidneys, heart and brain) from ischemic injury, and may reduce the risk of heart attack [5]. It also normalises the concentration of glucose in diet-induced obese and diabetic mice suggesting a similar eventual effect in humans [6].

RSV significantly exhibits anti-inflammatory and anti-angiogenic activities and regulates many cellular signaling pathways associated with cell metabolism, proliferation, differentiation, migration, and invasiveness of normal and cancer cells [7]. It also affects the in vitro and in vivo formation of new vessels [8]. Currently, RSV is widely studied as a potential antitumor therapeutic, in which its antigenicity-related and anti-proliferative activities take part [4]. In vitro studies showed that RSV significantly decreases the proliferation of many types of cancer cells including pancreatic cancer [9], breast cancer [10], and colon cancer [11]. Similarly, in vivo RSV could modulate tumorigenesis, affecting angiogenesis, growth, and development of tumors [8]. Next to anticancer therapy, the anti-angiogenic activity of RSV may potentially be used to inhibit pathologic neovascularisation in age-related eye diseases such as age-related macular degeneration (AMD) or diabetic retinopathy; the main diseases responsible for vision loss in the 65 and older age population. AMD is recognised as a chronic and progressive degeneration of the macula, resulting in loss of central vision in the elderly [12], significantly deteriorating the quality of life and increasing depression levels compared to without AMD elderly [13]. This disorder is marked by the presence of drusen in the macula followed by geographic atrophy or choroidal neovascularisation (CNV) [12] and classified in terms of expected invasion side into the retina. Because the development of neovascularisation is strictly correlated with vascular endothelial growth factor (VEGF), an anti-VEGF therapy has found its place in AMD treatment and it is considered to be a current gold standard therapy [14]. Targeted immunotherapy against VEGF is currently used in AMD treatments, and some new VEGF inhibitors are in clinical trials [15]. However, some authors revealed that a long-term incubation with anti-VEGF agents may induce epithelial to mesenchymal transition in ARPE-19 cells, which provides a novel insight into the pathogenesis of intravitreal anti-VEGF-associated complications [16].

Taking into account the anti-angiogenic activities of RSV, it may decrease the VEGF expression leading to inhibition of neovascularisation development and therefore affecting pathological angiogenesis. Seong et al. demonstrated that RSV inhibits the hypoxia-induced VEGF in ARPE-19 cells by CXCR4 expression suppression through decreased NF-κB phosphorylation [17].

Subramani et al. showed that RSV may reverse the adverse effects of anti-VEGF therapy as bevacizumab on cultured ARPE-19 cells. Epithelial to mesenchymal transition was reported to be lower in combination agents (bevacizumab + RSV) treated cultures compared to bevacizumab-only treated cultures. The proliferation was similar in both groups, in opposition to phagocytosis, which was increased in the bevacizumab + RSV group vs. bevacizumab group [18]. It may give new insight for a concept using anti-VEGF agent with RSV supplementation in patients with wet AMD or proliferative diabetic retinopathy and which deserves further investigation.

Moreover, RSV has been shown to suppress proliferation, migration, and fibronectin synthesis induced by transforming growth factor-β2, inhibiting epithelial to mesenchymal transition of retinal pigment epithelial cells and consequently the development of proliferative vitreoretinopathy, as the sirtuin1 activation effect [19].

Chen et al. (2017) demonstrated RSV inhibition on the decrease of zona occludens-1 and the increase of mesenchymal marker vimentin expression by suppressing Smad2 and Smad3 phosphorylation [20].

The excessive proliferation and differentiation of endothelial cells are responsible for several eye diseases. Our previous studies showed that RSV inhibits proliferation, migration, and invasiveness of the endothelial cell line HECa10 [21]. The HECa10 cells are murine high endothelial cells (HEC) from the peripheral lymph nodes, established as a non-transformed cell line, displaying and maintaining the characteristic phenotype
of HEC cells of the tissue of origin. Besides their characteristic ability to recognise and select lymphocytes and specifically adhering cells, HECa10 produce specific blood vessels proteins such as the E-selectin, angiotensin-converting enzyme, factor VIII-related antigen, von Willebrand factor and VE-cadherin [22,23]. Based on previous results [21], here we asked if the effects of RSV are cell-type specific and how epithelial cells, here, eye-derived cell line ARPE-19, respond to RSV treatment. Therefore, we evaluated, in parallel, the effects of 1–100 µM RSV on these two cell lines’ proliferation, death type: necrosis vs. apoptosis, cell cycle progression, and secretion of proangiogenic factors VEGF and bFGF.

2. Results
2.1. RSV Differently Affects Endothelial and Epithelial Cells Proliferation and Morphology

As shown in Figure 1A–F, RSV applied for 24 h at low concentrations (1, 5, and 10 µM) did not affect either HECa10 cell proliferation, as measured by neutral red or sulforhodamine B assays, or cell morphology. Higher concentrations of RSV (50 and 100 µM) caused a significant decrease in HECa10 cell number to about 20% (50 µM, \( p < 0.01 \)) and to 40% (100 µM, \( p < 0.001 \)) when compared to the control cells. HECa10 cells treated with RSV at 50 µM did not induce any morphological changes (Figure 1G). RSV addition only at 100 µM concentration changed the cell shape and induced apoptotic phenotype (Figure 1H). The incubations of cells with RSV in doses mentioned above and extended to 48 h caused a much deeper decrease in cell proliferation of HECa10.

![Figure 1](image.png)

Figure 1. Effect of different RSV concentrations on HECa10 cell proliferation as analysed by (A) NR assay and (B) SRB assay. Cell were incubated for 24 h with RSV (1, 5, 10, 50, and 100 µM). Control cells were cultured with 0.1% DMSO and without RSV. (C–H) Light microscopy images of control and RSV-treated cells (Delta Optical, magnification 100×). The results are presented as mean ± SD at least of three experiments with ** \( p < 0.01 \), *** \( p < 0.001 \) compared to control.
The treatment of ARPE-19 cells with low concentrations (1, 5, and 10 µM) of RSV did not reduce either proliferation or cell morphology (Figure 2A–F). Interestingly, the NR assay, but not the SRB assay, showed a significant increase in the cell number after treatment with 5, 10, and 50 µM of RSV ($p < 0.001$). The disparity between the results obtained with these two assays, and the fact that the NR assay measures the red dye uptake by lysosomes, suggests that RSV at 5, 10, and 50 µM concentrations did not induce an increase in the cell number but either increased the lysosome number or provoked lysosomal swelling, or increased the uptake of the dye by other means. Higher concentrations of RSV (50 and 100 µM) had no effect on ARPE-19 cells morphology (Figure 2G,H).

![Figure 2. Effect of different RSV concentrations on ARPE-19 cell proliferation as analysed by (A) NR assay and (B) SRB assay. Cell were incubated for 24 h with RSV (1, 5, 10, 50, 100 µM). Control cells were cultured with 0.1% DMSO and without RSV. (C–H) Light microscopy images of control and RSV-treated cells (Delta Optical, magnification 100×). The results are presented as mean ± SD at least of three experiments with *** $p < 0.001$ compared to control.]

2.2. RSV Effect on Cell Viability

Untreated HECa10 endothelial cells were used as a control group and have shown the presence of 90.5%, 5.4%, and 3.9% of living, apoptotic and necrotic cells, respectively (Figure 3A–C). RSV treatment caused a significant decrease in HECa10 cell viability only in the highest concentrations (50 and 100 µM) and caused approximately a 5-fold increase in
apoptosis as compared to the control cells (50 µM, \(p < 0.05\); 100 µM, \(p < 0.01\)). However, non-significant changes in the percentage of necrotic cells were observed.

2.3. RSV Differently Affects Cell Cycle Distribution in the Two Cell Lines

Figure 3A of HECa10 cells shows that in the control group the mean percentage of cells in the G0/G1 phase was 41.2%, in the S phase 42.2% and in the G2/M phase 16.4%, respectively. RSV at concentrations 1, 5, and 10 µM did not cause significant changes in the cell cycle distribution. In contrast, RSV used at 50 µM increased the number of cells in the G2/M phase (\(p = 0.049\)), while the treatment with RSV at 100 µM concentration significantly increased the percentage of cells in G0/G1 phase (about 1.5-fold) and at the same time reduced about 3-fold the proportion of cells in the S and G2/M phases.

In ARPE-19 untreated cells, the mean percentage of cells in the G0/G1 cell cycle phase was 65%, in the S phase 17.7%, and in the G2/M phase 17.3% (Figure 4B). The RSV treatment in all concentrations did not cause significant changes in the cell cycle distribution in these cells.
Figure 4. Effect of different RSV concentrations on the cell cycle of (A) HECa10 cells and (B) ARPE-19 cells. Cell were incubated for 24 h with RSV (1, 5, 10, 50, 100 µM). Control cells were cultured with 0.1% DMSO and without RSV. Analysis of the cell cycle was performed using flow cytometry (FACS Calibur, BD) and calculated using ModFit LT. The results are presented as mean ± SD in at least of three experiments with * $p < 0.05$, ** $p < 0.01$ compared to the control.

2.4. Impact of RSV on the Secretion of Angiogenic Cytokines

The addition of RSV to HECa10 cells induced inhibition of VEGF secretion by the treated cells as compared to the untreated control. The highest, about 20% decrease, in VEGF secretion was observed after the treatment of these cells with 1 µM RSV ($p < 0.01$) (Figure 5A). As shown in Figure 5B, only the treatment of HECa10 cells with 50 µM RSV significantly increased the concentration of bFGF ($p < 0.05$). Concomitantly, in ARPE-19 cells, RSV in all tested concentrations (1, 5, 10, 50, and 100 µM) caused the reduction of VEGF secretion and the increase in bFGF concentration (Figure 5C,D).
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Figure 5. Secretion of angiogenic cytokines by the RSV treated HECa10 and ARPE-19 cells. VEGF and bFGF concentration in the supernatants from (A,B) HECa10 and (C,D) ARPE-19 cell lines cultures after 24 h treatment with RSV at 1, 5, 10, 50, and 100 µM were measured using ELISA test. Control cells were cultured with 0.1% DMSO and without RSV. The results are presented as mean ± SD in at least three experiments with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to the control.

3. Discussion

The increase in the human lifespan causes a rapid increase in age-related eye diseases, such as retinal vein occlusions, age-related macular degeneration, and retinopathy of prematurity (ROP) [24]. It is predicted that in 2040, there will be 14.9–21.5 million individuals in Europe with early AMD and 3.9–4.8 million with late one [25]. It is known that in all these eye-related diseases, pathological angiogenesis is the main culprit. Angiogenesis is a physiological process in which endothelial cells over-proliferate and forms new blood vessels. The critical factors in the initiation and development of the angiogenesis process are proangiogenic cytokines, such as VEGF. Blocking VEGF by anti-VEGF is one of the therapeutic strategies for the prevention of pathological angiogenesis. However, the administration of anti-VEGF does not fully inhibit the process of pathological angiogenesis due to the increase in the expression of other angiogenic factors in the eye (e.g., bFGF) [26].
Therefore, there is a need for novel therapeutics which will not only attenuate or inhibit the angiogenesis process but will also be able to operate through different or complementary mechanisms. One such compound is RSV. It affects endothelial cell proliferation and migration capacity, which we demonstrated previously [21]. Here, we assess the in vitro study effect of RSV on two different cells found in the eye structure: endothelial cells (involved in neoangiogenesis in the eye) and retinal pigment epithelial (damage of the cells caused AMD) cells. We want to check if the effect of RSV is the effect induced by RSV is selective and if it has the same effect on both types of cells.

It is well documented that RSV causes a decrease in proliferation and induces apoptosis in numerous cancer cell lines, such as human prostate carcinoma LNCaP cells, human breast cancer cells, mouse JB6 epidermal cell line, and HL60 human leukemia cell line [27–30]. The antitumor concentration of RSV ranges from 10 to 300 \( \mu M \) and the degree of its action is time and cell-type dependent [31,32]. Here, the obtained results point out that the RSV in high concentrations (50 and 100 \( \mu M \)) was affecting the growth of endothelial cells but not retinal pigment. The cause by which RSV induces endothelial cell death was apoptosis. In contrast to the HECa10 cells, RSV at 1–100 \( \mu M \) concentration did not affect apoptosis and necrosis of the ARPE-19 cells. Taken together, RSV at 50 and 100 \( \mu M \) concentrations promoted endothelial cells elimination by apoptosis while did not affect the retinal pigment epithelial cells. The effect of RSV on endothelial cells is in agreement with our previous study [21] and others. Trapp et al. (2010) observed a decrease in endothelial cell viability (by apoptosis) after RSV treatment (50 \( \mu M \), 48 h) when the cell was co-cultured with A375, YUZAZ6, or WM3211 melanoma cells [33]. Mohammadi and Arablou in 2017 reviewed the effect of RSV on endometriosis and found that the polyphenol not only decreased the proliferation of endometrial cells (through apoptosis induction) but also affected endothelial cells (decreased proliferating activity associated with decreased VEGF level) [34]. Contrarily, some studies indicate that RSV may reveal proangiogenic properties in other cell lines. Indeed, it has been reported that RSV at 10 and 50 \( \mu M \) concentrations significantly increased tube formation in the HCAEC-human coronary arteriolar endothelial cells on the matrigel, and enhanced the expression of the VEGF protein [35]. Simão et al. [36] described similar properties of RSV. The treatment of cerebral endothelial cells (THBMEC) with RSV promoted proliferation, migration, and tube formation in the matrigel assays. However, in these studies, RSV was applied at 5 \( \mu M \) concentration. Conversely, RSV did not affect the proliferation and apoptosis of retinal pigmented cells (ARP-19 cell line). It was also confirmed by Kang and Choung [37]. It still remains unexplained why and how RSV induces apoptosis in cancer cell lines and endothelial HECa10 cell lines, but not in the non-tumor cells.

RSV induces cell cycle arrest in many cancer cell lines, such as A549, U87, U138, PC-3, and primary cell cultures, such as vascular smooth muscle cells and primary rat hepatocytes [38–42]. Here, we did not find significant changes in cell cycle phases in both endothelial and retinal pigment cells; however, we noticed a dose-dependent tendency for cell accumulation in the S phase. We observed about 10% more cells in the S phase upon 100 \( \mu M \) RSV treatment than in the control. Simão et al. [36] also showed that, in the human cerebral microvascular endothelial cell line, the increase in the proportion of the S phase and the decrease in the G0/G1 phase proportion in the cell cycle of these cells was observed. Ahmad et al. [43] reported that the RSV induced the G1-phase arrest in A431 cells (human epidermoid carcinoma) mediated by WAF-1/p21, which blocked the following cyclin-cdk complexes: cyclin E-cdk2, cyclin D1/D2-cdk6, and cyclin D1/D2-cdk4. We observed a similar increased cell percentage in the G0/G1 phase in HECa10 cells at 100 \( \mu M \) RSV concentration. However, this effect seems to be related rather to cell death and not to cell cycle arrest.

We showed that the RSV treatment (1–10 \( \mu M \)) reduced the VEGF concentration in both analysed cell lines. Moreover, 50–100 \( \mu M \) RSV reduced the VEGF level secreted by the epithelial ARPE-19 cells (significant reduction) but not by the endothelial HECa10 cells. Interestingly, the retinal pigment epithelial ARPE-19 cells produced significantly
higher levels of VEGF than endothelial HECa10 cells. These differences between the two cell types can be extremely important in the eye’s microenvironment reorganisation and intercellular interactions. These results are similar to the data obtained from other laboratories. RSV, in a dose-dependent manner in concentrations between 10 and 50 µM, suppressed VEGF-A and VEGF-C secretion induced by inflammatory cytokines in the RPE cells and decreased VEGF mRNA level [44]. Also, Tino et al. [45] observed that the 10–30 µM RSV decreased the concentration of VEGF in the ovarian cancer SKOV-3 cell line. Moreover, RSV may have negated improvement of the myocardial perfusion and the arteriolar density achieved by the VEGF treatment in the hypercholesterolemic swine model of chronic ischemia [46]. RSV may also affect the biological activity of VEGF. Recently, Hu et al. (2019) showed that RSV might attenuate angiogenesis by inhibiting receptor signaling [47]. In contrast to the VEGF, the bFGF concentration in HECa10 cells was not affected by the RSV treatment (except for 50 µM concentration) and it was increased in epithelial cells at 1–100 µM concentrations of RSV. The elevated level of the FGF2 mRNA was also observed by Strunz et al. [48] in rats supplemented daily for 6 weeks with 22.04 mg/kg of RSV. Furthermore, Uchiyama et al. [49] observed that RSV (5–15 µM) inhibited the angiogenic response of cultured endothelial F-2 cells to the vascular endothelial growth factor but not to the basic fibroblast growth factor.

In conclusion, the different effects of RSV on endothelial cells (decreased proliferation and increased apoptosis) and retinal pigment epithelial cells (increased proliferation and lack of increase of cell death) and significantly decreased concentration of VEGF indicate that the RSV might be used as the anti-angiogenic factor in the management of eye diseases with pathological angiogenesis [50]. However, to fully understand the mechanism of the RSV effects, further studies are necessary, especially taking into account the fact that RSV does not target some angiogenesis mechanisms.

4. Materials and Methods
4.1. Chemicals
RSV purchased from Sigma Aldrich (3,4′, 5-trihydroxy-trans-stilbene, 5-[(1E)-2-(4-hydroxyphenyl) ethenyl]-1,3-benzenediol; purity ≥ 99%; #R5010, Sigma-Aldrich, St. Louis, MO, USA) was diluted in DMSO to give a 100 mM stock solution, aliquoted and stored at −80 °C until further use.

4.2. Cell Culture
The murine endothelial cell line (HECa10) was kindly gifted from the Laboratory of Glycobiology and Cell Interactions, Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences, Wroclaw. Cells were grown in DMEM supplemented with 4.5 g/mL of glucose with L-glutamine, 10% FBS, 50 U/mL penicillin, and 50 µg/mL streptomycin (all from Thermo Fisher Scientific, Waltham, MA, USA). They were maintained at 37 °C in a 5% CO2 and 95% humidity with a medium changed every 2–3 days. Cells used for the experiments were issued from the 5th to 10th passage. Before experimental treatments, cells in the logarithmic phase of growth were trypsinized (0.05%, 5 min, 37 °C), centrifuged (300 × g, 5 min), and counted in the Bürker chamber.

Human retinal pigment epithelial cell line (ARPE-19) was purchased from the ATCC collection (ATCC® CRL-2302™, Manassas, VA, USA). Cells were grown in DMEM, 4.5 g/mL glucose with L-glutamine, 10% FBS, 50 U/mL penicillin, and 50 µg/mL streptomycin (all from Thermo Fisher Scientific, Waltham, MA, USA). They were maintained at 37 °C in a 5% CO2 and 95% humidity with a medium changed every 2 to 3 days. Cells for experiments were issued from the 5th to 10th passage. Before experimental treatments, cells in the logarithmic phase of growth were trypsinized (0.05%, 5 min, 37 °C), centrifuged (300 × g, 5 min), and counted in the Bürker chamber.

Both HECa10 and ARPE-19 cell lines were free of mycoplasma, determined by MycoFluor Mycoplasma Detection Kit (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer’s procedure.
4.3. Proliferation Assays

HeCa10 and ARPE-19 cells were seeded in a 96-wells culture plate at a concentration of $1 \times 10^4$ cells/well. After 24 h they were rinsed twice with PBS (Thermo Fisher Scientific, Waltham, MA, USA) and resuspended in a fresh growth medium. RVS in DMSO was added at 1, 5, 10, 50, 100 µM concentration. The final concentration of DMSO was 0.1%. The same concentration of DMSO was added to the control samples (cells cultured in a growth medium without RSV). After 24 h incubation with RSV, cells were imaged under the Delta Optical microscope, magnification 100×, and the uptake of the neutral red (NR) and total protein (SRB) tests were performed. Each assay was performed in triplicate ($n = 15$).

4.3.1. Neutral Red (NR) Assay

The assays were performed as previously described [51]. Briefly, the cells were rinsed twice with PBS, and a new growth medium with 2.5 µg of NR per well was added (37 °C, 5% CO₂, 95% humidity). After the 1.5 h incubation period, cells were rinsed four times with PBS. Subsequently, the solution of 49% ethanol and 1% acetic acid in distilled water was added to the cells and the plates were shaken for 5 min. at 100 rpm. The absorption was measured at a wavelength of 540 nm with the background cutoff at 690 nm (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). The results are presented as the percentage of the control values (mean ± SD).

4.3.2. Sulforhodamine B (SRB) Assay

The assays were performed as previously described [48]. Briefly, HeCa10 and ARPE-19 cells were rinsed twice with PBS. Cells were fixed in cold 50% trichloroacetic acid (TCA) in PBS (4 °C, 1 h). Fixed cells were rinsed four times with distilled water and subsequently in SRB solution (0.4 mg SRB/well in 1% of acetic acids in PBS) for 30 min. at room temperature. Next, the cells were rinsed three times with 1% of acetic acids in PBS and the amount of dye bound to cell proteins was assessed by fluorescence readout (excitation-560 nm; emission-590 nm FLUOstar Omega, BMG Labtech). Results are presented as the percentage of the control values (mean ± SD).

4.4. Viability Assay

The assays were performed as previously described [52]. Briefly, cells were seeded at density $8 \times 10^4$ in a 6-well plate. After 24 h incubation the cells (about 60 % confluent) were treated with different RSV concentrations (1, 5, 10, 50, and 100 µM) for next 24 h. After the treatment, floating cells were collected for future analysis and adherent cells were washed with PBS and harvested by trypsinization. Subsequently, all cells (floating and adherent) were washed twice with PBS, centrifuged (500× g, 5 min) and resuspended in the annexin binding buffer with annexin V-FITC antibody (5 µL, eBioscience, San Diego, CA, USA) and with propidium iodine (PI, 5 µL, Sigma-Aldrich, St. Louis, MO, USA). After 20 min. incubation, cells were washed twice with PBS and analysed by flow cytometry (FACS Calibur, Becton Dickinson, USA, Franklin Lakes, NJ, USA. The evaluation of apoptosis, necrosis, and viability was performed using Cell Quest software (BD). The assay was performed in triplicate ($n = 6$).

4.5. Cell cycle Analysis

HECa10 and ARPE-19 cells were plated in a 6-well plate for 24 h, and next, the cells at 60–70% confluence were treated with different RSV concentrations (1, 5, 10, 50, and 100 µM) for 24 h. After the treatment, floating cells were collected for future analysis and adherent cells were washed with PBS and harvested by trypsinization. Subsequently, all cells (floating and adherent) were washed twice with PBS, centrifuged (500× g/ 5 min), and fixed with 70% cold methanol at −20 °C for 24 h. Fixed cells were resuspended in PBS and centrifuged at 500× g for 5 min. Cell pellets were resuspended in PBS with propidium iodine and RNase A solution according to the manufacturer protocol (Cayman Chemical
Company, Ann Arbor, MI, USA) and incubated for 30 min. in the dark at room temperature. The stage of the cell cycle was assessed by flow cytometry (FACS Calibur, Becton Dickinson, USA, Franklin Lakes, NJ, USA) and calculated by ModFit LT 4.1 software (Verity Software House, Topsham, ME, USA). The assay was performed in triplicate ($n = 6$).

4.6. Cytokines Concentration Measurement

The cells were seeded at density $8 \times 10^4$ in a 6-well and culture for 24 h. Next, cells (about 60 % confluent) were treated with different RSV concentrations (1, 5, 10, 50, and 100 µM) for 24 h. After the treatment, 1 mL of the samples of the culture medium supernatant was collected and stored at $-80 \degree$ C until further analysis. 100 µL of the supernatant of each kind was used for further analysis.

The concentration of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were assessed in cell supernatant using mouse/human ELISA kits (R&D Systems, Minneapolis, MN, USA). The absorbance of the samples was measured in a FLUOstar Omega multi-sensing reader (BMG Labtech) at 450 nm with a reference cutoff at 570 nm and converted into concentration units by incorporating the results from the multi-point calibration curve. Analyses of cytokines concentrations were performed in duplicate from three independent experiments ($n = 6$).

4.7. Statistical Analysis

Statistical evaluation of the results was performed using T-tests and one-way ANOVA with Bonferroni correction (in the case of a normal distribution) or non-parametric Kruskal–Wallis and Mann–Whitney U tests (in the case of abnormal distribution). Assessment of the distribution of the data was evaluated using the Shapiro–Wilk test. GraphPad Prism software was used to carry out these tests (version 7; GraphPad Software, Inc., La Jolla, CA, USA). $p < 0.05$ was considered a statistically significant difference.

5. Conclusions

The differential effects of RSV treatment on epithelial and endothelial cell lines may suggest the usefulness of this compound in the prevention or treatment of pathological angiogenesis in the eye.


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