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Original article

Synergistic interactions between resveratrol and doxorubicin inhibit angiogenesis both in vitro and in vivo

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Abstract

Resveratrol is a polyphenolic compound which is found in many nutrients including grapes, peanuts, raspberries, and apples. Anti-proliferative, anti-angiogenic and apoptotic effects of resveratrol have been shown on various cancer cells. Doxorubicin is considered as one of the most effective anticancer agents and reveals its antitumor activity by induction of apoptosis and inhibition of angiogenesis. Our study reports for the first time the potent ability of resveratrol in combination with doxorubicin to inhibit angiogenesis in vitro and in vivo. The cytotoxic effect of resveratrol (1.56-100 μ M), doxorubicin (0.01-0.92 μ M) and their combination were analyzed in the human umbilical vein endothelial cells (HUVECs) by ATP assay. In vitro angiogenesis was evaluated using tube formation assay in HUVECs. In vivo anti-angiogenic activity was assessed in a chick chorioallantoic membrane (CAM) model using fertilized chicken eggs. All test groups were compared to thalidomide as a positive control, three concentrations of resveratrol (10-5-2.5 μ g/pellet) and a 2 μ g/pellet concentration of doxorubicin was examined. All data were evaluated statistically. Resveratrol and doxorubicin alone displayed inhibitory effects on angiogenesis and cell viability at higher doses. However, the combination of resveratrol and doxorubicin exhibited a significant dose-dependent inhibition of CAM angiogenesis in vivo as well as proliferation and tube formation in HUVECs compared to the positive control (\pm)-thalidomide. Our results suggest that resveratrol in combination with doxorubicin is a novel strategy in the prevention and treatment of angiogenesis

Key words: resveratrol, chick chorioallantoic membrane (CAM) assay, doxorubicin, HUVEC, tube formation, anti-angiogenic activity

Introduction

Angiogenesis can be described as the formation of new capillaries from pre-existing vessels (Yoo and Kwon 2013) and is a complex physiological event that is expected to occur in processes such as growth, development and wound healing. Furthermore, angiogenesis can occur pathologically in cancer, rheumatoid arthritis, inflammatory bowel diseases and some eye diseases (Yoo and Kwon 2013). The presence of angiogenesis in complex diseases, such as cancer, can cause tumor growth by provoking metastasis (Rouhi et al. 2010, Trapp et al. 2010).

Studies are increasingly aimed at determining the anti-angiogenic effect of plant-derived active substances in cancer treatment (Singh et al. 2015). The potent anti-angiogenic effects of phenol and polyphenolic compounds have been studied for many years. Several studies have been carried out on the useful and protective properties of these compounds on the process of impaired angiogenesis (Sun et al. 2015).

Resveratrol is a polyphenolic compound found in many foods from grapes to peanuts (Aggarwal et al. 2004). Resveratrol has antioxidant, anticancer, anti-aging and anti-inflammatory properties. Moreover, resveratrol has been reported to inhibit the proliferation of a wide variety of cancer cells. Anti-proliferative, apoptotic and anti-inflammatory effects of resveratrol have been shown on different types of cancer cells (Catalgol et al. 2012).

According to large-scale studies, resveratrol has been reported to reduce cardiovascular disease risk and show a cardioprotective effect by regulating vascular cell functions (Penumathsa and Maulik 2009). Studies have shown that resveratrol has a dose-dependent modulating effect on angiogenesis (Wang et al. 2010). However, the dose-dependent effect of resveratrol on angiogenesis has not been studied in combination with any chemotherapeutics.

Doxorubicin is a first-generation anthracycline chemotherapeutic which is frequently preferred in various cancer models. Despite the strong anticancer activity of doxorubicin on cancer cells, its administration is restricted because of acute and chronic toxicities (especially cardiotoxicity). Therefore, researchers are investigating alternative methods to suppress the severe side effects of doxorubicin and to highlight their positive effects. One of the methods is the reduced treatment dose of doxorubicin used with the combination of various compounds (Giordano et al. 2012, Gurel-Gurevin et al. 2018).

In vivo CAM (chick embryo chorioallantoic membrane) assay is an easy and cheap method for the detection of the anti-angiogenic property of drugs. The CAM

assay is one of the alternative models approved for replacement from the 3R theory (Ikitimur-Armutak et al. 2017). There is a limited number of studies showing the effect of resveratrol on the suppression of angiogenesis by CAM assay (Wang et al. 2010).

Therefore, in this study, cytotoxic effects of resveratrol and doxorubicin combinations administered in varying doses have been investigated on HUVEC cells by ATP viability assay. We also aimed to analyze its anti-angiogenic effect by tube formation and in vivo CAM assay.

Materials and Methods

Cell culture and chemicals

Human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in EGM-2 Endothelial Cell Growth Medium-2 BulletKit (Lonza, Basel, Switzerland) in a humidified incubator at 37°C and 5% CO₂. Resveratrol (Sigma, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) at 50 mM concentration as a stock solution. Doxorubicin (Kocak Farma, Turkey) was dissolved in Molecular Biology water at 2 mg/ml concentration as a stock solution. Reference anti-angiogenic (thalidomide; Sigma-Aldrich Chemie GmbH, Munich, Germany) was dissolved in dimethylsulfoxide (DMSO) as a stock solution (500 mM). The highest concentration of DMSO did not exceed 0.1% (v/v) in the cell culture.

ATP viability assay

The cytotoxic effects of resveratrol (1.56-100 µM), doxorubicin (0.01-0.92 µM) and their combination were analyzed in HUVECs by ATP assay kit (ATP Bioluminescent Somatic Cell Assay Kit; Sigma, Steinheim, Germany). Briefly, HUVECs were seeded at a density of 5×10^3 cells per well of 96-well plates in triplicate. The cells were treated with resveratrol (1.56-100 µM concentration), doxorubicin (0.01-0.92 µM) and their combinations for 48h. After 48h treatment, 150 µl of media was removed from each well and 50 µl of somatic cell ATP-releasing agent (ATP Bioluminescent Somatic Cell Assay Kit; Sigma, Steinheim, Germany) was added. The plate was well agitated and allowed to stand for 30 min at room temperature. At the end of the incubation, 50 µl of a mixture from each well was transferred to a white non-translucent plate. 50 µl of ATP Assay Mix (ATP Bioluminescent Somatic Cell Assay Kit, Sigma, Steinheim, Germany) was added to each well and the emitted light was measured using a 96-well Microplate Luminometer (Bio-Tek,

Table 1. Semi-quantitative scoring system for the evaluation of anti-angiogenic effects on Chick embryo chorioallantoic membrane (CAM).

Scale	Anti-angiogenic effect	Effects observed on CAM after treatment
<0.5	Inactive	No change (Normal embryo growth)
0.5-0.75	Weak	No capillary free area The area with a reduced density of capillaries around the pellet not larger than its area
>0.75-1	Strong	Small capillary free area or area with significantly reduced density of capillaries Effects not larger than double the size of the pellet
>1	Very strong	The capillary free area around the pellet at least doubles the size of the pellet

Average score=[Number of eggs (score >1)×2+number of eggs (score >0.75-1)×1]/(total number of eggs scored)

Winooski, VT, USA). ATP viability assay was performed at least twice and the results are given as mean±SD of independent experiments.

Tube formation assay

The effect of doxorubicin and resveratrol combination on in vitro angiogenesis of endothelial cells was evaluated using the tube formation assay on Matrigel (BD Biosciences, MA, USA). Briefly, Matrigel (50 µl) was added to wells of a pre-chilled 96-well plate and incubated at 37°C for 30 min and allowed to polymerize the Matrigel. HUVECs were resuspended in culture medium and added to the Matrigel containing wells at a density of 1.5×10^4 cells/well and treated with doxorubicin and resveratrol alone or their combination at different concentrations. Tube formation was assessed after 4, 8, 12 and 16 h using an inverted microscope. DMSO and reference anti-angiogenic thalidomide were included as negative and positive controls, respectively.

Chick embryo chorioallantoic membrane (CAM) assay

Resveratrol (0.25, 0.5 and 1 mg/ml) (R5010, Sigma-Aldrich, Germany), Doxorubicin (0.2 mg/ml) (Kocak Farma, Turkey), 0.25 mg/ml Resveratrol+0.2 mg/ml Doxorubicin and (±)-Thalidomide (as positive control) (Sigma-Aldrich, Germany) were dissolved in a 2.5% (w/v) agarose (UltraPure Agarose, Invitrogen) solution. To simplify the application, pellets of these solutions (10 µl) were prepared and applied dropwise on circular stainless steel supports of 5 mm diameter. It was allowed to solidify at room temperature and applied to the chick chorioallantoic membrane (CAM).

The in vivo CAM assay was performed as described previously by our research group (Gurel-Gurevin et al. 2018). Fertilized eggs were supplied from Mudurnu Piliç- Pak Tavuk Company (İstanbul, Turkey). All studies were performed in an incubator with fertilized chicken eggs for 7 days at 36.5°C and relative humidity of

80%. Fertilized eggs were previously incubated for 72 h at 36.5°C. 8-10 ml albumin was aspirated by injector needle in the bottom of the egg. A hole on the top of the eggshell was opened around 2 cm diameter using forceps, then covered with stretch film and the eggs were incubated again in the same conditions for 72 h. Pellets (1 pellet/egg) were placed on the CAM and the hole covered with stretch film. After 24 h of incubation, the anti-angiogenic effect on capillaries was evaluated under a stereo-microscope with Leica Application Suite, Las V4.7 (Leica). 10-15 eggs were evaluated for each test group (control, doxorubicin and resveratrol alone or their combination, thalidomide). Independent experiments of all groups were performed in triplicate. Anti-angiogenic effects were determined using a scoring system (see Table 1) (Bürgermeister et al. 2002, Ikitimur-Armutak et al. 2017). The anti-angiogenic scores were calculated using the following formula (Krenn and Paper 2009, Gurel-Gurevin et al. 2018). It was confirmed that this study did not need to be approved by the Istanbul University Ethics Committee (18.04.2018-146226).

Statistical analysis

All statistically significant differences were analyzed using one-way analysis of variance (ANOVA) as implemented in GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). All pairwise comparisons were performed within different groups with the Tukey's multiple comparisons test and $p \leq 0.001$ indicated statistical significance.

Results

Resveratrol enhances doxorubicin-induced cytotoxicity of HUVECs

To measure the cytotoxicity of resveratrol and doxorubicin, HUVECs were cultured either with various concentrations of resveratrol (1.56 µM-100 µM)

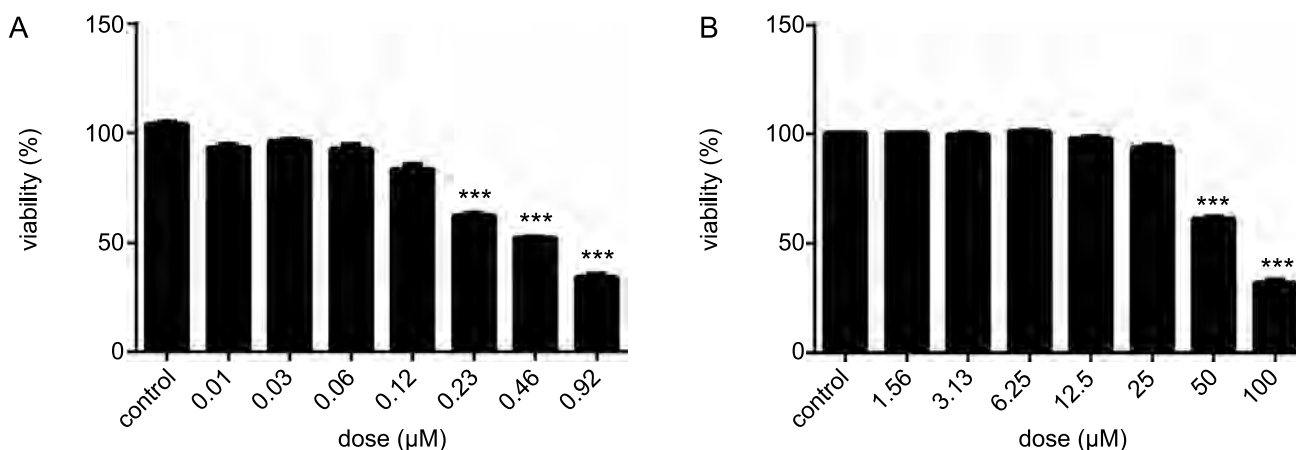


Fig. 1. Cell viability of resveratrol and doxorubicin on HUVECs. HUVECs were treated with doxorubicin (0.01–0.92 μM) (A) and resveratrol (1.56–100 μM) (B) for 48 h. The cell viability was measured using the ATP assay. Data are mean ± standard deviation (SD) of two separate experiments; significance was determined using Tukey's multiple comparisons tests *** p<0.001 compared with control.

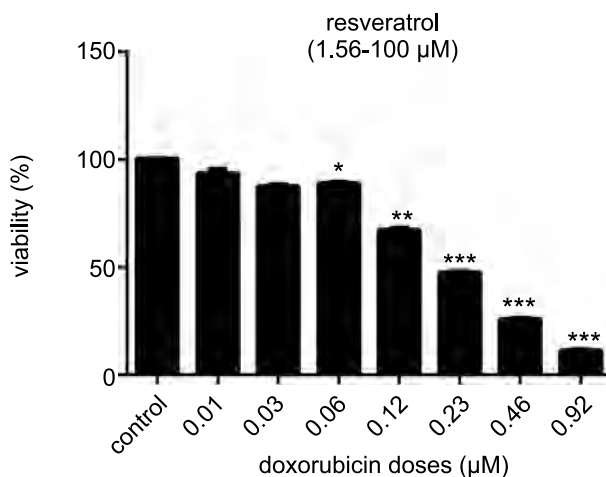


Fig. 2. Effect of resveratrol and doxorubicin combination on HUVEC cell viability evaluated by ATP assay. HUVECs were treated with resveratrol (1.56–100 μM) and doxorubicin (0.01–0.92 μM) for 48 hours. Data are mean ± standard deviation (SD) of two separate experiments; significance was determined using Tukey's multiple comparisons tests *** p<0.001 compared with control.

or doxorubicin (0.01–0.92 μM) alone for 48 h. Cell viability was evaluated using the ATP assay, as shown in Fig. 1. Resveratrol (50–100 μM) produced a remarkable reduction in the viability of HUVECs at 48h (Fig.1A). Doxorubicin significantly reduced the HUVECs viability in a concentration-dependent manner (Fig.1B). The results showed that treatment with resveratrol (1.56 μM–100 μM) in combination with doxorubicin (0.01–0.92 μM) resulted in a significantly enhanced inhibition of HUVECs proliferation compared with doxorubicin and resveratrol alone at equal equivalent concentrations (Fig. 2). This result indicates that resveratrol potentiates the efficacy of doxorubicin compared to its administration alone on HUVECs.

Doxorubicin in combination with resveratrol suppresses the tube formation in vitro

The effects of Doxorubicin (0.23–0.92 μM), resveratrol (25–100 μM) and their binary combination

on HUVECs were examined using an in vitro tube formation model at 4, 8, 12 and 16 h (Fig. 3 and Fig. 4). The effect of resveratrol (50–100 μM) alone was more effective than the thalidomide (400 μM) on disturbing the formation of a tube-like structure (Fig. 3). Compared with the positive control, at 16 h resveratrol at 50 and 100 μM doses resulted in significant inhibition of tube structures; however, resveratrol at 25 μM had no significant effect on tube formation. Doxorubicin treatment resulted in tube disruption only at the highest doses (0.46–0.92 μM) at 16h compared to thalidomide and control and we observed no reduction in total tube length at different doxorubicin treatment times and doses (Fig. 3). Doxorubicin in combination with resveratrol exhibited a very low or unobserved tube structure at almost whole treatment doses. Noneffective doses of resveratrol and doxorubicin were significantly inhibited tube formation of HUVECs when they used as a combined treatment (Fig. 4). Our data

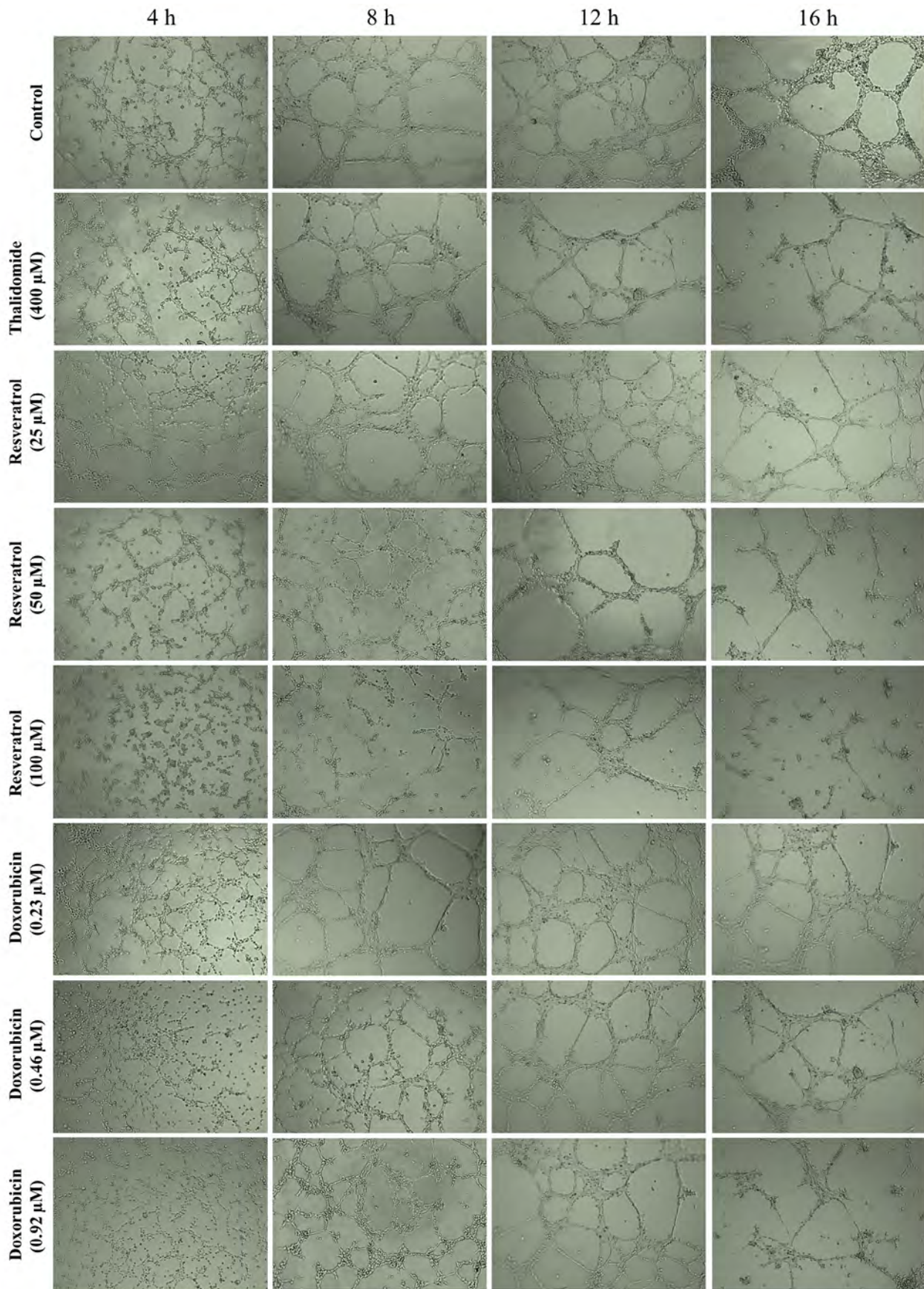


Fig. 3. The effect of resveratrol and doxorubicin alone on capillary tube structures in HUVECs at different times and doses. Capillary tube structures were photographed with a digital camera attached to a microscope. Each experiment was repeated at least two times. Thalidomide (400 μM) used as a positive control. All images are at x10 magnification.

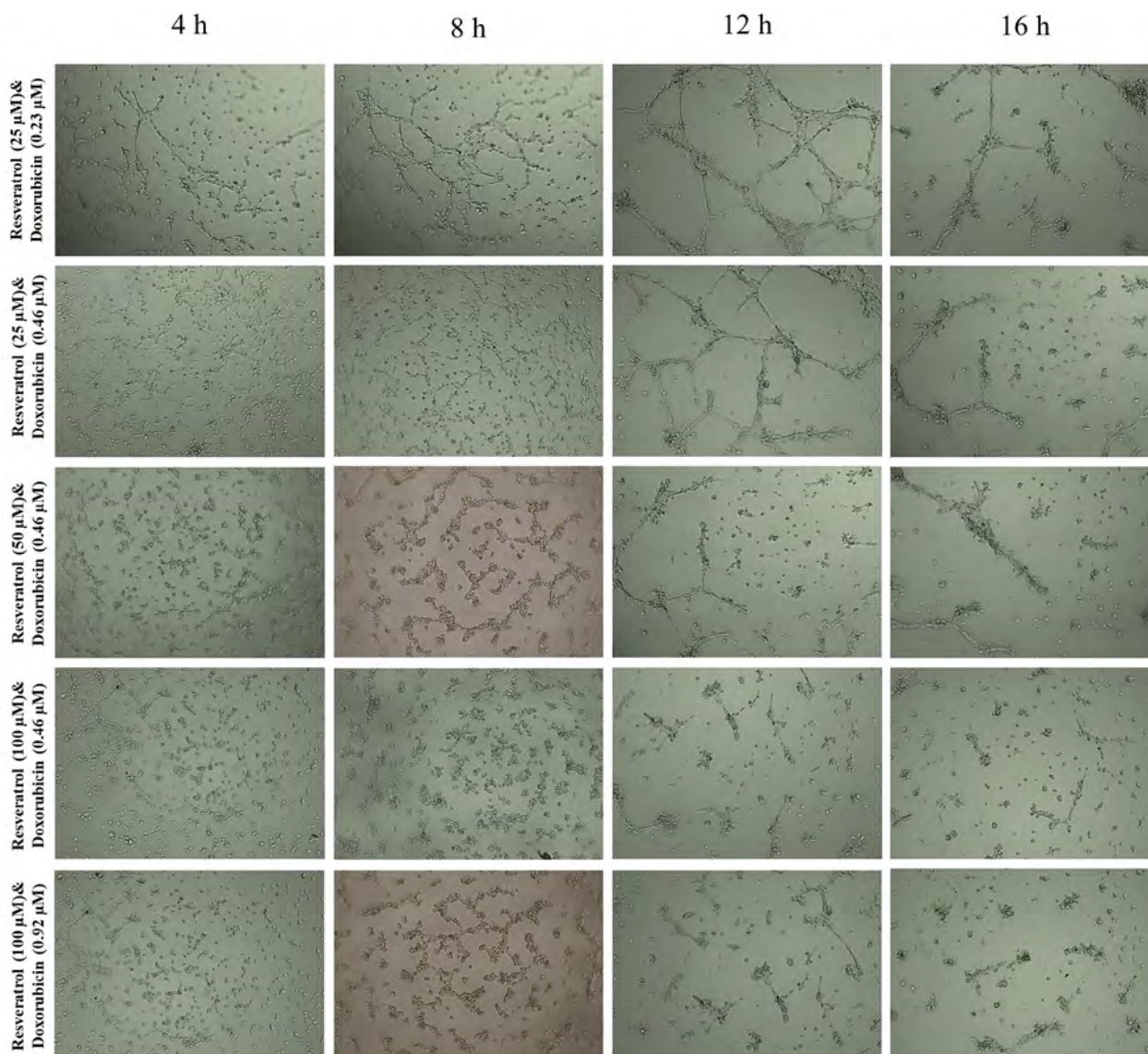


Fig. 4. Effect of resveratrol in combination with doxorubicin on the formation of tube-like structures in HUVECs in a time- and dose-dependent manner. Each experiment was repeated at least two times. All images are at x10 magnification.

indicate that all combination groups showed a highly antiangiogenic effect on HUVECs in vitro.

Doxorubicin in combination with resveratrol modulates angiogenesis on CAM assay

We further used the chick chorioallantoic membrane assay to test the angiogenic effect of doxorubicin in combination with resveratrol in vivo. Treatment using resveratrol (10-5-2.5 $\mu\text{g}/\text{pellet}$) and doxorubicin (2 $\mu\text{g}/\text{pellet}$) alone were examined compared to thalidomide as a positive control. Consistent with the in vitro data, resveratrol showed a dose-dependent anti-angiogenic effect (0.25-1 mg/ml) (Fig. 5). Compared with the control and thalidomide, resveratrol administration increased the anti-angiogenic effect at higher doses (defined as a very strong anti-angiogenic effect)

(Table 2). Doxorubicin concentration at 0.2 mg/ml was chosen from our previous study (Gurel-Gurevin et al. 2018) and slightly inhibited vascular formation. While resveratrol (1 mg/ml) and doxorubicin (0.2 mg/ml) treatment alone did not cause significant change in the formation of microvessel structures, 0.25 mg/ml resveratrol in combination with 0.2 mg/ml doxorubicin led to a significant reduction in angiogenesis (average score=1.70 \pm 0.142) (Fig. 5) (Table 2).

Discussion

Angiogenesis is a complex process involved in the production of new blood vessels and plays an important role in cancer. The increase of vascular formation in tumor tissues contributes to tumor cell proliferation,

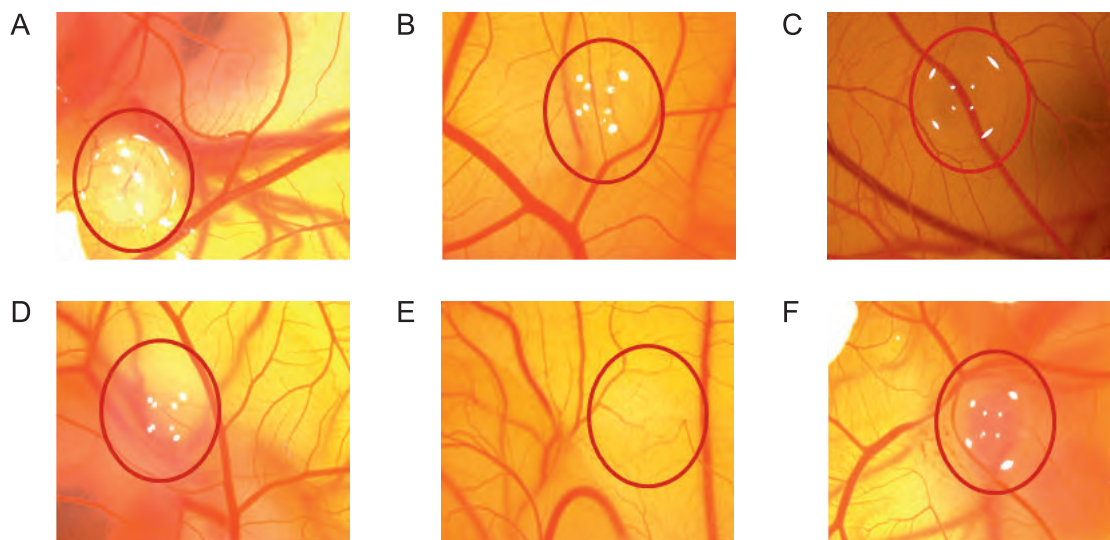


Fig. 5. Effect of resveratrol in combination with doxorubicin on the branching blood vessels in CAMs. (A) Thalidomide treatment as a positive control (5 mg/ml), (B) Doxorubicin treatment (0.2 mg/ml), (C) Resveratrol treatment (1 mg/ml), (D) Resveratrol treatment (0.5 mg/ml), (E) Resveratrol treatment (0.25 mg/ml), (F) Doxorubicin 0.2 mg/ml + Resveratrol 0.25 mg/ml combined treatment were evaluated according to stereo microscopic evaluation of CAM assay. Representative pictures at 10x magnification.

Table 2. Anti-angiogenic effects of thalidomide, doxorubicin, resveratrol treatment alone and their combination in the chick embryo chorioallantoic membrane assay.

Treatment Groups	Concentration (µg/pellet)	Average score ^a	Anti-angiogenic effect	Tox (%)
Resveratrol 1 mg/ml	10	1.08±0.083	Very strong effect	-
Resveratrol 0.5 mg/ml	5	0.81±0.035	Strong effect	-
Resveratrol 0.25 mg/ml	2.5	0.62±0.042	Weak effect	-
Doxorubicin 0.2 mg/ml	2	1.16±0.112 **	Very strong effect	-
Resveratrol 0.25 mg/ml + Doxorubicin 0.2 mg/ml	2.5 + 2	1.70±0.142 ***	Very strong effect	-
Agarose (blank)	2.5%, w/v	0.2 ± 0.2	No effect	-
Thalidomide (Positive control)	50	0.84±0.038	Strong effect	-

growth, and metastasis (Yoo and Kwon 2013). Since many studies have focused on developing new strategies to suppress angiogenesis in cancer treatment (Ma and Waxman 2008, Krenn and Paper 2009, Trapp et al. 2010), our study showed the effects of resveratrol in combination with doxorubicin on cell viability and angiogenesis. First, we examined endothelial cell proliferation in response to combinatorial treatment (Fig. 2). Endothelial cells are known to display significant angiogenic potential. Therefore, we used macrovascular endothelial cells namely umbilical-vein derived cells (HUVEC) which is also commonly used in angiogenesis research. Moreover, we were able to show that resveratrol and doxorubicin together are effective against the angiogenic activity of endothelial cells in vitro and in vivo.

Resveratrol is one of the natural substances associated with prominent anticancer, anti-oxidant, anti-aging

and anti-inflammatory activity (Penumathsa and Maulik 2009). It is known to have a multifaceted regulation mechanism on the cardiovascular system. Resveratrol affects cell growth and division, inflammation, apoptosis, metastasis, and angiogenesis in addition to its cardioprotective effects (Szende et al. 2000, Garvin et al. 2006). Bråkenhielm et al. (2001) asserted that resveratrol consumption might be effective in preventing angiogenesis-induced diseases (Bråkenhielm et al. 2001). Additionally, a study on athymic female mice found that resveratrol accelerated the growth of GFP-MDA-MB-231 cells in breast tissue at low doses (0.5 mg/kg) (Castillo-Pichardo et al. 2013); however, the life span of the animals was increased by 70%, the effectiveness of doxorubicin was increased, and cardiotoxic side effects were reduced at higher doses (10 mg/kg) (Osman et al. 2013). Several in vitro studies reported that resveratrol has the potential

to reduce angiogenic responses, proliferation, and migration in various cell types (Knobloch et al. 2010, Núñez et al. 2010, Trapp et al. 2010).

Recently, doxorubicin was used in combination with different active compounds due to its high cytotoxicity and undesirable side effects (Koceva-Chyła et al. 2005, Aydinlik et al. 2017, Gurel-Gurevin et al. 2018). It has been reported that when resveratrol is combined with doxorubicin the results show synergistic effects on various cancer cells in vivo and in vitro (Osman et al. 2013, Kim et al. 2014, Gu et al. 2015). Resveratrol is also reported to have a protective effect against doxorubicin cardiotoxicity (Koceva-Chyła et al. 2005). In another study, a combination of doxorubicin and resveratrol increased the cytotoxic effect of doxorubicin by exhibiting an accumulative effect on MDA-MB-231 and MCF-7 cancer cells (Kim et al. 2014). A combination of doxorubicin and resveratrol has been studied several times on different cancer cell lines, but no studies have examined the anti-angiogenic effects of this combination on HUVEC cell lines. Therefore, for the first time, the anti-angiogenic effect of doxorubicin and resveratrol combination was demonstrated in this study.

Doxorubicin and resveratrol were shown to have a dose-dependent cytotoxic effect in the range of 0.1-0.92 μM and 1.56 μM -100 μM respectively. Cytotoxicity was performed by combining the increasing doses of doxorubicin and increasing doses of resveratrol to demonstrate the synergistic effect of doxorubicin (0.1-0.92 μM) and resveratrol (1.56 μM -100 μM). Resveratrol (25-100 μM) in combination with doxorubicin (0.23-0.92 μM) was found to be effective compared to resveratrol and doxorubicin treatment alone. Additionally, cell viability decreased respectively, from 58.23% to 26.62% and from 33.24% to 11.64% after resveratrol (50-100 μM) and doxorubicin combinatorial treatment. According to these in vitro results, the combination of resveratrol and doxorubicin demonstrated a higher cytotoxic effect even at lower doses (Fig. 2). Chen et al. (2011) showed that resveratrol had a dose-dependent cytotoxic effect in doses of 100-1.56 μM and had dose dependent blocking effects on the formation of tube-like structures, as in our results (Chen and Easton 2011). Furthermore, our study demonstrated that resveratrol has an anti-angiogenic effect at 50 μM and 100 μM doses on HUVECs and provoked angiogenesis at doses less than 25 μM (Fig. 3). Wang et al. (2010) investigated the resveratrol effects on the ability to form tube-like structures and observed in vitro angiogenesis inhibition at 20 μM concentration, similarly to our results (Wang et al. 2010). Our results showed that a 50 μM concentration of resveratrol significantly reduced tube formation, and a 100 μM concentration

of resveratrol blocked the formation of tube-like structures. In this study, HUVECs were treated with doxorubicin in the range of 0.23-0.92 μM concentration to perform in vitro tube formation assay (Fig. 3). Doxorubicin has no strong anti-angiogenic effect on tube formation in the range of 0-1 μM concentration (Gurel-Gurevin et al. 2018). Resveratrol (25-100 μM) in combination with doxorubicin (0.23-0.92 μM) suppressed capillary tube formation (Fig. 4). Our data show that lower doses of resveratrol and doxorubicin have synergistic anti-angiogenic effects.

The CAM assay is an alternative in vivo method that is easy and quick in demonstrating an anti-angiogenic effect without the need for ethical permission (Cimpean et al. 2008). In this study, the effect of resveratrol on angiogenesis was examined in the range of 0.25, 0.5 and 1 mg/ml concentrations by in vivo CAM assay (Fig. 5). A 1 mg/ml concentration of resveratrol exhibited a stronger antiangiogenic effect than positive control thalidomide (0.84 ± 0.038) (Table 2). Similarly Bråkenhielm et al. (2001) and Chen et al. (2013), resveratrol has a dose-dependent antiangiogenic effect evaluated by in vivo CAM assay (Bråkenhielm et al. 2001, Chen et al. 2013).

Doxorubicin has been demonstrated to have a strong angiogenic effect in previous CAM assay studies (Gurel-Gurevin et al. 2018). The convenient dose of doxorubicin was determined as 0.2 mg/ml for the in vivo CAM assay regarding our previous study (Gurel-Gurevin et al. 2018). The score of resveratrol alone was determined as weak anti-angiogenic (0.62 ± 0.042) and the score of doxorubicin alone was determined as very strong anti-angiogenic (1.16 ± 0.112). In this study, Doxorubicin 0.2 mg/ml and resveratrol 0.25 mg/ml were combined to investigate the angiogenic effect by CAM assay. Resveratrol in combination with doxorubicin exhibited a synergistic effect similar to in vitro results and potentiated the anti-angiogenic effect of doxorubicin at the indicated doses. These results show a correlation with the recent studies, in that resveratrol increases angiogenesis at low doses and suppresses angiogenesis at high concentrations by exhibiting anti-angiogenic effects (Szende et al. 2000, Boncler et al. 2014). It has also been shown in other studies that resveratrol inhibited angiogenic responses in vitro and also reduced vascularisation in vivo on endothelial cell lines (Garvin et al. 2006, Mikuła-Pietrasik et al. 2012). There are also many studies describing the importance of combinatorial treatment with chemotherapy in angiogenesis. Combined treatment with resveratrol and chemotherapeutic agent 5-FU induced anti-angiogenic and anti-proliferative effects in cancer cells (Lee et al. 2015). Recent studies were reported that anti-angiogenic agents have a synergistic effect on the

prognosis of the disease when combined with chemotherapy or radiotherapy (Zhang et al. 2006, Ma and Waxman 2008, Comunanza and Bussolino 2017).

In conclusion, combining resveratrol with doxorubicin is a novel strategy that has the potential for improving anti-angiogenic activity and reducing the side effects of doxorubicin. Therefore, these results demonstrate that combination strategies are important in anti-angiogenic therapies. Further investigation at the molecular level is needed to clarify the mechanism of this synergistic effect.

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