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RESEARCH ARTICLE

# Biological effects on granulosa cells of hydroxylated and methylated resveratrol analogues

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Several resveratrol analogues have been designed to improve bioactivity: among these polymethoxystilbenes appear to be particularly promising. The present study was set up to investigate the biological functions of polymethoxystilbenes **2** and **3**, recently found in our lab as antiangiogenic agents, on a well-defined swine granulosa cell model. Proliferative activity and effects on steroidogenesis were evaluated, as well as the effect on granulosa cell vascular endothelial growth factor (VEGF) production, since these cells in basic conditions synthesize the main proangiogenic peptide. Moreover, we considered the effect of these two resveratrol analogues on granulosa cell redox status. Analogue **3** inhibited granulosa cell growth, while it stimulated steroidogenesis. A similar effect was displayed by **2** on estradiol 17β production and cell proliferation at the highest concentration tested. On the other hand, at the same dosage **2** decreased progesterone levels. Both analogues inhibited VEGF output. Granulosa cell redox status was unaffected by resveratrol analogue **2** while the highest concentration of **3** stimulated free radicals generation and scavenging enzyme activities. The overall results indicate that analogue **3** is the more powerful compound, thus suggesting that a slight modification in the structure markedly increases effectiveness. These data could be useful to develop more active resveratrol analogues for therapeutic use.

**Keywords:**
Free radicals / Polymethoxystilbenes / Resveratrol analogues / Steroids / VEGF

## 1 Introduction

Apples, onions, chocolate, green tea and other plant extracts are good sources of phenolics. Resveratrol (3,5,4′-trihydroxystilbene **1**; Fig. 1) is a naturally occurring stilbene derivative, found in mulberries, peanuts, in some medicinal plants and mainly present in skin of grapes and thus in red wine [1]. Resveratrol is reported to be a natural chemopreventive agent against cancer, a potent antioxidant and an anti-inflammatory molecule [2]. However, the concentrations required to exert these effects may be difficult to achieve by drinking only one or two glasses of red wine a day. Therefore, developing more potent analogues of resveratrol may provide a feasible means of achieving effective concentrations. The available *in vivo* studies indicate that resveratrol, although absorbed in high extent by the organism, has a poor bioavailability and may be converted *in vivo* into compounds that sometimes, as for piceatannol, are effective [3] but usually lack of its activities [4]. Thus, many resveratrol analogues have been recently synthesized in the hope to increase the activity and/or the bioavailability [5]. Among these, polymethoxystilbenes are particularly interesting since the presence of a 3,5-dimethoxy moiety appears to be associated with noticeable biological activity. Polymethoxystilbenes appear as a sub-group of great interest among the resveratrol analogues [6] and a deeper evaluation of these compounds may supply new lead compounds; *in vivo* studies indicate that three- or tetramethoxystilbenes are characterized by a higher bioavailability

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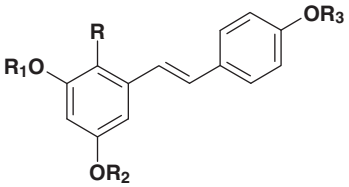
**Abbreviations: CM,** culture medium; **E2,** estradiol 17β; **P4,** progesterone; **SOD,** superoxide dismutase; **VEGF,** vascular endothelial growth factor




**1** R = $R_1$ = $R_2$ = $R_3$ = H
**2** R = H, $R_1$ = $R_2$ = $R_3$ = Me
**3** R = OH, $R_1$ = $R_2$ = $R_3$ = Me

**Figure 1.** Structures of compounds **1–3**.

than resveratrol [7, 8]. In a recent paper, we have documented that the resveratrol analogues 3,5,4′-trimethoxystilbene (**2**) and 2-hydroxy-3,5,4′-trimethoxystilbene (**3**) possess a powerful antiangiogenic activity [9] as evaluated by an angiogenesis bioassay set up in our lab (Fig. 1) [10]. The present research was undertaken to get an insight into the biological functions of compounds **2** and **3**. In order to study the effects of both 3,5,4′-trimethoxystilbene and its hydroxylated analogue on parameters related to cell growth, hormone production, angiogenesis and redox status swine granulosa cells were used as a previously well-defined experimental model [11–15]. Granulosa cells were chosen since they are true endocrine cells and are involved in several physiological processes such as angiogenesis. Moreover they are easy to recover. In particular, the main features of granulosa cell function, proliferation and steroidogenesis, were evaluated. In addition, we also tested the effect of these polymethoxystilbenes on vascular endothelial growth factor (VEGF) production, since we previously demonstrated [16] that granulosa cell produce this pivotal angiogenic peptide. Finally, redox status of granulosa cells was explored after treatment with resveratrol analogues.

## 2 Materials and methods

### 2.1 Chemicals

All reagents used in this study were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified.

Compound **2** was synthesized through an Arbuzov rearrangement followed by a Horner–Emmons–Wadsworth reaction, as previously reported [17]. According to a previously reported method, this procedure affords the *E*-stilbenoid with a minimal percentage of its *Z*-isomer [18].

Compound **3** was synthesized through a mild hydroxylation with *m*-chloroperoxybenzoic acid of **2** as previously reported [9].

### 2.2 Granulosa cell collection

Swine ovaries were collected at a local slaughterhouse from Large White cross-bred gilts, parity = 0. The stage of the cycle was unknown. Follicles were classified on a dimension-based fashion [14]. The ovaries were placed into cold PBS (4°C) supplemented with penicillin (500 IU/mL), streptomycin (500 μg/mL) and amphotericin B (3.75 μg/mL), maintained in a freezer bag and transported to the laboratory within 1 h. After a series of washings with PBS and ethanol (70%), granulosa cells were aseptically harvested by aspiration of large follicles (>5 mm) with a 26-gauge needle and released in medium containing heparin (50 IU/mL), centrifuged for pelleting and then treated with 0.9% prewarmed ammonium chloride at 37°C for 1 min to remove red blood cells. Cell number and viability were estimated using a haemocytometer under a phase contrast microscope after vital staining with trypan blue (0.4%) of an aliquot of the cell suspension. Cells were seeded in culture medium (CM) M199 supplemented with sodium bicarbonate (2.2 mg/mL), BSA (0.1%), penicillin (100 IU/mL), streptomycin (100 μg/mL), amphotericin B (2.5 μg/mL), selenium (5 ng/mL) and transferrin (5 μg/mL). Once seeded, cells were incubated in the presence or absence of compounds **2** or **3** (0.1, 1, 10 and 100 μM) and maintained for 48 h at 37°C under humidified atmosphere (5% $CO_2$). The treatment was identical for all experiments performed in this study.

### 2.3 Granulosa cell viability

A total of $2 \times 10^5$ cells were seeded in 96-well plates in 200 μL CM. Cell viability was assayed using a bioluminescent assay (ATP-lites; Packard Bioscience, Groningen, The Netherlands), which measured intracellular ATP levels as an indicator of cell numbers. ATP is a cell viability marker because it is present in all metabolically active cells and the concentration declines very rapidly when the cells undergo necrosis or apoptosis. The ATP lite-M assay system is based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin. This is illustrated in the following reaction scheme: ATP + D-luciferin + $O_2$ → luciferase, $Mg^{2+}$ → oxyluciferin + AMP + PPi + $CO_2$ + light. The emitted light is proportional to the ATP concentration. Briefly, 50 μL of mammalian cell lysis solution were added to 100 μL of cell suspension and the plate was shaken for 5 min in an orbital shaker at 700 rpm in order to lyse the cells and stabilize ATP. Then 50 μL of substrate solution were added to the wells and the microplate was shaken for 5 min in an orbital shaker at 700 rpm. The plate was placed in the dark for 10 min and the luminescence was measured in a luminometer (Victor, Packard Bioscience). The results were recorded in counts *per* second and the percentage of cell viability was calculated with reference to the negative control (cells without resveratrol analogues) for presentation purposes.

### 2.4 Granulosa cell steroid production

A total of $10^4$ cells/well were seeded in 96-well plates in 200 μL CM supplemented with androstenedione (28 ng/

mL). Culture media were then collected, frozen and stored at −20°C until progesterone (P4) and estradiol 17β (E2) determination by validated Radio Immuno Assays [19]. P4 assay sensitivity and $ED_{50}$ were 0.24 and 1 nmol/L, respectively; E2 assay sensitivity and $ED_{50}$ were 0.05 and 0.2 nmol/L. The intra- and inter-assay coefficients of variation were less than 12% for both assays.

### 2.5 Granulosa cell VEGF production

A total of $10^6$ granulosa cells in 1 mL CM+1% fetal calf serum were seeded in 24-well plates and incubated for 48 h. VEGF in culture media was quantified by an ELISA (Quantikine, R&D Systems, Minneapolis, MI, USA). This assay, developed for human VEGF detection, has been validated for pig VEGF [20]. The assay sensitivity was 8.74 pg/mL, the inter- and intra-assay Coefficients of Variation were always less than 7%. Victor Reader set to read at a wavelength of 450 nm emission was used to quantify the reaction product.

### 2.6 Granulosa cell superoxide ($O_2^-$) production

$O_2^-$ production was evaluated by WST-1 (4 - [3 - (4 - iodophenyl)–2 - (4 - nitrophenyl) - 2H–5 - tetrazolio] - 1,3-benzene disulfonate) test (Roche, Mannheim, Germany). The assay is based on the cleavage of the water-soluble tetrazolium salt, WST-1 to a yellow-orange, water-soluble formazan. Evidence exists that tetrazolium salts can be used as a reliable measure of intracellular $O_2^-$ production [21, 22]. A total of $10^4$ cells/200 μL CM were seeded in 96-well plates and incubated for 48 h. In total, 20 μL WST-1 were added to cells during the last 4 h of incubation and absorbance was then determined using the Victor reader at a wavelength of 450 nm against 620 nm.

### 2.7 Granulosa cell hydrogen peroxide ($H_2O_2$) production

$H_2O_2$ production was measured by an Amplex Red Hydrogen Peroxide Assay Kit (Molecular Probes, PoortGebouw, The Netherlands); the Amplex Red reagent reacts with $H_2O_2$ to produce resorufin, an oxidation product. Briefly, $2 \times 10^5$ cells/200 μL CM were seeded in 96-well plates and incubated for 48 h. After centrifugation for 10 min at 400 × *g*, the supernatants were discarded and cells were lysed adding cold Triton 1% in TRIS HCl (100 μL/well) and incubating on ice for 30 min. Undiluted cell lysates were used to perform the test and read against a standard curve of $H_2O_2$ ranging from 0.195 to 12.5 μM. The absorbance was determined with the Victor Reader using a 540 nm filter.

### 2.8 Granulosa cell scavenging enzyme activity

A total of $2 \times 10^5$ cells/200 μL CM were seeded in 96-well plates and incubated for 48 h. After centrifugation for 10 min. at 400 × *g*, the supernatants were discarded and cells were lysed adding cold Triton 1% in TRIS HCl (100 μL/$10^5$ cells) and incubating on ice for 30 min. Superoxide dismutase (SOD), catalase and peroxidase activities were assessed in cell lysates as described below.

SOD activity was determined by a SOD Assay Kit (Dojindo Molecular Technologies, Japan). Cell lysates were tested without dilution and a standard curve of catalase ranging from 0.156 to 20 U/mL was prepared. The colorimetric assay was performed measuring formazan produced by the reaction between tetrazolium salt (WST-1) and superoxide anion ($O_2^-$), produced by the reaction of an exogenous xantine oxidase. The remaining $O_2^-$ is an indirect hint of the endogenous SOD activity. The absorbance was determined with Victor Reader reading at 450 nm against 620 nm.

Catalase activity was measured by an Amplex Red Catalase Assay Kit (Molecular Probes) based on the formation of an oxidation product (resorufin) derived from the reaction between $H_2O_2$ given in excess, and the Amplex Red reagent in the presence of horseradish peroxidase. Cell lysates were diluted 1:10 to perform the test and read against a standard curve of catalase ranging from 62.5 to 1000 mU/mL. The absorbance was determined with Victor Reader using a 540 nm filter.

Peroxidase activity was measured by an Amplex Red Peroxidase Assay Kit (Molecular Probes) based on the formation of an oxidation product (resorufin) derived from the reaction between $H_2O_2$ given in excess and the Amplex Red reagent. Cell lysates were used undiluted to perform the test and read against a standard curve of peroxidase ranging from 0.039 to 5 mU/mL. The absorbance was determined with Victor Reader using a 540 nm filter.

### 2.9 Statistical analysis

Data are presented as means ± SEM. Statistical analysis was performed by means of ANOVA using Statgraphics package (STSC, Rockville, MD, USA). When significant differences were found, means were compared by Scheffè's F test. *p*-Values $< 0.05$ were considered to be statistically significant.

## 3 Results

### 3.1 Granulosa cell viability

Resveratrol analogue **2** significantly ($p < 0.05$) decreased ATP content only at the highest concentration tested, while analogue **3** displayed an inhibitory action at 1, 10, 100 μM ($p < 0.001$) (Fig. 2).

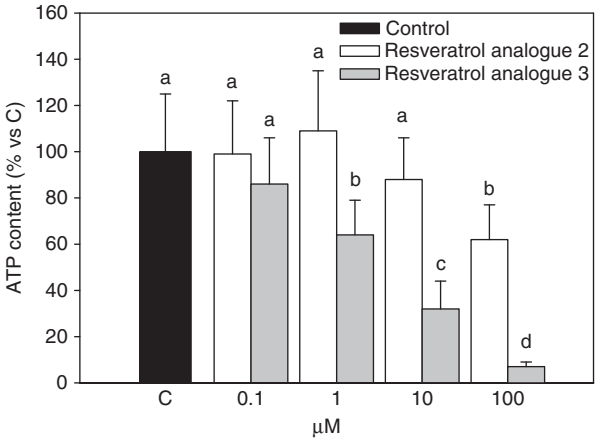



**Figure 2.** Effect of the 48 h treatment with or without (C) resveratrol analogue **2** or **3** at concentrations of 0.1, 1, 10, 100 μM on granulosa cell proliferation using ATP content assay test. Data, expressed as percentage *versus* respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters on the bars representing the same analogue indicate a significant difference ($p < 0.05$).

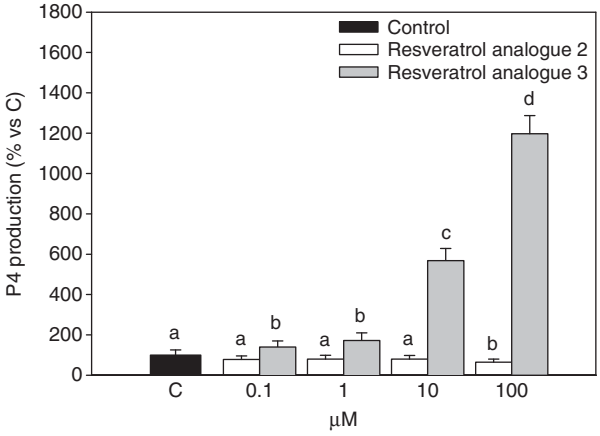



**Figure 3.** Effect of the 48 h treatment with or without (C) resveratrol analogue **2** or **3** at concentrations of 0.1, 1, 10, 100 μM on P4 production in swine granulosa cell culture using RIA. Data, expressed as percentage *versus* respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters on the bars representing the same analogue indicate a significant difference ($p < 0.05$).

### 3.2 Granulosa cell steroid production

P4 production (basal = 70 ± 9 ng/mL; means ± SEM) appeared significantly reduced only by the highest dosage of resveratrol analogue **2** ($p < 0.05$); on the contrary, resveratrol analogue **3** displayed a significant stimulatory effect on P4 levels at all concentrations tested, with a dose-dependent action at 10 and 100 μM ($p < 0.01$, Fig. 3).

As for E2 (3 ± 0.5 ng/mL), only 100 μM resveratrol analogue **2** enhanced the steroid production ($p < 0.01$), while resveratrol **3** was effective at all dosages in increasing E2 levels, with a dose-dependent action at 10 and 100 μM ($p < 0.001$, Fig. 4).

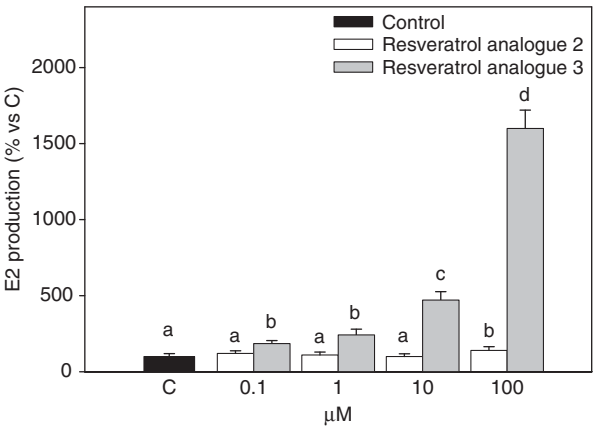



**Figure 4.** Effect of the 48 h treatment with or without (C) resveratrol analogue **2** or **3** at concentrations of 0.1, 1, 10, 100 μM on E2 production in swine granulosa cell culture using RIA. Data, expressed as percentage *versus* respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters on the bars representing the same analogue indicate a significant difference ($p < 0.01$).

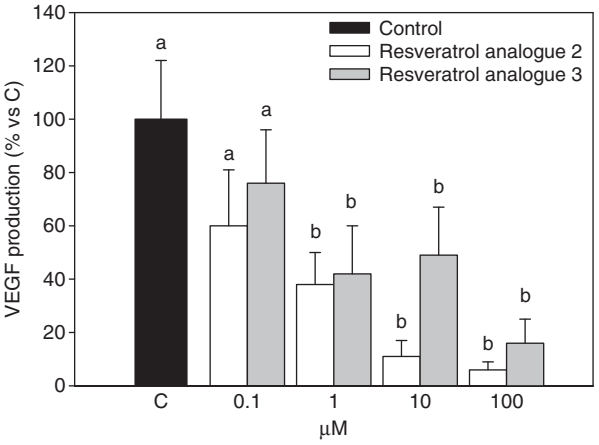



**Figure 5.** Effect of the 48 h treatment with or without (C) resveratrol analogue **2** or **3** at concentrations of 0.1, 1, 10, 100 μM on VEGF production in swine granulosa cell culture using ELISA. Data, expressed as percentage *versus* respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters on the bars representing the same analogue indicate a significant difference ($p < 0.01$).

### 3.3 Granulosa cell VEGF production

Both reseveratrol analogues significantly reduced VEGF levels (900 ± 50 pg/mL) at 1, 10 and 100 μM ($p < 0.01$) without any significant difference among the concentrations tested ($p < 0.01$) (Fig. 5).

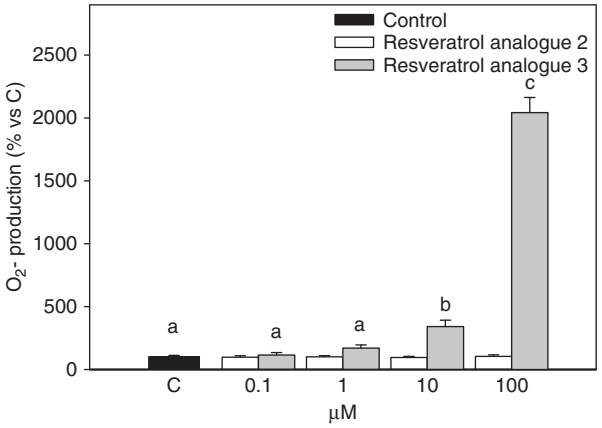



**Figure 6.** Effect of the 48 h treatment with or without (C) resveratrol analogue **2** or **3** at concentrations of 0.1, 1, 10, 100 μM on $O_2^-$ concentration in swine granulosa cell culture using WST-1 test. Data, expressed as percentage *versus* respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters on the bars representing the same analogue indicate a significant difference ($p < 0.05$).

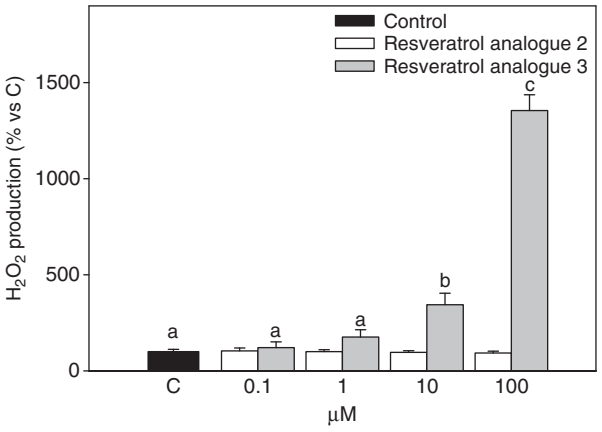



**Figure 7.** Effect of the 48 h treatment with or without (C) resveratrol analogue **2** or **3** at concentrations of 0.1, 1, 10, 100 μM on $H_2O_2$ concentrations in swine granulosa cell culture using colorimetric method. Data, expressed as percentage *versus* respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters on the bars representing the same analogue indicate a significant difference ($p < 0.001$).

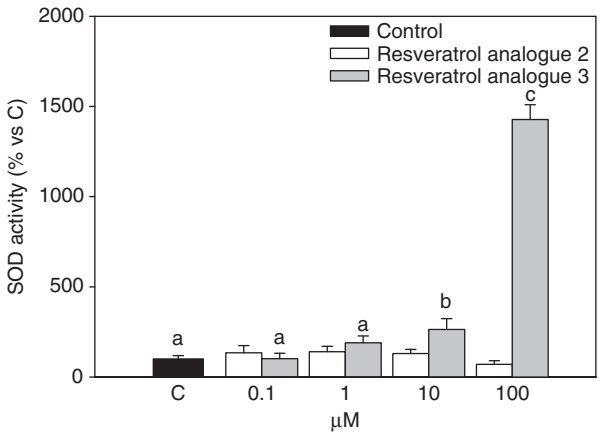



**Figure 8.** Effect of the 48 h treatment with or without (C) resveratrol analogue **2** or **3** at the concentrations of 0.1, 1, 10, 100 μM on SOD activity in swine granulosa cell culture using colorimetric method. Data, expressed as percentage *versus* respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters indicate a significant difference ($p < 0.001$).

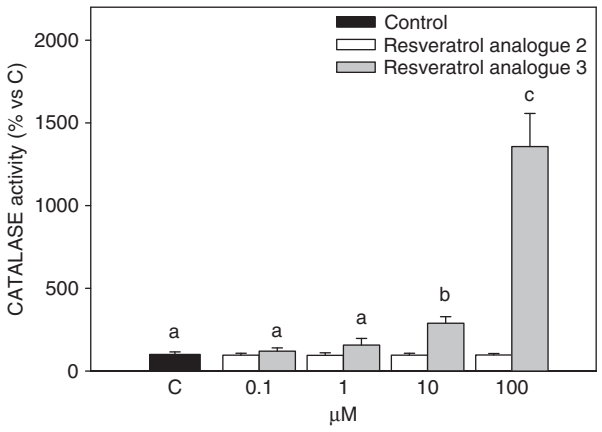



**Figure 9.** Effect of the 48 h treatment with or without (C) resveratrol analogue **2** or **3** at concentrations of 0.1, 1, 10, 100 μM on Catalase activity in swine granulosa cell culture using colorimetric method. Data, expressed as percentage *versus* respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters on the bars representing the same analogue indicate a significant difference ($p < 0.001$).

### 3.4 Granulosa cell redox status

Neither free radicals levels nor scavenging enzyme activity were significantly affected by resveratrol analogue **2** (Figs. 6–10). On the contrary, 10 and 100 μM resveratrol analogue **3** significantly ($p < 0.001$) increased both free radicals production (Figs. 6 and 7) and at the same time potentiated ($p < 0.001$) the activity of scavenging enzymes SOD (320 ± 10 mU/mL), peroxidase (1420 ± 220 mU/mL) and catalase (32 ± 7 mU/mL) (Figs. 8–10).

## 4 Discussion

Resveratrol is a phytoalexin found in a wide variety of dietary sources including grapes berries and peanuts. A primary impetus for research on resveratrol was from the paradoxical observation that a low incidence of cardiovascular diseases may co-exist with a high-fat diet intake and moderate consumption of red wine, a phenomenon known as the French paradox. During the last years, this substance has been the focus of many *in vitro* and *in vivo* studies investi-

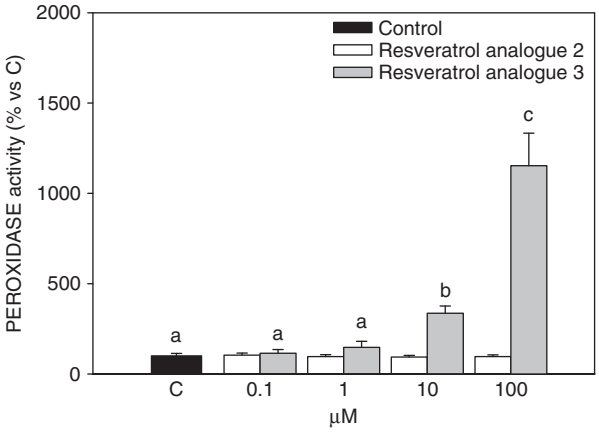



**Figure 10.** Effect of the 48 h treatment with or without (C) resveratrol analogue **2** or **3** at concentrations of 0.1, 1, 10, 100 μM on Peroxidase activity in swine granulosa cell culture using colorimetric method. Data, expressed as percentage *versus* respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters on the bars representing the same analogue indicate a significant difference ($p < 0.001$).

gating its biological attributes, which include mainly antioxidant and anti-inflammatory activities, anti-platelet aggregation effect, anti-atherogenic properties and cancer chemoprevention. Some of these effects may be due in part to resveratrol being a phytoestrogen, with biological properties similar to those of estrogens [23]. However, resveratrol is characterized by low bioavailability [24], short half-life [25] and extensive conjugation [26] and consequently needs to be used in high doses for possible efficacy of therapeutic treatments. Thus, a number of synthetic analogues of resveratrol have already been synthesized and tested for their potential anticancer, anti-inflammatory and anti-angiogenetic effects [6, 9, 17]. Among the resveratrol analogues, polymethoxystilbenes represent a sub-group of great interest due to their higher bioavailability, at least in intestinal and colonic mucosae and in the brain, with respect to resveratrol [7]. Recently, the pharmacokinetics of 3,5,4′-trimethoxystilbene (**2**) has been studied [8]: in comparison with **1**, **2** showed greater plasma exposure, longer half-life and lower clearance. Thus, as a first step in a deeper evaluation of methylated analogues of resveratrol, we have recently [9] demonstrated the antiangiogenic effect of several polymethoxystilbenes; among these, the most effective appeared to be 3,5,4′-trimethoxystilbene and most of all its 2-hydroxy analogue. Therefore, these molecules were tested in the present study in order to unravel their biological action on different granulosa cell functions. Our overall results clearly show that the 2-hydroxy analogue appears the most potent compound in affecting cell functional parameters, except for VEGF production, which was inhibited similarly by both resveratrol analogues. This would suggest that the biological activity can be effectively modulated by a slight modification of the chemical structure: in particular, the improved effectiveness may be related to an increased diffusion rate in a hydrophilic medium or in a better interaction with a receptor/enzyme active site. In this regard, it is worth noting that subtle modifications in the structure of resveratrol analogues may noticeably affect their tubulin-inhibitory activity, due to the strict requirements for an effective interaction with the colchicine-binding pocket of tubulin [27]. Moreover, different biological effects related to the slight different structures of polyhydroxylated stilbenoids have been reported [28].

In agreement with our previous paper [9] in which we documented a marked inhibition of endothelial cell growth, also granulosa cell viability was almost dose-dependently inhibited by the 2-hydroxy analogue. This feature could make this analogue an interesting compound for further evaluation as a potential cancer chemopreventive agent.

Data concerning the effects of the hydroxylated analogue **3** on steroidogenesis are remarkable, in that a potent stimulation of both P4 and E2 production was induced in granulosa cells. A similar effect was displayed by **2** on E2 production at the highest concentration tested. On the other hand, at the same dosage this analogue decreased P4 levels. At the moment the reason for these marked discrepancies are not clear. 3,5,4′-Trimethoxystilbene biological action appears similar to that of resveratrol [29] on different steroid-producing cells; due to its stilbene structure, resveratrol has been related to the synthetic estrogen diethylstilbestrol and included into endocrine disrupting compounds, chemicals with the potential to elicit negative effects on endocrine system of human and animal [23].

As suggested above, we could presume that the hydroxylation of the analogue 3,5,4′-trimethoxystilbene has a significant impact on biological function: in particular, the hydroxyl group could act ahead of steroids biosynthetic pathway, thus stimulating both P4 and E2 production. Further studies will be necessary to verify this hypothesis.

The interplay between free radicals and antioxidant scavenging enzymes is important in maintaining health, and in determining the rate of aging and age-related diseases [30]. Free radicals induce oxidative stress, which is balanced by the body's endogenous antioxidant systems and by the ingestion of exogenous antioxidants. If the generation of free radicals exceeds the protective effects of antioxidants this can cause oxidative damage, which accumulates during the life cycle, and can be implicated in aging and age-dependent diseases such as cardiovascular disease, cancer, neurodegenerative disorders and other chronic conditions [31–33]. Recent results have provided interesting insight into the effect of resveratrol on intracellular redox status. These results seem to support both anti- and pro-oxidant activities of this compound [34]. As for polymethoxystilbenes, present data demonstrate that 3,5,4′-trimethoxystilbene does not affect in any way cellular redox status while the 2-hydroxy analogue stimulates the production of radical species and, most interestingly, potentiates the activity of all the scavenging enzymes. This ability may be caused by the

presence of additional phenolic groups in its chemical structure as already suggested by Olas *et al.* [35] who evidenced a potent antioxidant effect by a naturally occurring resveratrol analogue *trans*-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene. In addition, Murias *et al.* [28] demonstrated that hydroxystilbenes with a different number of hydroxyl-groups and different substitution patterns exert different antioxidant potency.

Taken together, present data improve the knowledge about biological effects of polymethoxystilbenes and may be useful in order to develop resveratrol analogues for medical use.

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*The authors have declared no conflict of interest.*

## 5 References

[1] D'Archivio, M., Filasi, C., Di Benedetto, R., Gargiulo, R. *et al.*, Polyphenols, dietary sources and bioavailability. *Ann. Ist. Super. Sanità* 2007, *43*, 348–361.

[2] Nassiri-Asl, M., Hosseinzadeh, H., Review of the pharmacological effects of *Vitis vinifera* (Grape) and its bioactive compounds. *Phytother. Res.* 2009, *2*, 1197–1204.

[3] Potter, G. A., Patterson, L. H., Wanogho, E., Perry, P. J., *et al.*, The cancer preventative agent resveratrol is converted to the anticancer agent piceatannol by the cytochrome P450 enzyme CYP1B1. *Br. J. Cancer* 2002, *86*, 774–778.

[4] Espín, J. C., García-Conesa, M. T., Tomás-Barberán, F. A., Nutraceuticals: facts and fiction. *Phytochemistry* 2007, *68*, 2986–3008.

[5] Fang, J. G., Zhou, B., Structure-activity relationship and mechanism of the tocopherol-regenerating activity of resveratrol and its analogues. *J. Agric. Food Chem.* 2008, *56*, 11458–11463.

[6] Chillemi, R., Sciuto, S., Spatafora, C., Tringali, C., Anti-tumor properties of stilbene-based resveratrol analogues: recent results. *Nat. Prod. Commun.* 2007, *2*, 499–513.

[7] Sale, S., Verschoyle, R. D., Boocock, D., Jones, D. J. *et al.*, Pharmacokinetics in mice and growth-inhibitory properties of the putative cancer chemopreventive agent resveratrol and the synthetic analogue *trans* 3,4,5,4′-tetramethoxystilbene. *Br. J. Cancer* 2004, *90*, 736–744.

[8] Lin, H. S., Ho, P. C., A rapid HPLC method for the quantification of 3,5,4′-trimethoxy-*trans*-stilbene (TMS) in rat plasma and its application in pharmacokinetic study. *J. Pharm. Biomed. Anal.* 2009, *49*, 387–392

[9] Spatafora, C., Basini, G., Baioni, L., Grasselli, F. *et al.*, Antiangiogenic resveratrol analogues by mild *m*-CPBA aromatic hydroxylation of 3, 5-dimethoxystilbenes. *Nat. Prod. Commun.* 2009, *4*, 239–246.

[10] Basini, G., Bussolati, S., Santini, S. E., Bianchi, F. *et al.*, Hydroxyestrogens inhibit angiogenesis in swine ovarian follicles. *J. Endocrinol.* 2008, *199*, 127–135.

[11] Basini, G., Bianco, F., Grasselli, F., Tirelli, M. *et al.*, The effects of reduced oxygen tension on swine granulosa cell. *Regul. Pept.* 2004, *120*, 69–75.

[12] Basini, G., Grasselli, F., Bianco, F., Tirelli, M. *et al.*, Effect of reduced oxygen tension on reactive oxygen species production and activity of antioxidant enzymes in swine granulosa cells. *Biofactors* 2004, *20*, 61–69.

[13] Basini, G., Bianco, F., Grasselli, F., Epigallocatechin-3-gallate from green tea negatively affects swine granulosa cell function. *Domest. Anim. Endocrinol.* 2005, *28*, 243–256.

[14] Basini, G., Bussolati, S., Santini, S. E., Bianchi, F. *et al.*, Antiangiogenesis in swine ovarian follicle: a potential role for 2-methoxyestradiol. *Steroids* 2007, *72*, 660–665.

[15] Basini, G., Bussolati, S., Santini, S. E., Grasselli, F., Reactive oxygen species and anti-oxidant defences in swine follicular fluids. *Reprod. Fertil. Dev.* 2008, *20*, 269–274.

[16] Bianco, F., Basini, G., Grasselli, F., Angiogenic activity of swine granulosa cells: effects of hypoxia and vascular endothelial growth factor Trap R1R2, a VEGF blocker. *Domest. Anim. Endocrinol.* 2005, *28*, 308–319.

[17] Cardile, V., Chillemi, R., Lombardo, L., Sciuto, S. *et al.*, Antiproliferative activity of methylated analogues of E- and Z-resveratrol. *Z. Naturforsch. C* 2007, *62*, 189–195.

[18] Bachelor, F. W., Loman, A. A., Snowdon, L. R., Synthesis of pinosylvin and related heartwood stilbenes. *Can. J. Chem.* 1970, *48*, 1554–1557.

[19] Grasselli, F., Baratta, M., Tamanini, C., Effects of a GnRH analogue (buserelin) infused via osmotic minipumps on pituitary and ovarian activity of prepuberal heifers. *Anim. Reprod. Sci.* 1993, *32*, 153–161.

[20] Barboni, B., Turriani, M., Galeati, G., Spinaci, M. *et al.*, Vascular endothelial growth factor production in growing pig antral follicles. *Biol. Reprod.* 2000, *63*, 858–864.

[21] Benov, L., Fridovich, I., Is reduction of the sulfonated tetrazolium 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2-tetrazolium 5-carboxanilide a reliable measure of intracellular superoxide production? *Anal. Biochem.* 2002, *310*, 186–190.

[22] Ukeda, H., Shimamura, T., Tsubouchi, M., Harada, Y. *et al.*, Spectrophotometric assay of superoxide anion formed in Maillard reaction based on highly water-soluble tetrazolium salt. *Anal. Sci.* 2002, *18*, 1151–1154.

[23] Hanet, N., Lancon, A., Del mas, D., Jannin, B. *et al.*, Effects of endocrine disruptors on genes associated with 17β-estradiol metabolism and excretion. *Steroids* 2008, *73*, 1242–1251.

[24] Wenzel, E., Somoza, V., Metabolism and bioavailability of *trans*-resveratrol. *Mol. Nutr. Food Res.* 2005, *49*, 472–481.

[25] Asensi, M., Medina, I., Ortega, A., Carretero, J., Inhibition of cancer growth by resveratrol is related to its low bioavailability. *Free Rad. Biol. Med.* 2002, *33*, 387–398.

[26] Walle, T., Hsieh, F., DeLegge, M. H., Oatis, J. E. *et al.*, High absorption but very low bioavailability of oral resveratrol in humans. *Drug Metab. Dispos.* 2004, *32*, 1377–1382.

 

[27] Simoni, D., Invidiata, F. P., Eleopra, M., Marchetti, P. *et al.*, Design, synthesis and biological evaluation of novel stilbene-based antitumor agents. *Bioorg. Med. Chem.* 2009, *17*, 512–522.

[28] Murias, M., Jäger, W., Handler, N., Erker, T., *et al.*, Antioxidant, prooxidant and cytotoxic activity of hydroxylated resveratrol analogues: structure-activity relationship. *Biochem. Pharmacol.* 2005, *69*, 903–912.

[29] Chen, Y. C., Nagpal, M. L., Stocco, D. M., Lin, T., Effects of genistein, resveratrol, and quercetin on steroidogenesis and proliferation of MA-10 mouse Leydig tumor cells. *J. Endocrinol.* 2007, *192*, 527–537.

[30] Lu, T., Finkel, T., Free radicals and senescence. *Exp. Cell. Res.* 2008, *314*, 1918–1922.

[31] Khandrika, L., Kumar, B., Koul, S., Maroni, P. *et al.*, Oxidative stress in prostate cancer. *Cancer Lett.* 2009, *28*, 1248–1260.

[32] Zhang, H., Park, Y., Wu, J., Chen, X. *et al.*, Role of TNF-alpha in vascular dysfunction. *Clin. Sci. (Lond.)* 2009, *116*, 219–230.

[33] Aliev, G., Obrenovich, M. E., Reddy, V. P., Shenk, J. C. *et al.*, Antioxidant therapy in Alzheimer's disease: theory and practice. *Mini Rev. Med. Chem.* 2008, *8*, 1395–1406.

[34] Alarcon de la Lastra, C. A., Villegas, I., Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications. *Biochem. Soc. Trans.* 2007, *35*, 1156–1160.

[35] Olas, B., Wachowicz, B., Nowak, P., Stochmal, A. *et al.*, Comparative studies of the antioxidant effects of a naturally occurring resveratrol analogue*trans*-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene and resveratrolagainst oxidation and nitration of biomolecules in blood platelets. *Cell. Biol. Toxicol.* 2008, *24*, 331–340.

 