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Regulation of Vascular Endothelial Growth Factor in endometrial tumour cells by resveratrol and EGCG

James M. Dann [a,b,*], Peter H. Sykes [a], Drusilla R. Mason [b], John J. Evans [a,c,d]

[a] Laboratory for Cell and Protein Regulation, Department of Obstetrics and Gynaecology, University of Otago, Christchurch, New Zealand
[b] School of Biological Sciences, University of Canterbury, Christchurch, New Zealand
[c] Centre for Neuroendocrinology, University of Otago, New Zealand
[d] MacDiarmid Institute for Advanced Materials and Nanotechnology, New Zealand

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ABSTRACT

Objective. Our purpose was to establish whether resveratrol and (−)-epigallocatechin-3-gallate (EGCG), two compounds extracted from food, would reduce the amount of Vascular Endothelial Growth Factor (VEGF) secreted into the supernatants of cultured endometrial cancer cells.

Study design. Endometrial cancer samples were collected from 19 consenting women who were undergoing hysterectomy operations to remove tumours. Tumour cells were dispersed into single cell suspensions and cultured. Two immortalised cell lines were also studied. After incubating cells under various test and control conditions, ELISA was used to measure VEGF levels in the supernatants.

Results. VEGF was measurable at varied concentrations in the supernatants of cultured cells, from both cell lines and primary cultures. Cobalt chloride ($CoCl_2$), a hypoxia mimic, increased the measured secretion of VEGF from these cells. In contrast, treatment with either resveratrol or EGCG significantly reduced secretion of VEGF. Further, resveratrol and EGCG inhibited release from cells that were also exposed to $CoCl_2$.

Conclusion. Both resveratrol and EGCG induced significant reductions in the amount of VEGF secreted into the supernatant of cultured endometrial cancer cells. These results suggest that resveratrol and EGCG may have the potential to inhibit angiogenesis in endometrial tumours. Further investigation of these substances in endometrial cancer is warranted.

Introduction

Endometrial carcinoma is one of the most common gynaecologic cancers in Western countries. Hysterectomy is a highly effective treatment for the majority of women. However disseminated endometrial carcinoma is difficult to treat. A systemic non-toxic treatment is required.

Angiogenesis is a key step in the development of most solid tumours, including endometrial carcinoma. The ability of a tumour to develop vasculature in order to respond to its metabolic demands is key to its progression. Many studies have focussed on angiogenesis, with the aim of finding molecules that can halt or reverse the development of new blood vessels [1–3]. Vascular Endothelial Growth Factor (VEGF) is one of the key effector molecules of the angiogenic process. VEGF secretion is elevated in tumour cells and, following occupation of specific receptor molecules on endothelial cells, VEGF stimulates molecules and pathways involved in the co-ordinated formation of new blood vessels. Expression of VEGF itself is stimulated by hypoxia inducible factor (HIF-1) [4–7], a transcription factor that is increased under the conditions of hypoxia that are present in the cores of solid tumours [8–11]. In normoxic tissues the subunit HIF-1α is at low levels because it is rapidly prolyl-hydroxylated, ubiquitinated and then undergoes proteosomal degradation [8–12]. In hypoxic cells, however, as a result of decreased phosphorylation and ubiquitination there is reduced degradation of HIF-1α and consequent accumulation [13,14]. The HIF-1α combines with HIF-1β, which is constitutively expressed, and the heterodimer acts as a transcription factor activating genes that include some involved in angiogenesis. VEGF is one such gene and thus a hypoxic environment results in increased expression.

One molecule investigated here, resveratrol, is a phytoalexin that is found at significant concentrations in red wine, as well as other food substances. It has been shown to have chemoprotective effects in each of the three stages of cancer — carcinogenesis, tumour growth and metastasis [15]. Resveratrol has been shown to lower the levels of intracellular HIF-1α and VEGF in ovarian cancer cells [5], human papillomavirus-transfected cervical cancer cells [16], human tongue squamous carcinoma cells and hepatoma cells [17]. The other molecule investigated in this study is the polyphenol (−)-epigallocatechin-3-gallate (EGCG) which is found in substantial amounts in green tea.

* Corresponding author. Laboratory for Cell and Protein Regulation, Department of Obstetrics and Gynaecology, University of Otago, Christchurch, New Zealand. Fax: +64 3 364 0525.
E-mail address: james.dann@otago.ac.nz (J.M. Dann).

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Both *in vitro* and *in vivo* studies have shown that EGCG has anti-angiogenic properties [18]. The compound has been shown to inhibit angiogenesis and restrain growth in tumour models [19]. Drinking green tea has been reported to be protective against oesophageal cancer [20], colorectal cancers in females from Hebei Province, China [21], stomach cancers in Nagoya, Japan [22], gastric cancer in Kyushu, Japan [23], pancreatic and colorectal cancers in Shanghai, China [24] and breast cancer in Saitama, Japan [25]. The success of these studies encouraged the current investigation of the effect of EGCG on the regulation of VEGF secretion by endometrial cancer cells. Here, we report the effects of that investigation. Both resveratrol and EGCG have properties that appear to be favourable in regard to preventing the growth of many cancers. However there is a scarcity of information on their effects on the uterus. In this study we investigated the effects of the two food compounds on the secretion of VEGF from primary cultures of endometrial tumour cells.

Materials and methods

Cell culture

Following ethical approval from the South Island Board of Ethics (New Zealand), endometrial cancer tissue samples were collected from consenting women with endometrial cancer who were undergoing hysterectomy operations. Tumour cells were dispersed from the tissue using mechanical shearing followed by collagenase digestion. Resuspended cells were then passed through a 70 μm filter, and plated into a flask with α-Minimum Essential Medium (MEM) with 10% fetal bovine serum (FBS). Cells were then incubated at 37 °C with 5% CO_2. The culture medium was changed every second day, until the cells reached confluence and were then lifted using trypsin (2.5% in PBS) and passaged into a larger flask. Once the cells had reached confluence again, they were lifted and transferred to a 24-well plate prior to the experiment. Twenty four hours prior to stimulation, the culture medium was replaced with phenol-red free MEM (PRF-MEM). Cells were then subjected to the experimental treatment in 500 μl of PRF-MEM. After 24 h, cell supernatant was removed from the well, transferred to an Eppendorf tube and frozen until required for ELISA. Of the 20 tumours analysed, 11 were grade I, and the remaining 9 were grade II and III. All the tumours were endometroid adenocarcinomas, with the exception of one tumour, which was a carcinosarcoma. The qualitative response observed in our experimental work was similar across all tumours. RL952 and Ishikawa endometrial cancer cell lines were cultured as per the methods outlined for primary cell cultures.

VEGF ELISA

VEGF ELISA was performed using the DuoSet Human VEGF ELISA Kit (R&D Systems) that detected VEGF-A. A nine-point standard curve (to 1000 ng/ml) of human VEGF was constructed. Assays were performed as described by the manufacturer. The optical density of each well was determined using a microplate reader (Spectra Max 190, Molecular Devices). Optical density was recorded at 450 nm, with wavelength correction set to 540 nm. Software (Soft Max Pro, version 2.6.1, Molecular Devices) was used to create a 4 parameter logistic (4-PL) standard curve. The amount of VEGF in each sample was then found by comparing the absorbance of the unknowns to the standard curve.

MTT viability assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) tests were performed after the supernatants had been removed and stored. Each well of the plate was washed twice with 500 μl of PBS, which was added to each well, then removed by aspiration. This wash step was then repeated. After 900 μl of PRF-MEM had been added to each well of the plate, 100 μl of MTT (5 ng/ml) was added, and the plate was placed in the 37 °C incubator for 2 h for the colour to develop.

Following the incubation, 1 ml of 10% DMSO in isopropanol was added to each well. Crystals produced during the incubation were then dislodged from the plate using a pipette. The contents of each well were transferred into an Eppendorf tube and centrifuged for 5 min at 10,000 rpm. An aliquot of the supernatant from each tube was then transferred into a 96-well plate. Absorbance was measured at 570 nm (wavelength reduction at 690 nm), using water as a blank.

Data analysis and statistics

Graphing, data analysis and statistics were performed using GraphPad Prism 5 for Mac. Data were statistically analysed using paired or unpaired Student's *t*-test, and one-way or two-way ANOVA. $p<0.05$ was considered significant. All data were reported as mean ± SEM.

Results

Secretion of VEGF by tumour cells in basal conditions

The level of VEGF in the supernatant of endometrial cell cultures varied substantially between tumours (Fig. 1). Most primary endometrial cell cultures had an unstimulated VEGF level of below 1000 ρg/ml. Cell type JD44 was the one exception to this. The concentrations of VEGF in the supernatants of all of the primary tumour cultures were significantly less than the unstimulated VEGF levels found in the two endometrial cancer cell lines, Ishikawa and RL952.

Effect of incubation with resveratrol

Cobalt chloride ($CoCl_2$) was used to induce an increase in the level of HIF-1α and to thus mimic the increase seen in hypoxic cancer cells. $CoCl_2$ is known to be effective in increasing VEGF secretion from endometrial cancer cells by inhibiting HIF-1α degradation [4].

Cells were exposed to selected concentrations of $CoCl_2$ for 24 h. The amount of VEGF secreted into the culture medium was then assayed (Fig. 2). Stimulation of the cell cultures with 50 μM $CoCl_2$ caused a small increase in the amount of VEGF, relative to the control, but this was not significant. However, 100 μM $CoCl_2$ significantly stimulated VEGF secretion compared with controls ($p<0.05$, $n=12$). In subsequent experiments, 100 μM $CoCl_2$ was chosen to stimulate cells, as while both the 200 μM and 400 μM concentrations caused a greater increase in VEGF secretion, this increase was associated with a higher

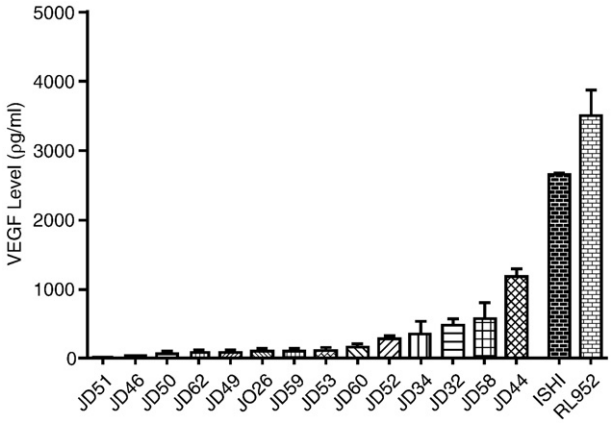

Fig. 1. Level of VEGF in supernatant of unstimulated endometrial cancer cells in culture. Cultures are arranged from lowest mean VEGF level to highest.

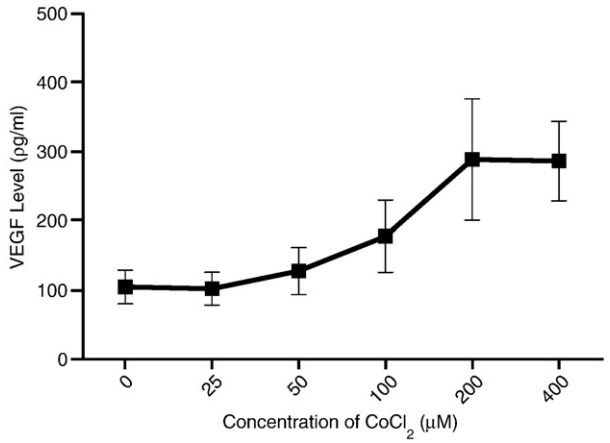

Fig. 2. Change in VEGF levels with exposure to $CoCl_2$. The mean VEGF level for each of the concentrations was the average of quadruplicate assays of 3 different tumours.

variation. A concentration of 100 μM is also consistent with conditions in previously reported studies in the literature [4,26].

We initially investigated the effects of resveratrol on an endometrial carcinoma cell line, RL952, which has well documented characteristics. The mean secretion of VEGF from cells incubated with $CoCl_2$ alone was significantly higher than that of the control group ($p<0.0001$, $n=26$, paired t-test) (Fig. 3). In contrast the VEGF levels in the supernatants of cells exposed to resveratrol alone were significantly lower than that of the supernatants of control cells ($p<0.0001$, $n=26$, paired t-test). When resveratrol was added with $CoCl_2$, the resulting secretion of VEGF was lower than for cells exposed to $CoCl_2$ alone ($p=0.0006$, $n=26$, paired t-test). In fact, resveratrol suppressed the $CoCl_2$-induced increase so that the VEGF concentration was not significantly different to that seen in control cells that were not exposed to the hypoxia mimic (NS, $n=26$, paired t-test).

The effects of resveratrol (100 μM) on basal and $CoCl_2$-stimulated VEGF release were then measured in primary endometrial cancer cells. $CoCl_2$ caused an increase in the VEGF level that was more than double that seen in controls (control, 350.4 ρg/ml ± 57.46 vs $CoCl_2$, 926.3 ρg/ml ± 125.4, $p<0.0001$, $n=108$, paired t-test) (Fig. 4). Again, resveratrol significantly reduced VEGF secretion (to 120.6 ρg/ml ± 30.3)

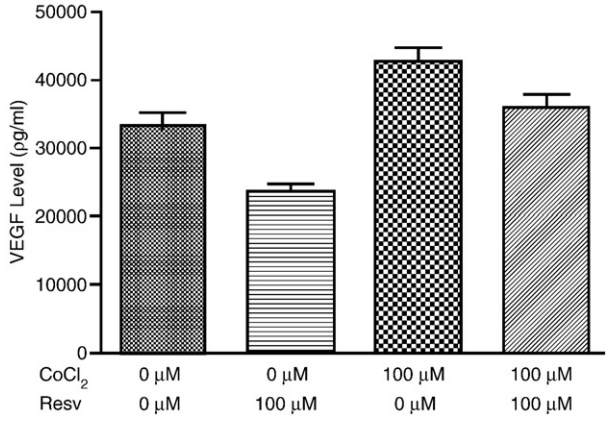

Fig. 3. VEGF levels in RL952 endometrial carcinoma cell line when exposed to resveratrol. Labels on the X-axis detail the concentrations of cobalt chloride ($CoCl_2$) and resveratrol (Resv) that each group was administered. The mean of the $CoCl_2$ only group was significantly higher than that of the control group ($p<0.0001$, $n=26$, paired t-test). The mean of the resveratrol only group was significantly lower than that of the VEGF than control group ($p<0.0001$, $n=26$, paired t-test). Resveratrol with $CoCl_2$ was not significantly different than the mean of control group (ns, $n=26$, paired t-test). The mean of the $CoCl_2$ only group was significantly higher than that of the resveratrol and $CoCl_2$ group ($p=0.0006$, $n=26$, paired t-test).

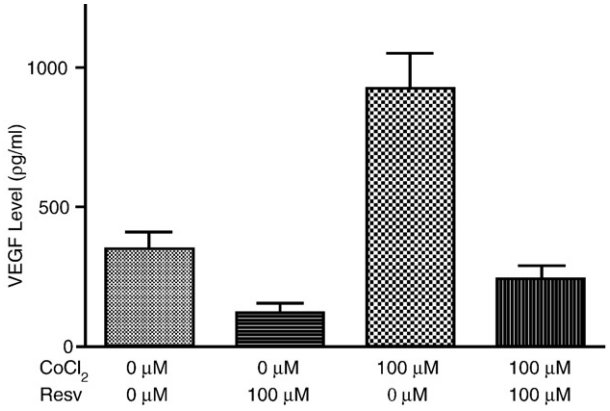

Fig. 4. VEGF levels in primary endometrial carcinoma culture cells when exposed to resveratrol. Labels on the X-axis detail the concentrations of cobalt chloride ($CoCl_2$) and resveratrol (Resv) that each group was administered. The mean of the $CoCl_2$ only group was significantly higher than that of the control group ($p<0.0001$, $n=108$, paired t-test). The mean of the resveratrol only group was significantly lower than that of the VEGF than control group ($p<0.0001$, $n=105$, paired t-test). Resveratrol with $CoCl_2$ was significantly lower than the mean of control group ($p<0.0056$, $n=103$, paired t-test). The mean of the $CoCl_2$ only group was significantly higher than that of the resveratrol and $CoCl_2$ group ($p<0.0001$, $n=103$, paired t-test).

compared to controls ($p<0.0001$, $n=113$, t-test). Further, VEGF release from cells exposed to resveratrol plus $CoCl_2$ (mean = 245.6 ρg/ml ± 45.45) was significantly lower ($p<0.0001$, $n=111$, t-test) than that from the $CoCl_2$ group (mean = 926.3 ρg/ml ± 125.4). The value for the resveratrol plus $CoCl_2$-treated cells was lower than that of the control, though this difference was not significant (NS, $n=111$, t-test). The results clearly establish that the resveratrol treatment reversed the $CoCl_2$-induced increase in VEGF secretion and also inhibited VEGF secretion that occurred in basal conditions.

The cell viabilities of cultures exposed to resveratrol were measured using the MTT assay. The measurements showed that resveratrol did not have a significant effect on the viability of the cultures, indicating that the reduction in VEGF was a direct result of resveratrol treatment, rather than a cytotoxic effect of the treatment.

It was hypothesized that the amount of VEGF secreted by the primary tumours used in this study would be correlated with the grade of each of the tumours. To test this hypothesis, we tabulated the concentration of VEGF secreted by cells from different grades of cancer for each of the experimental treatments (Fig. 5). Although cells from

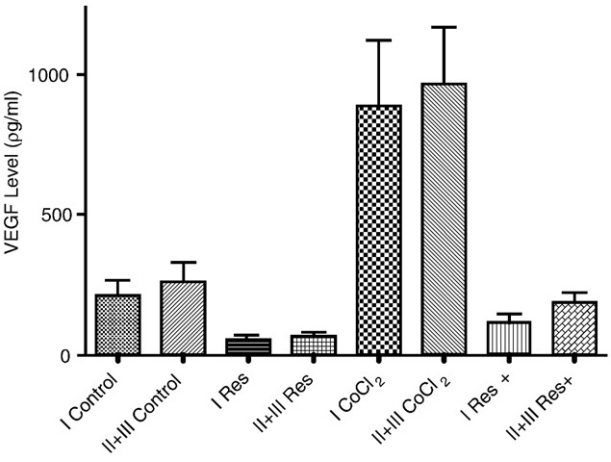

Fig. 5. VEGF levels in primary endometrial carcinoma cell cultures separated by grade. Tumours have been grouped by grade, either grade I or grade II + III. There was no significant difference observed between the means of the different tumour grades, across any of the 4 treatments used.

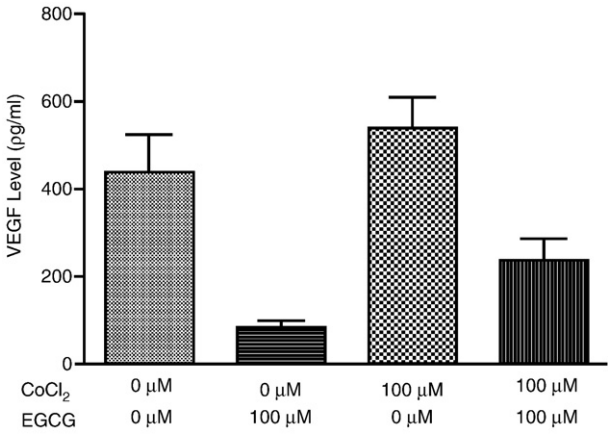

Fig. 6. VEGF levels in primary endometrial carcinoma cell lines when exposed to EGCG. Labels on the *X*-axis detail the concentrations of cobalt chloride ($CoCl_2$) and (−)-epigallocatechin-3-gallate (EGCG) that each group was administered. The mean of the $CoCl_2$ only group was not significantly higher than that of the control group (ns, $p<0.0001$, $n=22$, paired *t*-test). The mean of the EGCG only group was significantly lower than that of the VEGF than control group ($p=0.0044$, $n=22$, paired *t*-test). EGCG with $CoCl_2$ was not significantly different than the mean of control group (ns, $p=0.3713$, $n=22$, paired *t*-test). The mean of the $CoCl_2$ only group was significantly higher than that of the EGCG and $CoCl_2$ group ($p<0.0001$, $n=22$, paired *t*-test).

the Grade II + III tumours have a higher level of VEGF secretion than Grade I in each of the four treatments, the differences were not statistically significant. Further study is warranted to confirm whether the trend points to an important characteristic in VEGF regulation of endometrial tumours.

Effect of incubation with EGCG

Cells treated with 100 μM of EGCG showed a significant reduction in the amount of VEGF in the supernatant (EGCG, 82.39 ρg/ml ± 19.54 vs control, 436.06 ρg/ml ± 86.74; $p<0.0001$, $n=22$, *t*-test) (Fig. 6). As was seen in previous experiments, 100 μM $CoCl_2$ caused an increase in the amount of VEGF in the supernatant (537.65 ρg/ml ± 70.26) when compared to the control (436.06 ρg/ml ± 86.74). However, unlike the previous graphs, this result is not significant (NS, $n=22$, *t*-test), possibly because the smaller sample size in this experiment resulted in larger standard errors.

Cells treated with both EGCG and $CoCl_2$ showed a significant reduction in measured VEGF (235.65 ρg/ml ± 47.42) when compared to the VEGF secretion from cells treated with $CoCl_2$ alone (537.65 ρg/ml ± 70.26, $p<0.0001$, $n=36$, *t*-test). This showed that EGCG was a potent inhibitor of VEGF secretion from endometrial cancer cells, and significantly reduced the effects of $CoCl_2$.

Discussion

Here we have reported that the food extracts resveratrol and EGCG can cause a significant reduction in the amount of VEGF found in the supernatant of endometrial carcinoma cells cultured *in vitro*. Further, we have shown that these substances can counter the increase in VEGF caused by $CoCl_2$, a substance that can be used to mimic the effects of hypoxia. Both resveratrol and EGCG exhibited a concentration-dependent effect on the amount of VEGF secreted from the cells. Higher levels of the food compound induced more reduction in VEGF secretion. Toxicity effects became apparent at very high concentrations.

Although resveratrol has been previously reported to have anti-cancer effects in endometrial carcinoma, this study was the first to show that resveratrol reduces the secretion of VEGF in endometrial cancer cells. By using resveratrol at a concentration of 100 μm, the amount of VEGF released into the supernatant of endometrial cancer cells was significantly reduced, when compared to the VEGF levels in the supernatant of endometrial cancer cells without treatment. When compared to cells which had increased VEGF release induced by $CoCl_2$, resveratrol again significantly reduced the amount of VEGF in the supernatant. This suggests that resveratrol may reduce the angiogenic effects induced by hypoxia in endometrial cancer cells. This supports research previously published that has investigated the effect of resveratrol on VEGF and HIF-1α in other tumour types. While this study did not measure cellular HIF-1α levels directly, studies reported in the literature suggest that resveratrol reduces VEGF by inhibiting HIF-1α. Increased levels of VEGF and HIF-1α are found in ovarian cancer [7] and resveratrol has been shown to inhibit HIF-1α and reduce VEGF levels in that cancer [27]. As VEGF has been shown to correlate with HIF expression in endometrial cancers [28], it is possible that inhibition of HIF-1α by resveratrol is the mechanism by which the compound reduces VEGF release. However, as other intermediaries may be involved, direct measurement of HIF-1α levels in endometrial cancer cells treated with resveratrol is still required to confirm this.

Studies that have investigated the role of VEGF in endometrial cancer have shown that the levels of VEGF and HIF-1α were significantly elevated in these cancers [6]. A large study that looked at rates of breast cancer – another cancer that shows elevated HIF-1α levels [29] – found that women who consumed more resveratrol in their diets had lower risks of developing breast cancer [30]. Given the significant reductions in VEGF caused by resveratrol in this study, further investigation into the effects of dietary resveratrol on endometrial cancer rates are warranted.

This study is the first to show that EGCG, a catechin extract from green tea, reduces the secretion of VEGF in endometrial cancer cells. By using EGCG at a concentration of 100 μm, the amount of VEGF released into the supernatant of endometrial cancer cells was significantly reduced, when compared to the VEGF levels in the supernatant of endometrial cancer cells without treatment. When compared to cells which had increased VEGF release induced by $CoCl_2$, EGCG again significantly reduced the amount of VEGF in the supernatant. This suggests that EGCG can reduce the angiogenic effects induced by hypoxia in endometrial cancer cells. This result is consistent with previously reported work investigating the effects of EGCG on VEGF release in other cancer cell types, including cervical and hepatoma cells [31]. However, replication of this result is required, as it has been reported that EGCG can actually lead to an increase in VEGF [32] and to cell proliferation [33]. The intermediate compounds involved in the effects are not yet defined and these details require further investigation. Nevertheless the results in this study indicate the possibility of further studies to demonstrate whether resveratrol or EGCG have an *in vivo* effect at physiological levels.

In this study, we have reported that VEGF levels in endometrial carcinoma cultures can be reduced by the addition of 100 μM resveratrol or EGCG. This reduction was also shown to be great enough to counter the increase in VEGF levels seen with the addition of the hypoxia mimetic $CoCl_2$. Our results suggest that resveratrol and EGCG may have potent anti-angiogenic effects in endometrial cancers, and should be investigated further as a potential nutraceutical treatment for this disease.

Conflict of interest statement
The authors declare that there are no conflicts of interest.

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