Human Reproduction, Vol.28, No.1 pp. 178–188, 2013
Advanced Access publication on October 18, 2012 doi:10.1093/humrep/des369

human reproduction

ORIGINAL ARTICLE *Infertility*

Natural therapies assessment for the treatment of endometriosis

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Submitted on April 26, 2012; resubmitted on September 9, 2012; accepted on September 14, 2012

STUDY QUESTION: Can resveratrol and epigallocatechin-3-gallate (EGCG) inhibit the growth and survival of endometriotic-like lesions *in vivo* in a BALB/c model of endometriosis, and *in vitro* in primary cultures of human endometrial epithelial cells (EECs)?

SUMMARY ANSWER: Resveratrol and EGCG exerted a potent inhibitory effect on the development of endometriosis in a BALB/c murine model and on the survival of EECs.

WHAT IS KNOWN ALREADY: Endometriosis is a common condition associated with infertility and pelvic pain in women of reproductive age. Resveratrol and EGCG are two polyphenols with anticarcinogenic and antioxidant properties that have been proposed as natural therapies to treat endometriosis.

STUDY DESIGN, SIZE, DURATION: Fifty-six 2-month-old female BALB/c mice underwent surgical induction of endometriosis. Treatments with resveratrol or EGCG started 15 days post-surgery and continued for 4 weeks. Human biopsies were taken with a metal Novak curette from the posterior uterine wall from 16 patients with untreated endometriosis and 15 controls who underwent diagnostic laparoscopy for infertility.

MATERIALS, SETTING, METHODS: After the treatments, animals were sacrificed and lesions were counted, measured, excised and fixed. Immunohistochemistry for proliferating cell nuclear antigen and CD34 was performed for cell proliferation and vascularization assessment in the lesions. The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique was performed for apoptosis evaluation. Peritoneal fluid was collected to analyze vascular endothelial growth factor levels. Human EECs were purified from proliferative-phase endometrial biopsies and cultured. The effect of both polyphenols on cell proliferation was determined by a colorimetric assay using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit and on apoptosis by the TUNEL technique, using an *In Situ* Cell Death Detection Kit with Fluorescein.

MAIN RESULTS: In the mouse model, both treatments significantly reduced the mean number ($P < 0.05$ versus control) and the volume of established lesions ($P < 0.05$ versus control). Treatments consistently statistically significantly diminished cell proliferation (resveratrol $P < 0.01$ and EGCG $P < 0.05$, versus control), reduced vascular density (resveratrol $P < 0.01$ and EGCG $P < 0.001$, versus control) and increased apoptosis within the lesions (resveratrol $P < 0.01$ and EGCG $P < 0.05$, versus control). Both compounds induced reduction in human EEC proliferation ($P < 0.05$ versus basal) and increased apoptosis ($P < 0.05$ versus basal) in primary cultures.

LIMITATIONS: *In vitro* studies were only carried out in epithelial cells from human eutopic endometrium.

WIDER IMPLICATIONS OF THE FINDINGS: The present findings are promising and will assist the development of novel natural treatments for endometriosis.

STUDY FUNDING: This study was supported by ANPCYT (PICT 6384 BID 1201 OC-AR) and CONICET (PIP 5471), Argentina. None of the authors has any conflict of interest to declare.

Key words: endometriosis / resveratrol / EGCG / human eutopic endometrium / BALB/c mice

Introduction

Endometriosis is one of the most common benign disorders and affects 6–10% of women of reproductive age (Giudice and Kao, 2004). The disease is defined as the presence of endometrial glands and stroma outside the uterine cavity and patients with endometriosis often suffer from dysmenorrhea, dyspareunia, dysuria and chronic abdominal or pelvic pain, as well as infertility, resulting in a limited quality of life (Giudice and Kao, 2004). As the disease is estrogen-dependent, medical therapies are principally aimed at down-regulating ovarian estrogen production (Valle and Sciarra, 2003; Mihalyi *et al.*, 2006). However, it is well known that current medical therapies available for the treatment of endometriosis have adverse effects that limit their long-term use. Moreover, recurrence of the disease after the cessation of therapy is very frequent and most patients need to extend treatment to maintain a hypoestrogenic environment until conception. Therefore, it is important to keep on looking for new safe and effective long-term treatments.

Nowadays, natural compounds found in food and plants are being considered for the treatment of diverse diseases, like cancer, for example, which shares important similarities with endometriosis even though the latter is a benign disease (Varma *et al.*, 2004). The health benefits of green tea, red wine, garlic and fresh fruits have been described for many years, and their efficiency in the prevention of diseases has been confirmed by several molecular studies (Cucciolla *et al.*, 2007; Khan and Mukhtar, 2008). Catechins in green tea and polyphenols in red wine are responsible for the observed benefits of these products that are part of our daily diet habits. Epigallocatechin-3-gallate (EGCG) is the major catechin found in green tea and has been considered in the last years for the treatment of different types of cancer, based on its antioxidant, antiangiogenic and antiproliferative effects (Zaveri, 2006; Khan and Mukhtar, 2008). Its antimitotic properties lead to the idea that EGCG could be useful for the treatment of endometriosis. Recent studies showed encouraging results in this area (Laschke *et al.*, 2008; Xu *et al.*, 2009, 2011). However, until now there is no evidence of the systemic effect of oral administration, neither of this compound nor of the direct effect, on the survival of endometrial epithelial human cells in primary cultures. One of the aims of our study was to evaluate the effects of oral administration of EGCG on the growth and survival of surgically induced endometriosis in immunocompetent BALB/c mice, considering the importance of the immune system in this pathology (Tariverdian *et al.*, 2007, 2009). On the other hand, another effective compound is resveratrol (*trans*-3,4′,5-trihydroxystilbene), a natural phytoalexin produced by some grape species, peanuts and berries in response to fungal infections or UV radiation (Cucciolla *et al.*, 2007). The most significant concentrations of resveratrol are found in the skin of grapes and therefore in red wines but not white wines. Evidence indicates that this compound has anticarcinogenic, anti-inflammatory and antioxidant properties as well as pro-apoptotic and antiangiogenic effects (Brakenhielm *et al.*, 2001; Garvin *et al.*, 2006). There is only one very recent study that evaluated the effect of resveratrol on experimental endometriosis *in vivo* and on the invasiveness of endometrial stromal cells *in vitro* (Bruner-Tran *et al.*, 2011). The limited evidence available leads to the need of more studies to elucidate the effect of this polyphenol on the survival of endometrial epithelial human cells, as well as its effect on endometriosis development in an immunocompetent mouse model of the disease.

The aim of our study was to evaluate the effects of these two natural compounds both *in vivo* and *in vitro* on the growth and survival of experimental endometriosis in a BALB/c mouse model and on primary cultures of human endometrial epithelial cells (EECs), respectively.

Materials and Methods

Animals

In this study, 56 2-month-old female BALB/c mice were used. All procedures were performed according to the NIH guidelines for the care and the use of laboratory animals and approved by the Ethics and Research Committee from the Biology and Experimental Medicine Institute of Buenos Aires, Argentina. A total of five animals died or had to be sacrificed between 2 and 3 days after surgery because they did not fully recover from the intervention.

Surgical induction of endometriosis and treatment

Endometriotic-like lesions were induced through transplantation of one of the uterine horns to the bowel mesentery as previously described (Bilotas *et al.*, 2010). Briefly, animals were deeply anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Afterwards, mice underwent laparotomy by a mid-ventral incision to expose the uterus and the intestine. The right uterine horn was removed and placed in a Petri dish containing DMEM-F12 (Gibco, Paisley, UK) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco). The uterine horn was opened longitudinally and then cut into square pieces measuring ~4 mm^2. Three equal pieces of tissue were then sutured onto the serosal layer with a single 6–0 nylon suture (Supralon, Ethicon, Somerville, NJ, USA). Endometrial tissue was sutured facing the serosa. The abdomen was then closed with a 5–0 nylon suture.

After surgery, animals were randomly assigned to experiments to test the effect of resveratrol (R5010, Sigma-Aldrich™ Co., Saint Louis, MO, USA), or EGCG (E4268, Sigma-Aldrich). Treatment was initiated 2 weeks after surgery and continued for 4 weeks. Mice were monitored daily and no evidence of toxicity was noted based on body weight, food consumption, grooming behavior or activity levels compared with respective controls. Besides the estrous cycles were followed cytologically during treatments to ensure that they do not interfere with reproductive activity. No differences were detected between controls and treated mice (data not shown).

Resveratrol treatment

Resveratrol was dissolved in 100% ETOH to make a stock solution of 50 mg/ml. Twenty-nine animals were randomly assigned to three experimental groups: Res I (resveratrol 10 mg/kg in water), Res II (resveratrol 25 mg/kg in water) and control (10% ETOH vehicle). The treatments were administered daily intraperitoneally.

EGCG treatment

EGCG was dissolved in water to make a stock solution of 20 mg/ml. Twenty-seven animals were randomly assigned to three experimental groups: EGCG I (EGCG 20 mg/kg in water), EGCG II (EGCG 100 mg/kg in water) and control (vehicle only).The treatments were administered daily by esophageal gavage.

Evaluation of endometriotic-like lesions

After 4 weeks of treatment, animals were sacrificed by cervical dislocation. The abdomen was opened by a ventral midline incision. Implantation sites were localized by the presence of a lesion or by a suture alone. Lesions were counted and measured in two perpendicular diameters ($d < D$) using a caliper. Lesion volumes were determined using the formula: $V = (4/3)\pi r^2 R$ (r and R are the radii, $r < R$) (Ricci *et al.*, 2011). Then lesions were excised and fixed in 10% formaldehyde for histological analysis. Formalin-fixed specimens were paraffin-embedded and cut into 5 μm serial sections. Several sections from each specimen were stained with hematoxylin-eosin and examined microscopically for the presence of histological hallmarks of endometriosis. The evaluation of the established endometriotic-like lesions was performed by two independent observers, blinded to the treatment condition.

Immunohistochemistry for proliferating cell nuclear antigen and CD34

Serial sections of the endometriotic-like lesions were subjected to standard immunohistochemistry (Meresman *et al.*, 2000). Tissue sections were incubated overnight with the primary antibody [rabbit antimouse proliferating cell nuclear antigen (PCNA) polyclonal, 1:300, FL-261, Santa Cruz Biotechnology, Santa Cruz, CA; or rat antimouse CD34 monoclonal, 1:50, ab8158 Abcam, Cambridge, MA, USA] at 4°C. After that, sections were treated for 60 min with the proper secondary biotinylated antibody (antirabbit IgG, 1:200, B7389 Sigma-Aldrich; or goat antirat IgG 1:500, B7139 Sigma-Aldrich) followed by incubation with streptavidin-peroxidase (LSAB + System, Dako, Carpinteria, CA, USA). Binding was visualized by incubating sections with diaminobenzidine and lightly counterstaining with hematoxylin. As a negative control, immunoglobulin of the same immunoglobulin class and concentration as the primary antibodies was used.

PCNA positive cells were identified by the presence of brown nuclear reactivity. A total of 300 epithelial cells were counted from representative fields, considering all lesions and the percentage of PCNA positive cells was established per mouse, blinded to the treatment condition. All percentages were used to get the mean value per group. Any nuclear staining was regarded as positive.

Staining for CD34 was assessed to study vascular density identifying blood vessels. The percentage of total area expressing immunoreactivity for CD34 was established blindly to the treatment condition, by analyzing 10 representative fields from all lesions with the Image J 1.33u software (NIH, USA). The CD34 positive area was calculated per field and then averaged per mouse. All percentages were used to get the mean value per group.

TUNEL assay

For apoptosis quantification, sections were processed for *in situ* immunolocalization of nuclei exhibiting DNA fragmentation, by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique, using the apoptosis detection kit Apoptag Plus (Chemicon International, Temecula, CA, USA). Sections were treated according to the manufacturer's instructions as previously described (Meresman *et al.*, 2000). The positive control was provided by the supplier of the kit. As a negative control, a number of tissue samples were subjected to treatment without TdT. Finally, sections were counterstained with hematoxylin. A total of 300 epithelial cells were counted from representative fields, considering all lesions, and the percentage of TUNEL positive cells was established per mouse, blinded to the treatment condition. All percentages were used to get the mean value per group.

Quantification of vascular endothelial growth factor

Peritoneal fluid was collected by rinsing the abdominal cavity with 1.5 ml of saline and centrifuged at 4°C during 10 min at 1500 rpm. Supernatants were stored at −20°C until assayed for vascular endothelial growth factor (VEGF) using a commercial enzyme-linked immunoassay (ELISA) kit according to the manufacturer's instructions (QIA52, Calbiochem, EMD Chemicals Inc., USA). The sensitivity level for the VEGF ELISA was 3 pg/ml. The intra-assay variability for VEGF was ±4.3% while the inter-assay variability was ±5.7%. All samples were assessed in triplicate.

Collection of human biopsies

A total of 31 patients in reproductive age who underwent diagnostic laparoscopy for infertility participated in this study: 16 with untreated endometriosis (Stages I and II, according to the Revised American Society for Reproductive Medicine Classification [ASRM, 1997]) and 15 controls. Confirmation of the disease was performed by histological documentation. Control subjects were infertile women without endometriosis or any infectious or non-infectious pathology that could affect the evaluated cell population, with tubal factor or unexplained infertility, undergoing diagnostic laparoscopy. To avoid false negatives, only patients who did not complain of pelvic pain were considered for the control group. All patients were infertile, showed regular menstrual cycles and had not received any hormonal medical treatment for the last 6 months. All subjects signed informed consent prior to evaluation. Biopsies of eutopic endometrium were obtained from all subjects in the proliferative phase, as described previously (Meresman *et al.*, 2000). Biopsies were taken with a metal Novak curette from the posterior uterine wall. This study was approved by the Ethics and Research Committee from the Biology and Experimental Medicine Institute of Buenos Aires, Argentina.

Isolation and culture of EECs

EECs were obtained from eutopic endometrial biopsies of patients with endometriosis and control subjects. The tissue was immediately placed into a sterile tube with culture medium and processed within 3 h of collection. Epithelial cells were enzymatically separated and isolated by successive centrifugation, and primary cultures were established for *in vitro* studies as previously described (Meresman *et al.*, 2003a,b). Briefly, tissue was minced, washed and placed in MEM Eagle D-Valine Modified w/L-Glutamine, D-Valine medium (USBiological, MA, USA) supplemented with penicillin 100 IU/ml, streptomycin 100 mg/ml and amphotericin B 25 mg/ml (Gibco) with collagenase 0.5 mg/ml (type I, Gibco). After 2 h of incubation at 37°C in a 5% CO_2 atmosphere, the resulting suspension was centrifuged at 100 g for 5 min. The pellet containing epithelial glands was resuspended in culture medium and spun again at 100 g for 5 min. The final pellet mainly contained epithelial cells. The enriched epithelial fraction was cultured with MEM D-Val supplemented with 10% fetal bovine serum (FBS; Gibco) and grown to sub-confluence (70–80%) at 37°C for 48 h before the experiments. In all cases, each biopsy was utilized to run one or more experiments and there was no pooling of samples.

It has been previously shown that this method guarantees purity of EECs in culture (Meresman *et al.*, 2003a,b). Culture's purity was 85–90% for epithelial cells as determined by cytokeratin staining (Supplementary data, Fig. S1).

Cell proliferation assay in primary cultures

After purification, EECs (5×10^4/well) were plated in 96-well culture plates with MEM D-Val supplemented with 10% FBS and incubated at 37°C in a 5% CO_2 atmosphere. After 48 h, cultures were washed and incubated with different concentrations of resveratrol (0, 25, 50 and

100 μM) or EGCG (0, 20, 40, 80 and 100 μM) in fresh medium supplemented with 2.5% FBS. Basal conditions (0μ M) were performed by incubating EECs with the final concentration of dimethyl sulfoxide (DMSO; D5879, Sigma-Aldrich), the vehicle used to reconstitute both compounds. EEC proliferation was determined by a colorimetric assay using the CellTiter 96®AQueous One Solution Cell Proliferation Assay kit (Promega Corp., Madison, WI, USA). After 24 h of treatment, 20 μl of One Solution Reagent was added to each well and incubated for 2 h at 37°C. Absorbance was measured at 490 nm using a multi-well plate reader. The One Solution Reagent contains MTS tetrazolium (3-[4,5-dimethylthiazol-2-yl] -5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium), which is bioreduced by cells into a formazan product. The absorbance of the formazan product is directly proportional to the number of living cells. Experiments were conducted with replicates of six wells per treatment condition. Similar experiments were conducted with cells purified from nine biopsies from women with endometriosis and seven biopsies from control subjects. Cell proliferation was expressed as a percentage of basal conditions in each experiment.

Apoptosis assay in primary cultures

EECs (1×10^5/well) were plated in Lab-Tek eight-well culture chambers (Nalge Nunc, Naperville, IL, USA) with MEM D-Val supplemented with 10% FBS. After 48 h, cultures were washed and incubated with different concentrations of resveratrol (0, 25, 50 and 100 μM) or EGCG (0, 20, 40, 80 and 100 μM) in medium supplemented with 2.5% FBS. Basal conditions (0 μM) consisted of the final concentration of DMSO as explained above. Cells were incubated for 24 additional hours and then were fixed in 4% paraformaldehyde to assess the percentage of apoptotic cells by the TUNEL technique, using an *In Situ* Cell Death Detection Kit with Fluorescein (Roche 11684795910, Roche Diagnostics GmbH Roche Applied Science, Mannheim, GE, USA) according to the manufacturer's protocol. Lab-Tek slides were stained with 4′-6′ diamino-2-phenyllindole (DAPI) to label nuclei and then mounted in Vectashield mounting medium (H-1000, Vector Laboratories Inc., Burlingame, CA, USA). The apoptotic index was evaluated using a fluorescence microscope. The total number of fluorescein isothiocyanate (FITC)-positive nuclei from 300 DAPI-positive nuclei was determined by analyzing representative fields under each treatment condition. Similar experiments were conducted with cells purified from seven biopsies from women with endometriosis and eight biopsies from control subjects. Results were expressed as a percentage of FITC/TUNEL positive to total DAPI-stained nuclei.

Statistical analysis

Statistical analyses were performed using GraphPad PRISM Software V4.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical comparisons between groups were performed using parametric one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, or non-parametric one-way ANOVA followed by Dunn's multiple comparison test. TUNEL and PCNA positive cells were counted blindly, by two independent observers. Results were expressed as mean ± SEM. In all cases, statistical significance was considered to be $P < 0.05$.

Results

Effect of resveratrol and EGCG on endometriotic-like lesion growth

The number of established lesions observed per mouse was significantly reduced by the higher (25 mg/kg/day) dose of resveratrol ($P < 0.05$ versus control). However, EGCG was able to reduce the number of established lesions at either 20 or 100 mg/kg/day ($P < 0.05$ versus control). The results are displayed in Fig. 1A. Regarding the volume of developed lesions, both treatments caused a statistically significant reduction in lesion size. As seen in Fig. 1B, resveratrol only diminished their volume at 25 mg/kg/day ($P < 0.05$ versus control), whereas EGCG treatment was able to significantly diminish lesion size at either 20 or 100 mg/kg/day (I $P < 0.05$ versus control; II $P < 0.01$ versus control).

Effect of resveratrol and EGCG treatments on cell proliferation and apoptosis in endometriotic-like lesions

Cell proliferation was evaluated by immunohistochemistry for PCNA (Fig. 2). Treatment with both doses of resveratrol and EGCG caused a decrease in epithelial cell proliferation compared with the control group (Res I $P < 0.01$ versus control; Res II $P < 0.001$ versus control; EGCGI $P < 0.05$ versus control; EGCGII $P < 0.01$ versus control).

Complementary to the observed results for cell proliferation, all treatments significantly increased the apoptotic index in epithelial cells of endometriotic-like lesions (Res I $P < 0.01$ versus control; Res II $P < 0.001$ versus control; EGCGI $P < 0.05$ versus control; EGCGII $P < 0.01$ versus control), assessed by the TUNEL technique (Fig. 3).

Effect of resveratrol and EGCG on VEGF levels and vascular density

To determine the vascular density, blood vessels in endometriotic-like lesions were immunostained with CD34 antibody, and the percentage of vascular area was quantified (Fig. 4). In both cases, the treatment-inhibitory effect was clearly associated with an important decrease in the vascular density (Res I/II $P < 0.01$ versus control; EGCGI/II $P < 0.001$ versus control).

After treatments, peritoneal fluid was collected and levels of VEGF were assessed by ELISA. Treatment with EGCG (both doses) significantly decreased VEGF levels in the peritoneal fluid (I $P < 0.001$ versus control; II $P < 0.05$ versus control). However, resveratrol did not significantly reduce the peritoneal VEGF levels at any of the doses tested (Fig. 5).

Effects of resveratrol and EGCG on EEC proliferation and apoptosis

The effects of different concentrations of resveratrol and EGCG on EEC proliferation are displayed in Fig. 6A and B. After exposure to 50 and 100 μM of resveratrol, the EECs showed a significantly lower percentage of cell proliferation ($P < 0.05$ and $P < 0.01$ respectively, versus basal) in cultures from both control women (Fig. 6A(i)) and endometriosis patients (Fig. 6B(i)). EGCG treatment showed a significant inhibition with all assayed concentrations (40, 80 and 100 μM), excluding the lowest one (20 μM), which had no significant effect on basal cell proliferation (Fig. 6A(ii)–B(ii)). The same significant effect was observed in cultures from endometriosis patients ($P < 0.05$, $P < 0.01$, $P < 0.001$ versus basal, respectively) and from control women ($P < 0.01$ versus basal).

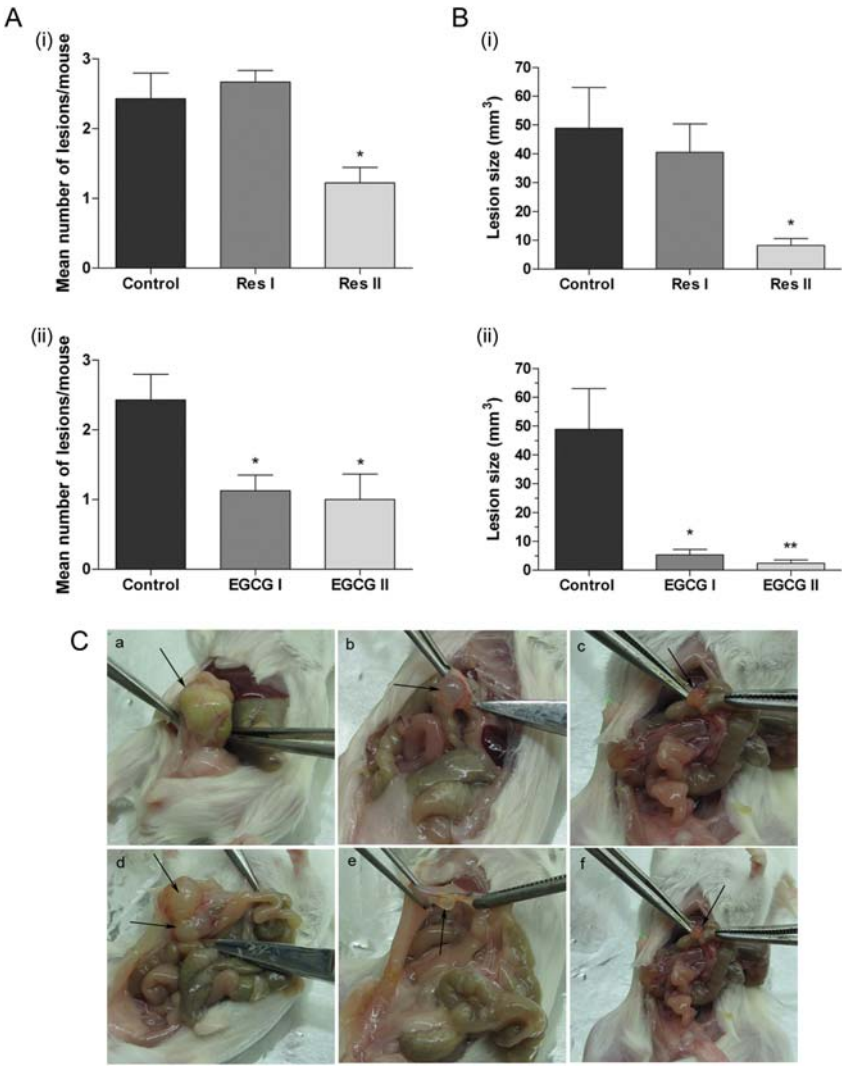

Figure 1 The effect of resveratrol and EGCG on the number (**A**) and volume (**B**) of endometriotic-like lesions. Mice with surgically induced endometriosis were treated everyday starting on post-surgery day 15 with: (i) resveratrol (Res I 10 mg/kg–Res II 25 mg/kg) or vehicle (control); (ii) EGCG (EGCG I 20 mg/kg–EGCG II 100 mg/kg) or vehicle (control). After 4 weeks of treatment, mice were sacrificed and established lesions were counted and measured with calipers. The number of established lesions (>0 mm^3) per mouse was calculated for each treatment group. The volume for each lesion was calculated with the formula $V = (4/3)\pi r^2 R$. Volume values of all developed lesions per mouse were averaged and the mean was calculated for each treatment group. Results are expressed as mean $\pm$ SEM. $^*P < 0.05$/$^{**}P < 0.01$ versus control. $n = 8$ (Res control), 10 (Res I), 10 (Res II), 8 (EGCG control), 9 (EGCG I), 6 (EGCG II).(**C**) Photographs illustrating the endometriotic-like lesions of (a) Res control, (b) Res I, (c) Res II, (d) EGCG control, (e) EGCG I and (f) EGCG II are displayed. Black arrow heads indicate the established lesions at the end of treatments.

The effects of different concentrations of resveratrol and EGCG on EEC apoptosis are displayed in Fig. 7A and B. Complementary to the results obtained from cell proliferation experiments, resveratrol 50 and 100 μM enhanced apoptosis in EEC from control women ($P < 0.05$ and $P < 0.01$ versus basal, respectively). However, in endometriosis EEC cultures, 100 μM was the only concentration that showed a significant effect ($P < 0.01$ versus basal). Regarding EGCG treatment, 40, 80 and 100 μM assayed doses showed a significant effect on endometrial growth enhancing apoptosis in EEC from control subjects (40/80 μM $P < 0.05$ versus basal; 100 μM $P < 0.01$ versus basal), as well as in EEC from endometriosis patients ($P < 0.01$ versus basal).

Discussion

Treatment of endometriosis-associated symptoms typically requires surgical as well as medical intervention (Crosignani *et al.*, 2006). Even though current available medical therapies are not curative *per se*, they are a mainstay of pain suppression and lesion regression in women suffering from this pathology. However, efforts are still being focused on the improvement and promotion of new treatments with higher efficacy and fewer side effects.

Over the last decade, the use of herbal compounds, multivitamin supplements, minerals and other plant-derived components to treat different diseases has been growing more prevalent. In the last

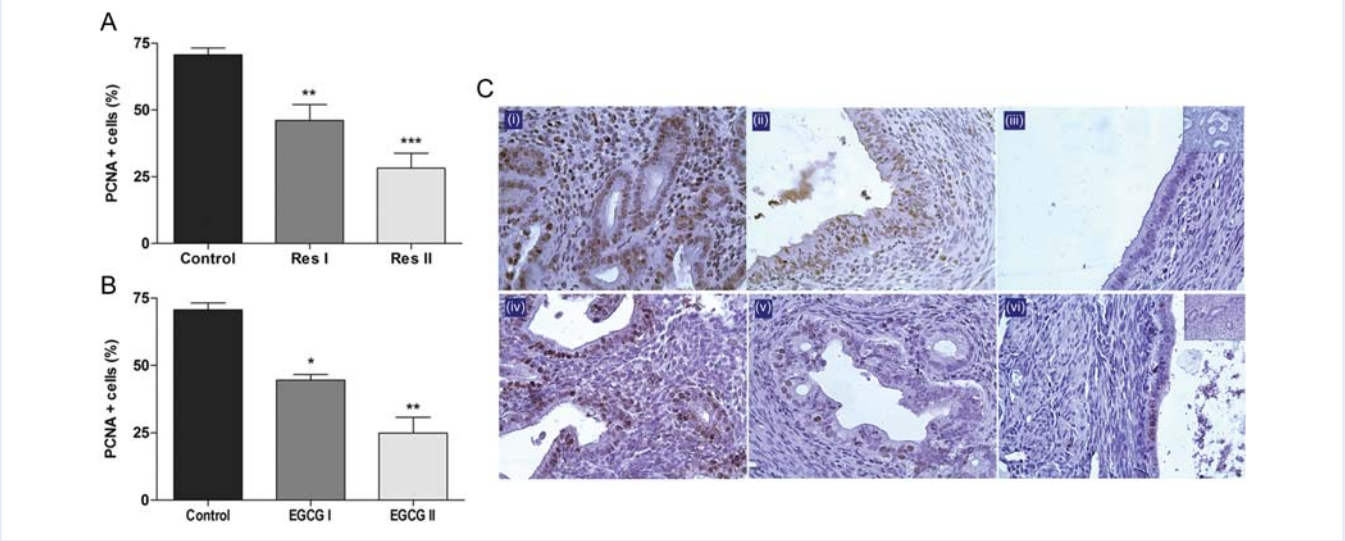

Figure 2 The effect of resveratrol and EGCG on endometriotic-like lesion cell proliferation. Mice with surgically induced endometriosis were treated everyday starting on post-surgery day 15 with: (**A**) resveratrol (Res I 10 mg/kg–Res II 25 mg/kg) or vehicle (control), (**B**) EGCG (EGCG I 20 mg/kg–EGCG II 100 mg/kg) or vehicle (control). After 4 weeks of treatment, mice were sacrificed and lesions were dissected away, formalin-fixed, paraffin-embedded and cut into sections. Cell proliferation was evaluated by immunohistochemistry for PCNA and was quantified in the epithelial fraction as a percentage of PCNA positive cells. Results are expressed as mean ± SEM. $*P < 0.05$/$**P < 0.01$/$***P < 0.001$ versus control. $n = 6$ (Res control), 6 (Res I), 5 (Res II), 6 (EGCG control), 6 (EGCG I), 5 (EGCG II). (**C**) Photomicrographs of PCNA immunostaining of (i) Res control, (ii) Res I, (iii) Res II, (iv) EGCG control, (v) EGCG I and (vi) EGCG II are displayed. As a negative control (inset (iii) and (vi)) an immunoglobulin of the same class and concentration as the primary antibody was used. Magnification: 400×.

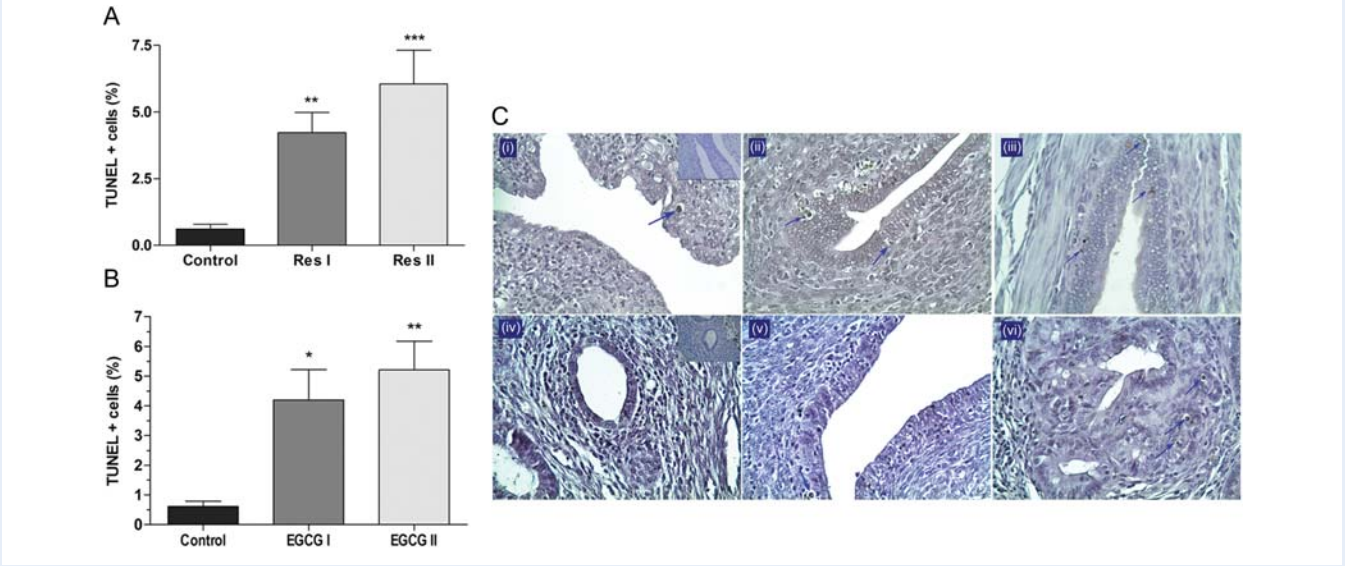

Figure 3 Effect of resveratrol and EGCG on endometriotic-like lesion apoptosis. Mice with surgical induced endometriosis were treated everyday starting on post-surgery day 15 with: (**A**) resveratrol (Res I 10 mg/kg–Res II 25 mg/kg) or vehicle (control), (**B**) EGCG (EGCG I 20 mg/kg–EGCG II 100 mg/kg) or vehicle (control). After 4 weeks of treatment, mice were sacrificed and lesions were dissected away, formalin-fixed, paraffin-embedded and cut into sections. Apoptosis was assessed by TUNEL technique and was quantified in the epithelial fraction as percentage of TUNEL positive cells. Results are expressed as mean ± SEM. $*P < 0.05$/$**P < 0.01$/$***P < 0.001$ versus control. $n = 6$ (Res control), 6 (Res I), 5 (Res II), 6 (EGCG control), 6 (EGCG I), 5 (EGCG II). (**C**) Photomicrographs of TUNEL staining of (i) Res control, (ii) Res I, (iii) Res II, (iv) EGCG control, (v) EGCG I and (vi) EGCG II are displayed. Blue arrow heads indicate apoptotic cells in the epithelial fraction. Negative controls (inset (i) and (iv)) were incubated in the absence of TdT. Magnification: 400×.

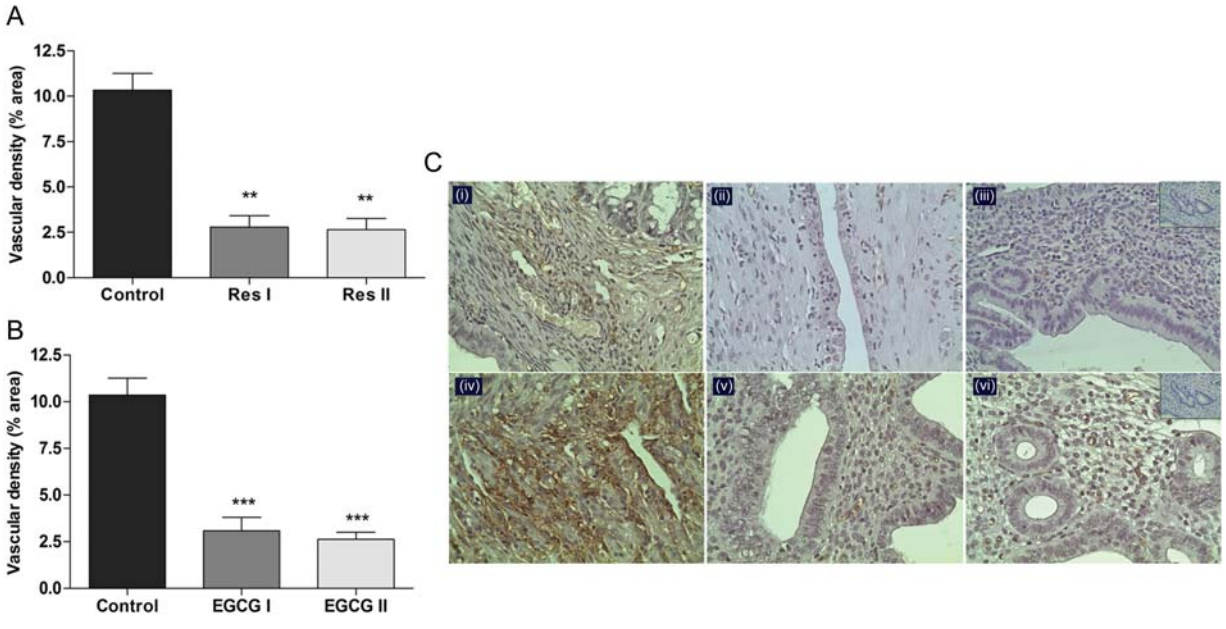

Figure 4 Effect of resveratrol and EGCG on endometriotic-like lesion vascular density. Mice with surgical induced endometriosis were treated everyday starting on post-surgery day 15 with: (**A**) resveratrol (Res I 10 mg/kg–Res II 25 mg/kg) or vehicle (control), (**B**) EGCG (EGCG I 20 mg/kg–EGCG II 100 mg/kg) or vehicle (control). After 4 weeks of treatment, mice were sacrificed and lesions were dissected away, formalin-fixed, paraffin-embedded and cut into sections. Vessels were identified using CD34 staining of the vascular endothelium and quantified as percentage area. Results are expressed as mean ± SEM. $^{**}P < 0.01$/$^{***}P < 0.001$ versus control. $n = 5$ (Res control), 6 (Res I), 6 (Res II), 5 (EGCG control), 6 (EGCG I), 5 (EGCG II). (**C**) Photomicrographs of CD34 immunostaining of (i) Res control, (ii) Res I, (iii) Res II, (iv) EGCG control, (v) EGCG I and (vi) EGCG II are displayed. As a negative control (inset (iii) and (vi)) an immunoglobulin of the same class and concentration as the primary antibody was used. Magnification: 400×.

years, it has been shown that complementary and alternative medicine such as medicinal herbs and other botanicals with anti-inflammatory properties could be useful in the treatment of endometriosis-associated pain (Wieser *et al.*, 2007).

EGCG from green tea and resveratrol from red wine and grapes are some of the natural options that have lately been considered to treat different types of cancer (Aggarwal *et al.*, 2004; Zaveri, 2006; Cucciolla *et al.*, 2007; Khan and Mukhtar, 2008; Chen *et al.*, 2011).

Nowadays green tea is considered one of the most promising dietary agents for the prevention and treatment of cancer. Previous studies have shown that EGCG inhibited the growth of various human cancer cells such as cervical cancer cells (Qiao *et al.*, 2009) and ovarian cancer cells (Rao and Pagidas, 2010), among others, as well as uterine leiomyoma cells (Zhang *et al.*, 2010a,b). Not many works have been reported on the EGCG ability to inhibit the onset of endometriosis (Xu *et al.*, 2009, 2011; Bruner-Tran *et al.*, 2011). However, these recent few studies have been developed in a nude mouse model; hence the immune system's role was not considered.

Resveratrol affects a large array of biological activities and its mechanisms of action are clearly dose dependent (Szende *et al.*, 2000; Bhat *et al.*, 2001; Harikumar and Aggarwal, 2008; Athar *et al.*, 2009). Resveratrol's effects have been studied in chemoprevention and treatment of hepatocellular carcinoma (Bishayee *et al.*, 2010), breast cancer (Garvin *et al.*, 2006), Lewis lung carcinoma (Kimura and Okuda, 2001) and liver cancer (Wu *et al.*, 2004), with positive results.

Considering that patients usually consult for pain symptoms or difficulty in achieving pregnancy, after endometriotic lesions have already established, we look for possible therapies to treat established lesions. Taking all these data into account, one of the aims of the present study was to evaluate the effect of two natural therapies with resveratrol and EGCG, during the development of established endometriotic-like lesions experimentally induced in BALB/c mice.

We observed that both resveratrol and EGCG exerted a significant inhibition on the development of endometriotic-like lesions, reducing the size of the lesions, by diminishing cell proliferation and increasing apoptotic levels. Moreover, both treatments were able to decrease the number of established lesions per mouse. It is remarkable that a treatment that begins 15 days after surgery can affect the development and maintenance of already established endometriotic-like lesions rather than just their establishment.

Although EGCG has previously been reported to exert distinct anticancer effects (Khan and Mukhtar, 2008; Zhang *et al.*, 2010a), and some recent works showed its antiangiogenic effects on endometriosis (Xu *et al.*, 2009, 2011), this is to our knowledge, the first study demonstrating the effectiveness of oral administration in a murine model of endometriosis using immunocompetent BALB/c mice. In addition, we proved the EGCG antiproliferative and proapoptotic effects on endometriosis in accordance with those previously observed in other models (Rao and Pagidas, 2010; Zhang *et al.*, 2010b), and confirmed the EGCG antiangiogenic effects, reflected

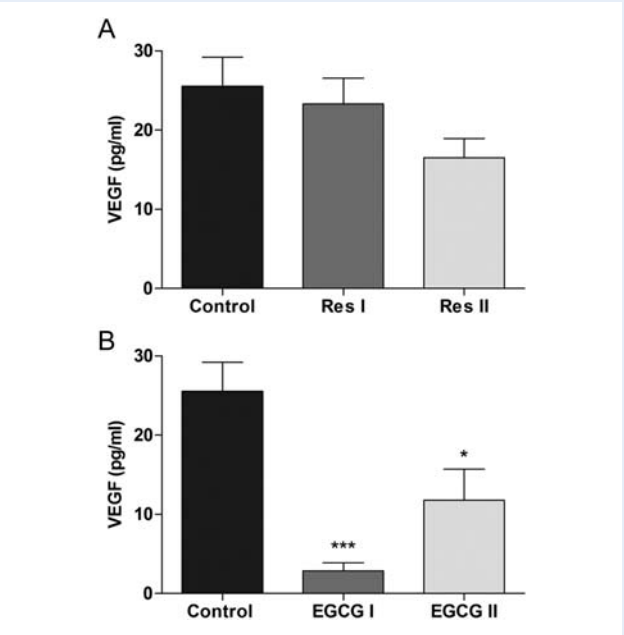

Figure 5 VEGF peritoneal fluid levels. Mice with surgically induced endometriosis were treated everyday starting on post-surgery day 15 with: (**A**) resveratrol (Res I 10 mg/kg–Res II 25 mg/kg) or vehicle (control), (**B**) EGCG (EGCG I 20 mg/kg–EGCG II 100 mg/kg) or vehicle (control). After 4 weeks of treatment, mice were sacrificed and the peritoneal fluid was collected. Levels of VEGF were assessed by ELISA. $*P < 0.05$/$***P < 0.001$ versus control. $n = 8$ (Res control), 10 (Res I), 9 (Res II), 8 (EGCG control), 7 (EGCG I), 6 (EGCG II).

by a reduction in the levels of VEGF in peritoneal fluid and in the vascular density of lesions.

Bruner-Tran *et al.* (2011) have recently demonstrated that resveratrol is able to reduce the development of experimental endometriosis in a nude mouse model. They related these protective effects of resveratrol to a reduction in endometrial cell proliferation and an increase in cell death. However, the effects of resveratrol on the proliferation of endometriotic tissues were not so clear: they found a decrease of MKI67 expression but an increase in PCNA staining, both proliferation markers. In this study, we observed and quantified a clear and significant decrease in cell proliferation by PCNA staining after treatment with resveratrol.

We and other authors have described the importance of angiogenesis for the development of endometriosis and it was proposed that the suppression of blood vessel development by the inhibition of VEGF may be a novel therapeutic approach for this pathology (Nap *et al.*, 2004; Ricci *et al.*, 2011). In an earlier study, Brakenhielm *et al.* (2001) showed that the suppression of angiogenesis could be at least one of the mechanisms involved in the antitumor effect of resveratrol and posited that the consumption of resveratrol could be beneficial in the prevention of angiogenesis-dependent diseases. There are extensive studies showing the antiangiogenic effect of resveratrol in different cancer models (Garvin *et al.*, 2006; Hu *et al.*, 2007; Trapp *et al.*, 2010) but this is the first report on endometriosis. In this study, we confirmed its antiangiogenic properties, obtaining a reduction in the vascular density of the endometriotic-like lesions. Even though we did not observe a significant decrease in VEGF levels in the peritoneal cavities of treated mice, the antiangiogenic effects of resveratrol could be mediated by other proangiogenic factors. Moreover, resveratrol could be regulating the expression of VEGF receptors instead of VEGF levels.

On the other hand, according to our knowledge, this is the first report evaluating the effects of resveratrol and EGCG on the survival of EECs from human biopsies, and it clearly demonstrates that treatment with both polyphenols effectively reduce cell proliferation and induce cell death. Only one study carried out by Bruner-Tran *et al.* studied the effect of resveratrol on the invasiveness of endometrial stromal cells, but in this work only endometrial tissue from women without endometriosis was used. The authors made clear the need of further studies to also evaluate the effects of resveratrol on eutopic endometrial tissues from women with endometriosis (Bruner-Tran *et al.*, 2011).

In the present study and in previous works performed in our laboratory (Meresman *et al.*, 2003a; Olivares *et al.*, 2008), we found no significant differences in the percentage of cell proliferation and apoptotic cells between endometrial cell cultures from endometriosis patients and control women. In spite of the fact that the *in vitro* response of the cells employed in this investigation may not be identical to its physiological response, the use of endometrial eutopic cells in short-term culture as a model for endometriosis research has been well approved and documented previously (Olivares *et al.*, 2008).

Green tea represents one of the most widely consumed beverages in the world; however, green tea extracts are also available as nutrient supplements, offering an easy way of achieving the required EGCG doses without having to take innumerable tea cups. In the same way, long-term consumption of resveratrol-enriched wine products is not a recommendable treatment due to the alcohol content; so other food products should be considered as alternative resveratrol sources, as well as compressed pills with the necessary dose. On the other hand, more studies are needed to determine the effective human plasma concentrations of EGCG or resveratrol to achieve the inhibition of endometriotic-like lesion development.

Better understanding of the basic mechanisms of action of EGCG and resveratrol, as well as their bioavailability is needed to determine the potential usefulness of these natural compounds as endometriosis preventive agents.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors' roles

A.G.R. had full responsibility for the study design, performed the experiments, analyzed data, wrote the manuscript and had primary responsibility for the final content. C.N.O. contributed with experimental work and data collection. A.G.R., C.N.O., M.A.B., J.I.B., G.F.M. and R.I.B. participated in data interpretation and critical discussion. G.F.M. and R.I.B. conducted the research. J.J.S. provided the human biopsies. All authors read and approved the final manuscript.

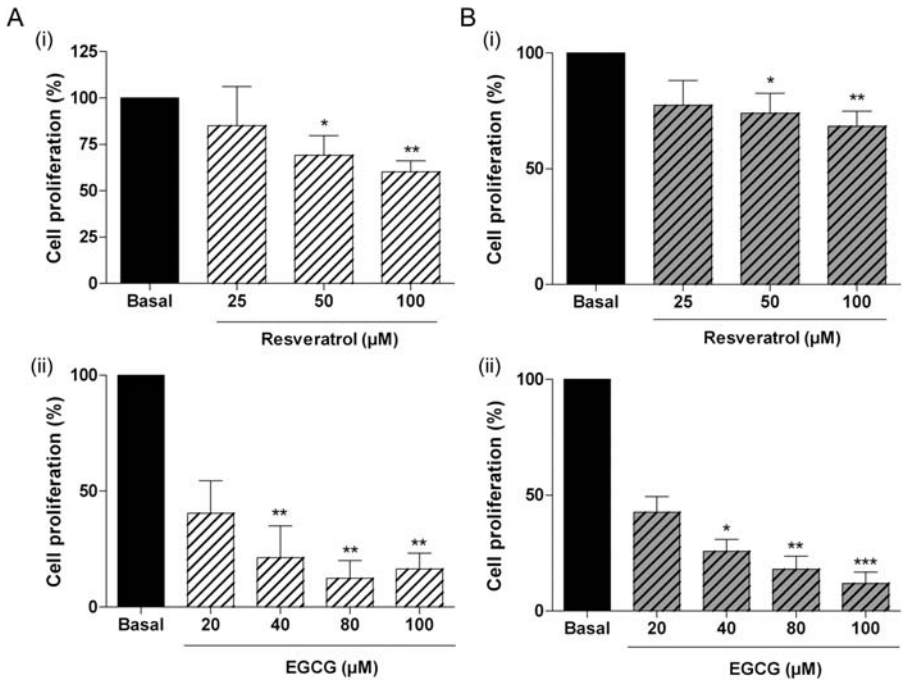

Figure 6 The effect of resveratrol and EGCG on cell proliferation in EEC cultures. EECs from seven control women (**A**) and nine patients with endometriosis (**B**) were grown for a pre-confluent period of 48 h and then treated for 24 h with resveratrol (i), EGCG (ii) or vehicle control (basal) (i and ii). Cell proliferation was assessed by an MTS assay. Values of each treatment condition are expressed as a percentage of basal cell proliferation (set as 100%). Results are expressed as mean ± SEM. $*P < 0.05$/$**P < 0.01$/$***P < 0.001$ versus basal.

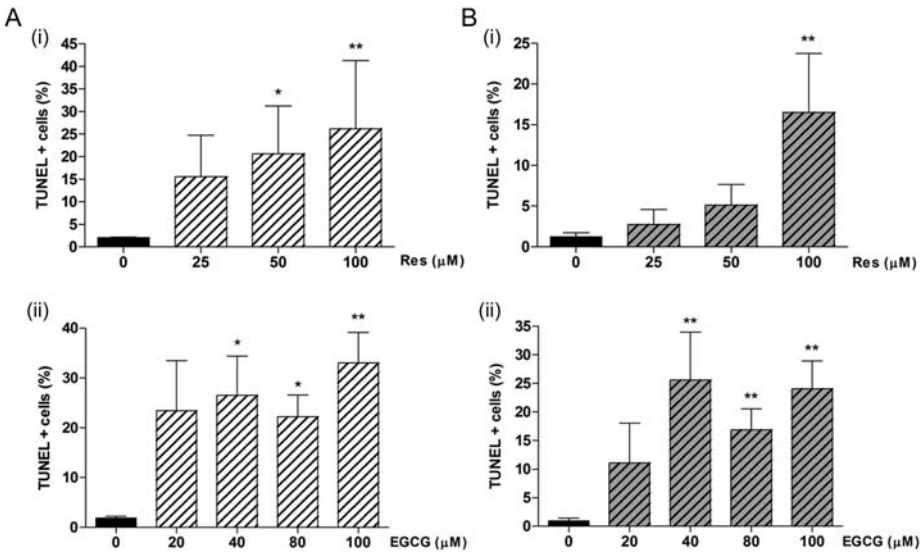

Figure 7 The effect of resveratrol and EGCG on apoptosis in EEC cultures. EECs from eight control women (**A**) and seven patients with endometriosis (**B**) were grown for a pre-confluent period of 48 h and then treated for 24 h with resveratrol (i), EGCG (ii) or vehicle control (0 μM) (i and ii). Apoptosis was assessed by the TUNEL technique. Values of each treatment condition are expressed as a percentage of FITC/TUNEL positive nuclei from ~300 total DAPI-stained nuclei. Results are expressed as mean ± SEM. $*P < 0.05$/$**P < 0.01$ versus control (0 μM).

Funding

This study was supported by ANPCYT (PICT 6384 BID 1201 OC-AR) and CONICET (PIP 5471), Argentina.

Conflict of interest

None declared.

References

Aggarwal BB, Bhardwaj A, Aggarwal RS, Seeram NP, Shishodia S, Takada Y. Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. *Anticancer Res* 2004;**24**:2783–2840.

ASRM. Revised American Society for Reproductive Medicine classification of endometriosis: 1996. *Fertil Steril* 1997;**67**:817–821.

Athar M, Back JH, Kopelovich L, Bickers DR, Kim AL. Multiple molecular targets of resveratrol: anti-carcinogenic mechanisms. *Arch Biochem Biophys* 2009;**486**:95–102.

Bhat KP, Lantvit D, Christov K, Mehta RG, Moon RC, Pezzuto JM. Estrogenic and antiestrogenic properties of resveratrol in mammary tumor models. *Cancer Res* 2001;**61**:7456–7463.

Bilotas M, Meresman G, Stella I, Sueldo C, Baranao RI. Effect of aromatase inhibitors on ectopic endometrial growth and peritoneal environment in a mouse model of endometriosis. *Fertil Steril* 2010;**93**:2513–2518.

Bishayee A, Politis T, Darvesh AS. Resveratrol in the chemoprevention and treatment of hepatocellular carcinoma. *Cancer Treat Rev* 2010;**36**:43–53.

Brakenhielm E, Cao R, Cao Y. Suppression of angiogenesis, tumor growth, and wound healing by resveratrol, a natural compound in red wine and grapes. *FASEB J* 2001;**15**:1798–1800.

Bruner-Tran K, Osteen KG, Taylor HS, Sokalska A, Haines K, Duleba AJ. Resveratrol inhibits development of experimental endometriosis in vivo and reduces endometrial stromal cell invasiveness in vitro. *Biol Reprod* 2011;**84**:106–112.

Chen D, Wan SB, Yang H, Yuan J, Chan TH, Dou QP. EGCG, green tea polyphenols and their synthetic analogs and prodrugs for human cancer prevention and treatment. *Adv Clin Chem* 2011;**53**:155–177.

Crosignani P, Olive D, Bergqvist A, Luciano A. Advances in the management of endometriosis: an update for clinicians. *Hum Reprod Update* 2006;**12**:179–189.

Cucciolla V, Borriello A, Oliva A, Galletti P, Zappia V, Della RF. Resveratrol: from basic science to the clinic. *Cell Cycle* 2007;**6**:2495–2510.

Garvin S, Ollinger K, Dabrosin C. Resveratrol induces apoptosis and inhibits angiogenesis in human breast cancer xenografts in vivo. *Cancer Lett* 2006;**231**:113–122.

Giudice LC, Kao LC. Endometriosis. *Lancet* 2004;**364**:1789–1799.

Harikumar KB, Aggarwal BB. Resveratrol: a multitargeted agent for age-associated chronic diseases. *Cell Cycle* 2008;**7**:1020–1035.

Hu Y, Sun CY, Huang J, Hong L, Zhang L, Chu ZB. Antimyeloma effects of resveratrol through inhibition of angiogenesis. *Chin Med J (Engl)* 2007;**120**:1672–1677.

Khan N, Mukhtar H. Multitargeted therapy of cancer by green tea polyphenols. *Cancer Lett* 2008;**269**:269–280.

Kimura Y, Okuda H. Resveratrol isolated from polygonum cuspidatum root prevents tumor growth and metastasis to lung and tumor-induced neovascularization in Lewis lung carcinoma-bearing mice. *J Nutr* 2001;**131**:1844–1849.

Laschke MW, Schwender C, Scheuer C, Vollmar B, Menger MD. Epigallocatechin-3-gallate inhibits estrogen-induced activation of endometrial cells in vitro and causes regression of endometriotic lesions in vivo. *Hum Reprod* 2008;**23**:2308–2318.

Meresman GF, Vighi S, Buquet RA, Contreras-Ortiz O, Tesone M, Rumi LS. Apoptosis and expression of Bcl-2 and Bax in eutopic endometrium from women with endometriosis. *Fertil Steril* 2000;**74**:760–766.

Meresman GF, Bilotas M, Buquet RA, Baranao RI, Sueldo C, Tesone M. Gonadotropin-releasing hormone agonist induces apoptosis and reduces cell proliferation in eutopic endometrial cultures from women with endometriosis. *Fertil Steril* 2003a;**80**(Suppl 2):702–707.

Meresman GF, Bilotas MA, Lombardi E, Tesone M, Sueldo C, Baranao RI. Effect of GnRH analogues on apoptosis and release of interleukin-1beta and vascular endothelial growth factor in endometrial cell cultures from patients with endometriosis. *Hum Reprod* 2003b;**18**:1767–1771.

Mihalyi A, Simsa P, Mutinda KC, Meuleman C, Mwenda JM, D'Hooghe TM. Emerging drugs in endometriosis. *Expert Opin Emerg Drugs* 2006;**11**:503–524.

Nap AW, Griffioen AW, Dunselman GA, Bouma-Ter Steege JC, Thijssen VL, Evers JL, Groothuis PG. Antiangiogenesis therapy for endometriosis. *J Clin Endocrinol Metab* 2004;**89**:1089–1095.

Olivares C, Bilotas M, Buquet R, Borghi M, Sueldo C, Tesone M, Meresman G. Effects of a selective cyclooxygenase-2 inhibitor on endometrial epithelial cells from patients with endometriosis. *Hum Reprod* 2008;**23**:2701–2708.

Qiao Y, Cao J, Xie L, Shi X. Cell growth inhibition and gene expression regulation by (-)-epigallocatechin-3-gallate in human cervical cancer cells. *Arch Pharm Res* 2009;**32**:1309–1315.

Rao SD, Pagidas K. Epigallocatechin-3-gallate, a natural polyphenol, inhibits cell proliferation and induces apoptosis in human ovarian cancer cells. *Anticancer Res* 2010;**30**:2519–2523.

Ricci AG, Olivares CN, Bilotas MA, Meresman GF, Baranao RI. Effect of vascular endothelial growth factor inhibition on endometrial implant development in a murine model of endometriosis. *Reprod Sci* 2011.

Szende B, Tyihak E, Kiraly-Veghely Z. Dose-dependent effect of resveratrol on proliferation and apoptosis in endothelial and tumor cell cultures. *Exp Mol Med* 2000;**32**:88–92.

Tariverdian N, Theoharides TC, Siedentopf F, Gutierrez G, Jeschke U, Rabinovich GA, Blois SM, Arck PC. Neuroendocrine-immune disequilibrium and endometriosis: an interdisciplinary approach. *Semin Immunopathol* 2007;**29**:193–210.

Tariverdian N, Siedentopf F, Rucke M, Blois SM, Klapp BF, Kentenich H, Arck PC. Intraperitoneal immune cell status in infertile women with and without endometriosis. *J Reprod Immunol* 2009;**80**:80–90.

Trapp V, Parmakhtiar B, Papazian V, Willmott L, Fruehauf JP. Anti-angiogenic effects of resveratrol mediated by decreased VEGF and increased TSP1 expression in melanoma-endothelial cell co-culture. *Angiogenesis* 2010;**13**:305–315.

Valle RF, Sciarra JJ. Endometriosis: treatment strategies. *Ann N Y Acad Sci* 2003;**997**:229–239.

Varma R, Rollason T, Gupta JK, Maher ER. Endometriosis and the neoplastic process. *Reproduction* 2004;**127**:293–304.

Wieser F, Cohen M, Gaeddert A, Yu J, Burks-Wicks C, Berga SL, Taylor RN. Evolution of medical treatment for endometriosis: back to the roots? *Hum Reprod Update* 2007;**13**:487–499.

Wu SL, Sun ZJ, Yu L, Meng KW, Qin XL, Pan CE. Effect of resveratrol and in combination with 5-FU on murine liver cancer. *World J Gastroenterol* 2004;**10**:3048–3052.

Xu H, Lui WT, Chu CY, Ng PS, Wang CC, Rogers MS. Anti-angiogenic effects of green tea catechin on an experimental endometriosis mouse model. *Hum Reprod* 2009;**24**:608–618.

Xu H, Becker CM, Lui WT, Chu CY, Davis TN, Kung AL, Birsner AE, D'Amato RJ, Wai Man GC, Wang CC. Green tea epigallocatechin-3-gallate inhibits angiogenesis and suppresses vascular endothelial growth factor C/vascular endothelial growth factor

receptor 2 expression and signaling in experimental endometriosis in vivo. *Fertil Steril* 2011;**96**:1021–1028.

Zaveri NT. Green tea and its polyphenolic catechins: medicinal uses in cancer and noncancer applications. *Life Sci* 2006;**78**:2073–2080.

Zhang D, Al Hendy M, Richard-Davis G, Montgomery-Rice V, Sharan C, Rajaratnam V, Khurana A, Al Hendy A. Green tea extract inhibits proliferation of uterine leiomyoma cells in vitro and in nude mice. *Am J Obstet Gynecol* 2010a;**202**:289.e1–289.e9.

Zhang D, Al-Hendy M, Richard-Davis G, Montgomery-Rice V, Rajaratnam V, Al-Hendy A. Antiproliferative and proapoptotic effects of epigallocatechin gallate on human leiomyoma cells. *Fertil Steril* 2010b;**94**:1887–1893.