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ORIGINAL PAPER

Prodrug of green tea epigallocatechin-3-gallate (Pro-EGCG) as a potent anti-angiogenesis agent for endometriosis in mice

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Abstract Green tea epigallocatechin-3-gallate (EGCG) can inhibit angiogenesis and development of an experimental endometriosis model in mice, but it suffers from poor bioavailability. A prodrug of EGCG (pro-EGCG, EGCG octaacetate) is utilized to enhance the stability and bioavailability of EGCG in vivo. In this study, the potential of pro-EGCG as a potent anti-angiogenesis agent for endometriosis in mice was investigated. Homologous endometrium was subcutaneously transplanted into mice to receive either saline, vitamin E, EGCG or pro-EGCG treatment for 4 weeks. The growth of the endometrial implants were monitored by IVIS® non-invasive in vivo imaging during the interventions. Angiogenesis of the endometriotic lesions was determined by Cellvizio® in vivo imaging and SCANCO® Microfil microtomography. The bioavailability, anti-oxidation and anti-angiogenesis capacities of the treatments were measured in plasma and lesions. The implants with adjacent outer subcutaneous and inner abdominal muscle layers were collected for histological, microvessel and apoptosis examinations. The result showed that EGCG and pro-EGCG significantly decreased the growth of endometrial implants from the 2nd week to the 4th week of intervention. EGCG and pro-EGCG significantly reduced the lesion size and weight, inhibited functional and structural microvessels in the lesions, and enhanced lesion apoptosis at the end of interventions. The inhibition by pro-EGCG in all the angiogenesis parameters was significantly greater than that by EGCG, and pro-EGCG also had better bioavailability and greater anti-oxidation and anti-angiogenesis capacities than EGCG. Ovarian follicles and uterine endometrial glands were not affected by either EGCG or pro-EGCG. Vitamin E had no

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effect on endometriosis. In conclusion, pro-EGCG significantly inhibited the development, growth and angiogenesis of experimental endometriosis in mice with high efficacy, bioavailability, anti-oxidation and anti-angiogenesis capacities. Pro-EGCG could be a potent anti-angiogenesis agent for endometriosis.

Keywords Endometriosis · Anti-angiogenesis · Green tea · Epigallocatechin-3-gallate

Introduction

Angiogenesis has an essential role in development, reproduction and repair. However, pathological angiogenesis occurs not only in tumor formation but also in a range of non-neoplastic diseases that could be classed together as "angiogenesis-dependent diseases" [1, 2]. Endometriosis is a chronic disorder characterized by the implantation of endometrial glands and stroma outside the uterine cavity. Despite different hypotheses regarding the pathogenesis of endometriosis, it is widely accepted that endometriosis is an angiogenesis-dependent disorder and that angiogenesis plays an essential role in the growth and survival of endometriotic lesions [3]. Endometriotic lesions require new vessel formation to deliver the oxygen and nutrient supply that are essential to the development and progression of endometriosis [4]. Dense vascularization is a typical pathological feature of endometriosis. Numerous peritoneal blood vessels can be observed around the endometriotic lesions during laparoscopy, and ectopic endometrium is highly vascularized under histological examination [5]. Animal models have confirmed that angiogenesis occurs in endometriosis by demonstrating the development of adjacent blood vessels from the surrounding vasculature into the endometriotic implants [6, 7]. Anti-angiogenesis therapy offers a new opportunity for the treatment of endometriosis [8].

Tea is the second most popular beverage worldwide after water [9]. It is consumed in different parts of the world as black, green and oolong tea. Green tea is prepared from the leaves of the tea plant *Camellia sinensis,* with its high nutraceutical values [10]. The polyphenols found in green tea, especially epigallocatechin-3-gallate (EGCG), have potent anti-oxidative, anti-mitotic and anti-angiogenic properties [11]. The anti-angiogenic activity of EGCG has been widely demonstrated in vitro and in vivo [12]. In our previous studies, we demonstrated that EGCG suppressed the angiogenesis signaling pathway and inhibited neovascularization and the growth of experimental endometriosis in mice [7, 13]. However, EGCG is notably unstable and is known to have poor bioavailability [14]. A synthetic derivative of EGCG, obtained by acetylation of EGCG, can act as a prodrug of EGCG (pro-EGCG) with enhanced stability and improved bioavailability [15]. In this study, we investigated the potential of pro-EGCG as a novel anti-angiogenesis agent for endometriosis in mice.

Materials and methods

Chemicals

Pro-EGCG was synthesized from (-)-EGCG as previously described [15]. HPLC-graded EGCG, vitamin E and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). D-Luciferin (Caliper Life Sciences, MA, USA), FITC-Dextran MW150,000 (PolySciences Inc., PA, USA), YO-PRO®-1 iodide (Invitrogen, CA, USA) and Microfil MV-120 Blue (Flow Tech, MA, USA), used for in vivo imaging, were purchased from their respective manufacturers.

Animals

Transgenic luciferase-expressing mice (*CMV-Luc*) were obtained from Xenogen (Xenogen Cor., CA, USA), and immunocompromised mice (NOD-SCID) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). All animals were housed in disease free conditions in a separate barrier facility with individually ventilated filter cages at university hospital. Ambient temperature was monitored and cycles of light and darkness were regulated. Mice were fed with irradiated laboratory chow and sterile water ad libitum for at least 1 week of acclimatization before the surgery and experiments. All of the animal research was approved from the Animal Experimentation Ethics Committee (07/010/MIS) and was performed in accordance with the institutional guidelines.

Experimental endometriosis

Based on our previous study, an immune-compromised mouse model of experimental endometriosis was created by subcutaneous transplantation of human or mouse tissues to assess the anti-angiogenic effects and mechanisms of green tea catechin [7, 13]. Either subcutaneous or intraperitoneal inoculations of the endometrial tissues in mice effectively resulted in the formation of endometriotic lesions at both locations [16], but subcutaneous implantation allowed for more accurate quantification of size and number of the lesions and also easy access for in vivo imaging. In this study, endometrial tissues from eight-week-old female *CMV-Luc* mice ($n = 15$) were acquired for transplantation into non-luminescent NOD-SCID mice ($n = 32$). The mouse endometrium was obtained during the proliferating estrous stage, and biopsies 2 mm in diameter were obtained with a sterile dermal biopsy punch (Miltex, NY, USA). The biopsies were

immediately washed in prewarmed, phenol-red-free DMEM/F-12 medium (Sigma) to remove residual blood and mucus, and the samples were maintained in serum-free DMEM/F-12 medium before transplantation [17]. Cultured endometrial biopsies were randomly allocated for transplantation. Five mouse endometrial biopsies per treatment group were implanted into a single subcutaneous pocket created in the abdominal wall of the mice along the ventral midline immediately below the umbilicus. All of the surgical procedures were performed under general anesthesia with isoflurane (Baxter, IL, USA), and the mice were monitored until they fully recovered. Immediately after the transplantation, the mice were randomly assigned to receive either vehicle ($n = 8$; sham control), vitamin E ($n = 8$; anti-oxidant control, 20 mg/kg), EGCG ($n = 8$; anti-angiogenesis control, 50 mg/kg, $\approx$1.5 mg/30 g) or pro-EGCG ($n = 8$; 50 mg/kg). Dulbecco's phosphate-buffered saline (PBS, Sigma) was used as solvent to dissolve the drugs. These doses were selected because their significant anti-angiogenic and anti-oxidative effects have been reported in mice [18, 19]. The treatments were administered once per day via intraperitoneal injections for a consecutive 4-week period. At the end of intervention, size of the endometriotic lesions was determined as the longest length and perpendicular width according to a venire caliper and was calculated as $0.52 \times \text{width}^2 \times \text{length in mm}^3$. The endometrial implants, together with subcutaneous and muscle layers, were removed from the abdomen wall for detailed histological examination and specific staining analysis.

In vivo imaging

Development and angiogenesis of the experimental endometriosis were monitored using various in vivo imaging systems. During the intervention, growth of the endometriotic lesions ($n = 8$ per group) was monitored each week by measuring the bioluminescence of the subcutaneous luciferase-positive implants using a non-invasive IVIS 200 live animal imaging system (Xenogen) as previously described [20]. A total of 150 mg/kg of luciferin was injected intraperitoneally into the mouse tail vein 10 min prior to imaging. An image from each animal was captured at bin size 4 in triplicate, and the bioluminescence signal intensities were averaged for comparison. At the end of intervention, functional angiogenesis of the endometriotic lesions ($n = 8$ per group) was monitored by measuring the flow of fluorescence dye in the new microvessels developed in the implants using a Cellvizio® LAB LSU-488 system with a ProFlex Microprobe S1500 (Mauna Kea Technologies, Paris) as previously described [13]. A total of 10 mg/ml of FITC-Dextran was injected intravenously into each mouse prior to imaging. Total vessel length (μm) and area (μm^2) and functional capillary length (μm^{-1}) and area density were measured using Cellvizio® LAB Vessel Detection software, and averaged from 4 perpendicular regions of the lesions in replicates. At the same time, apoptotic cells in the lesions were captured by Cellvizio-LAB 488 with a ProFlex MiniO/100 microprobe using Cellvizio® LAB I/C Viewer software (Mauna Kea Technologies). A total of 10 μl of 10 μM YO-PRO®-1 iodide in DSMO was injected directly into the lesion area. The total number of apoptotic cells was counted and averaged from the 4 perpendicular regions of the lesions in replicates as described above. Both IVIS and Cellvizio imaging procedures were performed under general anesthesia with isoflurane (Baxter, IL, USA), and the mice were observed until fully recovered. After live animal imaging, structural angiogenesis in the endometriotic lesions ($n = 4$ per group) was further examined by detecting the Microfil in the new microvessels developed in the implants using a VivaCT 40 μCT system (Scanco Medical, Switzerland) as previously described [21]. The mice were sacrificed by cervical dislocation and were immediately perfused with 4 % paraformaldehyde and Microfil MV-120 Blue. Three-dimensional microvessel architecture was constructed, and absolute and relative Microfil-filled microvessel volumes were determined using the built-in software.

Histology and immunostaining

Paraffin sections ($n = 4$ per group) were prepared for standard hematoxylin and eosin (H&E) staining, angiogenesis immunostaining and TUNEL staining as previously described [7]. Immunohistochemistry staining of in situ angiogenesis was performed by the immunoperoxidase method using a MACH 3 HRP Polymer Kit (Biocare Medical, Concord), and counterstained using methyl green or blue solution. Antibodies against mouse CD31 and alpha-smooth muscle actin (αSMA) (Abcam Inc., MA, USA) were used to double fluorescence stain the microvessels developed in the endometriotic lesions, and CD34 (Abcam Inc.,) was used to stain the microvessels in the ovaries and uterus. The samples without primary antibodies served as negative controls. The total number of positively stained new and old microvessels in the lesions was counted, respectively. The mean microvessel numbers and sizes were calculated from 5 separated sections from each animal within the treatment group and are presented as numbers per lesion and μm^2 per lesion, respectively. TUNEL labeling for in situ apoptotic cells was performed using an ApopTag Peroxidase In Situ Apoptosis Detection Kit (Temecula, CA) according to the manufacturer's instructions. The total numbers of TUNEL-positive stained cells were counted. The mean numbers of apoptotic cells were calculated from 5 separated sections from each animal within the treatment group and are presented as numbers per lesion as above. For all of the examinations,

five best sections showed complete histological features of the outer skin and subcutaneous layers; the middle endometriotic lesion and the inner abdominal muscles and peritoneum were first evaluated and were selected for detailed examination by two experienced pathologists. Each section was evaluated by both examiners, who were blinded to the experimental groups and results.

Analytical measurements

Pharmacokinetics of EGCG and pro-EGCG in the animals were studied by measuring the corresponding EGCG and pro-EGCG concentrations (EGCG in the EGCG group, pro-EGCG in the pro-EGCG group) in plasma during the intervention and also in lesions at the end of intervention. EGCG and pro-EGCG were quantified by analytical methods as previously described [15, 22, 23]. Anti-oxidative and anti-angiogenesis capacities of the samples were also studied by measuring oxygen radical absorbance capacity (ORAC) using a modified phycoerythrin fluorescence decay assay as previously described [24], and vascular endothelial growth factor (VEGF) concentrations were measured using a commercial ELISA kit (Uscn Life Science Inc., TX, USA) according to the manufacturer's manual. Blood samples ($n = 8$ per group) were collected from the tail vein once per week, and plasma was prepared by immediate centrifugation. Implants without subcutaneous and muscle layers ($n = 4$ per group) were removed from the abdominal wall, and the endometriotic lesions were frozen. All of the samples were kept in −80 °C prior to analysis.

Eutopic endometrium and ovary

Reproductive effects of EGCG and pro-EGCG on the endometrium and ovaries of each animal were also examined. After the 4-week intervention, the uterine horns and ovaries ($n = 8$ per group) were removed and fixed for histological examination and angiogenesis immunostaining as described above. Endometrial glands, ovarian follicles and vascularization in endometrial and ovarian stroma were assessed.

Statistics

Sample size for animals was calculated according to our previous studies [7]. The anticipated difference in reduced size in the experimental endometriotic lesions after EGCG treatment in mice was 10 mm^2, and the anticipated standard deviation was 3.5 mm^2, so at least 8 mice per treatment group were required for a type I error of 0.01 with power of 99 %. The results are expressed as means ±SEMs. Longitudinal comparisons between the groups during the intervention were performed using ANOVA followed by post hoc comparisons of individual groups using Bonferroni correction. Final comparisons between the groups after the intervention were performed using Student's parametric independent t test. Statistical analysis was performed with PASW Statistics version 18 (SPSS Inc., IL, USA). P values <0.05 were considered to be significant.

Results

Endometriosis growth and development

To determine the therapeutic effects of pro-EGCG on the growth and development of experimental endometriosis in mice, we monitored the endometriotic lesions with non-invasive in vivo imaging. Lesion growth was indicated by the increased bioluminescent signals from the implants (Fig. 1a). In the control saline group, the lesion bioluminescence was slightly increased in the first 2 weeks; then it rapidly increased at the 3rd week and maintained at high levels at the 4th week (Fig. 1b). With EGCG treatment, the lesion bioluminescence was maintained around the baseline levels, and the intensity was significantly lower than that of the saline group at the 2nd, 3rd and 4th weeks of the intervention. With pro-EGCG treatment, the lesion bioluminescence decreased further below the baseline levels, and the intensity was significantly lower than that of both the saline group and the EGCG group at the 2nd, 3rd and 4th weeks of the intervention. In the vitamin E group, the lesion bioluminescent intensity was significantly lower than that of the saline group at the 3rd week, but it returned to normal growth, as in the saline group, at the 4th week of the intervention.

At the terminal surgery, the implanted endometriotic lesions were identified as being located between the host abdominal muscle and the subcutaneous layers (Fig. 2a). The lesions were smooth and well-defined. Under microscopic examination, the lesions were situated between the inner abdominal muscle and the peritoneal layers and the outer subcutaneous and skin layers. Compared to the control group, the lesion size and weight were significantly decreased in both the EGCG and pro-EGCG groups (Fig. 2b). The lesions from the pro-EGCG treated mice were significantly smaller and lighter than the lesions from the EGCG-treated mice. The endometriotic lesions after EGCG and pro-EGCG treatment were poorly developed. The endometrial tissues were small and loosely attached to the adjacent tissues in the EGCG group but were degraded and almost detached from the peritoneal muscle layers in the pro-EGCG group. In contrast, systemic treatment with vitamin E did not significantly decreased the lesions' size or weight, and a large cyst-like endometrial gland structure was formed.

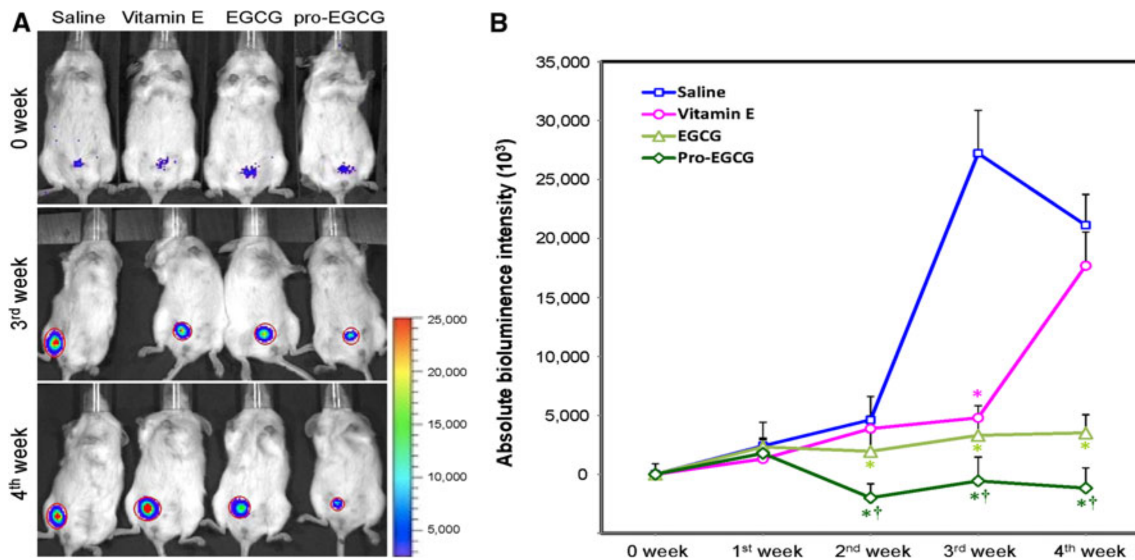

Fig. 1 Pro-EGCG suppresses the growth of experimental endometriosis in mice. **a** Growth of the luminescent endometriotic lesions was monitored in every week during interventions. Representative captures at baseline (0 week), 3 and 4 week are shown. The intensity of the luminescent signal denotes as in a *color scale* extending from luminescence intensity of 0 (*blue*, low signal, no growth), 15,000 (*green*, moderate signal, slow growth) to 25,000 (*red*, high signal, fast growth). **b** Longitudinal changes of absolute luminescence intensities were recorded in every week during interventions. Mean ± SEM, On-way ANOVA and multiple comparisons by post hoc Bonferroni test, $*P < 0.05$ compared with saline group; $\dagger P < 0.05$ compared with EGCG group

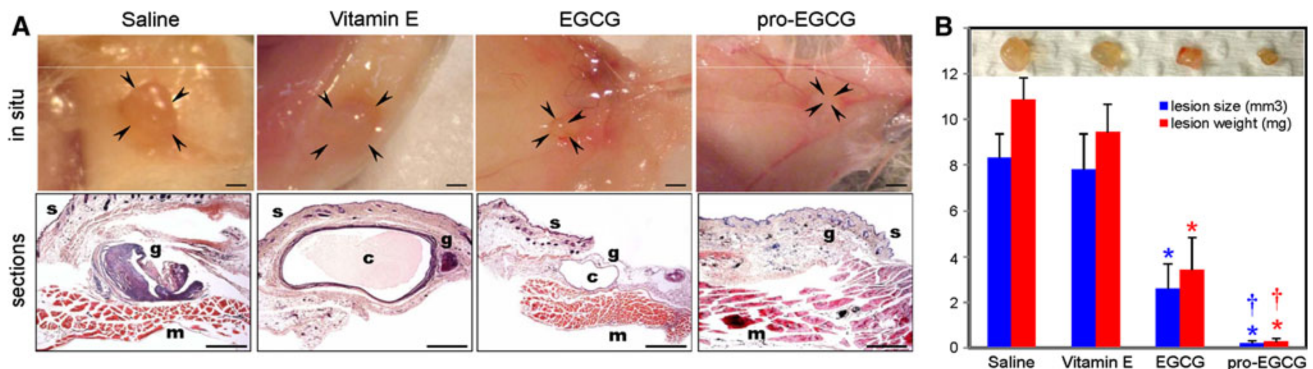

Fig. 2 Pro-EGCG limits the development of experimental endometriosis in mice. **a** *Upper panels* show the endometrial implants developed in the right ventral abdominal wall under laparotomy. *Arrows* indicate the greatest length and perpendicular width of the lesions for lesion size calculation. *Lower panels* shows the sandwich structures of outer skin and subcutaneous layers (*s*), middle endometriotic lesions with endometrial glands (*g*) and endometrial cyst-like structures (*c*), and inner abdominal muscle and peritoneum (*m*). *Scale bars*: 0.5 mm. **b** *Bar charts* of the lesion size and weight in different groups and representative lesion *pictures* are shown. Mean ± SEM, student's t test, $*P < 0.05$ compared with saline group; $\dagger P < 0.05$ compared with EGCG group

Anti-angiogenesis and apoptosis

In vivo angiogenesis imaging by Cellvizio and microCT and in situ angiogenesis immuno-histochemistry analysis by CD31 and αSMA staining showed that lesion neovascularization was poorly developed in the EGCG- and pro-EGCG-treated mice (Fig. 3a). When compared to the saline group, both EGCG and pro-EGCG, but not vitamin E, significantly inhibited the total vessel length and area, the functional capillary length, density and area, the vessel volume and density, and both the CD31-positively and αSMA-negatively stained new microvessel numbers and the CD31-positively and αSMA-positively stained old microvessel numbers in the lesions (Fig. 3b). All of the microvessel parameters in the pro-EGCG group were significantly lower than those in the EGCG group. In vivo apoptosis imaging by Cellvizio and in situ dUTP nick end labeling by TUNEL staining showed that lesion apoptosis increased in the EGCG- and pro-EGCG-treated mice (Fig. 4a). Compared to the saline group, both EGCG and pro-EGCG but not vitamin E significantly increased the total apoptotic cell numbers in the lesions (Fig. 4b). The

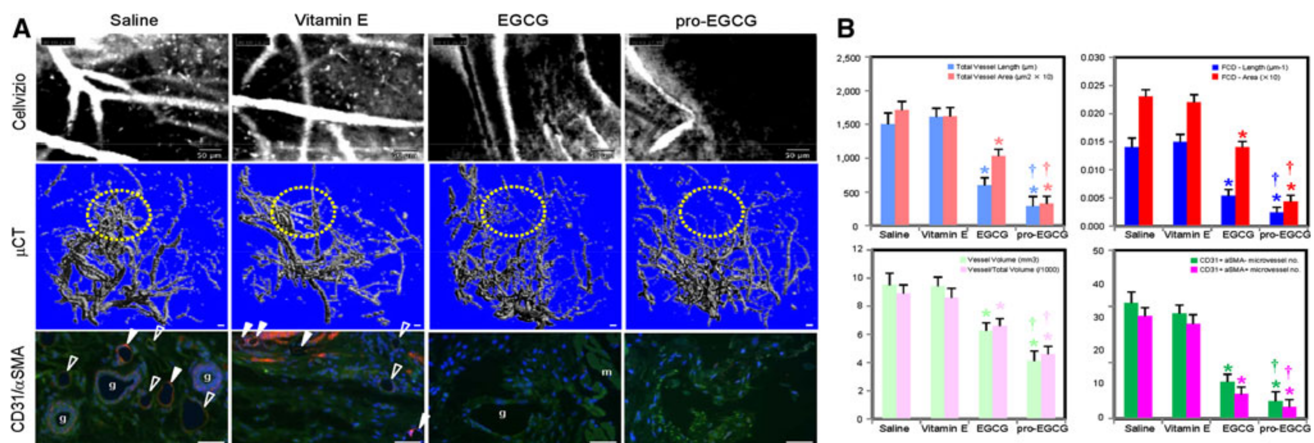

Fig. 3 Pro-EGCG inhibits the angiogenesis of experimental endometriosis in mice. **a** *Upper panels*: Microvessels in the endometriotic implants were perfused with FITC-Dextran and captured by Cellvizio (*white colour*). *Middle panels*: Microvessel architectures surrounding the lesions and within the lesions were perfused with microfil contrast medium and captured by µCT (*yellow dots*). *Lower panels*: Microvessels in the endometriotic lesions were determined by specific anti-mouse antibodies CD31 for endothelial cells in red, αSMA for smooth muscles in *green*, and DAPI for nuclei in *blue*. New microvessels are CD31-positively and αSMA-negatively stained (*closed arrows*), old microvessels are CD31-positively and αSMA-positively stained (*opened arrows*). *g*: endometrial glands; *c*: endometrial cyst-like structures; *m*: abdominal muscle. *Representative images* in different groups are shown. *Scale bars*: 10 µm. **b** *Bar charts* of the lesion microvessel parameters in different groups are presented. Mean ± -SEM, student's t test, $*P < 0.05$ compared with saline group; † $P < 0.05$ compared with EGCG group

apoptotic cell numbers in the pro-EGCG group were also significantly higher than the number in the EGCG group.

Pharmacokinetics, anti-oxidation and anti-angiogenesis capacities

To compare the pharmacokinetics and the anti-oxidation and anti-angiogenesis capacities of the treatment in mice, we measured the EGCG and pro-EGCG concentrations and the ORAC and VEGF concentrations in plasma during the intervention and in endometriotic lesions after the intervention. The bioavailability of the treatment in plasma and in endometriotic lesions with pro-EGCG was significantly higher than that with EGCG (Fig. 5a). ORAC in plasma and endometriotic lesions significantly increased in the vitamin E, EGCG and pro-EGCG groups compared to the saline group (Fig. 5b). ORAC in plasma and endometriotic lesions in the pro-EGCG group was significantly higher than in the EGCG and vitamin E groups, but the EGCG group was not significantly different from the vitamin E group. VEGF concentrations in plasma and endometriotic lesions significantly decreased in the EGCG and pro-EGCG groups compared to the saline group, while the vitamin E group was not significantly different from the saline group (Fig. 5c). VEGF concentrations in plasma and endometriotic lesions in the pro-EGCG group were significantly lower than those in the EGCG group.

Side effects

All of the animals were healthy and active after the endometrium transplantation. No signs of stress, intolerance to anesthesia, surgery or implantation or any toxic responses to saline, vitamin E, EGCG and pro-EGCG administration were observed during the interventions. No significant changes in weight were observed over the experimentation period within and among the groups. There was no observable swelling, inflammation or hair loss at the abdominal implantation sites. The histological examination revealed no obvious reproductive effects on ovarian follicles and endometrial glands under the different treatments (Fig. 6). Also, vascularization of the ovaries and the uterus was not affected in any of the treatment groups.

Discussion

Tea polyphenols are the main chemical constituents of green tea [25]. These polyphenols are composed of various kinds of catechin derivatives, mainly catechin (C), epicatechin (EC), gallocatechin (GC), epigallocatechin (EGC), epicatechin-3-gallate (ECG), gallocatechin-3-gallate (GCG) and epigallocatechin-3-gallate (EGCG). In particular, EGCG is the most abundant catechin and has the most potent biological properties among the other catechins [26]. However, the bioavailability of EGCG is poor because it is (1) unstable in neutral or slightly alkaline conditions in the

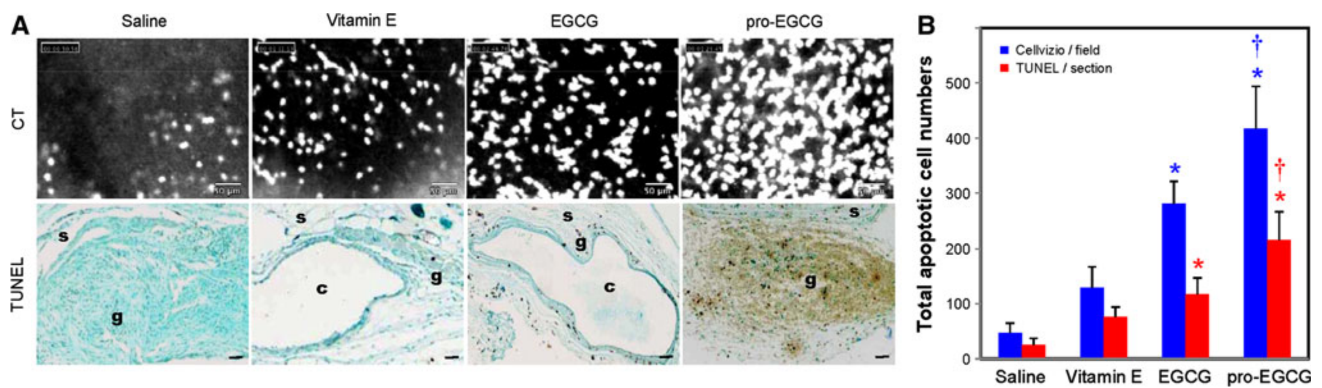

Fig. 4 Pro-EGCG enhances the apoptosis of experimental endometriosis in mice. **a** *Upper panels*: Apoptotic cells in the endometriotic implants were labeled with YO-PRO®-1 iodide and captured (*white colour*). *Lower panels*: Apoptotic cells in the endometriotic lesions were determined by TUNEL staining (*dark brown stains*). *s*: skin and subcutaneous layers, *g*: endometrial glands; *c*: endometrial cyst-like structures. *Representative images* in different groups are shown. *Scale bars*: 50 µm. **b** *Bar charts* of the lesion apoptotic cells in different groups are presented. Mean ± SEM, student's t test, $^{*}P < 0.05$ compared with saline group; †$P < 0.05$ compared with EGCG group

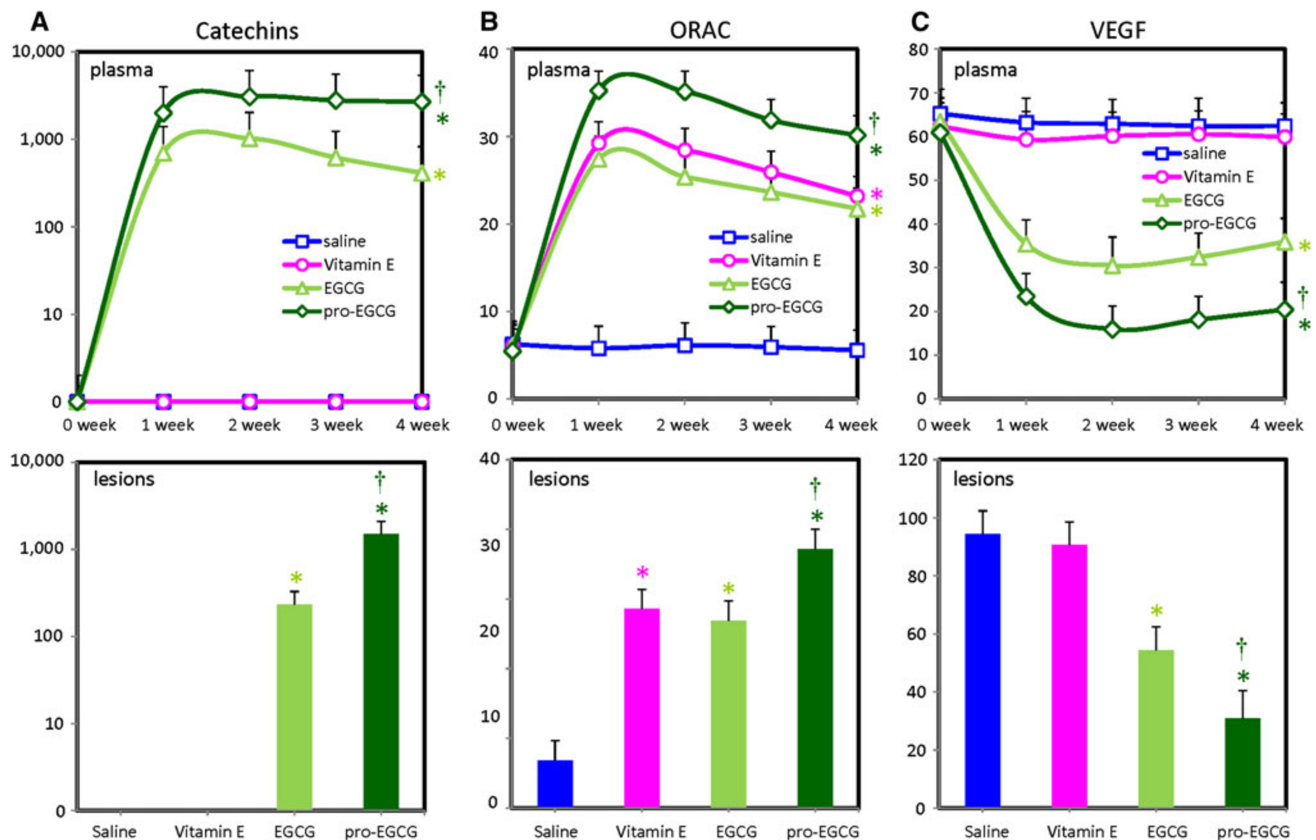

Fig. 5 High bioavailability, anti-oxidation and anti-angiogenesis capacities of pro-EGCG. *Upper panels* show the changes of corresponding EGCG and pro-EGCG concentrations (ρmol/ml), oxygen radical absorbance capacity (ORAC, mM Trolox-equiv/ml) and vascular endothelial growth factor (VEGF, pg/ml) concentrations in plasma during intervention. Mean ± SEM, On-way ANOVA and multiple comparisons by post hoc Bonferroni test, $^{*}P < 0.05$ compared with saline group; †$P < 0.05$ compared with EGCG group. *Lower panels* show the final EGCG and pro-EGCG concentrations (ρmol/mg), ORAC (mM Trolox-equiv/mg) and VEGF concentrations (pg/mg) in the lesions after intervention. Mean ± SEM, student's t test, $^{*}P < 0.05$ compared with saline group; †$P < 0.05$ compared with EGCG group

intestine and in body fluid; (2) poorly absorbed due to the high hydrophilicity of EGCG; and (3) modified through biotransformation reactions, including methylation, glucuronidation, and sulfate formation [27]. Pro-EGCG is a protected EGCG analogue created by the acetylation of the reactive hydroxyl groups, which prevents generation of the

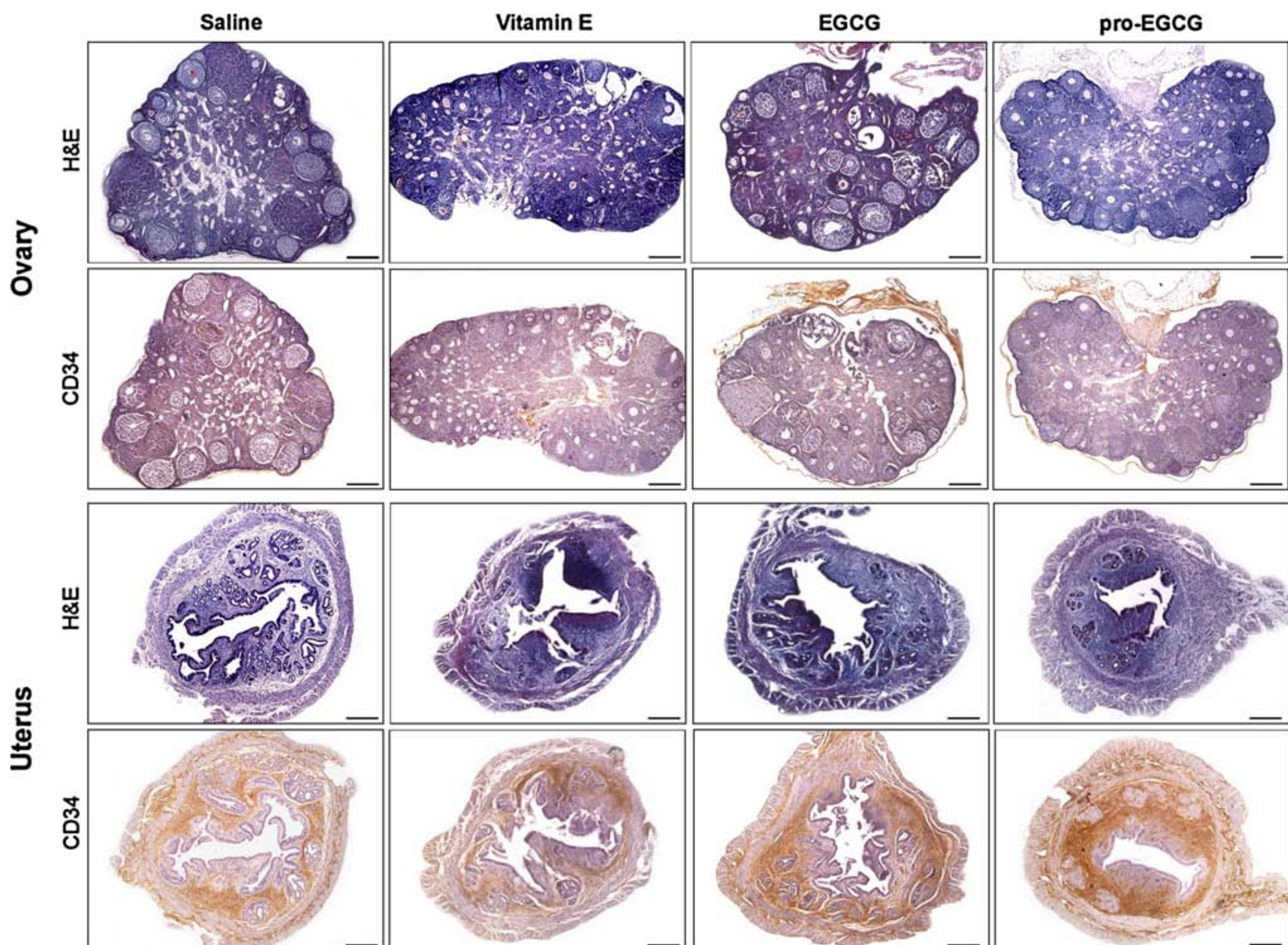

Fig. 6 Pro-EGCG preserves normal ovarian follicles and endometrial glands. Ovarian follicles and endometrial glands were determined by H&E staining and microvessels in ovarian and endometrial stroma were determined by anti-mouse CD34 immunostaining in ovaries (*upper panels*) and uterus (*lower panels*). *Representative images* in different groups are shown. *Scale bars*: 0.5 mm

more reactive phenoxide anions and radicals for dimerization and metabolism [14]. In addition, pro-EGCG remains biologically inactive until it is deacetylated by esterases and converted into its parent EGCG compound in cells [15]. Furthermore, it has also been shown that intragastric administration of pro-EGCG in CF-1 mice results in better bioavailability than with administration of equimolar doses of EGCG [28].

Anti-angiogenesis is one of the most well-characterized biological properties of green tea polyphenols. Experimental studies using a variety of tumor angiogenesis models have shown that green tea extract and EGCG in particular have potent anti-angiogenic effects [29, 30]. The underlying mechanism is still not fully understood, although the inhibitory effects of EGCG on VEGF expression and on receptor activity in cancer, as well as in endometriosis, have been extensively investigated [13, 31–35]. Recently, the anti-angiogenic activity of pro-EGCG was demonstrated in a prostate cancer model [36]. In our study, pro-EGCG also showed strong anti-angiogenic effects on the experimental endometriosis model in mice. As in the prostate model, the inhibitory effects of pro-EGCG on the angiogenesis of endometriosis were even more potent than those of EGCG. Because pro-EGCG has higher plasma and tissue anti-oxidation and anti-angiogenesis capacities than EGCG, the enhanced bioactivity of pro-EGCG in the endometriosis model might have been due to its better bioavailability in vivo. Although the anti-angiogenesis mechanism of pro-EGCG was not investigated in the present study, similar inhibitory effects on VEGF expression and receptor activity could be expected. EGCG and pro-EGCG are also potent anti-oxidants. EGCG and pro-EGCG significantly increased their total anti-oxidation capacities in plasma and lesions, as did Vitamin E, but vitamin E had no effects on endometriotic angiogenesis and growth, suggesting that the anti-angiogenic effects of pro-EGCG on experimental endometriosis were independent from its anti-oxidative properties.

In contrast, pro-EGCG exhibited proteasome inhibition and cell death induction in cancer cells [15]. Pro-EGCG showed much higher potency than EGCG in the inhibition of proliferation and transforming activity and the induction of apoptosis in human prostate, breast, leukemic, and simian virus 40-transformed cells [37]. In our study, pro-EGCG also demonstrated greater efficacy than EGCG in the inhibition of the development and growth of experimental endometriosis in mice and in the apoptosis of endometriotic lesions. In contrast to the prostate cancer inoculation model in mice [36], the significant inhibition of endometriotic growth in our study was observed after only 2 weeks of pro-EGCG treatment, while the inhibition of tumor growth in Lee's study was observed as early as in the 1st week of treatment. The delayed efficacy might have been due to the larger tissues (5 pieces in 2 mm^3 each) used in this study compared to the single piece of 1 mm^3 used in Lee's study. It has been demonstrated that bioluminescence was strongly correlated with lesion size from the first week [38]. The significant decrease in bioluminescence after 2 weeks of pro-EGCG treatment is considered to be evidence of the efficacy of pro-EGCG in the inhibition of the development and growth of endometriosis in mice. Unfortunately, we did not monitor the microvessels at different time points directly; therefore, we cannot exclude whether inhibition is associated with a reduction of blood vessels in the lesions.

Current treatments for endometriosis consist of various hormonal therapies aimed at interrupting the cycles of stimulation and bleeding of endometriotic lesions. However, this approach is deemed not very satisfactory [39]. Anti-angiogenesis for the treatment of endometriosis has the potential advantage of lower recurrence rates and less endocrine side effects compared to conventional surgical and hormonal therapies [40]. Common angiostatic compounds, such as VEGF antibody, TNP-470, endostatin and anginex, have significantly decreased microvessel density and have inhibited established endometriosis lesions [16, 41, 42]. To date, the only clinical trial that has been conducted was on thalidomide [43]. Although the results showed promising pain relief in endometriosis patients, thalidomide is a potential teratogen, which prohibits its use in women who want to become pregnant. Nevertheless anti-angiogenic agents could also serve to limit physiological angiogenesis, such as in ovulation and menstruation [44]. These agents' possible adverse effects on reproductive functions and on offspring must be addressed in this vulnerable population [45, 46]. In our study, ovarian follicles and uterine endometrial glands were not affected by either EGCG or pro-EGCG treatment, suggesting these natural anti-angiogenic agents might not alter normal ovulation and menstruation. Pro-EGCG is therefore a more stable and potent green tea polyphenol with the potential of becoming a novel anti-angiogenesis agent for endometriosis [47]. This polyphenol can be easily manufactured at a relatively low cost and might already have a toxicity profile similar to that of EGCG [48]. Because the pathological angiogenesis of endometriosis is still not fully understood, the specific angiogenesis signaling pathway and anti-angiogenesis targeting of pro-EGCG must be further characterized in animal studies and in clinical trials to confirm the efficacy and safety pro-EGCG for endometriosis.

There were 2 major limitations of the current study. The experimental endometriosis model was established by subcutaneous rather than intraperitoneal transplantation. Because most endometriosis occurs in the peritoneal cavity, an intraperitoneal approach can represent the growth and development of endometriosis as it occurs in endometriosis patients. However, intraperitoneal transplantation results in random implantation sites, which increase the variation of bioluminescence images for accurate quantitation and for consistent comparisons within and among groups. Although endometrial tissues can be fixed in the sub-peritoneal wall by sutures, as previously described [38], the sutures themselves can further damage the tissues and affect the angiogenesis process. Therefore, a subcutaneous transplantation protocol and suture-free procedure are preferable. Furthermore, the animals were administered EGCG and pro-EGCG by an intraperitoneal rather than an oral route. Because detailed pharmacokinetic data on the oral administration of EGCG and pro-EGCG are still unavailable, further studies are necessary to confirm the bioavailability of EGCG and pro-EGCG in endometriotic lesions and also the efficacy of oral EGCG and pro-EGCG in the treatment of endometriosis.

Conclusion

In conclusion, pro-EGCG significantly inhibited the development, growth and angiogenesis of experimental endometriosis in mice with greater efficacy, better bioavailability, and greater anti-oxidation and anti-angiogenesis capacities than EGCG. Pro-EGCG could be a potent anti-angiogenesis agent for endometriosis.

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Conflict of interest T.H.C. declares that he is the inventor of the issued patent claiming the use of pro-EGCG for proteasome inhibition and the treatment of cancer. The other authors declare there is no conflict of interest.

References

1. Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. Nature 407:249–257
2. Simons M (2005) Angiogenesis: where do we stand now? Circulation 111:1556–1566
3. Taylor RN, Lebovic DI, Mueller MD (2002) Angiogenic factors in endometriosis. Ann N Y Acad Sci 955:89–100
4. Groothuis PG, Nap AW, Winterhager E, Grümmer R (2005) Vascular development in endometriosis. Angiogenesis 8:147–156
5. Nisolle M, Casanas-Roux F, Anaf V, Mine JM, Donnez J (1993) Morphometric study of the stromal vascularization in peritoneal endometriosis. Fertil Steril 59:681–684
6. Becker CM, Rohwer N, Funakoshi T, Cramer T, Bernhardt W, Birsner A, Folkman J, D'Amato RJ (2008) 2-Methoxyestradiol inhibits hypoxia-inducible factor-1α and suppresses growth of lesions in a mouse model of endometriosis. Am J Pathol 172:534–544
7. Xu H, Lui WT, Chu CY, Ng PS, Wang CC, Rogers MS (2009) Anti-angiogenic effects of green tea catechin on an experimental endometriosis mouse model. Hum Reprod 24:608–618
8. Hull ML, Charnock-Jones DS, Chan CL, Bruner-Tran KL, Osteen KG, Tom BD, Fan TP, Smith SK (2003) Antiangiogenic agents are effective inhibitors of endometriosis. J Clin Endocrinol Metab 88:2889–2899
9. Heiss ML, Heiss RJ (ed) The story of tea, Ten Speed Press, Berkeley, 2007
10. Yamamoto T (ed) Chemistry and applications of green tea, CRC Press, Boca Raton, 1997
11. Nagle DG, Ferreira D, Zhou YD (2006) Epigallocatechin-3-gallate (EGCG): chemical and biomedical perspectives. Phytochemistry 67:1849–1855
12. Cao YH, Cao RH (1999) Angiogenesis inhibited by drinking tea. Nature 398:381
13. Xu H, Becker CM, Lui WT, Chu CY, Davis TN, Kung AL, Birsner AE, D'Amato RJ, Wai Man GC, Wang CC (2011) 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 96:1021–1028
14. Chen Z, Zhu QY, Tsang D, Huang Y (2001) Degradation of green tea catechins in tea drinks. J Agric Food Chem 49:477–482
15. Lam WH, Kazi A, Kuhn DJ, Chow LM, Chan AS, Dou QP, Chan TH (2004) A potential prodrug for a green tea polyphenol proteasome inhibitor: evaluation of the peracetate ester of (-)-epigallocatechin gallate [(-)-EGCG]. Bioorg Med Chem 12:5587–5593
16. Nap AW, Griffioen AW, Dunselman GAJ, Bouma-Ter Steege JCA, Tijssen VJL, Evers JLH, Groothuis PG (2004) Antiangiogenesis therapy for endometriosis. J Clin Endocrinol Metab 89:1089–1095
17. Bruner-Tran KL, Osteen KG, Duleba AJ (2009) Simvastatin protects against the development of endometriosis in a nude mouse model. J Clin Endocrinol Metab 94:2489–2494
18. Sang S, Lambert JD, Hong J, Tian S, Lee MJ, Stark RE, Ho CT, Yang CS (2005) Synthesis and structure identification of thiol conjugates of (-)-epigallocatechin gallate and their urinary levels in mice. Chem Res Toxicol 18:1762–1769
19. Villayandre BM, Paniagua MA, Fernandez-Lopez A, Chinchetrua MA, Calvo P (2004) Effect of vitamin E treatment on *N*-methyl-d-aspartate receptor at different ages in the rat brain. Brain Res 1028:148–155
20. Song C, Xiang J, Tang J, Hirst DG, Zhou J, Chan KM, Li G (2011) Thymidine kinase gene modified bone marrow mesenchymal stem cells as vehicles for antitumor therapy. Hum Gene Ther 22:439–449
21. Sun MH, Leung KS, Zheng YP, Huang YP, Wang LK, Qin L, Leung AH, Chow SK, Cheung WH (2012) Three-dimensional high frequency power Doppler ultrasonography for the assessment of microvasculature during fracture healing in a rat model. J Orthop Res 30:137–143
22. Chu KO, Wang CC, Chu CY, Rogers MS, Choy KW, Pang CP (2004) Method for determination of catechins and catechin gallates in tissues by HPLC with coulometric array detection and selective solid-phase extraction. J Chromat B 810:187–195
23. Chu KO, Wang CC, Rogers MS, Choy KW, Pang CP (2004) Determination of catechins and catechin gallates in biological fluids by liquid chromatography with coulometric array detection and solid-phase extraction. Anal Chim Acta 510:69–76
24. Wang CC, Chu CY, Chu KO, Choy KW, Rogers MS, Khaw KS, Pang CP (2004) Trolox-equivalent antioxidant capacity (TEAC) assay versus oxygen radical absorbance capacity (ORAC) assay in plasma. Clin Chem 50:952–954
25. Graham HN (1992) Green tea composition, consumption and polyphenol chemistry. Preventive Med 21:334–350
26. Lea CH, Swoboda PAT (1957) The antioxidant action of some polyphenolic constituents of tea. Chem Ind 1073–1074
27. Zhu QY, Zhang A, Tsang D, Huang Y, Chen ZY (1997) Stability of green tea catechins. J Agric Food Chem 45:4624–4628
28. Lambert JD, Sang S, Hong J, Kwon SJ, Lee MJ, Ho CT, Yang CS (2006) Peracetylation as a means of enhancing in vitro bioactivity and bioavailability of epigallocatechin-3-gallate. Drug Metab Dispos 34:2111–2116
29. Fassina G, Vene R, Morini M, Minghelli S, Benelli R, Noonan DM, Albini A (2004) Mechanisms of inhibition of tumor angiogenesis and vascular tumor growth by epigallocatechin-3-gallate. Clin Cancer Res 10:4865–4873
30. Liao J, Yang GY, Park ES, Meng X, Sun Y, Jia D, Seril DN, Yang CS (2004) Inhibition of lung carcinogenesis and effects on angiogenesis and apoptosis in A/J mice by oral administration of green tea. Nutr Cancer 48:44–53
31. Lamy S, Gingras D, Beliveau R (2002) Green teas catechins inhibit vascular endothelial growth factor receptor phosphorylation. Cancer Res 62:381–385
32. Kojima-Yuasa A, Hua JJ, Kennedy DO, Matsui-Yuasa I (2003) Green tea extract inhibits angiogenesis of human umbilical vein endothelial cells through reduction of expression of VEGF receptors. Life Sci 73:1299–1313
33. Kondo T, Ohta T, Igura K, Hara Y, Kaji K (2002) Tea catechins inhibit angiogenesis in vitro, measured by human endothelial cell growth, migration and tube formation, through inhibition of VEGF receptor binding. Cancer Lett 180:139–144
34. Jung YD, Kim MS, Shin BA, Chay KO, Ahn BW, Liu W, Bucana CD, Gallick GE, Ellis LM (2001) EGCG, a major component of green tea, inhibits tumor growth by inhibiting VEGF induction in human colon carcinoma cells. Br J Cancer 84:844–850
35. Sartippour MR, Shao ZM, Heber D, Beatty P, Zhang L, Liu C, Ellis L, Liu W, Go VL, Brooks MN (2002) Green tea inhibits vascular endothelial growth factor (VEGF) induction in human breast cancer cells. J Nutr 132:2307–2311
36. Lee SC, Chan WK, Lee TW, Lam WH, Wang X, Chan TH, Wong YC (2008) Effect of a prodrug of the green tea polyphenol (-)-epigallocatechin-3-gallate on the growth of androgen-independent prostate cancer in vivo. Nutr Cancer 60:483–491
37. Kuhn D, Lam WH, Kazi A, Daniel KG, Song S, Chow LM, Chan TH, Dou QP (2005) Synthetic peracetate tea polyphenols as potent proteasome inhibitors and apoptosis inducers in human cancer cells. Front Biosci 10:1010–1023
38. Becker CM, Wright RD, Satchi-Fainaro R, Funakoshi T, Folkman J, Kung AL, D'Amato RJ (2006) A novel noninvasive model of endometriosis for monitoring the efficacy of antiangiogenesi therapy. Am J Pathol 168:2074–2084

39. Rice VM (2002) Conventional medical therapies for endometriosis. Ann N Y Acad Sci 955:343–352
40. Xu H, Wang CC (2010) Angiogenesis and anti-angiogenesis therapy of endometriosis. In: Mitchell LA (ed) Endometriosis: symptoms, diagnosis and treatments, Chapter 1. Nova Science, New York, pp 1–40
41. Dabrosin C, Gyorffy S, Margetts P, Ross C, Gauldie J (2002) Therapeutic effect of angiostatin gene transfer in a murine model of endometriosis. Am J Pathol 161:909–918
42. Yagyu T, Kobayashi H, Matsuzaki H, Wakahara K, Kondo T, Kurita N, Sekino H, Inagaki K, Suzuki M, Kanayama N, Terao T (2005) Thalidomide inhibits tumor necrosis factor-alpha-induced interleukin-8 expression in endometriotic stromal cells, possibly through suppression of nuclear factor-kappaB activation. J Clin Endocrinol Metab 90:3017–3021
43. Scarpellini F, Sbracia M, Lecchini S, Scarpellini L (2002) Anti-angiogenesis treatment with thalidomide in endometriosis: a pilot study. Fertil Steril 78:S87
44. Nyberg P, Xie L, Kalluri R (2005) Endogenous inhibitors of angiogenesis. Cancer Res 65:3967–3979
45. Klauber N, Rohan RM, Flynn E, D'Amato RJ (1997) Critical components of the female reproductive pathway are suppressed by the angiogenesis inhibitor AGM-1470. Nat Med 3:443–446
46. Becker CM, Sampson DA, Rupnick MA, Rohan RM, Efstathiou JA, Short SM, Taylor GA, Folkman J, D'Amato RJ (2005) Endostatin inhibits the growth of endometriotic lesions but does not affect fertility. Fertil Steril 84(Suppl 2):1144–1155
47. Kazi A, Wang Z, Kumar N, Falsetti SC, Chan TH, Dou QP (2004) Structure-activity relationships of synthetic analogs of (-)-epigallocatechin-3-gallate as proteasome inhibitors. Anticancer Res 24:943–954
48. Cao YH, Cao RH, Bråkenhielm E (2002) Antiangiogenic mechanisms of diet-derived polyphenols. J Nutr Biochem 13:380–390