Therapeutic potential of andrographolide for treating endometriosis

Yu Zheng¹, Xishi Liu¹, and Sun-Wei Guo¹,²,*

¹Shanghai OB/GYN Hospital, Fudan University, Shanghai 200011, China ²Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Shanghai 200011, China

*Correspondence address. E-mail: hoxial0@gmail.com

Submitted on January 18, 2011; resubmitted on January 21, 2012; accepted on February 7, 2012

BACKGROUND: Mounting evidence shows that nuclear factor-κB (NF-κB) plays an important role in endometriosis. We therefore evaluated the therapeutic potential of andrographolide, an NF-κB inhibitor.

METHODS: Primary cell cultures were performed using ectopic endometrial tissue specimens and their homologous eutopic endometrial specimens from 16 women with endometriosis, as well as control samples from 4 women without endometriosis. Andrographolide was evaluated for an effect on cell proliferation and cell cycle, DNA-binding activity of NF-κB and expression of cyclooxygenase-2 (COX-2) and tissue factor (TF). In a rat model of endometriosis, andrographolide treatment was evaluated for an effect on lesion size, hotplate response latency and expression of phosphorylated p50 and p65, COX-2 and nerve growth factor (NGF) in ectopic endometrium.

RESULTS: Andrographolide dose dependently suppressed proliferation and cell cycle progression, attenuated DNA-binding activity of NF-κB in endometriotic stromal cells and inhibited COX-2 and TF expression. In the rat experiment, induced endometriosis resulted in reduced response latency. Andrographolide treatment significantly reduced lesion size in a dose-dependent manner and significantly increased response latency. Andrographolide treatment also significantly reduced immunoreactivity of COX-2, phosphorylated p50 and p65, and NGF in ectopic endometrium.

CONCLUSIONS: Treatment with andrographolide significantly suppresses the growth of ectopic endometrium in vitro and in vivo, and results in a significant improvement in generalized hyperalgesia in rats with induced endometriosis. Therefore, andrographolide may be cytoreductive and may relieve pain symptoms in women with endometriosis. With excellent safety and cost profiles, andrographolide could be a promising therapeutic agent for endometriosis.

Key words: andrographolide / endometriosis / hyperalgesia / NF-κB / valproic acid

Introduction

Endometriosis is characterized by the ectopic presence of estrogen-responsive endometrial tissues and is a common and debilitating gynaecological disorder with an enigmatic pathogenesis (Giudice and Kao, 2004). Among all its presenting symptoms, pain of various kinds, dysmenorrhea, pelvic pain and dyspareunia top the list of complaints from afflicted women, and are the symptoms that impact most negatively on the quality of life (Giudice and Kao, 2004; Olive et al., 2004; Gao et al., 2006; Fourquet et al., 2010; Tripoli et al., 2011). Consequently, one primary goal in treating women with endometriosis is to alleviate pain. While the current treatment of choice for endometriosis is surgery, medical treatment is often needed either as the first-line therapy or due to the high recurrence risk after surgery (Giudice, 2010). The current medical treatment modalities for endometriosis are somewhat effective in relieving endometriosis-associated pain, yet the relief of pain appears to be relatively short term (Waller and Shaw, 1993). In addition, they have many undesirable, and sometimes severe, side effects (Kiilholma et al., 1995, Lessey, 2000, Bulun et al., 2005). Consequently, a more efficacious medical treatment, preferably with a more favourable side effect and cost profile, is urgently needed (Nothnick and D’Hooghe, 2003).

One notable hallmark of endometriosis is inflammation, manifested by defective immunosurveillance and inflammatory hyperresponsiveness. The former is characterized by increased macrophage activation (Halme et al., 1984) and decreased NK cell cytotoxicity against endometrial and haematopoietic cells (Oosterlynck et al., 1991; Oosterlynck et al., 1993), while the latter is characterized by increased production of proinflammatory cytokines such as interleukin (IL-1β; Keenan et al., 1995), tumour necrosis factor-α (TNFα; Keenan...
et al., 1995), RANTES (CCL5; Khorraram et al., 1993), IL-8 (Ryan et al., 1995) and monocyte chemotactic peptide-1 (Akourm et al., 1996), among others (Harada et al., 2001). The activated macrophages also secrete prostaglandins, proinflammatory cytokines and angiogenic and neurotrophic peptides which, collectively, promote proliferation, angiogenesis, invasion and inflammation. It is generally viewed that local inflammation is the major cause of pain and infertility in endometriosis (Bulun et al., 2005; Minici et al., 2007).

One important, and very likely major, culprit in the pathogenesis of endometriosis, is that behind this hallmark of inflammation and is probably the nexus linking proliferation, inflammation, angiogenesis and invasion, is nuclear factor-κB (NF-κB). NF-κB is an important transcription factor which has recently attracted much attention (Guo, 2007; Celik et al., 2008; Gonzalez-Ramos et al., 2010) and has been shown to be constitutively activated in endometriosis (Gonzalez-Ramos et al., 2007; Grund et al., 2008; Lousse et al., 2008). Growing evidence has now shown that NF-κB is involved in many aspects of the development of endometriosis, including inflammation, proliferation, angiogenesis, oxidative stress and invasion (Guo, 2007; Gonzalez-Ramos et al., 2010). The NF-κB-induced proteins, such as proinflammatory cytokines IL-1β and TNFα, are also potent NF-κB inducers, thus resulting in a vicious cycle that likely maintains, perpetuates or even augments the chronic inflammatory state that defines endometriosis (Guo, 2007). Fairly extensive in vitro and in vivo studies have also shown that NF-κB inhibitors appear to be a promising therapeutic agent for endometriosis. In fact, one commonality shared by all mainstay and investigated drugs for treating endometriosis is that they all suppress NF-κB activation (Guo, 2007).

The canonical NF-κB activation pathways, for example that TNFα-induced, are through the p65 and p50 heterodimers (Bonizzi and Karin, 2004). This suggests that the NF-κB p50 subunit is also involved in endometriosis. Indeed, we recently reported, through the use of the p50 knockout mice, that p50 is involved in the development of endometriosis. Indeed, we recently reported, through the use of the p50 knockout mice, that p50 is involved in the development of endometriosis (Karin, 2004). This suggests that the NF-κB p50 subunit is also involved in endometriosis. Indeed, we recently reported, through the use of the p50 knockout mice, that p50 is involved in the development of endometriosis (Liu et al., 2012). We also carried out immunohistochemistry (IHC) analysis of COX-2, the activated forms of p50 and of p65, and nerve growth factor (NGF) in ectopic endometrium in the rat model of endometriosis.

Materials and Methods

Tissue samples

This study was approved by the institutional ethics review board of Shanghai OB/GYN Hospital, Fudan University. All specimens were obtained after informed consent. Sixteen patients (33.6 ± 5.2 years old, all in their later proliferative phase at the time of surgery) with histologically confirmed ovarian endometriomas and who underwent laparoscopy were recruited for this study. From eight of them (aged 33.5 ± 6.8 years), the ectopic endometrial tissue specimens and their homologous eutopic endometrial specimens were obtained at the time of laparoscopy. For the other eight patients (aged 33.8 ± 6.8 years), their ectopic endometrial tissue samples were obtained for the electrophoretic mobility shift assay (EMSA, detailed below).

For control samples, we collected endometrial tissue samples through curettage from four similarly aged patients (33.5 ± 8.27 years old), who were diagnosed with hydrosalpinx (n = 1), fallopian tube obstruction (n = 1), teratoma (n = 1), and cervical intraepithelial neoplasia (CIN 2–3, n = 1) but having no endometriosis, adenomyosis, leiomyoma or malignant tumour. All diagnoses were confirmed histologically after the operation. All specimen donors were premenopausal and had regular and normal menstrual cycles, and had received no hormone therapy or intrauterine device for at least 6 months prior to surgery.

Isolation, identification and culture of endometriotic and endometrial stromal cells

The isolation and culture of endometriotic and endometrial stromal cells were carried out following previously reported protocols (Chatzaki et al., 2001) with minor modifications. Briefly, endometriotic and endometrial tissues were minced into small pieces, immersed in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Shiyi Biotechnology, Inc., Shanghai, China) and gently shaken for 1 h for enzymolysis at 37°C. The filtrated cells were centrifuged, and the sedimentation was further subjected to collagenase (2.5 mg/ml; Sigma-Aldrich, St. Louis, USA) and gently shaken for 1 h for enzymolysis at 37°C, and separated by the means of serial filtration through two consecutive gauge sieves of 149 and 37 μm. The filtrated cells were centrifuged, and the sedimentation containing stromal cells was suspended in DMEM/F12 and plated onto 6-cm dishes. After 2 h at 37°C allowing for stromal cell adherence, other suspending components were rinsed away with phosphate-buffered saline (PBS).

The cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, USA). When grown to ~80% confluence, the cells were dissociated with 0.25% trypsin and re-plated to new dishes at 5 × 10³ cells/well, and incubated at 37°C in a humidified 5% CO₂–95% air environment. The purification of stromal cell populations was confirmed by immunocytochemical staining for

Potential of andrographolide for treating endometriosis

Andrographolide (C₂₀H₂₀O₅, Andro hereafter) is an active ingredient chemical extracted from Andrographis (Andrographis paniculata), which has been used as a medicinal herb in traditional Chinese medicine for alleviation of inflammatory disorders for thousands of years. Andro is known to be anti-inflammatory (Abu-Ghefreh et al., 2009) and to interfere with NF-κB binding to DNA (Hidalgo et al., 2005), and the underlying mechanism has recently been shown to result from the suppression of NF-κB activation (Ota et al., 2001; Krukun et al., 2008). Andro has also been shown to be antinociceptive in animals (Lin et al., 2009; Sulaiman et al., 2010). Most remarkably, Andro, unlike many NF-κB inhibitors, is already commercially available with an excellent safety profile and is a non-prescription medication in China, indicative for upper respiratory tract infection. A recent clinical trial on the use of A. paniculata extract containing 30% of total Andros to treat rheumatoid arthritis reported promising results (Burgos et al., 2009).

In view of the above information, we evaluated, in this study, the in vitro and in vivo effects of Andro in endometriosis. We hypothesized that Andro treatment inhibits the proliferation of endometriotic cells, suppresses COX-2 and TF expression, reduces lesion size and also improves generalized hyperalgia in rats with induced endometriosis. Besides a negative control, we used valproic acid (VPA) treatment as a positive control, since emerging data strongly suggest that endometriosis is an epigenetic disease (Guo, 2009) and that VPA treatment results in the retardation of endometriosis and improvement in pain behaviour in rats with induced endometriosis (Liu et al., 2012). We also carried out immunohistochemistry (IHC) analysis of COX-2, the activated forms of p50 and of p65, and nerve growth factor (NGF) in ectopic endometrium in the rat model of endometriosis.
vimentin, a marker for stromal cells, and cytokeratin, a marker for epithelial cells (see Supplementary data, Fig. S1). The primary cells used in all experiments were within 3–10 passages of the start of culture.

Drug reconstitution
Both Andro and its solvent, dimethyl sulfoxide (DMSO), were purchased from Sigma-Aldrich (St. Louis, USA), and used for in vitro experiments. The Andro stock concentration was 15 mM. In all experiments, the Andro stock would be diluted correspondingly by culture medium and the controls would be carried out with the same medium containing DMSO of identical volume to the Andro stock.

For animal experiments, Andro, which is a commercial, non-prescription drug in China, was obtained from the Andrographolide Pill, manufactured by Tasly Pharmaceutical Co., Ltd. (Tianjin, China). VPA was also purchased from Sigma-Aldrich.

Sulforhodamine B assay
The effect of Andro treatment on the viability of endometriotic stromal cells (EmSCs) was determined by the sulforhodamine B (SRB) assay following a published protocol (Vichai and Kirtikara, 2006) with minor modification. Since there was no published report on the effect of Andro on EmSC per se, we first investigated whether Andro treatment has a time-dependent effect on EmSC viability at a concentration reported to be effective on other cell types (Xia et al., 2004). Briefly, ~5000 cells/well were seeded in 96-well plates. After 24 h, the medium was replaced by 350 µl medium with 0 (as control), 5, 10, 15, 20, 25 and 30 µM Andro added. In experiments with lower Andro concentrations (0–25 µM), different volumes of DMSO were added to ensure that the final concentration of DMSO was identical in each medium of different Andro concentrations. After 48 h, 150 µl of pre-cooled 30% [w/v] trichloroacetic acid (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) was added to all plates, which were then cooled down at 4°C for 1 h. After rinsing 5 times with distilled water, the plates were laid at room temperature for dehydration. Each well was then coloured with 0.4% SRB in 1% acetic acid [w/v] for 0.5 h at room temperature, after which uncombined SRB would be washed away by 1% acetic acid. Before optical density (OD) measurement at 540 nm, the plates were dehydrated at room temperature prior to reconstitution of the dye in 150 µl of 10 mM Tris. The relative growth rate was calculated as the ratio of the average OD values of experimental wells versus the average OD value of control wells.

Flow-cytometry assay of cell cycles
The primary EmSCs were treated with 0, 15 and 30 µM of Andro for 48 h, and then subjected to trypanosinization which was terminated by the medium. Suspended cells were centrifuged at 500g and washed with cold PBS (Sinopharm Chemical Reagent Co., Ltd.). After fixation in 70% pre-cooled ethanol (Sinopharm Chemical Reagent Co., Ltd.) at 4°C for 1 h, cells were concentrated to ~5 x 10^5/ml. The cell nuclei were then stained with propidium iodide and subjected to fluorescence-activated cell sorting analysis using the FACScan system (Becton Dickinson, Franklin Lakes, New Jersey, USA). The data were analysed using ModFit V.1.2. software (Venty Software House, Topsham, USA).

Western blot analyses
For the nuclear phospho-p50 (p-p50) assay, the nuclear protein of primary-cultured EmSCs and the eutopic endometrial stromal cells were extracted by following the protocol of Celllytic™ NuCLEAR™ Extraction Kit (NXTRACT, Sigma-Aldrich).

To evaluate the effect of Andro on basal and TNFα-induced expression of COX-2 and TF in EmSC, we pretreated EmSC with phenol red- and serum-free medium for 24 h when cells reached ~80% confluence. Then the cells were treated with one of the following compounds for 72 h: (i) the vehicle (DMSO) of the same volume of the Andro solution, (ii) 30 µM Andro, (iii) 5 ng/ml TNFα along with DMSO of the same volume as the Andro solution for 48 h following 24 h of incubation with media containing only DMSO of the same volume as that of the Andro solution or (iv) 5 ng/ml TNFα along with 30 µM Andro treatment for 48 h following pretreatment for 24 h with 30 µM Andro.

For total protein extraction, all cells were scraped and lysed in commercial lysis buffer (P0013C, Beyotime Institute of Biotechnology, Nantong, China) supplemented with 1% [v/v] phosphatase and protease inhibitor cocktail (78440, Pierce Biotechnology, Rockford, USA), agitated for 30 min on ice and then centrifuged for 30 min at 15 000g at 4°C to remove the pellet. Protein concentration was determined using a bicinchoninic acid protein quantitative analysis kit (P0010S, Beyotime Institute of Biotechnology). All proteins mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (P0015, Beyotime Institute of Biotechnology) were heated for 5 min at 95°C for denaturation. For all assays, 30 µg of protein sample was separated on an 10% SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA).

All blots were blocked in 5% non-fat milk reconstituted in tris-buffered saline with Tween 20 [0.15 M NaCl, 0.05% Tween-20, 10 mM Tris—HCl (pH 8.0)] for 2 h and subsequently incubated at 4°C overnight with the following primary antibodies: p-p50 (ser 337, 1:300; sc-101744, Santa Cruz Biotechnology, Santa Cruz, USA), COX-2 (1:300; sc-19999, Santa Cruz Biotechnology), TF (1:2000; #612161, Merck KGaA, Darmstadt, Germany) and Lamin B1 and GAPDH served as the loading controls for nuclear protein amount and total protein amount, respectively. After the primary and secondary antibodies were added, the signal was detected using enhanced chemiluminescence (ECL) on hyperfilms. Finally, the integrated optical density (IOD) of each band was evaluated with Gel-pro 4.5 Analyzer (Media Cybernetics, Inc., USA). Briefly, films of western blots were scanned and the images were processed by the software, yielding the IOD value.

Electrophoretic mobility shift assay
EmSCs were pretreated with phenol red- and serum-free medium for 24 h when cells reached ~80% confluence. Prior to the preparation of nuclear protein of EmSC, the cells were treated with 30 µM of Andro or DMSO of an identical volume for 72 h. The consensus oligonucleotide NF-κB probes end-labelled with biotin or unlabelled (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) were purchased from Beyotime Institute of Biotechnology. The nuclear extracts were prepared with Celllytic™ NuCLEAR™ Extraction Kit (NXTRACT, Sigma-Aldrich). The nuclear extracts, 10 µg for each treatment, were pre-incubated with the reaction mixture [10 mM Tris—HCl (pH 7.5), 50 mM KCl, 2.5% glycerol, 5 mM MgCl2, 10 mM ethylenediaminetetraacetic acid (EDTA), 1 mM DL-dithiothreitol and 50 µg/ml protein (dilution of the crude nuclear extract) for 10 min at room temperature. In a cold probe competitive reaction, 100-fold unlabelled probe was added for a pre-incubation. Then the biotin-labelled probe (10 fmol) was added to each of the reaction mixtures and incubated at room temperature for 30 min. The protein–DNA complexes were separated from free DNA probes through 6% non-denaturing polyacrylamide gels in 0.5× Tris–boric acid–EDTA buffer at 15 V/cm and electrophoretic transferred onto a nylon membrane (Pierce Biotechnology, Rockford, USA) for 45 min at 380 mA. The transferred DNA was cross-linked on the membrane by illuminating with the UV light for 20 min at a distance of 10 cm. Then the membrane was processed by following the protocol of nucleic acid detection kit (Pierce Biotechnology, Rockford, USA). Briefly, the membrane was incubated in conjugate/blocking buffer for 15 min
after 15-min pre-incubation in blocking buffer. The membrane was then washed four times (5 min each) and transferred to substrate equilibration buffer for another 5 min. Finally, the signal was detected with ECL and hyperfilms. The IOD of each shift band was measured with a Gel-pro 4.5 Analyzer as described above.

**Animals**

Fifty Sprague–Dawley virgin female rats, 8–8 weeks old and 200–250 g in weight, were purchased from the Laboratory Animals Facility of Fudan University (Shanghai, China) and used for this study. They were maintained under controlled conditions with a light/dark cycle of 12/12 h at 25°C and had access to chows and water ad libitum. All experiments in this study were performed under the guidelines of the National Research Council’s Guide for the Care and Use of Laboratory Animals and approved by the institutional experimental animals review board of Shanghai OB/GYN Hospital, Fudan University.

**The animal experiment protocol**

The whole animal experiment process, including the surgery procedure, hotplate test (described below) and lesion size measurement, was similar to that presented in our previous report (Lu et al., 2010). Briefly, after 3 days of acclimatization and before the surgery, the rats were randomly divided into five equal-sized groups (10 each): (i) U, untreated group; (ii) L, low-dose Andro group; (iii) H, high-dose Andro group; (iv) V, VPA-treated group and (v) J, joint (low-dose Andro + low-dose VPA) treatment group. After grouping and tagging, a baseline hotplate test (Test 1) was administrated to all rats. Thereafter, all rats received endometriosis-inducing surgery through autologous transplant of endometrial tissue fragments. Briefly, the rats were anesthetized with 10% chloral hydrate (m/v) at 0.6 ml/200 g body weight. After laparotomy, the left uterine horn was excised and cut into four roughly equal-sized fragments. Each tissue fragment was sutured back to peritoneal wall of the rat itself with 6/0 braided silk suture. A schematic description of grouping information. Five different photomicrographs were captured in Image-Pro Plus, and the area containing glandular cells was manually depicted groups of data on hotplate latency, immunostaining levels or distribution of continuous variables between two or more groups was made using the Wilcoxon’s and Kruskal’s test, respectively, and the paired Wilcoxon test was used when the before–after comparison was made for the same group. Pearson’s or Spearman’s rank correlation coefficient was used when evaluating correlations between two variables when both variables were continuous or when at least one variable was ordinal.

**Statistical analysis**

For descriptive statistics, we used boxplot (Tukey, 1977) to graphically depict groups of data on hotplate latency, immunostaining levels or lesion sizes, in which the bottom and top of the box represent the lower and upper quartiles, respectively, the band near the middle of the box represents the median and the ends of the whiskers represent the smallest and the largest non-outlier observations. The comparison of distributions of continuous variables between two or more groups was made using the Wilcoxon’s and Kruskal’s test, respectively, and the paired Wilcoxon test was used when the before–after comparison was made for the same group. Pearson’s or Spearman’s rank correlation coefficient was used when evaluating correlations between two variables when both variables were continuous or when at least one variable was ordinal.
To see whether Andro and/or VPA treatment, and other possible factors were responsible for the change in hotplate latency before and after the treatment, a multiple linear regression model was used. The treatment of VPA or not was coded as 1 or 0, while for Andro the dosage was used as the covariate. \( P \)-values of <0.05 were considered statistically significant. All computations were made with R statistics software system version 2.14.0 (R Development Core Team, 2010).

**Results**

**Primary culture of stromal cell and identification**

We successfully established stromal cell monolayer cultures derived from normal endometrium and both eutopic and ectopic endometrial specimens of endometriosis patients. Under a phase-contrast microscope, the morphology of primary stromal cells was found to be characteristically flat and spindle-shaped and indistinguishable (Supplementary data, Fig. S1). The purity of the cultured stromal cells was found to be \( \approx 95\% \), as judged by the positive staining for vimentin and negative staining for cytokeratin. The morphology of stromal cells within the first 10 passages in the primary culture was found to be quite consistent and homogenous.

**Andro inhibits proliferation of EmSC and retards cell cycle progression**

Using the SRB assay, we evaluated the effect of Andro on the viability of EmSC. We found that Andro inhibited their proliferation in a dose-dependent manner (\( P = 2.7 \times 10^{-9} \); Fig. 2A), reducing the cellular proliferation by 21.7, 34.6, 44.9, 51.6, 54.5 and 63.5\%, respectively, at concentrations of 5, 10, 15, 20, 25 and 30 \( \mu \)M, as compared with untreated cells.

By flow cytometry, we found that percentage of EmSC cells in the G0/G1 phase was increased by 13.8\%, while that of S-phase cells was decreased by 21.3\% after being incubated with 15 \( \mu \)M of Andro. Yet the differences did not reach statistical significance (\( P = 0.09 \) and \( P = 0.07 \), respectively). When treated with 30 \( \mu \)M of Andro, however, the percentage of G0/G1-phase cells increased by 20.0\%, while that of S-phase cells decreased by 32.5\%, both reaching statistical significance (\( P = 0.036 \) and \( P = 0.020 \), respectively; Fig. 2B). These
data clearly show that Andro treatment significantly hindered the cell cycle progression of EmSC, and are consistent with anti-proliferative effect of Andro as seen in the SRB assay.

**Immunoreactivity to p-p50 in the EmSC nucleus**

Andro is known to suppress NF-κB activation through suppressing p50 (Xia et al., 2004). Consistent with our previous report, we found that p-p50, the activated form of p50, was found in the nuclear protein extracts of EmSc but not in that of normal or eutopic endometrial stromal cells (Fig. 3). The presence of p-p50 in EmSC is consistent with the published data that NF-κB is constitutively activated in pelvic endometriotic lesions (Gonzalez-Ramos et al., 2007), and provides a strong rationale for the use of Andro as a potential therapeutic agent for endometriosis.

**Effect of Andro on DNA-binding activity of NF-κB**

By EMSA, we found that NF-κB was constitutively activated in EmSC and Andro treatment significantly \( (P = 0.0001) \) attenuated its activation in terms of DNA-binding activity (Fig. 4), consistent with our previous report (Xia et al., 2004).

**Effect of Andro on COX-2 and TF expression in EmSC**

By western blotting, we found that both COX-2 and TF are constitutively expressed in EmSC, consistent with previous reports (Ota et al., 2001; Krikun et al., 2008). Their expression levels were significantly increased when stimulated with TNFα (both \( P \)-value were 0.008 for COX-2 and TF, paired Wilcoxon test; Fig. 5A and B). Treatment with Andro, however, inhibited not only the constitutive but also TNFα-induced expression of COX-2 and TF in EmSC (\( P = 0.008 \) and \( P = 0.008 \) for COX-2 and TF, respectively, for basal levels; both \( P \)-values = 0.01 for COX-2 and TF, respectively, for TNFα-stimulated levels; Fig. 5A and B).

**Treatment with Andro reduces lesion size in rats with induced endometriosis**

Using the protocol described above, we successfully induced endometriotic lesions in rats (Fig. 6A). The lesion sizes of all treated groups were significantly smaller than those of untreated rats (\( P = 0.002, 0.002, 0.006 \) and 0.006, respectively, for Groups L, H, V and J; Fig. 6B). The average lesion size in Groups L, H, V and J was reduced by 50.6, 55.8, 38.0 and 37.1%, respectively, as compared with untreated rats. Of note, the inhibition of lesion growth by Andro was dose dependent (\( P = 0.0005 \)), consistent with the in vitro results presented above.

**Effect of Andro treatment on the hotplate latency**

We subjected all rats to a hotplate test and measured their response latency prior to the endometriosis-induction surgery (baseline or Test 1), 2 weeks after surgery (pretreatment test or Test 2) and 4 weeks after the drug treatment started (after treatment test or Test 3).
As expected, no difference was found in Test 1 ($P = 0.83$) or Test 2 latencies ($P = 0.43$) among the five groups, indicating that these groups were comparable before and after the surgical induction of endometriosis (Fig. 7). However, Test 2 latency was significantly decreased by 22.8% when compared with the baseline level ($P = 1.9 \times 10^{-9}$, paired test), suggesting that the induction of endometriosis significantly increased the rats’ sensitivity to the noxious thermal stimulus.

Four weeks after drug treatment, however, the response latency in all four treatment groups (L, H, V and J) was significantly increased as compared with their Test 2 latency ($P = 0.014, 0.037, 0.014$ and $0.014$, respectively, by paired test; Fig. 7), suggesting that the treatment with either VPA, Andro or jointly significantly improved the response latency in rats with induced endometriosis. In contrast, no such improvement was found in the untreated rats ($P = 0.49$). In fact, the response latency in all treated rats more or less returned to the level that was comparable to the baseline level (all $P$-values $> 0.13$, by paired test). The untreated rats, however, had significantly reduced response latency (by 20.4% on average) as compared with the baseline level ($P = 0.01$, paired test; Fig. 7).

**Effect of treatment on the immunoreactivity of p-p50, p-p65, NGF and COX-2 in ectopic endometrium**

We also evaluated the immunoreactivity of p-p50, p-p65, NGF and COX-2 in the ectopic endometrial glandular areas in all groups of rats. We found that for all four proteins, treatment with Andro and/or VPA significantly reduced their immunoreactivity in ectopic endometrial glands (Figs 8 and 9). A multiple linear regression analysis indicated that treatment with Andro and VPA significantly reduced the p-p50 immunoreactivity ($P = 0.008$ and $P = 0.003$, respectively) and the joint treatment further reduced its immunoreactivity ($P = 0.002$). Similarly, treatment with Andro and VPA significantly reduced the p-p65 immunoreactivity ($P = 3.6 \times 10^{-7}$ and $P = 3.8 \times 10^{-5}$, respectively) and the joint treatment further reduced its immunoreactivity ($P = 0.003$). This suggests that Andro and VPA may act through different pathways to suppress NF-κB activation in ectopic endometrium.

For NGF, the result was similar, i.e. treatment with Andro and VPA significantly reduced the immunoreactivity to NGF ($P = 2.0 \times 10^{-4}$ and $P = 6.8 \times 10^{-6}$, respectively) but the joint treatment did not further reduce its immunoreactivity ($P = 0.05$). For COX-2, the treatment with Andro and VPA also significantly reduced the immunoreactivity to COX-2 ($P = 8.1 \times 10^{-6}$ and $P = 0.0056$, respectively), but the joint treatment did not further reduce its immunoreactivity ($P = 0.19$).

The immunoreactivity levels of the four proteins were all positively correlated: the correlation coefficient between p-p50 and p-p65 immunoreactivity was $0.76$ ($P = 1.7 \times 10^{-10}$); between p-p50 and NGF: $r = 0.54$ ($P = 4.8 \times 10^{-5}$); between p-p50 and COX-2: $r = 0.35$ ($P = 0.001$); between p-p65 and NGF: $r = 0.58$ ($P = 9.7 \times 10^{-5}$); between p-p65 and COX-2: $r = 0.50$ ($P = 0.0002$) and between NGF and COX-2: $r = 0.37$ ($P = 0.0077$).

**p-p50, p-p65, NGF and COX-2 immunoreactivity and their relationship with lesion size and pain**

We evaluated the relationship, if any, between p-p50, p-p65, NGF and COX-2 immunoreactivity and lesion size by multiple linear regression analysis. We found that, while p-p50, p-p65, NGF and COX-2 staining...
levels were all marginally yet significantly correlated with lesion size ($r = 0.33, P = 0.019, r = 0.46, P = 0.0007, r = 0.30, P = 0.033$ and $r = 0.42, P = 0.02$, respectively), only p-p65 immunoreactivity was found to be positively and significantly associated with lesion size ($P = 0.0007$).

While p-p65 immunostaining levels in ectopic endometrium correlated negatively with the hotplate latency after drug treatment ($r = 0.30, P = 0.037$), no significant correlation was found for p-p50, NGF and COX-2 (all $P$-values $>0.085$). A regression analysis using the change in the hotplate latency before and after drug treatment as the independent variable and lesion size, pretreatment latency and immunostaining levels as covariates indicated that only lesion size and the pretreatment latency were significantly and negatively associated with change in latency ($P = 0.018$ and $P = 5.0 \times 10^{-5}$, respectively).

**Discussion**

In this study, we found that Andro retards cell cycle progression, inhibits proliferation in a dose-dependent manner, attenuates NF-kB DNA-binding activity and also inhibits constitutive and TNFα-induced COX-2 and TF expression in primary EmSCs. In addition, we found that, in a rat model of endometriosis, Andro treatment results in reduced lesion size, improved sensitivity to a noxious thermal stimulus and decreased immunoreactivity to p-p50, p-p65, NGF and COX-2 in ectopic implants. These in vitro and in vivo data, taken together, strongly indicate that Andro is a promising therapeutic agent for endometriosis.

COX-2 is known to play a critical role in perpetuating the positive feedback loop in inducing inflammation and promoting proliferation in endometriosis (Bulun et al., 2005), and is possibly involved in invasion (Banu et al., 2008; Takenaka et al., 2010) and angiogenesis (Machado et al., 2010) as well. Its overexpression has been well documented in endometriosis (Ota et al., 2001; Chishima et al., 2002), and has been reported to correlate with the severity of dysmenorrhea (Matsuzaki et al., 2004) and non-menstrual chronic pelvic pain (Buchweitz et al., 2006) in women with endometriosis. COX-2 overexpression also has been identified recently as a putative biomarker for recurrence (Yuan et al., 2007).
The presence of p-p50 in the nucleus of EmSCs is consistent with our previous finding in mouse studies (Lu et al., 2011) and confirms that p50 subunit is involved in the NF-κB activation in endometriosis, likely through the canonical pathway. Since the promoter of COX-2 contains two NF-κB-binding sites (Kaltschmidt et al., 2002) and is thus an NF-κB target gene, it is perhaps not surprising that Andro treatment resulted in reduced expression of COX-2 since inhibition of NF-κB activation likely leads to COX-2 down-regulation (Kaltschmidt et al., 2002).

TF is a cell membrane-bound glycoprotein and a member of the cytokine receptor superfamily (Versteeg et al., 2001), and also an NF-κB target gene (Mackman et al., 1991). TF is constitutively expressed in a diverse array of extravascular cells, especially in many important organs and tissues such as heart and kidney, but not in liver and skeletal muscle. Following vascular injury, TF binds to FVII/ VIIa and the TF:FVIIa complex activates prothrombin to thrombin, triggering the clotting cascade. Besides its role in coagulation, TF also functions in many biological processes such as haemostasis, thrombosis, inflammation, angiogenesis and tumour growth (Versteeg et al., 2008). In the normal endometrium, TF has been shown to be expressed mainly in stromal cells of the secretory phase (Krikun et al., 1998; Krikun et al., 2000). Existing data suggest that TF may be involved in the pathogenesis of endometriosis, possibly in angiogenic and inflammatory signalling (Krikun et al., 2008). Li et al recently demonstrated the direct interaction of the p50/p65 heterodimer with the NF-κB site of the human TF promoter and showed that Andro-treated mice exhibited blunted TF expression and reduced venous thrombosis (Li et al., 2009). Our results are consistent with these results. It is a pity that we did not perform an IHC analysis for TF for the rat experiment, simply due to the lack of commercially available antibody to TF for rats.

Consistent with the reported suppression of NF-κB by Andro (Xia et al., 2004), we found that Andro retards cell cycle progression and inhibits proliferation of EmSCs dose dependently. In the in vivo study,
we also found that Andro treatment reduces both p-p50 and p-p65 immunoreactivity in ectopic endometrium and, consistent with our own in vitro results, dose dependently reduces the lesion size, as did VPA treatment.

Studies have shown that surgically induced endometriosis results in vaginal hyperalgesia (Berkley et al., 2001) and hypersensitivity to noxious thermal stimulus in rodents (Lu et al., 2010; Zhao et al., 2011). Consistent with these observations, we recently found that women with endometriosis have generalized hyperalgesia in body areas beyond the site of the ectopic implants without any significant change in pain sensory threshold (He et al., 2010). These results, taken together, suggest that the use of hotplate latency as proxy measure for endometriosis-associated generalized hyperalgesia in rodents is justifiable. Similar to previous findings in rats (Zhao et al., 2011), we found that Andro treatment results in improved hotplate latency in rats with induced endometriosis.

Andro treatment also reduced the immunoreactivity to NGF. It is well documented that NGF expression is increased in inflamed tissues, which can lead to hyperalgesia by both peripheral and central mechanisms (Pezet and McMahon, 2006). NGF can directly activate and sensitize primary afferent nociceptors (Rueff and Mendell, 1996) and may also promote neurite outgrowth in endometriosis (Barcena de Arellano et al., 2011). Conceivably, the increase in NGF expression in ectopic implants may directly activate nociceptors in lesions, contributing to endometriosis-induced hyperalgesia, consistent with the reports that NGF expression is elevated in women with endometriosis (Anaf et al., 2002) and also in ectopic endometrium from rats with induced endometriosis (Zhang et al., 2008).

Alternatively, the NGF overexpression may suggest roles in cellular proliferation, differentiation, angiogenesis and invasion in non-neuronal cells (Chen et al., 2010; Walsh et al., 2010), as well as in angiogenesis (Nico et al., 2008) or a regulatory role in inflammation and tissue remodelling (Lambiase et al., 2004). Indeed, NGF is recently reported to be increased in the peritoneal fluid from women with endometriosis (Barcena de Arellano et al., 2011). The elevated NGF levels may be due to an increased NGF production by immune-competent B and T cells in response to cytokine stimulation (Heese et al., 2006). Incidentally, treatment with NF-κB inhibitors results in reduced NGF production by B cells stimulated by cytokines (Heese et al., 2006).
Besides the apparent suppression of NF-κB activation and the inhibition of NF-κB target genes such as COX-2 and TF, Andro is very likely to have other desirable effects. Besides its hepatocyte- and cardiocyte-protective (Negi et al., 2008; Woo et al., 2008), anti-inflammatory (Wang et al., 2007) and anti-antiogenic (Sheeja et al., 2007) effects, Andro has been reported to have immune-stimulating or immunomodulatory properties (Sheeja and Kutan, 2007a,b; Sheeja and Kutan, 2010; Wang et al., 2010). In addition, it can inhibit lipopolysaccharide (LPS)-induced nitric oxide (NO) production through down-regulation of inducible NO synthase (Chiou et al., 1998; Chiou et al., 2000) and can also inhibit the production of reactive oxygen species (Shen et al., 2000, 2002). Moreover, it has been shown to down-regulate hypoxia-inducible factor-1α (Lin et al., 2011), a factor intimately involved in angiogenesis, and to modulate macrophage activation as well as cytotoxic T lymphocyte responses (Sheeja and Kutan, 2007a,b). More recently, Andro has been shown to reduce LPS/interferon (IFN)-γ-induced p65 nuclear translocation, DNA-binding activity, p65 Ser(536) phosphorylation and NF-κB reporter activity and suppress LPS/IFN-γ-induced inducible NO synthase and matrix metalloproteinase 9 expression in rat vascular smooth muscle cells (Hsieh et al., 2011). These properties, along with our in vitro and in vivo results shown in this study, strongly suggest that Andro may be a promising therapeutic candidate for endometriosis treatment. In addition, the fact that Andro has been proven to be a medication with excellent safety, side effect and cost profiles is also important for endometriosis, since for a non-fatal disease such as endometriosis, drug safety is an important consideration when it comes to the viability of a drug candidate (Guo, 2008). Moreover, one additional advantage to using Andro as a therapeutic agent is that the plant extracts have ancillary health benefits, such as hepatocyte and cardiocyte protection (Negi et al., 2008; Woo et al., 2008).

Our motivation of adding the joint treatment (J) group in the in vivo study was to see as whether or not the joint use of Andro and VPA, both at lower dosage, would be more effective than the use of a single agent alone. It is possible that the VPA and Andro may have different modes of actions and different side effect profiles. By using two agents both at lower dosage, we hoped to achieve desired therapeutic effects similar to or better than that when used alone without increasing the risk of side effects, since a combination of drugs from different classes could cancel out each others’ untoward effects (Rosenthal and Gavras, 2006). Indeed, we saw that the cytoreductive effect of joint treatment appears to be similar to that of a much higher dosage of VPA (Fig. 6B), although no greater than that of the low-dose Andro. In addition, the joint treatment appeared to yield the most reduction in p-p50 expression in ectopic endometrium (Fig. 9A), a similar reduction in p-p65 expression (Fig. 9B), and a comparable reduction in NGF expression when compared with high-dose Andro (Fig. 9C) and COX-2 expression similar to that with high-dose VPA (Fig. 9D). Consequently, the joint treatment, which used only 1/4 of dosage of VPA alone and the same dosage as low-dose Andro, improved endometriosis-associated pain behaviour comparable to that of VPA alone or Andro alone (Fig. 7). While the broad concept of combining several active ingredients into one medication, called a polypill, which is intended to reduce the number of medications that need to be taken by the patient, has existed for decades in the treatment of cardiovascular diseases (Wald and Law, 2003) and appears to be promising in preventing cardiovascular disease (Mayor, 2011) and also in treating adenomyosis in mice (Mao et al., 2011), there are many unresolved issues and challenges ahead and should warrant more research in the future.

Promising as it seems, one issue regarding the efficacy of Andro is whether the improvement in hotplate latency in rats with induced endometriosis is the result of suppression of ectopic implants consequent to Andro treatment, or merely the result of reduction of inflammatory response to the surgical trauma resulting from Andro treatment. In other words, it is unclear as whether or not the improvement in pain behaviour resulting from Andro treatment is specific to endometriosis-induced pain. Since we did not have the rats going through a sham surgery, this question, pertinent to all animal studies of endometriosis measuring endometriosis-related pain behaviour, cannot be addressed directly in our current experiment. However, given the role of NF-κB in inflammation and in life and death in ectopic endometrial cells (Guo, 2007), our in vitro results showing dose-dependent inhibition of proliferation and cell cycle progression of EmSCs, it is unlikely that the effect of Andro in improving pain behaviour in rats is non-specific, or merely the reduction of inflammatory response to surgical trauma. Furthermore, Andro has many aforementioned desirable properties and, more importantly, there is a lack of any documented evidence that Andro can be or has been used as a general analgesic. Indeed, the similar question was raised regarding our previous study on the use of histone deacetylase inhibitors to treat endometriosis, but our recent study shows that the effect is specific to endometriosis-induced pain (Liu et al., 2012). Future studies using sham surgery as well as an inactive structural analogue of Andro should completely resolve this issue.

To summarize, we have shown that Andro suppresses the growth of ectopic endometrial cells dose dependently, both in vitro and in vivo, and inhibits constitutive and TNFα-induced COX-2 and TF expression. In addition, Andro treatment results in improved sensitivity to noxious thermal stimulus. In light of these results, of other desirable properties of Andro, and in particular of its excellent safety profile, we believe that Andro is a promising therapeutic agent for endometriosis, and that it is ready to be tested clinically.

**Supplementary data**

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

**Acknowledgements**

We thank two anonymous reviewers for their helpful comments on earlier versions of this manuscript. The help from Dr Xiang Tao in reading IHC slides was very much appreciated.

**Authors’ roles**

Y.Z. performed all the experiments, and participated in writing and some data analysis. X.S.L. coordinated the study, helped design the study, participated in securing tissue samples as well as writing. S.W.G. conceived and designed the entire study, analysed and
interacted with data, drafted and revised the manuscript. All authors approved the manuscript.

**Funding**

This research was supported in part by grants 30872759 (SWG) from the National Science Foundation of China, grants 09PJ0015 and 10410700200 from the Shanghai Science and Technology Commission (SWG), grant 09-11 from the State Key Laboratory of Medical Neurobiology of Fudan University (SWG) and a grant from the Key Specialty Project of the Ministry of Health, People’s Republic of China.

**Conflict of interest**

None declared.

**References**


Mayor S. Four in one polypill halves predicted cardiovascular risk, international study shows. *BMJ* 2011; **342**:d3355.


Shen YC, Chen CF, Chiou WF. Andrographolide prevents oxygen radical production by human neutrophils: possible mechanism(s) involved in its anti-inflammatory effect. *Br J Pharmacol* 2002;135:399–406.
Wald NJ, Law MR. A strategy to reduce cardiovascular disease by more than 80%. *BMJ* 2003;326:1419.