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Comparative study of the short-term effects of a novel selective estrogen receptor modulator, ospemifene, and raloxifene and tamoxifen on rat uterus

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Abstract

To investigate the differential short-term effects of selective estrogen receptor (ER) modulators (SERMs) on uterus, we treated adult ovariectomized rats with a novel SERM, ospemifene (Osp), two previously established SERMs (tamoxifen and raloxifene (Ral)) and estradiol. The expression of two estrogen-regulated early response genes *c-fos* and vascular endothelial growth factor (VEGF), and DNA synthesis were analysed at 1–24 h after treatment of ovariectomized rats. Induction of *c-fos* mRNA by each of the SERMs showed a biphasic pattern with peaks at 3 and 20 h, respectively. The maximum level of VEGF mRNA was observed at 1 h after raloxifene and 6 h after tamoxifen or ospemifene treatment. Maximum levels of the *c-fos* and VEGF mRNA after raloxifene treatment were higher than those seen after treatments with E2 or a corresponding dose of tamoxifen or ospemifene. DNA synthesis was significantly increased by ospemifene, tamoxifen and raloxifene both in luminal and glandular epithelium. The stimulation was transient, peaking at 16 h. In comparison, the maximum level observed at 16 h after E2 treatment sustained at least until 24 h. DNA synthesis in stromal cells was increased by the SERMs but not by E2 at 24 h. When treated together with E2, the SERMs were able to antagonise E2-stimulated DNA synthesis at 16 h. Our results demonstrate that the initial response of uterus to ospemifene, raloxifene and tamoxifen includes activation of early response genes and even transient stimulation of DNA synthesis in spite of their different long-term effects. However, the early stimulatory events may be mediated by different mechanisms leading to diverging pathways in various tissue compartments and development of differential SERM-specific long-term responses of uterus.

Keywords: SERM; Ospemifene; Tamoxifen; Raloxifene; Uterus; *c-fos*; VEGF; DNA synthesis

1. Introduction

Selective estrogen receptor (ER) modulators (SERMs) are a group of structurally diverse compounds that bind to estrogen receptors and elicit agonist or antagonist responses depending on their chemical structure and the specific properties of the target tissues [1]. A number of chemically unrelated compounds have been demonstrated to function as SERMs. They are capable of interacting with the estrogen receptors, and producing a unique in vivo profile of effects in uterus and other organ [2,3]. We have recently identified a novel SERM, ospemifene (Osp), previously named FC1271a [chemical name: *Z*-2-(4-(4-chloro-1,2-diphenyl-but-1-enyl)phenoxy)ethanol] (Fig. 1), which is a triphenylethylene derivative [4]. It specifically binds to both ERα and ERβ with the affinity similar to that of tamoxifen. Ospemifene prevents bone loss and lowers the level of serum cholesterol [4]. It has a low uterotrophic activity and in contrast to tamoxifen it does not cause DNA adducts in rat liver [4]. Tamoxifen is widely used in the adjuvant therapy of breast cancer [5,6]. It is also efficient in prevention of breast cancer [2,7], but its use is complicated by an increased incidence of endometrial cancer [8–10]. Raloxifene (Ral), another SERM also prevents breast cancer and it is even more efficient in prevention of breast cancer than tamoxifen [11]. However, unlike tamoxifen, raloxifene has not been shown to stimulate proliferation of the endometrium [12–15]. As the in vivo effects of ospemifene, tamoxifen and raloxifene are similar (estrogen-like) in bone

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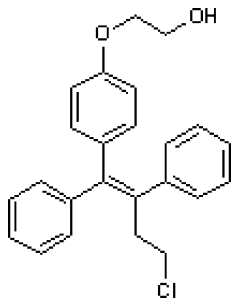


Fig. 1. The chemical structure of ospemifene [*Z*-2-(4-(4-chloro-1,2-diphenyl-but-1-enyl)phenoxy)ethanol].

but strikingly different in uterus [2,4], it would be of interest to analyse the short-term effects of SERMs on uterus.

Administration of 17β-estradiol (E2) to adult ovariectomized (OVX) rats evokes a cascade of stimulatory events in the uterus. Early responses observed within a time period of minutes to a few hours after E2 administration include increase in vascular permeability, water imbibition, increase in organ weight, and induction of expression of proto-oncogenes [16,17] and several growth factors [18,19] as well as stimulation of DNA synthesis [20]. These responses later lead to mitosis and cellular differentiation [21]. One of the characteristics of early estrogenic action in uterus is a rapid, transient rise in the expression of two early-response genes *c-fos* and vascular endothelial growth factor (VEGF) [16,22]. *C-fos* and VEGF are well-characterised primary response genes induced in uterus by estrogen treatment of immature or ovariectomized rats [16,22]. Their stimulation is believed to be associated with regulation of proliferation, vascular permeability, and angiogenesis. The direct estrogen regulation of these genes is confirmed by identification of functional estrogen responsive elements (ERE) in the *c-fos* gene [23,24] and VEGF gene [25,26]. Previous work also showed that other regulatory elements mediating estrogen action, such as AP-1 and Sp1 sites, were found in the promoter region of both *c-fos* and VEGF genes [27,28]. Therefore, we used them as marker genes to test the estrogen-like effects of SERMs.

In the present study, we examined the short-term effects of a novel SERM ospemifene on the expression of two early response genes *c-fos* and VEGF in comparison to two established SERMs raloxifene and tamoxifen and to E2 on the rat uterus. We also analysed the time course of DNA synthesis in different compartments of OVX rat uterus at 1–24 h after SERM and E2 treatments. Our data show that all three SERMs cause similar-type even if not identical stimulation of uterine metabolism during a short-term (up to 24 h) treatment of ovariectomized rats. The results suggest that a longer time period, probably involving a sequence of cell-specific responses and interactions of various tissue compartments is required to build up the differential long-term effects of SERMs on uterus.

2. Methods and materials

2.1. Compounds

17β-Estradiol and BrdU were purchased from Sigma, St. Louis, Mo, USA. Tamoxifen (*Z*-isomer), raloxifene (*Z*-isomer) and ospemifene (*Z*-isomer) were synthesised and purified in the Chemical Research Laboratory of Orion Corporation, Oulu, Finland. The purity of the compounds was 99%.

2.2. Animals and treatments

Female adult Sprague–Dawley rats (age 2.5–3 months) were from the Animal Quarters, BioCity, Turku, Finland. The Ethical Committee of the University of Turku approved the test protocols. The rats were housed in the animal rooms of the Animal Center with constant temperature 22 ± 2 °C and air humidity 50 ± 20%. The light/dark cycle was automatically controlled (a 12 h-light/dark cycle). Commercial laboratory rat food (Special Diet Services, Essex, England) and tap water were available ad libitum to the rats. For ovariectomy, rats were anaesthetised by giving an intraperitoneal (i.p.) injection (2 mg/kg) of diazepam (Diapam®, Orion Co., Finland) for muscle relaxation, which was then followed by a 0.1 ml/100 g i.p. injection of fentanyl-fluanisone anesthetic (Hypnorm®, Janssen Pharmaceuticals Ltd., Grove, Oxford) and 0.1 ml s.c. injection of buprenofin (Temgesic® 0.3 mg/ml) for postoperative analgesia. Bilateral OVX was performed using a dorsal approach. In this study, the rats were ovariectomized for 1 week before use. Four rats were included in each treatment group. In experiment 1, the rats received a single s.c. injection of either 50 μg/kg of E2, 3 mg/kg of each SERM compound (raloxifene, tamoxifen and ospemifene) or polyethylenglygol (PEG) solution (vehicle control). The PEG solution contains Methylparahydroxybenz 1.73 g, NaCl 8.65 g, Propylparahydroxybenz 0.19 g, Macrogol 3000 28.8 g, Polysorb 80 1.92 ml in 1 l sterile water (Orion Corporation, Turku, Finland). Following CO_2 asphyxiation, animals were killed by cervical dislocation at indicated times. In experiment 2, rats received a single s.c. injection of SERM (3 mg/kg of each) 30 min prior to E2 injection and were killed at 16 h after E2 injection. The uteri were excised and cleared from fat before weighting. One uterine horn was immediately frozen in liquid nitrogen for RNA isolation and the other was fixed in 4% buffered paraformaldehyde (pH = 7.4) to be processed for immunohistochemistry.

2.3. Analysis of DNA synthesis by immunohistochemical detection of incorporation of BrdU

To measure DNA synthesis in vivo at various time points following treatments, the animal received an i.p. injection of BrdU (Sigma) (50 mg/kg in saline) 1 h prior to kill. Five micrometres sections were mounted to silane-coated glass

slides and immunohistochemical staining of incorporated BrdU was performed as described [29]. Briefly, the sections were deparaffinized. Endogenous peroxides were blocked with 2% peroxides and antigen retrieval was performed by microwave heating the sections for 10 s at 700 W in 10 mM citrate buffer (pH = 6.0). The sections were then incubated with a monoclonal mouse anti-BrdU antibody (DAKO Corp., Glostrup, Denmark) at 1:100 dilution for 1 h at room temperature. Secondary antibody and visualisation of positive cells were performed using VectaStain Elite kit (Vector Laboratories, Burlingame, CA). The BrdU labelling index was calculated for the luminal and glandular epithelial cells separately by counting 500 nuclei in each category from randomly selected uterine cross-sections under the light microscope. The labelling index was expressed as the percentage of BrdU labelled nuclei of the total number nuclei examined.

2.4. RNA isolation and Northern blot analysis

Total RNA was extracted using the single step method [30] and were stored in RNase-free distilled water at −80 °C. Aliquots of total RNA (10 μg) were separated by electrophoresis on 1% agarose/formaldehyde gel and were vacuum transferred to nylon membranes (Genescreen, Du-Pont, NEN, Boston, MA). After UV cross-linking, the membranes were hybridised in buffer as suggested by the manufacturer. After hybridisation, the membranes were washed with 2× SSC, 0.1% SDS at room temperature and 0.2× SSC, 0.1% SDS at 65 °C, and exposed to X-OMAT AR film (Eastman Kodak, Rochester, NY) at −70 °C.

The *c-fos* and VEGF riboprobes were prepared as described previously [31,16,32]. The filters were subsequently stripped and reprobed with ^{32}P-dCTP labelled 28S rRNA cDNA probe to monitor RNA loading. Autoradiographic films were first scanned by a UMAX scanner (UMAX Inc., Fremont, CA) and a Binuscan® Photoperfect software package (Binuscan Inc., New York, NY). The images were saved as TIFF-type files (*.tif, Microsoft Co. and Aldus Co., New York, NY) and then quantified by the TINA 2.0 densitometric analytical system (Raytest Isotopenmeβ gerate GmbH, Straubenhardt, Germany) according to the manufacturer's instruction. After normalisation to the densitometric values of 28S, the lowest densitometric value was designated as 1 and other values were expressed as the fold of the lowest one.

2.5. Reverse transcription and real-time quantitative PCR (QT-PCR)

To remove genomic DNA, RNA samples were treated using RNase-free DNase I (Roche Diagnostics, GmbH, Germany) for 30 min at 37 °C followed by heat inactivation at 75 °C for 5 min and stored at −80 °C. RNA was reverse transcribed (RT) (including a control without reverse transcriptase added) in a final volume of 50 μl containing 1× reverse transcriptase buffer (Invitrogen, Carlsbad, CA), 1.25 mM each dNTPs, 5 mM $MgCl_2$, 2U RNase inhibitor, 5 pmol/μl random Hexamer, 10 U reverse transcriptase and 1 μg RNA. The RT reaction was performed at room temperature overnight. *C-fos* and VEGF primer/probe sets were run on the same cDNA samples prepared. PCR reactions were performed using the ABI Prism 7700 Reaction Sequence Detection System (Pekin-Elmer Applied Biosystems) with a total volume of 50 μl reaction mixture containing 5 μl cDNA from RT reaction, 3 mM $MgCl_2$, 200 μM dNTPs, 300 nM primer, 100 nM probe, 200 U/μl Tag polymerase (Invitrogen, Carlsbad, CA). The PCR condition was as follows: 95 °C 1 min, 95 °C 12 s, 60 °C 1 min for 40 cycles. The primers and probes for the *c-fos* and VEGF were designed with the assistance of a computer program Primer Express (Pekin-Elmer Applied Biosystems, Foster City, CA). The sequences of rat *c-fos* and VEGF primers and probes were listed in Table 1. The amplicon sequences for *c-fos* and VEGF were examined by BLAST analysis (NCBI) and no significant homology with any other genes was observed. Both probes were labelled with FAM as a reporter.

To monitor potential DNA contamination, negative controls were included in every amplification run. They include a RT negative control (samples without reverse transcriptase in RT) and a control without template added during PCR.

Table 1
Primers and probes for real-time quantitative PCR assays

Transcripts	Taqman primers and probes	Accession number
c-fos	220(+) ACTACCATTCCCCAGCCG 291(−) GATCTGCGCAAAAGTCCTGT 246(+) FAM-TCCAGCATGGGCTCTCCTGTCA	X06769
VEGF	37(+) CCTGGCTTTACTGCTGTACCTC 106(−) CTGCTCCCCTTCTGTCGTG 61(+) FAM-CCATGCCAAGTGGTCCCAGGC	AF222779
18S	535(+) GAGGGAGCCTGAGAAACGG 602(−) GTCGGGAGTGGGTAATTTGC 555(+) FAM-TACCACATCCAAGGAAGGCAGCAGG	M10098

2.6. Statistical analysis

Results are given as the means ± S.E.M. and were tested using one-way analysis of variance and Duncan's new multiple range test to determine the significant differences between different experimental groups by StartView 4.15 statistic program (Abacus Concepts Inc., Berkeley, CA, USA). The *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Time course of induction of DNA synthesis

Incorporation of BrdU into dividing cells was used to assess uterine cell proliferation in OVX rat treated with E2 or the SERMs. Immunohistochemical detection of BrdU-positive cells in luminal (LE) and glandular epithelium (GE) at 16 h after a single dose of E2 or each of the SERMs is shown in Fig. 2. Specific staining with the anti-BrdU antibody was confined to the nucleus of the cells (Fig. 2). Quantitative analysis of BrdU-positive cells in uterus at 12, 16, 20 and 24 h after treatments revealed that DNA synthesis in LE (Fig. 3A) and GE (Fig. 3B) reached a maximum level at 16 h after E2 treatment. The stimulation of DNA synthesis after injection of any of the three SERMs also peaked at 16 h after treatment. The labelling index in LE (Fig. 3A) was much higher than that of GE (Fig. 3B) in E2 treated uterus whereas in SERM-treated uterus the labelling index in LE and GE was at the similar level. At 24 h after treatment, the labelling index in LE still remained high in E2-treated uterus, while it declined to the control level in SERM-treated uterus (Fig. 3A). Concomitantly, DNA synthesis in stromal cells started to increase at 24 h in SERM-treated uterus, whereas hardly any labelling was observed in E2-treated uterus (Fig. 4). Taken together, all the SERMs studied here were able to induce transient stimulation of DNA synthesis in uterine epithelial cells at 16 h. There was also an obvious increase of DNA synthesis in stromal cells at 24 h after SERM treatments.

3.2. Effect of combined treatment of SERMs with E2 on DNA synthesis and uterine weight

The time course studies revealed that maximum DNA synthesis in the OVX uterus was observed at 16 h after E2 and SERM treatments (Fig. 3A and B). Therefore, this time point was chosen to study the ability of SERMs to antagonise or synergize the E2 stimulation of DNA synthesis. The BrdU labelling index of cells in LE (Figs. 5A and 6) and GE (Figs. 5B and 6) at 16 h after treatment was significantly lower when any of the SERMs was given together with E2 than that when E2 was administered alone (Figs. 5 and 6). It is notable that the labelling index of cells in both LE and GE after combined treatments (E2+ SERM) was also significantly lower than after a SERM only (Fig. 5A and B). For example, when treated with ospemifene alone, the significant induction of DNA synthesis was observed in both LE (Fig. 5A) and GE (Fig. 5B) but the induction was almost abolished when ospemifene was combined with E2.

E2 and tamoxifen (Tam) treatments significantly increased relative uterine weights (uterine weight/body, weight $\times 10^5$), which were 141% ($P < 0.05$) and 138% ($P < 0.05$) of the weight of OVX control (100%), respectively (Fig. 7). No statistically significant change in

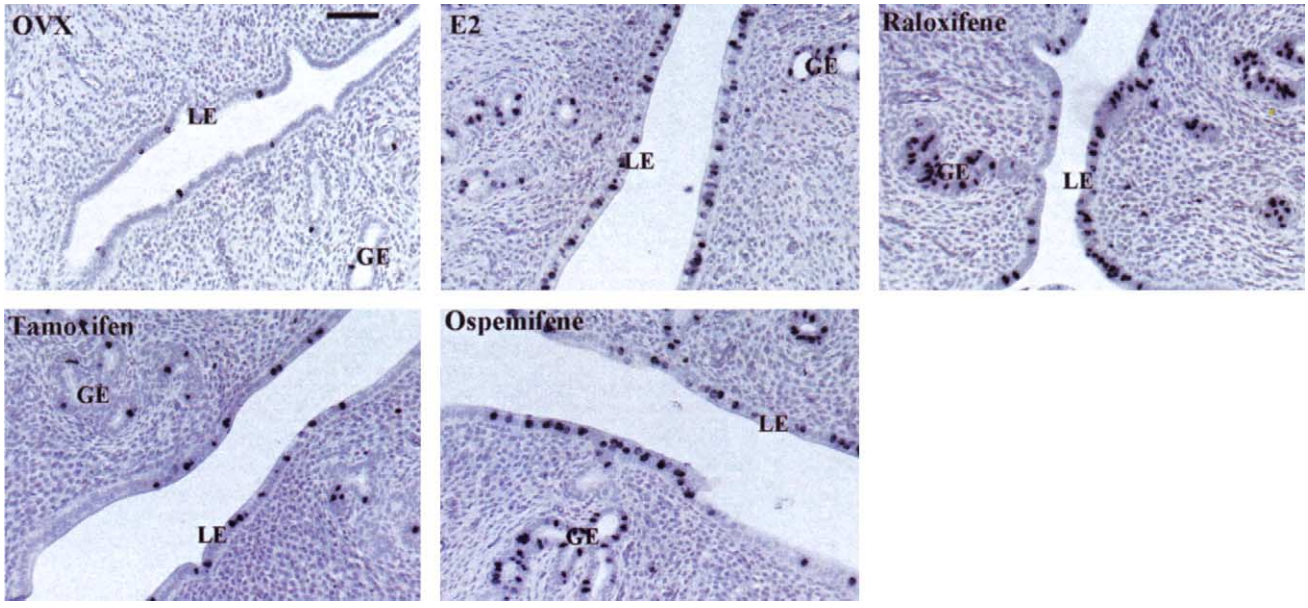


Fig. 2. The photomicrographs of BrdU-labelled cells in the uterus of OVX rat treated with E2, raloxifene, tamoxifen and ospemifene for 16 h. Luminal epithelium, LE; glandular epithelium, GE; bar = 10 μm.

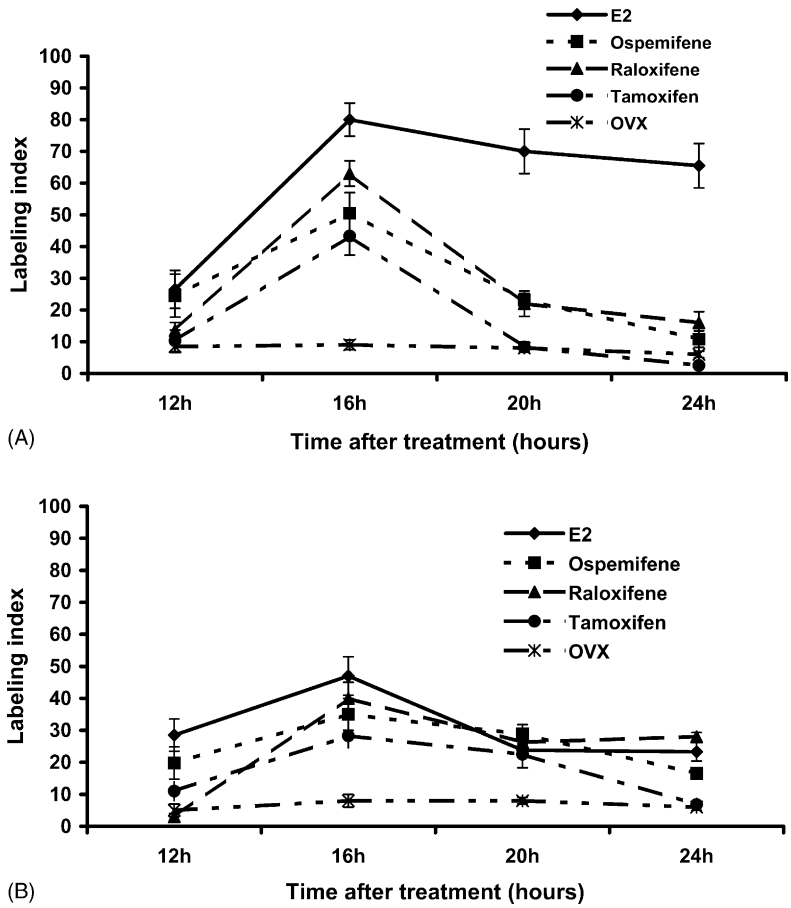


Fig. 3. The time course of induction of DNA synthesis by E2, raloxifene, tamoxifen and ospemifene in the LE (A) and GE (B) in the uterus of OVX rats. DNA synthesis was measured by counting the percentage of BrdU-labeled nuclei in LE and GE, respectively. The total number of nuclei calculated at random fields/slide was 500 at each category. Values are means ± S.E.M. ($n = 4$) at each time points.

the uterine weight was observed after raloxifene and ospemifene treatments (Fig. 7) indicating lower uterotrophic activity of these compounds. Raloxifene significantly inhibited the E2-stimulated increase of uterine weight (Fig. 7). No E2 antagonistic effects on uterine weight were observed after tamoxifen and ospemifene treatments (Fig. 7).

3.3. Induction of c-fos and VEGF mRNA

To estimate the ability of SERMs to evoke early gene responses known to be induced by E2, the mRNA levels of *c-fos* and VEGF were examined in the uterus of OVX rats at different time points after administration of E2 or any of the SERMs (Figs. 8 and 9). The Northern blot analyses showed that the uterine level of *c-fos* mRNA was low in control OVX rats and increased rapidly following an injection of E2 or the three SERMs (Fig. 8A and B) in a time-dependent manner. The level of *c-fos* mRNA showed a biphasic increase peaking approximately at 1–3 and 20 h after treatment with E2 or SERMs. E2 caused a robust induction of *c-fos* mRNA, with a maximum increase at 1 h. Another peak at 20 h was less prominent. In the uterus treated with raloxifene, tamoxifen or ospemifene, the first peak of *c-fos* mRNA induction was at 3 h. The second peak was also observed at 20 h after treatment was weaker and comparable to that observed after E2 administration. It is noteworthy that the maximum level of the *c-fos* mRNA (18-fold above the control value) after raloxifene treatment was higher than that seen after E2 (13-fold above control value), tamoxifen (13-fold above control value) or ospemifene (11-fold above control value) treatment. The level of *c-fos* mRNA after tamoxifen treatment remained elevated at 24 h (14-fold above control value), whereas that after E2, raloxifene or ospemifene treatment had declined.

The level of VEGF mRNA was not detectable in the uterus of OVX control rats but it was significantly increased after injections of E2 or any of the SERMs (Fig. 9). Two VEGF transcripts of approximately 3.7 and 4.5 kb were observed in the uterine RNA, as previously reported by Hyder et al. [22]. However, the relationship of these transcripts to the multiple forms of VEGF protein is presently unknown. The 3.7 kb transcript was quantified because it was the predominant form in our experiments. E2 caused a transient induction of VEGF mRNA, with a maximum level at 1 h (11-fold above the control values) and a decline to the control level by 12–24 h. Raloxifene caused a similar induction of VEGF

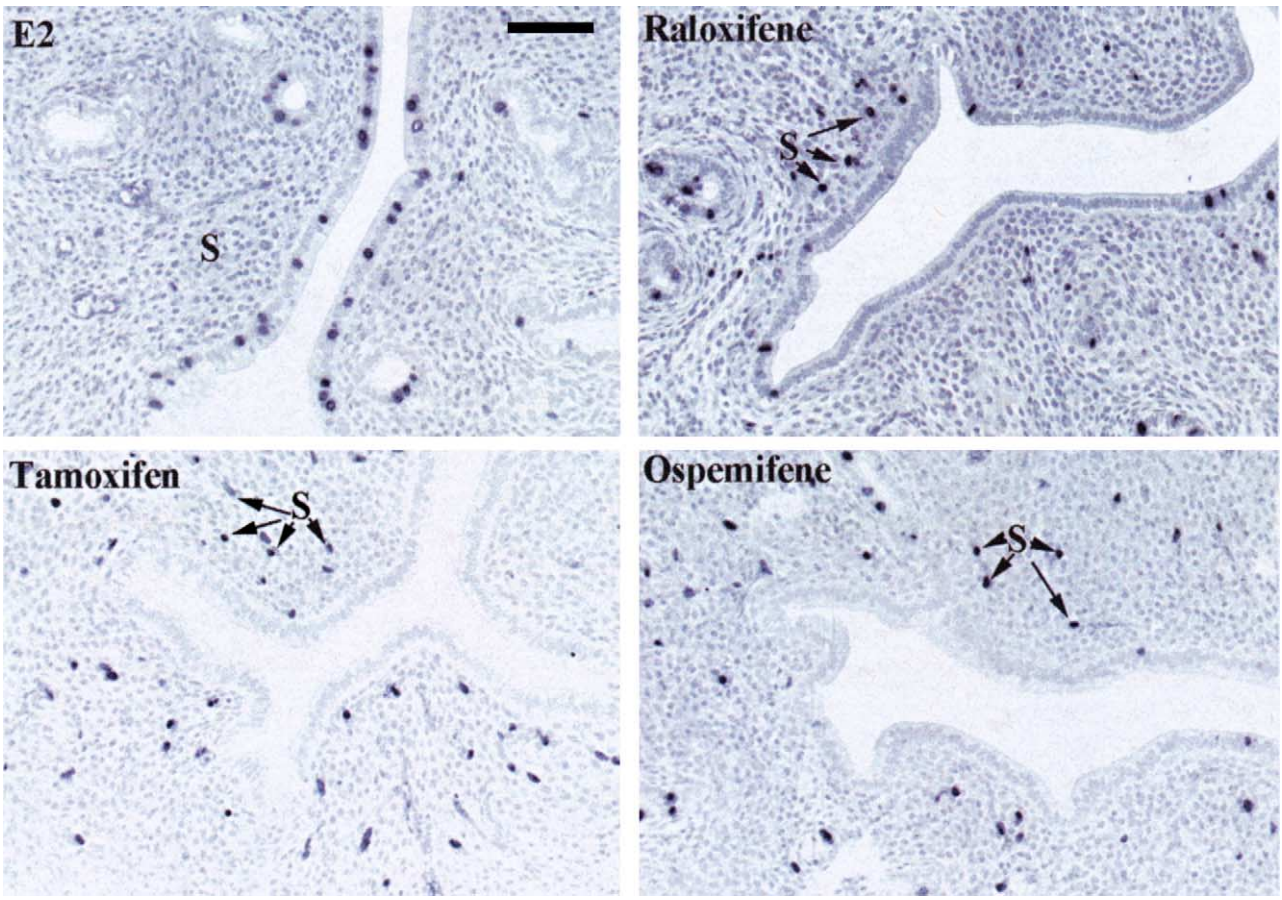


Fig. 4. The photomicrographs of BrdU-labeled cells in the uterus of OVX rat treated with E2, raloxifene, tamoxifen and ospemifene for 24 h. Stroma, S; bar = 10 μm.

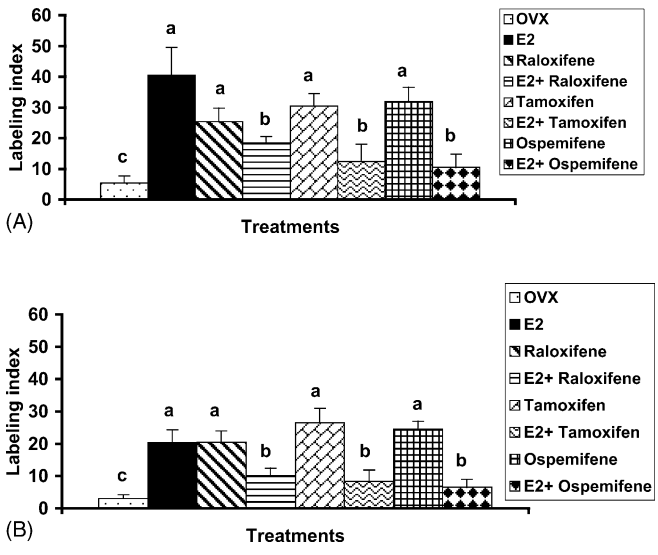


Fig. 5. DNA synthesis in the luminal (A) and glandular (B) epithelium of the uterus of ovariectomized rats treated with E2, raloxifene, tamoxifen or ospemifene with or without E2 for 16 h. DNA synthesis was measured by counting the percentage of BrdU-labeled nuclei. The total number of nuclei calculated at random fields/slide was 500 in each category. The columns are means ± S.E.M. ($n = 4$). Bars marked with different letters are significantly different ($P < 0.05$).

mRNA. The induction of VEGF mRNA by tamoxifen and ospemifene was slower than that by E2 and raloxifene. After tamoxifen or ospemifene treatment the maximum level was observed at 6 h. Interestingly, the level of VEGF mRNA (17-fold above control values) at 1 h after raloxifene administration was much higher than that seen after E2, tamoxifen (8-fold above control values) or ospemifene (4-fold above control values). Ospemifene increased expression of VEGF mRNA much less effectively than tamoxifen and raloxifene.

To more precisely quantify the changes of *c-fos* and VEGF mRNA levels, we performed QT-PCR assays (Fig. 10). Accurate quantification was achieved through generation of standard curves for *c-fos* and VEGF mRNAs. The standard curves for *c-fos* (Fig. 10A and B) and VEGF (Fig. 10C and D) were generated using the 1:10 (2.0×10^7–2.0×10^3 molecules) serially diluted solutions of the synthetic *c-fos* and VEGF single strand DNA amplicon in water containing 10 ng/μl yeast tRNA (Invitrogen, Carlsbad, CA). The amount of *c-fos* and VEGF mRNA expression were obtained from the threshold cycle (Ct) number at which a significant increase in fluorescent signal associated with an exponential increase of PCR products can be first detected using Applied Biosystems analysis software. Real-time PCR assays were

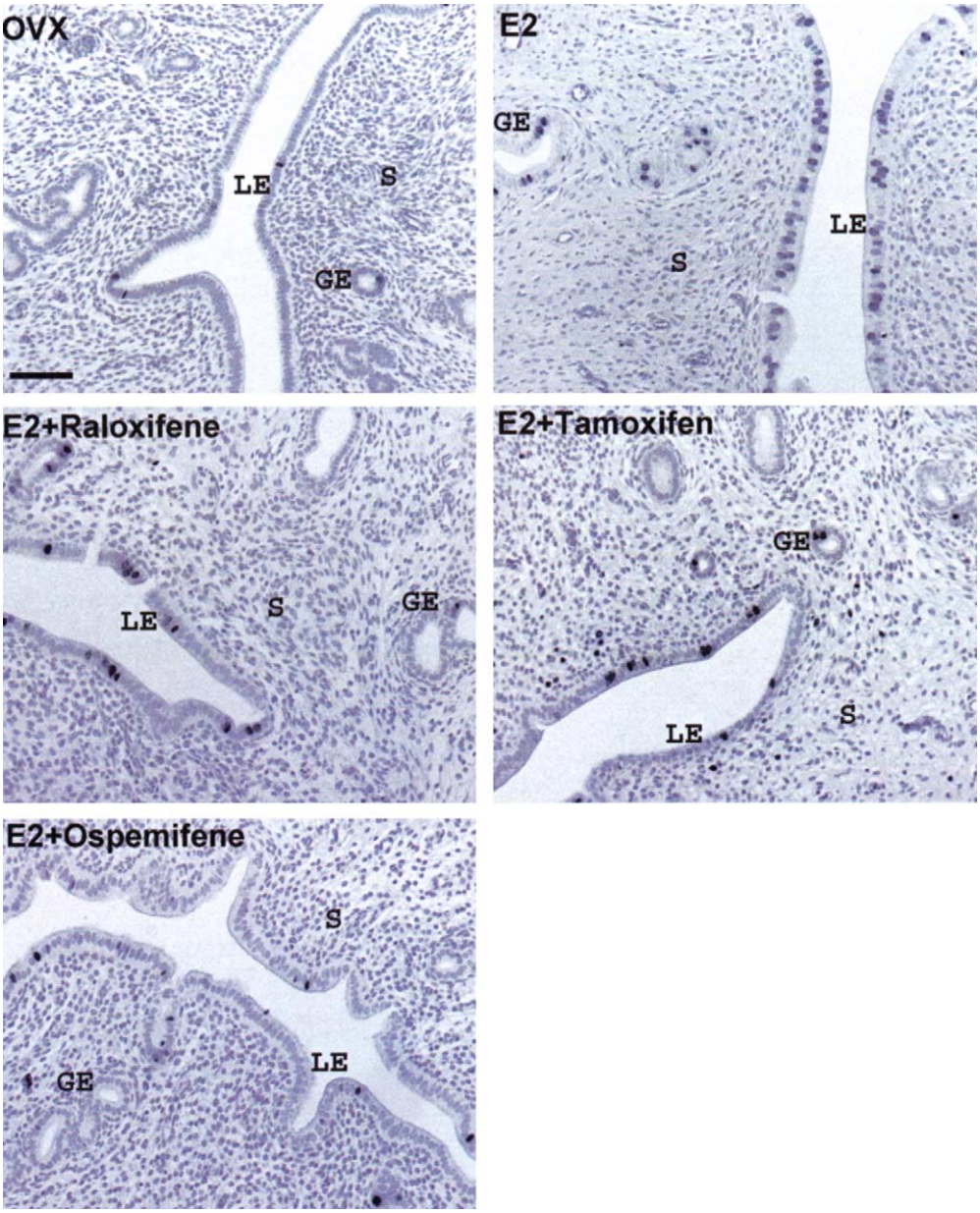


Fig. 6. Photomicrographs of BrdU-labeled cells in OVX rat uterus treated with E2 or E2 together with raloxifene, tamoxifen or ospemifene for 16 h. Luminal epithelium, LE; glandular epithelium, GE; stroma, S; bar = 10 μm.

conducted in triplicate for each sample, and mean value was used for calculation of the mRNA levels. To quantify the transcripts of *c-fos* and VEGF precisely, we used 18S ribosome RNA as an internal control.

The QT-PCR results (Fig. 10E and F) showed that the changes of levels of *c-fos* and VEGF mRNA following an injection of E2 or the three SERMs were in similar manners to that seen in Northern blot analyses (Figs. 8B and 9B). The levels of *c-fos* mRNA increased to the highest levels at 1–3 h after treatment with E2 or SERMs (Fig. 10E). The maximum levels of VEGF mRNA were observed at 1 h after raloxifene and 6 h after tamoxifen or ospemifene treatment (Fig. 10F).

4. Discussion

Estrogen induces a cascade of events that result in a synchronised wave of DNA synthesis and cell division in uterus [20,21]. In the mature ovariectomized rat, E2 treatment induces a maximum level of DNA synthesis 12–16 h after treatment, and mitosis follows at 18–24 h [33,34]. This is mediated by estrogen regulation of a specific genetic program that leads to recruitment of quiescent cells to cell cycle and progression from the G1 to the S phase [35]. Our data demonstrate that these SERMs studied similar to E2 could render uterine epithelial cells to progress to the S phase, suggesting that three SERMs exert estrogen-agonistic activity in

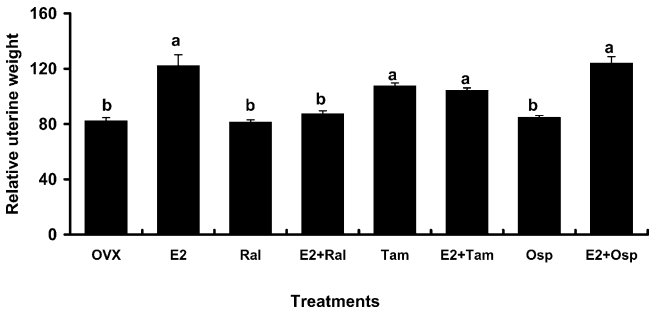


Fig. 7. Relative uterine weights in OVX rat treated with E2, or raloxifene, tamoxifen or ospemifene with or without E2 for 16 h. Bars represent means ± S.E.M. ($n = 4$). Bars with different letters are significantly different ($P < 0.05$).

the induction of DNA synthesis at an early time point. However, from 16 h onwards, DNA synthesis declined in luminal and glandular epithelium in SERM-treated OVX uterus comparing to E2. It seems that the sustained stimulation of DNA synthesis at 24 h (and probably later), as observed after E2, is correlated with increased epithelial cell number and a long-term stimulation of epithelial cell proliferation. When co-administered with E2, SERMs antagonised the stimulatory effects of E2 on DNA synthesis in epithelial cells, suggesting that the effect of SERMs is mediated by estrogen receptor and that they are also able to exert antagonistic activity. It is notable that E2 was also able to oppose the early stimulation of DNA synthesis by all of the SERMs studied here.

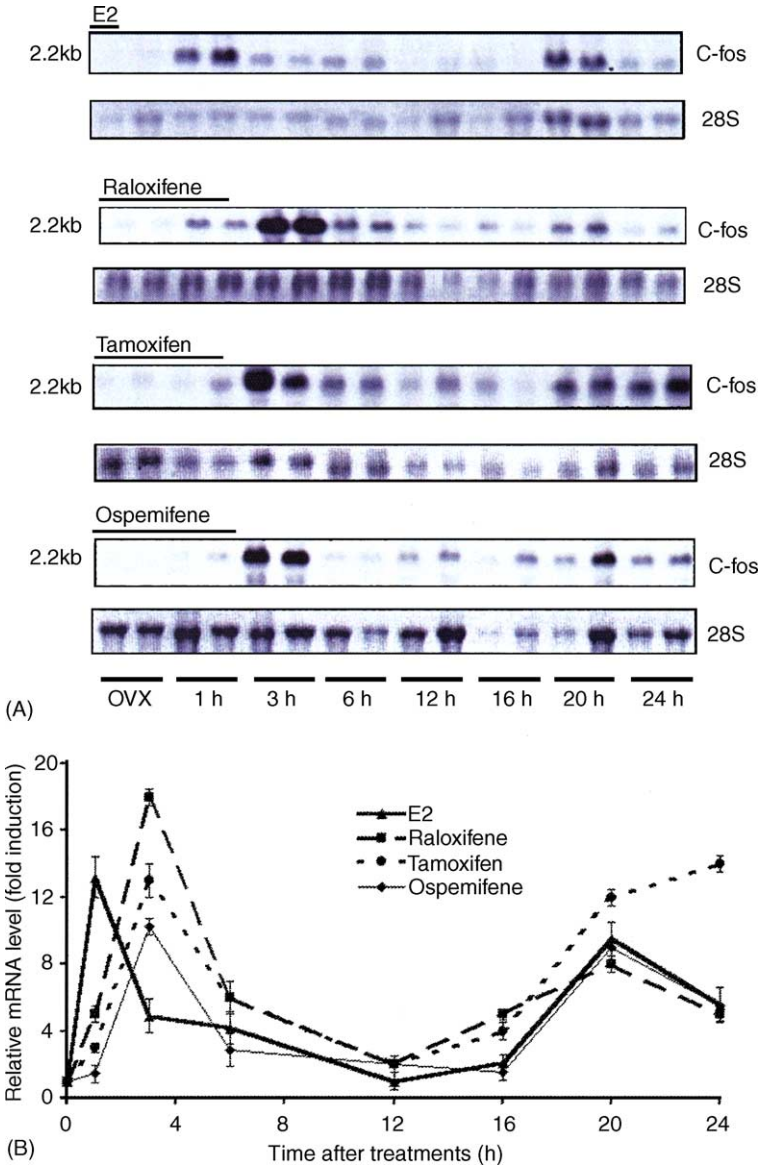


Fig. 8. Time-dependent induction of *c-fos* mRNA by E2, raloxifene, tamoxifen or ospemifene in the uterus of ovariectomized rats. (A) Uterine total RNA (10 μg/lane) was analysed by Northern blot. OVX rats were treated with E2, raloxifene, tamoxifen or ospemifene for 1, 3, 6, 12, 16, 20 and 24 h (four animals in each treatment group). Duplicated samples are shown in each treatment. 28S rRNA is used as control. (B) The signals for *c-fos* mRNA were normalised to 28S rRNA. The values representing means ± S.E.M. ($n = 4$) are expressed as fold induction over the OVX control.

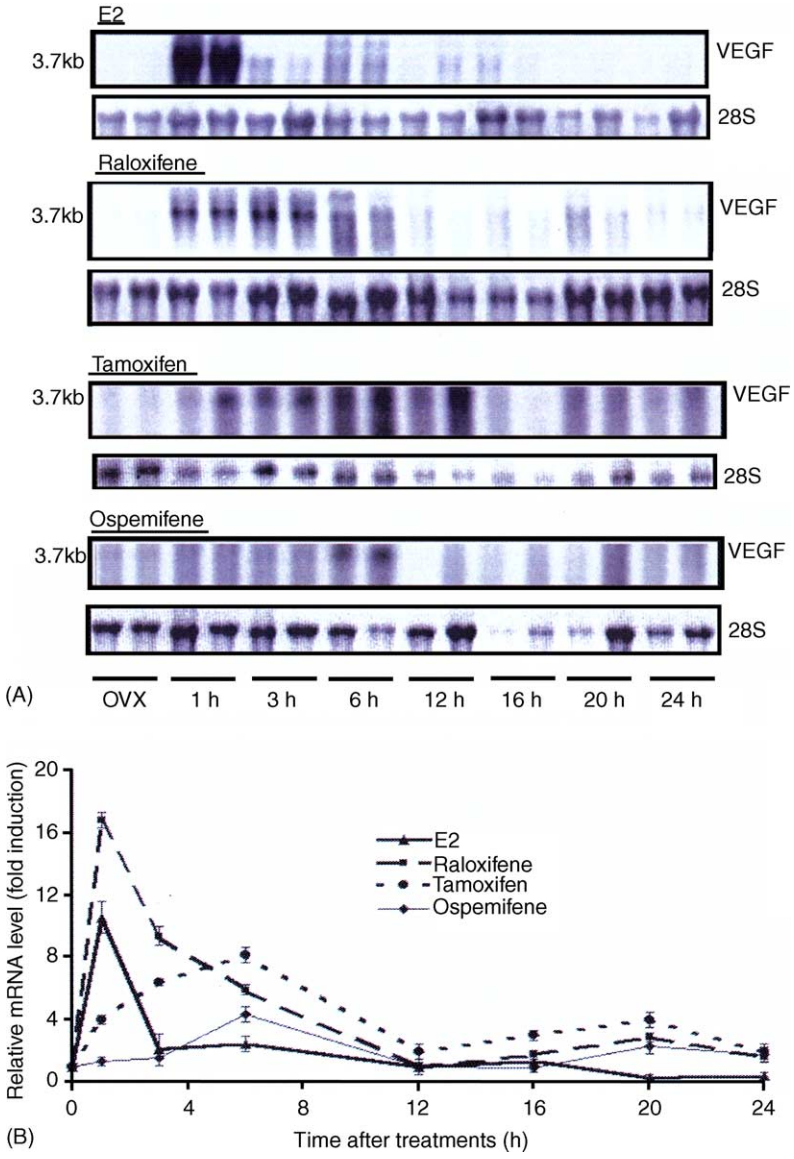


Fig. 9. Time-dependent induction of VEGF mRNA by E2, raloxifene, tamoxifen or ospemifene in the uterus of the ovariectomized rats. (A) Total uterine RNA (10 μg/lane) was analysed by Northern blot. OVX rats were treated with E2, raloxifene, tamoxifen or ospemifene for 1, 3, 6, 12, 16, 20 and 24 h. Duplicated samples are shown for each treatment. 28S rRNA is used as control. (B) The 3.7 kb VEGF mRNA was normalised to 28S rRNA. The values representing means ± S.E.M. ($n = 4$) are expressed as fold induction over the OVX controls.

Interestingly, BrdU-positive cells were observed in stromal compartment at 24 h after treatments of the three SERMs, but not after E2 treatment, although the labelling index in stromal cells is very low (data not shown). The stimulatory effect on stromal cell DNA synthesis may be unrelated to estrogen-like effect since no DNA synthesis was observed in stromal cells at the same time point after E2 treatment.

Although all three SERMs studied increased epithelial cell proliferation and antagonised E2-stimulated cell proliferation at 16 h after treatments, they seemed to differ in their ability to induce early weight increase and to antagonise the effect of E2 on uterine weight. The ability of ospemifene and raloxifene to stimulate DNA synthesis at 16 h was interesting considering their low estrogen-like effect on uterine weight at the same time point. Since epithelial cells have been estimated to represent only about 5% of total uterine cell population [36], epithelial DNA synthesis could have only a minor effect on uterine weight. It is thus more likely that the weight increase caused by tamoxifen at 16 h was mainly due to 'water imbibition', a rapid increase in vascular permeability observed after estrogenic stimulation [37].

The expression of *c-fos* and VEGF has been linked to the uterotrophic activity of estrogen and tamoxifen [22,38]. The induction of *c-fos* expression occurs primarily in epithelial cells [39–41] whereas VEGF is expressed in both epithelial [42] and stromal compartments [22,42]. The immediate early response gene *c-fos* together with jun encodes the AP-1 tran-

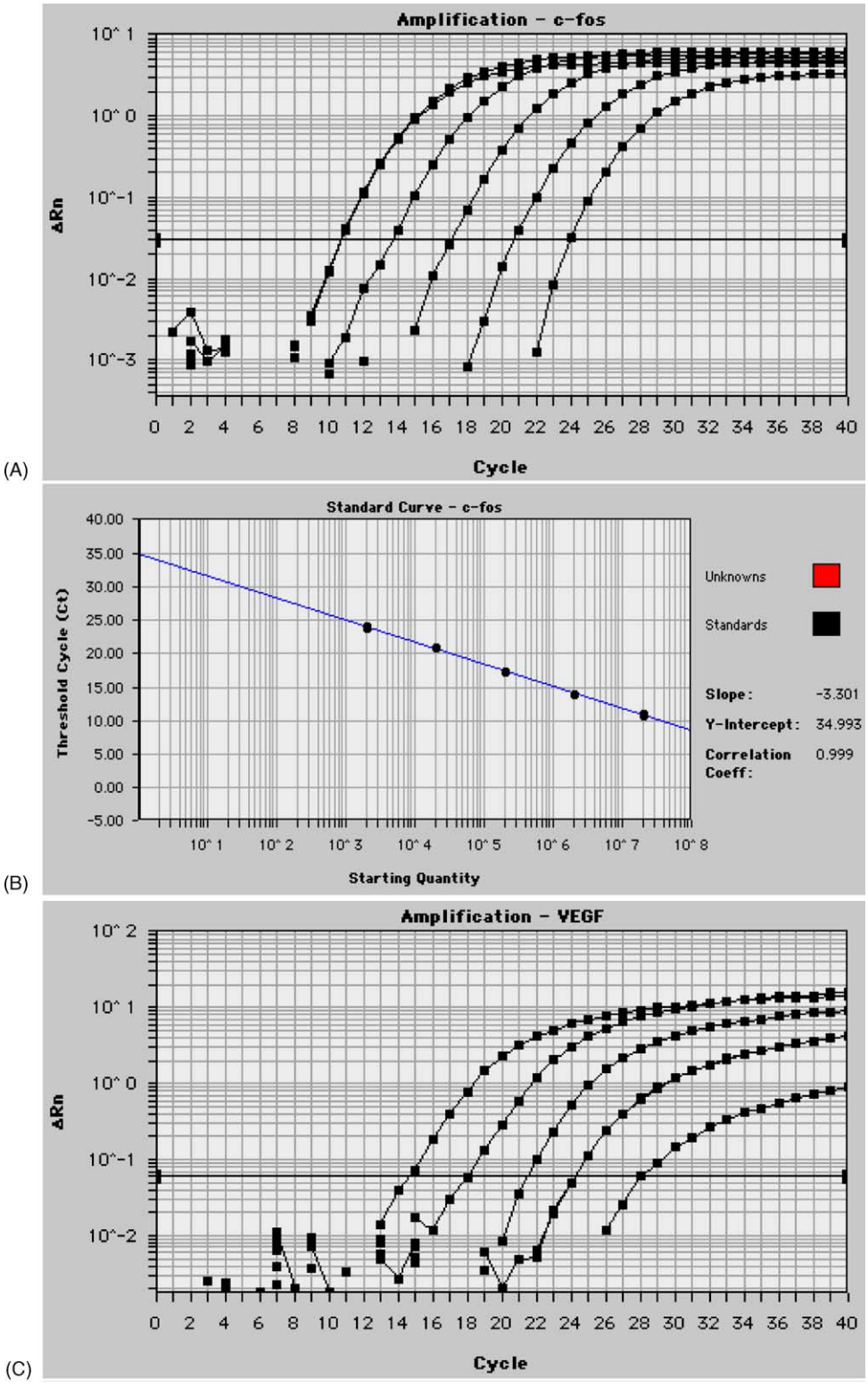
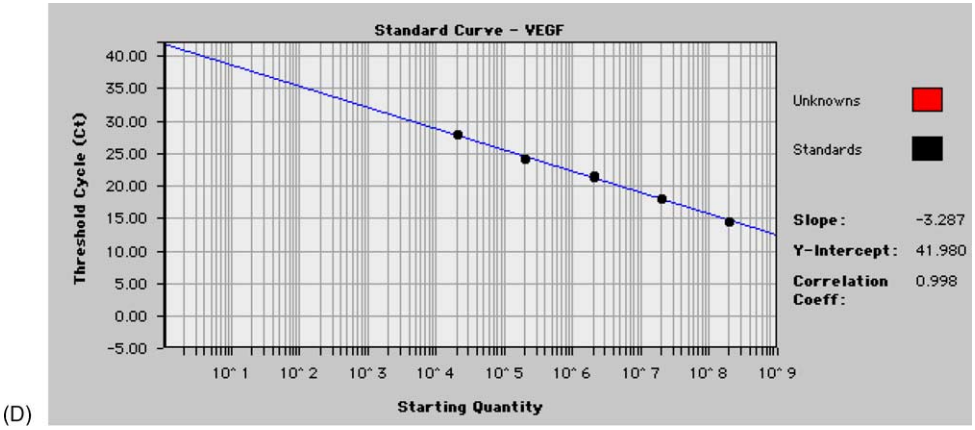
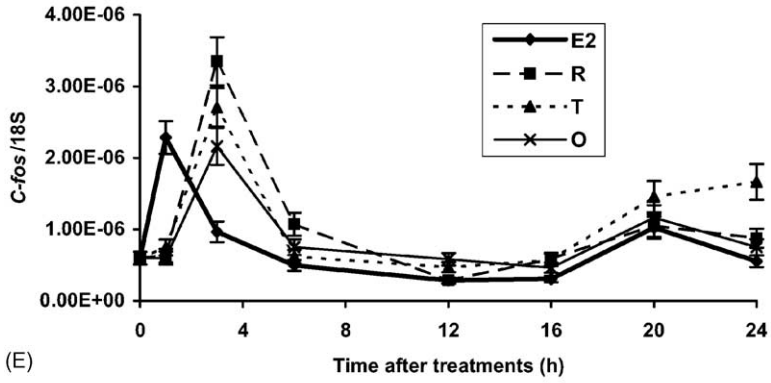


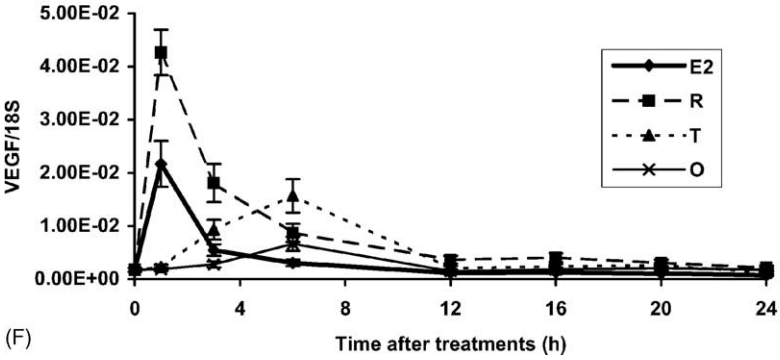
Fig. 10. Real-time quantitative PCR (QT-PCR) assays. (A) Amplification plots for *c-fos*. (B) *C-fos* standard curve plotting log starting copy number vs. Ct. (C) Amplification plots for VEGF. (D) VEGF standard curve plotting log starting copy number vs. Ct. (E) *C-fos* mRNA levels measured by QT-PCR in the uterus of ovariectomized rats at different time points after administration of E2, raloxifene, tamoxifen or ospemifene. The molecule numbers of *c-fos* in 100 ng total RNA were normalised to the corresponding values of 18S ribosome RNA. The values represent the mean ± S.E.M. in each time point ($n = 3$). (F) VEGF mRNA levels measured by QT-PCR in the uterus of ovariectomized rats at different time points after administration of E2, raloxifene, tamoxifen or ospemifene. The molecule numbers of VEGF in 100 ng total RNA were normalised to the corresponding values of 18S ribosome RNA. The values represent the mean ± S.E.M. in each time point ($n = 3$).



(D)



(E)



(F)

Fig. 10. (*Continued*).

scription factor, a key component of the signal transduction network that regulates cell proliferation and differentiation [43,44]. Our observation of a rapid E2 and tamoxifen induction of *c-fos* mRNA expression is in agreement with previous reports [17,38]. Interestingly, we found that ospemifene and raloxifene could also induce expression of *c-fos*. However, a long-term (10-day and longer) treatment with ospemifene or raloxifene had little effect on epithelial cell proliferation in uterus (our unpublished data) although *c-fos* mRNA expression remained upregulated (our unpublished data). This suggests that early induction of *c-fos* expression by SERMs is not necessarily associated with a subsequent stimulation of uterine growth and function. It is possible that *c-fos* is able to convert SERM signals to different signalling pathways. It has been shown that *c-fos* is a signal transducer, which may act either in transcriptional activation and repression [45].

All three SERMs and E2 caused a biphasic stimulation of *c-fos* mRNA within the first 24 h of treatment. This resembles the cyclic formation of ER transcription complex and the following cyclic transcription of target genes in the presence of E2 or the SERMs tamoxifen and raloxifene as reported previously [46].

One of the early events caused by estradiol is the increased vascular permeability and oedema in the endometrium [47]. This has been associated with the rapid induction of VEGF mRNA expression following administration of E2 in immature and OVX rats [48,49]. Hyder et al. have shown

that tamoxifen produces quantitatively similar increases in VEGF transcript levels as E2 [22].

The induction of VEGF expression by tamoxifen and novel triphenylethylene compound ospemifene in this study is in agreement with a previous study which demonstrates that the triphenylethylene class of antiestrogens is predominantly agonists for the induction of VEGF [50]. However, no uterine wet weight gain was observed after ospemifene treatment. Interestingly, we found that the benzophene class of antiestrogen raloxifene could also induce expression of VEGF, without increasing uterine wet weight in OVX rat at 16 h after treatment. Inhibitors in connective tissues may regulate in vivo angiogenic activity of VEGF since it was found recently that a connective tissue growth factor (CTGF) binds to VEGF and inhibits VEGF-induced angiogenesis [51]. It is possible the SERMs differentially regulates VEGF inhibitor expression and results in differential effects on uterine water imbibition and thus influences uterus weight gain. We would further explore this possibility. Three VEGF splice variants VEGF164, VEGF120 and VEGF188 have been found to be present in rat uterus [48], but the significance of these splice variants is not clear. It is also possible that different forms of VEGF are induced in rat uterus by different SERMs and they may have different biological functions.

SERMs, such as tamoxifen and raloxifene, act through interaction with the ER [1]. The novel SERM ospemifene binds to both ERα and ERβ with the affinity similar to that of tamoxifen [4]. The ER activate transcription not only via classical estrogen response elements (ERE) but also from various alternative response elements, such as AP-1, SP-1 and nuclear factor-κB (NF-κB) [52]. The presence of two ERs, ERα and ERβ, reveal that the mechanisms of action of estrogen and SERMs are more complex than previously thought. Tamoxifen activates AP-1 pathway for transcriptional activation and AP-1 proteins, such as Fos and Jun are needed for tamoxifen stimulation of uterus-derived endometrial cancer cells [53]. Recent studies have shown that SERMs may activate different signalling pathways by binding to ERα or ERβ. It has been shown that ERβ is a potent activator of AP-1 in the presence of SERMs, such as tamoxifen and raloxifene [54]. This mechanism could be involved in the estrogen-like agonistic effects of tamoxifen and the diverse effects of SERMs in uterus. We are currently investigating the signalling pathways of ospemifene.

In summary, we report here that a novel SERM ospemifene and two well-characterised SERMs tamoxifen and raloxifene are able to induce a rapid, even if transient stimulation of DNA synthesis and expression of two early response genes *c-fos* and VEGF in the uterus of ovariectomized rats. Administration of these SERMs together with E2 was able to antagonise E2-stimulated DNA synthesis. Similarly, E2 antagonised the effect of SERMs on DNA synthesis, both suggesting an estrogen receptor-mediated mechanism. Taken together, our experiments demonstrate that the activation of early response genes and transient stimulation of DNA synthesis are involved in the initial response of uterus to the SERMs tamoxifen, raloxifene and ospemifene, notwithstanding their very different long-term effects on uterus. However, it is possible that different molecular mechanisms underlie these early stimulatory events and eventually lead to diverging pathways. It is probable that after an initial stimulation a cascade of responses and interactions of various tissue compartments is required for development of SERM-specific effects on uterus.

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