Seasonal expressions of androgen receptor, estrogen receptors and cytochrome P450 aromatase in the uteri of the wild Daurian ground squirrels (*Spermophilus dauricus*)

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Abstract

The reproductive tissues including the uterus undergo dramatic changes in seasonal breeders from the breeding to non-breeding seasons. Classically, sex steroid hormones play important roles in the uterine morphology and functions. To clarify the relationship between sex steroid hormones and seasonal changes in the uterine morphology and functions, the wild Daurian ground squirrels (*Spermophilus dauricus*) were used as seasonal breeder model. And the immunolocalizations and expression levels of androgen receptor (AR), estrogen receptors α and β (ERα and ERβ) and cytochrome P450 aromatase (P450arom) were investigated in the uteri of the wild Daurian ground squirrels in the breeding (April) and the non-breeding (June) seasons *via* immunohistochemistry, Western blot and RT-PCR. Histologically, the uterine weight, the thickness of endometrium and the glandular density were significantly higher in the uteri of the breeding season than those of the non-breeding season. In both seasons, the immunostaining of AR was only presented in stromal cells of the uteri; the positive staining of ERα and ERβ were localized in stromal cells and glandular cells; P450arom was merely immunolocalized in glandular cells. The protein and mRNA expression levels of ERα, ERβ and P450arom were higher in the uteri of the breeding season than those of the non-breeding season; conversely, the expressions of AR were higher in the uteri of the non-breeding season comparing with those of the breeding season in both protein and mRNA levels. The AR: ER ratio in the uteri of the non-breeding season exceeded the AR:ER ratio in the uteri of the breeding season in the wild Daurian ground squirrels. These results suggested that seasonal changes in the expression levels of AR, ERs and P450arom might be correlated with the uterine morphology and histology changes, and estrogen may play an important autocrine/paracrine role in regulating the uterine functions of the wild Daurian ground squirrels.

Introduction

The uterus is one of the major components in female reproductive system, which is composed of endometrium and myometrium.[1] The endometrium, which consists of the luminal epithelial cells, the glandular cells and the stromal cells,[2-4] is the dominant functional unit of the uteri.[1] And the endometrium is a hormone-dependent multicellular tissue, whose compartments respond to hormonal cues in a coordinated spatial and temporal manner characterized by extensive cross-talk to regulate the development and homeostasis of the tissue.[2] During the estrous cycle and pregnancy, the uterus undergoes significant changes,[3,5] such as cell proliferation and differentiation to prepare the suitable environment for the development of fertilized oocyte and embryo.[1,2] Moreover, the annual changes in the uterine structure and functions are also observed in seasonal breeders including horses[6] and stray bitches.[7] The endometrium of seasonal breeders exhibits regeneration during the breeding season and degeneration during the non-breeding season.[1,8]

The endometrium is known to be one of the major target tissues of sex steroid hormones.[9,10] Sex steroid hormones including androgens and estrogens play key roles in the structural and functional changes of the endometrium in vertebrates.[5] Androgens and estrogens exert their physiological functions *via* androgen receptor (AR),[11-13] estrogen receptor α (ERα) and estrogen receptor β (ERβ)[10,14] respectively to mediate the signaling pathways.[4,9] After binding with the receptors, the ligand-receptor[12] complex enters the nuclei to regulate the expressions of target genes and influences a wide range of biological activities, for instance, the cell proliferation in the endometrium.[2,15] In addition, cytochrome P450 aromatase (P450arom) is the key limited enzyme in the procedure of estrogen synthesis,[14,16] which functions to aromatize androgens to estrogens.[4,11,17] P450arom is usually expressed in the gonadal tissue, for example, the granulosa cells in the ovary,[18] however, it is also found in extra-gonadal tissue, such as bones,[19] brains,[20-22] adipose tissues,[23] breasts,[24,25] skins[26] and uteri.[5,14,22] Extra-gonadal estrogens that are converted by P450arom from androgens exert important physiological functions in the local tissues, such as the role of local estrogen in brain sexual differentiation[20-22] and the protective effects on bones.[19]

The wild Daurian ground squirrel (*Spermophilus dauricus*) is a typical seasonal breeder, which has a strict and extremely compressed breeding season from April to May and a long period of non-breeding season from June to the following March.[8,20,21,27] In the wild Daurian ground squirrels, our previous studies have implicated that there were seasonal changes in the ovarian weight and follicular compositions and furthermore the circulating levels of estradiol-17β were also changed seasonally.[18] In a previous study of our group, there were statistical significant differences in the glandular nuclear numbers of endometrium between the breeding and non-breeding seasons.[8] The regulations of endometrium growth and regression were orchestrated by the growth factors and sex

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steroid hormones.[8] Our previous data has shown that the expressions of nerve growth factor (NGF) and its receptors TrkA and p75, which belonged to the family of proteins known as neurotrophins were found to be involved in the regulation of ovarian[28,29] and uterine[8] functional changes. In this study, we investigated the expression and distribution patterns of AR, ERs and P450arom in the uteri of the wild Daurian ground squirrels during the breeding and non-breeding seasons, in order to clarify the relationship between sex steroid hormones and seasonal changes in the uterine morphology and functions.

Materials and Methods

Animals

All the procedures on animals were carried out in accordance with the policy on the Care and Use of Animals by the Ethical Committee, Beijing Forestry University and approved by the Department of Agriculture of Hebei province, China (JNZF11/2007). The wild Daurian ground squirrels that were regarded as adults according to their body weights (242-412 g) were captured on April 19th 2017 in the breeding season (n=10) and on June 28th 2016 in the non-breeding season (n=11) in Hebei Province, China. The uterine samples were quickly removed and dissected, part of which were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 24 h, and then they were deposited in 70% ethanol for histological and immunohistochemical observation. Another part of uterine samples was dissected, snap frozen in liquid nitrogen and then kept in -80°C for protein and mRNA detections.

Histology

Uterine samples were dehydrated through ethanol-dimethylbenzene series and embedded in paraffin wax. Serial sections (5 μm) were mounted on poly-L-lysine (Sigma-Aldrich) coated slides. The sections were stained with hematoxylin-eosin (HE) for general histological observations. The number of uterine gland was assessed with NIH ImageJ software, with the method described by Kirby *et al*.[30]

Immunohistochemistry

Five-μm-thick serial sections made from 5 individuals' uterine tissues of each period were incubated with 10% normal goat serum to reduce background staining caused by the second antibody. The sections were then incubated with primary antibodies (1:200) raised against rabbit polyclonal anti-AR (sc-816, Santa Cruz Biotechnology Santa Cruz, CA, USA), rabbit polyclonal anti-ERα (sc-542, Santa Cruz Biotechnology), rabbit polyclonal anti-ERβ (sc-8974, Santa Cruz Biotechnology) or rabbit polyclonal anti-aromatase (ab18995, Abcam, Cambridge, UK) for 12 h under 4°C. The sections were then incubated with a secondary antibody, goat anti-rabbit lgG conjugated with biotin and peroxidase with avidin, using rabbit ExtrAvidin Peroxidase staining kit (Sigma) was performed, followed by visualizing with 30 mg 3,3-diaminobenzidine (Wako, Tokyo, Japan) solution in 150 mL of 0.05 M Tris-HCl buffer, pH 7.6, plus 30 μL H_2O_2. The specificity of AR, ERα, ERβ and P450arom antibodies has been described in our previous studies.[20,31] The control sections were treated with normal rabbit serum instead of the primary antisera.

Western blotting

Four individuals' uterine tissues of each period were diced into small pieces using a clean razor blade. The tissues were homogenized in a homogenizer containing 300 μL of 10 mg/mL PMSF stock and incubated on ice for 30 min while maintaining the temperature at 4°C throughout all the procedures. Homogenates were centrifuged at 12,000× g for 10 min at 4°C. Protein extracts (25 μg) were mixed with an equal volume of 2× Laemmli sample buffer. Equal amounts of each sample were loaded and ran on a 12% SDS-PAGE gel at 18 V/cm and transferred to nitrocellulose membranes using a wet transblotting apparatus (Bio-Rad, Richmond, CA, USA). The membranes were blocked in 3% BSA for 1 h at room temperature. Primary incubation of the membranes was carried out using a 1:500 dilution of rabbit anti-P450arom, anti-AR, anti-ERa or anti-ERβ antibody for 60 min. Secondary incubation of the membrane was then carried out using a 1:1000 dilution of goat anti-rabbit IgG tagged with horse radish peroxidase for 60 min. Finally, the membranes were colored with 25 mg 3, 3-diaminobenzidine (Wako) solution in 25 μL TBS-T buffer (0.02 M Tris, 0.137 M NaCl and 0.1% Tween-20, PH 7.6) plus 3 μl H_2O_2. β-actin was used for the endogenous control. Densitometric analysis of signals was quantified using Quantity One software (Version 4.5, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

RT-PCR

The first-strand cDNA from total RNA of 4 individuals' uteri of each period were synthesized using StarScript II Reverse Transcriptase and Oligo (dT)18 by TIANScript RT Kit (Tiangen, Beijing, China). The 20 μL of reaction mixture contained 3 μg of total RNA, 1 μL of Oligo (dT)18, 1 μL of 10 mM deoxyribonucleoside triphosphate (dNTP), 4 μL of 250 mM Tris–HCl (pH 8.3), 375 mM KCl and 15 mM $MgCl_2$, 2 μL of 0.1 M dithiothreitol, 0.5 μL of RNase Inhibitor and 200 U of StarScript II enzyme. The 25 μL of reaction mixture contained 2 μL of first-strand cDNA, 0.5 μM each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 20 mM Tris–HCl (pH 8.4) and 2.5 U of Taq polymerase (Tiangen). The amplification was under the following condition: 94°C for 3 min for the initial denaturation of the RNA/cDNA hybrid, 35 cycles of 94°C for 30 s, 51°C for 30 s and 72°C for 1 min with a final extension of 10 min at 72°C. The first-strand cDNA was used for PCR amplification with the following primers (Table 1). The PCR product was electrophoresed in the 1% agarose gel and individual bands were visualized by ethidium bromide (EB) staining. The housekeeping gene Actb was selected as the endogenous control. The bands were quantified using Quantity One software and the related expressions relative to Actb were calculated.

Table 1. Oligonucleotide primers used for RT-PCR.

	Sequence of primer	Product size (bp)
AR	F: 5'TGTCTCTCGCCCAGTTCATT 3' R: 5'AACCAGCCCAGAAGATGACA 3'	172
ER	F: 5' TTATGGGGTCTGGTCCTGTG3' R: 5'CATCTCTCTGACGCTTGTGC3'	230
ER	F: 5' TCTGGGTGATTGCGAAGAGT3' R: 5' CCCCGAGATTGAGGACTTGT3'	215
P450arom	F: 5'ATTTGGCAGCAAACTTGGGT3' R: 5' CAGTCTGTCCAGGTGCCTTA 3'	185
Actb	F: 5' GACTCGTCGTACTCCTGCTT 3' R: 5' AAGACCTCTATGCCAACACC 3'	223

Statistical analysis

Statistical comparisons were made with Student's *t*-test. A value of P<0.05 was considered indication of statistical significance.

Results

The observations of the uterine morphological and histological features

Morphological and histological features of the uteri were observed in the wild Daurian ground squirrels during the breeding and non-breeding seasons (Figures 1 and 2). The uteri of ten wild Daurian ground squirrels captured on April were regarded as in the breeding season (Figure 1a). The uteri of eleven wild Daurian ground squirrels captured on June were regarded as in the non-breeding season (Figure 1b). The weights of the uteri were significantly changed during the breeding season (696±69 mg) and the non-breeding season (214±19 mg) (Figure 1c). Marked histological changes in the uteri were also observed in the breeding and non-breeding seasons (Figure 2 a,b). The thickness of uterine endometrium went through remarkable increment during the breeding season (433±97 μm) and atrophy during the non-breeding season (175±23 μm) (Figure 2c). And there were more uterine glands in breeding season (69±12 mm^{-2}), whereas fewer glands were observed in the uteri of non-breeding season (30±6 mm^{-2}) (Figure 2d).

Immunolocalizations of AR, ERα, ERβ and P450arom in the uteri

Representative sections for immunohistochemical localizations of AR, ERα, ERβ and P450arom were shown in Figure 3 and the results were summarized in Table 2. In both seasons, the immunostainings of AR were only present in stromal cells of the uteri with stronger positive staining during the non-breeding season (Figure 3 a,b); the positive signal of ERα and ERβ were localized in stromal cells and glandular cells and were stronger immunostained during the breeding season (Figure 3 c-f); P450arom was immunolocalized in glandular cells, which were stronger positive-stained during the breeding season (Figure 3 g,h). No immunostaining was detected in negative control sections when normal rabbit serum was substituted for the primary antibody (Figure 3 i,j).

Expressions of AR, ERα, ERβ and P450arom proteins

The immunoreactivities of AR, ERα,

Table 2. Relative abundance of target proteins in the wild Daurian ground squirrels' uteri during the breeding and non-breeding seasons.

	Breeding season		Non-breeding season	
	SC	GC	SC	GC
AR	+	-	++	-
ERα	++	+++	+	+
ERβ	++	++	+	+
P450arom	-	++	-	+

The immunohistochemical staining was determined as positive (+), strongly positive (++), very strongly positive (+++), and negative (-). Staining that was weak but higher than that of the control was set as positive (+). The highest intensity staining was set as very strongly positive (+++). A staining intensity between + and +++ was set as strongly positive (++). SC, stromal cell; GC, glandular cell.

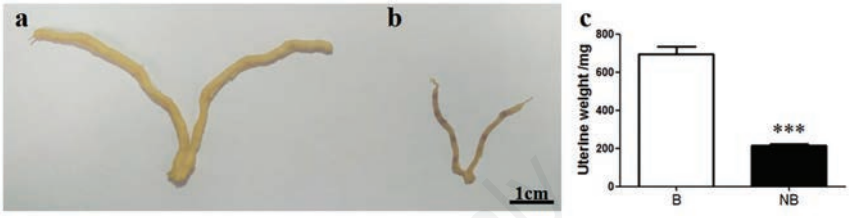

Figure 1. Anatomy and morphology of the uteri in the wild Daurian ground squirrels. Morphological features of uteri were observed in the wild Daurian ground squirrels during the breeding season (a) and the non-breeding season (b). The weight of uteri in the wild Daurian ground squirrels during the breeding season and the non-breeding season (c). Data were shown as the mean + SEM. ***P<0.001.

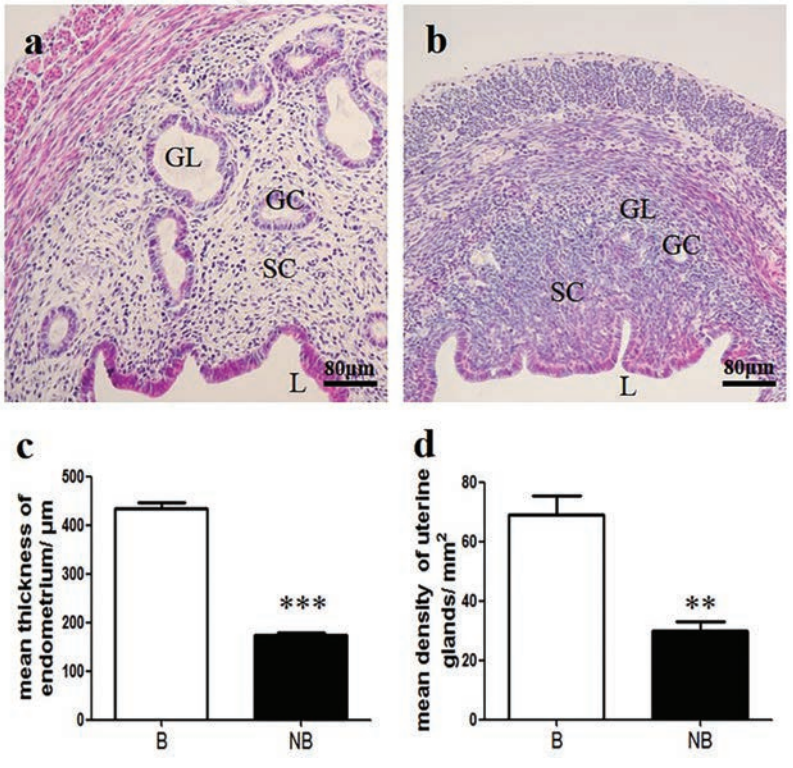

Figure 2. Histological structure of the wild Daurian ground squirrels' uteri by hematoxylin-eosin (HE). The myometrium and endometrium of uterine tissues were observed in the wild Daurian ground squirrels during the breeding (a) and non-breeding seasons (b). The mean thickness of endometrium in the wild Daurian ground squirrels during the breeding season and the non-breeding season (c). The mean density of uterine glands in the wild Daurian ground squirrels during the breeding season and the non-breeding season (d). In each period, n = 5. SC, stromal cell; GC, glandular cell; GL, glandular lumen; L, uterine lumen. Data were shown as the mean + SEM. **P<0.01; ***P<0.001.

ERβ and P450arom proteins positioned at 101 kDa, 66 kDa, 59 kDa and 55 kDa in different seasons, respectively (Figure 4). The results were normalized to the expression levels of β-actin and the water was used as a negative control (Figure 4, lane NC). While AR expression levels in the breeding season was significantly lower than the non-breeding season (Figure 4a), the expression of ERα, ERβ and P450arom in the breeding season were significantly higher than the non-breeding season (Figure 4 c-d).

Expressions of AR, ERα, ERβ and P450arom mRNA

AR, ERα, ERβ and P450arom mRNA levels were also detected in the uteri of the wild Daurian ground squirrels during the breeding and non-breeding seasons (Figure 5). The results were normalized to β-actin. A negative control, where water was substituted for the reverse transcriptase during cDNA synthesis, showed no DNA contamination (Figure 5, lane NC). Consistent with the protein levels, AR mRNA levels in the breeding season was significantly lower than in the non-breeding season (Figure 5a), while ERα, ERβ and P450arom mRNA levels peaked significantly in the breeding season, markedly dropped in the non-breeding season (Figure 5 c-d).

Ratio of AR to ERs

The ratio of AR to ERs was calculated on the basis of their mRNA levels in the uteri of the wild Daurian ground squirrels (Figure 6). Both the ratios of AR to ERα (Figure. 6a) and AR to ERβ (Figure 6b) were significantly high in the non-breeding season compared to the breeding season.

Discussion

This was the first study to investigate the expression patterns of AR, ERα, ERβ and P450arom in the uteri of the wild Daurian ground squirrels, which clearly demonstrated the presence of AR, ERα, ERβ and P450arom in the uteri of this wild rodent during the breeding and non-breeding seasons. These findings strongly suggested that seasonal alterations in the expression levels of AR, ERs and P450arom might be correlated with uterine morphology and histology changes, and estrogen may play an important autocrine/paracrine role in regulating uterine functions of the wild Daurian ground squirrels.

In response to the seasonal changes in the environment, the animal developed various strategies to adapt to nature, and the

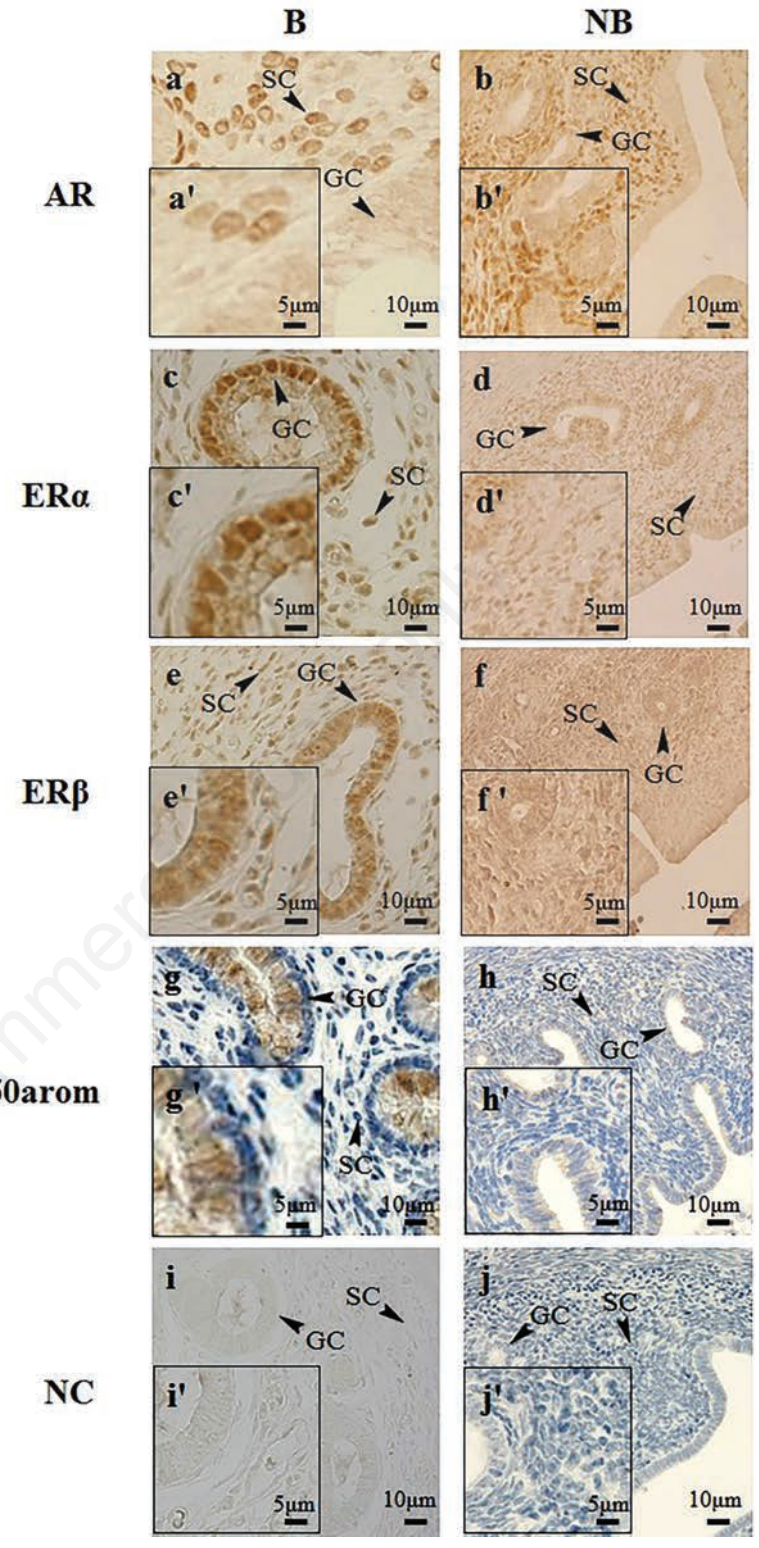

Figure 3. Immunohistochemistry of AR, ERα, ERβ and P450arom in the uteri of the wild Daurian ground squirrels during the breeding and non-breeding seasons. In the boxed area on the bottom left in x', immunoreactive cells were shown at higher magnification. The left column (a, c, e, g, i) represented staining in the breeding season, and the right column (b, d, f, h, j) represented immunostaining in the non-breeding season, respectively. The positive immunoactivities for AR (a,b), ERα (c,d) and ERβ (e,f) were observed in the nucleus, while the positive immunoactivities for aromatase (g,h) was in the cytoplasm. Negative controls (i,j) were counterstained with haematoxylin. In each period, n = 5. SC, stromal cell; GC, glandular cell.

annual changes in the growth and involution of organs were often observed the animals living in the temporal zone.[32,33] Size and weight of reproductive organs were generally related to their reproductive ability.[34] The seasonality of uterine mass shown in the present study was in agreement with extensive reports in this species,[8,27] and the present histological results showed that the uterine gland grew bigger, including nucleus growth and gland lumen extension in the breeding season, and the gland lumen shrunk remarkably and less nuclei were observed in the non-breeding season. These findings were similar to those observations in other seasonal breeders, such as possums and dunnarts.[3,35] In possums, the uteri underwent extensive morphological and histological changes during the estrous cycle. There was no embryonic diapause and both uteri are small and likely to be functionally dormant during anestrus, and the uteri in both pregnant and non-pregnant possums underwent significant linear weight increases to reach their maximum size early in the luteal phase.[35] Studies in the dunnarts has also shown that growth of the uterine wall and noted increased epithelial cell height were observed in the early stages of pregnancy.[36] Our previous studies have also indicated that plasma estradiol-17β and progesterone concentrations were significantly higher in the breeding season than those in the non-breeding season in this species.[8,18,27] Therefore, the present results suggested that seasonal changes of uterine morphology and histology in the wild Daurian ground squirrels might be dependent on ovarian steroid hormones cycles.

The endometrium is a major target tissue of ovarian steroid hormones. Proliferation, differentiation, and the cellular composition of endometrium depend on the rise and fall of circulating sex steroid hormones in normal ovulatory cycles.[9] It is well known that estrogens and androgens are essential for the regulation of growth and cell differentiation in the uteri.[13,36] The actions of androgens and estrogens are mediated *via* ERs and AR respectively, which belong to the superfamily of nuclear receptors.[37] The distribution of sex steroid hormone receptors in the different uterine cell types appears to be different among species. In this study, AR was only presented in stromal cells of the uteri, and ERα and ERβ were localized in stromal cells, luminal epithelial cells and glandular cells during the breeding and non-breeding seasons. These findings were in accordance with the views that differences in cellular distribution of sex steroid hormone receptors among species were probably associated with different roles in the uteri and indicated species specific reaction.[13] In the uteri, sex steroid action occurs *via* direct steroid hormone receptor signaling in target cells and indirect paracrine effects on neighboring cellular compartments.[2] Previous studies have reported that androgenic effects in the mouse uteri could be mediated *via* ERs signaling,[38,39] with extensive crosstalk between ERα and ERβ.[40] Moreover, study in the canine uteri have shown that the stroma and myometrium contain more cells that were positive for steroid receptors and their staining intensity was apparently greater, suggesting that these cell types were more sensitive to steroid hormones and were possibly more important in mediating hormonal signals to other uterine cell types.[41,42] The current results that ERα and ERβ expression levels were higher in the uteri of the breeding season than those of the non-breeding season although expression of AR were not different in the breeding and non-breeding seasons, which suggested that estrogen and androgen might play essential roles in modulating uterine cell proliferation, stromal-epithelial crosstalk and differentiation in preparation for pregnancy during the breeding season.

P450arom is the enzyme responsible for the conversion of androgens to estrogens. Although P450arom expression was detected mainly in the ovaries, other tissues have

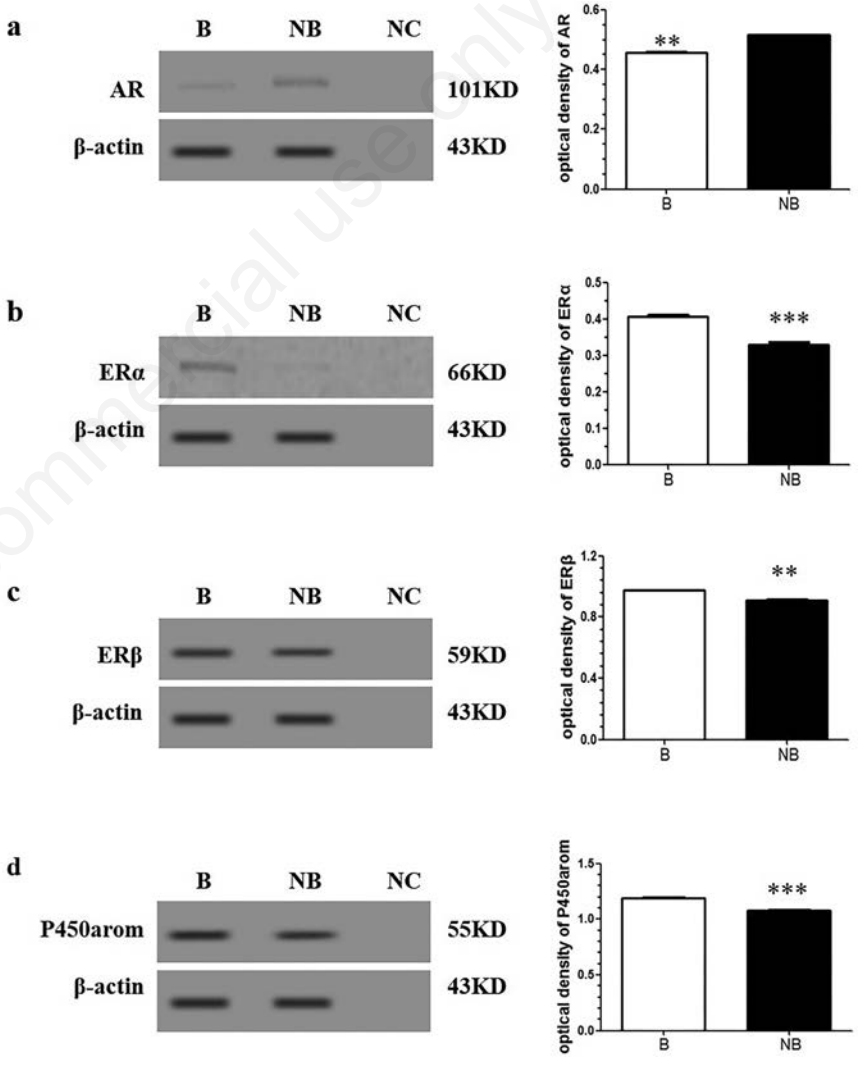

Figure 4. Western blot analysis of the protein levels of AR, ERα, ERβ and P450arom during the breeding and non-breeding seasons, respectively. β-actin blots were used as controls to correct for loading in each lane. The expression levels of AR (a), ERα (a), ERβ (c), P450 arom (d) in uteri of the wild Daurian ground squirrels in different seasons were determined by densitometric analysis. B, the breeding season; NB, the non-breeding season; NC, the negative control. In each period, n = 4. Data were shown as the mean + SEM. **P<0.01; ***P<0.001.

been shown to express this enzyme. The lines of evidence have reported the expression and cellular localization of P450arom mRNA and protein in eutopic endometrium.[43] In human, endometriotic stromal cells secreted estrogen and that this secretion could be increased by addition of testosterone to the media. Further, increased P450arom mRNA levels were confirmed in the endometriotic cell cultures and that this expression may be associated with epigenetic modifications of the P450arom gene.[44] Reduction of P450arom activity by P450arom inhibitors has been associated with reduced endometriotic lesion size in both animal models and in human studies.[43] For example, mouse model have shown a resolution of ectopic endometriotic lesions when P450arom was suppressed with inhibitors.[45] In this study, P450arom was immunolocalized in luminal epithelial cells and glandular cells, and the mean protein and mRNA levels of P450arom were significantly higher in the uteri of the breeding season than those of the non-breeding season. Together with ERα and ERβ local expressions in stromal cells, luminal epithelial cells and glandular cells, it suggested that the locally produced estrogen by the uteri might act in autocrine and/or paracrine manners *via* the interaction with locally expressed P450arom and might, therefore, mediate seasonal uterine functions of the wild Daurian ground squirrels.

Sex steroids have been shown to exert different effects in cell proliferation and apoptosis resulting from either acute or chronic stimulation.[46,47] It was interesting that there was no distinguishing change in the protein and mRNA levels of AR, however, the protein and mRNA levels of both ERs decreased markedly in the non-breeding season compared to the breeding season. In the present study, it was found in the wild Daurian ground squirrels that the AR: ER ratio in the uteri of the non-breeding season exceeded the AR: ER ratio in the uteri of the breeding season. Androgen might be predominantly converted into estrogen in order to regulate the follicular development *via* binding of ERs during the breeding season, whereas androgen might predominantly directly bind AR to regulate the follicular development during the non-breeding season in the ovaries of this species.[18] Therefore, the present results also implied that androgen might mainly directly bind AR to regulate uterine functions changes during the non-breeding season in the wild Daurian ground squirrels. Further studies are needed to investigate the circulating levels of androgens in the wild female Daurian ground squirrels during the

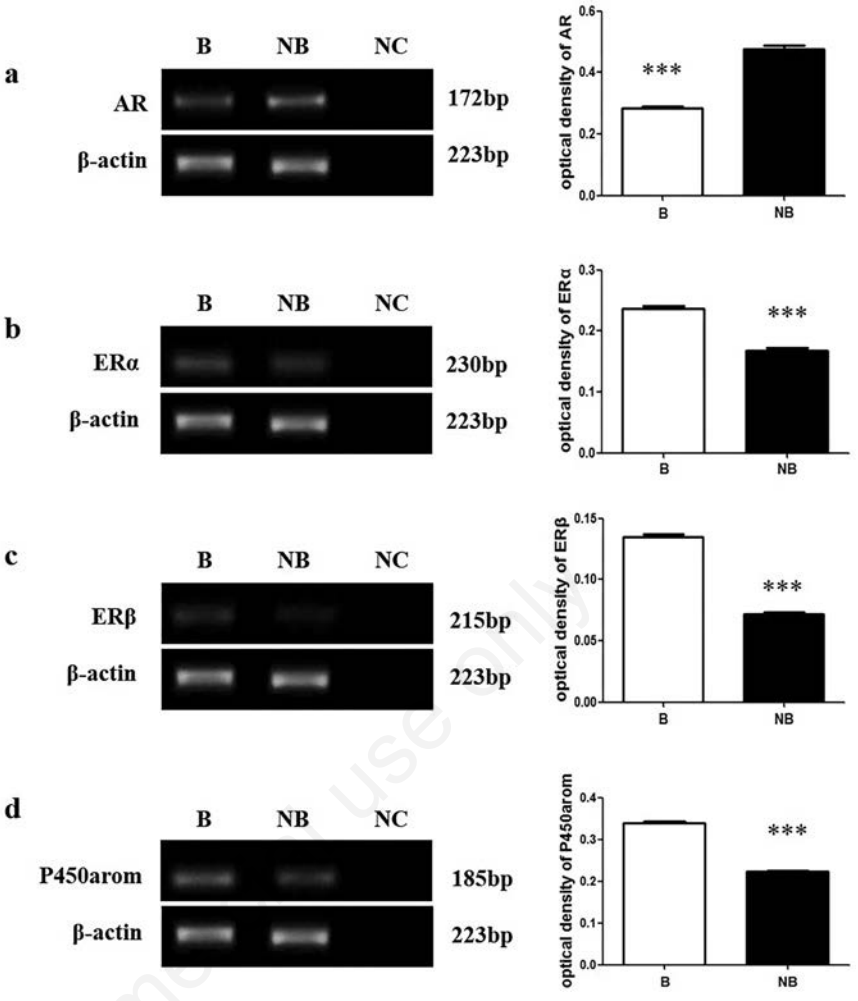

Figure 5. RT-PCR analysis of the mRNA levels of AR, ERα, ERβ and P450arom during the breeding and non-breeding seasons. The expressions of genes AR (a), ERα (b), ERβ (c) and CYP19 (d) showed the changes during the breeding and non-breeding seasons. B, the breeding season; NB, the non-breeding season; NC, the negative control. In each period, n = 4. Data were shown as the mean + SEM. ***P<0.001.

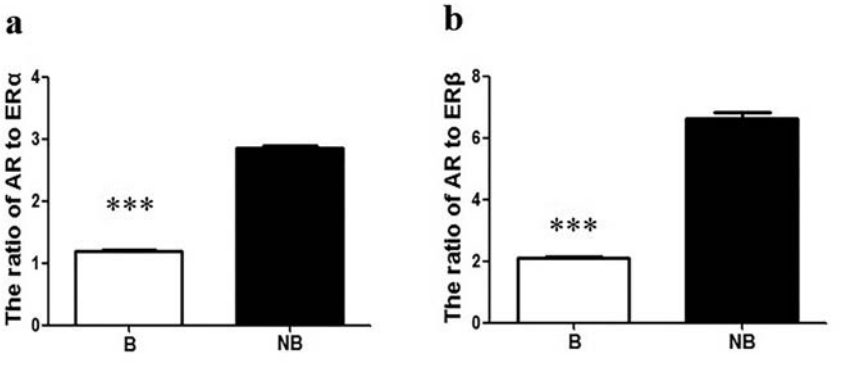

Figure 6. The ratio of AR to ERs in the uteri of the wild Daurian ground squirrels. The ratio of AR to ERα (a) and ERβ (b) in the uteri of wild Daurian ground squirrels according to the mRNA expression levels of AR and ERs. Data were shown as the mean + SEM. ***P<0.001.

breeding and non-breeding seasons in order to clarify the molecular mechanisms for androgens in the regulation of endometrial homeostasis.

In summary, the present results demonstrated that seasonal changes in uterine morphology and histology in the wild Daurian ground squirrels were correlated with changes in distribution of AR, P450arom and ERs during the breeding and non-breeding seasons. The data presented here will greatly aid the dissection of steroid hormones endocrine pathways in the wild Daurian ground squirrels, and population control of a fecund rodent species.

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