Effects of dehydroepiandrosterone, Premarin and Acolbifene on histomorphology and sex steroid receptors in the rat vagina

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Abstract

To assess the specific estrogenic and/or androgenic effects of a potential novel hormone replacement therapy, we have examined the morphology of the rat vagina 9 months after ovariectomy (OVX) and treatment of OVX animals with dehydroepiandrosterone (DHEA), conjugated estrogens Premarin and the selective estrogen receptor modulator Acolbifene. OVX led to atrophy and inflammatory changes while Acolbifene reduced the inflammation incidence and induced mucification of the vaginal epithelium. Premarin induced a typical keratinized stratified squamous epithelium while DHEA induced stimulation of the vaginal epithelium, with mucous cells typical of an androgenic effect, combined with increased collagen fiber compactness of the lamina propria. On the other hand, after OVX, the vaginal muscle layer decreased by 46%, an effect which was 41 and 100% reversed by DHEA and Premarin, respectively. The present data show particularly interesting effects of DHEA on the three layers of the vaginal wall, namely a highly mucified epithelium, an increased muscularis thickness and increased collagen fiber compactness in the lamina propria. DHEA exerts both androgenic and estrogenic effects on the vaginal mucosa, thus providing a more physiological replacement therapy.

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1. Introduction

Vaginal dryness affects about 50% of postmenopausal women at the age of 50–60 years and 72% after 70 years [1]. Of these women, about 80% experience urogenital disorders, especially vaginitis and dyspareunia [2]. Since these problems are believed to be at least partially related to the deprivation of sex steroids, appropriate local hormonal replacement therapy should be considered. In fact, postmenopausal women do not only lack all ovarian estrogens, but they are also progressively deprived of the androgens originating from the peripheral intracrine transformation of dehydroepiandrosterone (DHEA) into both androgens and estrogens [3–5]. In fact, serum DHEA and DHEA-S progressively decrease from the age of 30–50 years [5–7].

There is an increasing interest in the potential of combined estrogen–androgen replacement therapy [8,9], although the use of the estrogenic component is limited by the potential negative impact on breast cancer and cardiovascular events [10–12]. Based upon recent advances in our understanding of human sex steroid physiology, especially in postmenopausal women [3,5], the use of DHEA becomes, in this case, a possibility to provide with the appropriate levels of androgens and estrogens synthesized in specific tissues by intracrine mechanisms, while avoiding systemic effects [5,13–15].

The selective estrogen receptor modulator (SERM) Acolbifene (EM-652) is a benzopyran derivative originally developed for the prevention and treatment of breast cancer [16]. Acolbifene is the compound having the highest affinity of all known compounds for the estrogen receptor (ER) [16–18]. This compound displays a pure and highly potent antiestrogenic activity in the mammary gland and endometrium while decreasing serum cholesterol and triglycerides and...
preventing bone loss, at least in the rat [17]. On the other hand, the inhibitory effect of DHEA on the growth of human breast cancer xenografts in nude mice provides further support for its use in hormone replacement therapy [19,20]. In fact, combined treatment of DHEA and Acolbifene has been proposed as a beneficial chemopreventive and therapeutic approach in breast cancer [14]. It is possible that the combination of DHEA, Acolbifene and possibly an estrogen could provide optimal benefits for women at menopause. Recently, we found that the high potency of Acolbifene completely blocks the stimulatory effect of estradiol (E2) on the mammary gland and uterus in the rat and could thus avoid the risk of breast and uterine cancer [21].

In the present study, the ovariectomized (OVX) rat model was used to examine the effects of DHEA, Premarin and Acolbifene, alone or in combination, on the morphology and on the distribution of ERα, PR and AR after 9 months of treatment. Since the rodent adrenal does not secrete DHEA or DHEA-S [3,22], the percutaneous administration of DHEA in OVX animals, while preventing first pass of the orally administered steroid through the liver, is the only source of sex steroids in the model used, thus facilitating the interpretation of the data. As an estrogen, Premarin was chosen in this protocol because it is the most commonly used estrogen preparation in North America as hormone replacement therapy. In a previous study performed in our laboratory, the morphology observed following treatment of OVX rats with 17β-estradiol was identical to that seen with the use of Premarin.

2. Materials and methods

2.1. Animals and treatments

Ten to 12-week-old female Sprague–Dawley rats (Crl: CD®(SD)Br VAF/Plus®) (Charles River Laboratory, St-Constant, Canada) weighing approximately 220–270 g at the start of the experiment were used. The animals were acclimatized to the environmental conditions (temperature: 22 ± 3 °C; humidity: 50 ± 20%; 12-h light/12-h dark cycles, lights on at 07:15 h) for at least 1 week before starting the experiment. The animals were housed individually and were allowed free access to water and rodent food (Lab Diet 5002, Ralston Purina, St. Louis, MO). The experiment was conducted in accordance with the CCAC Guide for Care and Use of Experimental Animals in an animal facility approved by the Canadian Council on Animal Care (CCAC) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

A total of 126 female rats were randomly distributed into 9 groups of 14 animals each as follows: (1) intact control; (2) ovariectomized control; (3) OVX + Acolbifene (2.5 mg/kg); (4) OVX + Premarin (0.5 mg/kg); (5) OVX + Premarin + Acolbifene; (6) OVX + DHEA (80 mg/kg); (7) OVX + DHEA + Acolbifene; (8) OVX + DHEA + Premarin; (9) OVX + DHEA + Acolbifene + Premarin. On the first day of the study, the animals of all groups (except group one) were bilaterally ovariectomized under isoflurane-induced anesthesia. Premarin and Acolbifene were administered by oral gavage (0.5 mL/rat) as suspensions in 0.4% methylcellulose while DHEA was solubilized in 50% ethanol–50% propylene glycol and was topically applied (0.5 mL/rat) on a shaved area of 2 cm × 2 cm of the dorsal skin. Acolbifene was synthesized in the medicinal chemistry division of our laboratory, as described [16]. The compound had a purity of 99.2%. DHEA was obtained from Scheweizerhall Inc. at a purity of 100% while Premarin was a product of Wyeth Pharmaceuticals for intravenous human use containing 24.8 mg/vial (claimed for 25 mg) of total conjugated estrogens of which 54.4% were sodium estrone sulfate and 28.7% sodium equilin sulfate. At the DHEA administered dose, the DHEA blood level ranged between 70 and 100 nmol/L [7, and unpublished data]. Dosage selection for Premarin corresponds to the minimal dose sufficient to reverse OVX-induced uterine atrophy, while Acolbifene was administered at a dose sufficient to cause uterine atrophy similar to OVX after its administration to Premarin-treated OVX animals. Treatments were initiated on day 2 of the study and the compounds were administered once daily for 36 weeks. Animals from the intact and OVX control groups received the vehicle alone by oral gavage and topical application.

Twenty-four hours after the last dosing, overnight fasted animals were sacrificed under isoflurane anesthesia by exanguination at the abdominal aorta (nine animals per group) or by intracardiac perfusion with 10% neutral buffered formalin (five animals per group). Vaginae from non-perfused animals were collected and weighed, while the vaginae collected from perfused animals were marked with black ink on the ventral side and then trimmed as described below.

2.2. Histological procedures

The entire vagina of each perfused animal was postfixed in 10% neutral buffered formalin. Each vagina was then divided into seven equal cross-sections as illustrated in Fig. 1, routinely processed and embedded all together in the same paraffin block. Within the paraffin block, the seven vaginal cylindrical segments were positioned in a sequence corresponding to their original anatomical position and oriented perpendicular to the surface of the block, thus allowing the segments to be cut in cross-sections. For each animal, a 4-μm-thick paraffin section was cut and stained with haematoxylin–eosin for morphological examination.

2.3. Histomorphometry

Measurements of the different vaginal layers were performed on the fifth segment (Fig. 1), which is approximately halfway between the middle region and the portio vaginalis uteri (segment 7). This fifth segment was found to display a representative epithelial surface and a sufficient thickness...
Fig. 1. Division along the longitudinal axis of the rat vagina into seven cross-
segments, from the external orifice (ostium) (segment 1) to the cervix level
(segment 7) (portio vaginalis uteri) (modified from Popesko et al.[62]). Each
segment is about 3–4 mm long.

of smooth muscle. Images were captured with a DC-330
3CCD color camera (Dage-MTI, Michigan City, IN, USA)
and quantified using Image-Pro Plus 3.0 software (Media
Cybernetics, Silver Spring, MD, USA). Thus, using a 5×
objective (Leica Microsystems, Willowdale, Ont., Canada),
three to four thickness measurements per layer were obtained
from representative artifact free areas of the epithelium and
muscularis, as well as for the three vaginal layers together.
The thickness of the lamina propria was obtained by subtrac-
ting the thickness of the epithelium and muscularis from total
vaginal thickness.

2.4. Immunohistochemistry

Immunostaining was performed using Zymed SP kits
(San Francisco, CA). Paraffin sections (4 μm) were deparaf-
fined in toluene and rehydrated through ethanol. Endoge-
nous peroxidase activity was eliminated by preincubation
with 3% H2O2 in methanol for 30 min. A microwave
retrieval technique using 0.01 M citrate buffer for 15 min
was applied. After cooling the slides, non-specific bind-
ing was blocked using 10% goat serum for 20 min. Sec-
tions were then incubated for 1.5 h at room temperature
with ERα (AB-1, Calbiochem, CA), AR (N-20, Santa Cruz
Biotechnology, CA) or PR (Ab-4, NeoMarkers, CA) antibod-
ies, at 1:200, 1:250 and 1:250, respectively. After washing
in PBS buffer, sections were incubated with biotinylated
antirabbit secondary antibody for 10 min and thereafter with
streptavidin–peroxidase for another 10 min (Zymed, CA).
Diaminobenzidine was used as the chromogen to visual-
ize the biotin/streptavidin–peroxidase complex, under micro-
scope monitoring. Counterstaining was performed using #2
Gill’s hematoxylin for 30 s. For controls, immunabsorption
with an excess of the peptide used to raise the antibody, or
substitution with non-immune rabbit IgG, was performed.
Semi-quantitative evaluation of the number and intensity
of immunostained nuclei was performed as indicated in
Table 2.

2.5. Statistical analysis

Data are presented as means ± S.E.M. of eight to nine ani-
mals per group for vaginal weight or five animals per group
for vaginal layer thickness determinations. Statistical signif-
icance was determined according to the multiple-range test
of Duncan–Kramer [23].

3. Results

3.1. Morphology and thickness of the different layers of
rat vagina

To examine with precision the three layers of the rat
vaginal wall, namely the epithelium, lamina propria and mus-
cularis, seven segments obtained along the longitudinal axis
(Fig. 1) were first examined. While important morphologi-
cal differences were observed between the different groups,
in general, morphology was uniform in all animals of the
same group and segments 2–7 have shown similar epithelial
histological features. The few exceptions observed will be
mentioned later. Segment 5 was thus used to illustrate the
effect of the various treatments on the vaginal epithelium.

3.1.1. Epithelium

In intact animals at estrus, which represents the typ-
ical estrogenic pattern, a keratinized stratified squamous
epithelium was observed in all segments (Table 1; Fig. 2 A).
A similar epithelium characterized all segments of the
OVX + Premarin group (Fig. 2 E), with the exception of some
areas of large mucous cells in segments 2–5 (Table 1), a reac-
tion seen following long-term administration of an estrogenic
compound [24]. On the other hand, cycling rats at proestrus,
which are under a mixed estrogenic-progestational influ-
ence, show the presence of epithelial mucification (Fig. 2 B).
This effect is illustrated by a stratified squamous epithe-
lium covered by layers of mucified cells lining segments 2–7
(Table 1).

In the OVX group, the absence of ovarian stimulation led
to an atrophy of the vaginal epithelium, which characterized
all segments (Table 1; Fig. 2 C). Thus, this poorly stratified
Table 1

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E = epithelium morphology: KS (keratinized stratified squamous), LHM (large hypertrophied mucous cells), SCM (small aligned columnar or cuboidal mucous cells), A (atrophy: small cuboidal cells).
L = lamina propria thickness: T (thick), MT (moderately thick), t (thin).
Compactness of collagen fibers: H (high), M (moderate), L (low), M (muscularis thickness), T (thick), MT (moderately thick), t (thin), s (scarce).

epithelium consisted of one to two atrophied cuboidal or flattened squamous cell layer(s) with some small mucous cell areas, overlying a basa cell layer. Most importantly, moderate inflammation with many foci of intraepithelial microabcesses (small areas of agglomerated leucocytes within the epithelium) was a frequent finding. These changes were often accompanied by focal erosion (zones where the epithelial layer partially disappears) and ulceration (complete disappearance of the epithelial layer) (Fig. 3).

In OVX animals treated with Acolbifene, segment 1 showed a keratinized stratified squamous epithelium similar to that of the intact group, except that it included 9–11 cell layers compared to the 10–15 layers found in animals at estrus or when OVX animals received Premarin (Fig. 4). The epithelial thickness of Acolbifene-treated animals in segment 1 was thus higher than that of OVX rats, which comprised only four to six cell layers. In the other segments (Table 1), the basal layer was covered by a layer of low columnar mucous cells (Fig. 2D), which were more developed than in OVX animals. In the OVX + Acolbifene group, only three out of five animals showed signs of minimal inflammation while moderate inflammatory changes were general findings in all OVX animals. When the combination of Premarin and Acolbifene was used, the morphology was superimposable to that found in the OVX + Acolbifene group, namely the typical pattern of cylindrical mucous cells, more rarely seen in OVX animals (Table 1; Fig. 2F). No inflammation occurred in the group of animals receiving both Premarin and Acolbifene.

In all groups, segment 1 showed a comparable stratified squamous epithelium, except in the animals of the...
Fig. 2. Vaginal epithelium histomorphology of segment 5 in the nine groups of rats. Bar in (I) 40 μm. (A) As representative of the estrogenic effect, the stratified squamous epithelium of cycling rats at estrus consists of four main layers: one cell layer of stratum basale (b), six to seven cell layers of stratum spinosum (s) and a stratum granulosum of five to six layers (g) overlaid by the tightly packed flattened cornified cells that form the stratum corneum (c). (B) Cycling rats at proestrus, which are under an estrogenic-progestational influence, are used to illustrate mucification. In most segments 2–7, one basal cell layer (b) was overlaid by five to six cell layers of stratum spinosum (s) and a stratum mucification (m) consisting of three to four layers of mucous cells. (C) In the OVX control, a basal cell layer (b) was overlaid by one to two layer(s) of atrophic cuboidal or flattened cells (a). (D) The vaginal epithelium of OVX rats treated with a daily oral dose of Acolbifene (2.5 mg/kg) shows atrophy, but with an outer layer of low columnar mucous cells (m) overlying the basal cell layer (b). (E) Vaginal epithelium of OVX rats treated with a daily oral dose of Premarin (0.5 mg/kg). The OVX-induced atrophy was replaced by an estrogenic pattern similar to that found at estrus. (F) In OVX animals, which received Premarin + Acolbifene, atrophy predominated with a morphology similar to that of Acolbifene-treated animals, although larger mucous cells were seen. (G) Following treatment of OVX animals with a once daily cutaneous application of DHEA (80 mg/kg) on an area of 2 cm × 2 cm of the dorsal skin, an hypertrophic epithelium which consisted of three to five layers of mucous cells (m) was seen overlying a basal layer (b). Several invaginations characterized this epithelium (arrows). (H) In most areas of the vaginal epithelium of DHEA + Acolbifene-treated animals, a layer of mucous cells (m) rested on a basal cell layer (b), while in some areas, many layers of mucous cells overlaid the basal cell layer. Several invaginations characterized this epithelium (arrows). (I) Treatment with DHEA + Premarin led in three animals to a mixed epithelium composed of three to seven cell layer-thick stratified squamous epithelium (s) overlaid by three to five layers of mucous cells (m). In other two animals, areas of stratified squamous epithelium were predominant (insert). Bar (in insert) 30 μm. (J) When DHEA, Premarin and Acolbifene were combined, the epithelium was similar to that of the DHEA + Acolbifene group.
Fig. 3. Vaginal mucosa of OVX animals showing: (A) moderate inflammatory changes characterized by focal leukocyte infiltration with intraepithelial microabscess (IM) and (B) focal erosion (E) characterized by reduced epithelial thickness and ulceration (U) visualized as a complete disappearance of the epithelium. Bar in (B) 30 μm.

OVX group, where atrophy was a predominant characteristic (Fig. 4). Moreover, in the OVX + DHEA and OVX + DHEA + Acolbifene groups, mucous cells frequently accompanied the dominant stratified squamous epithelium of segment 1 (not shown).

The seven segments of the vaginal epithelium of OVX animals which received DHEA were composed of large multilayered columnar mucous cells with distended cytoplasmic vacuoles, a feature typical of an androgenic effect [25,26] (Table 1; Fig. 2G). Several large invaginations characterized the epithelium after DHEA treatment. A similar epithelial morphology was found in all segments of the OVX + DHEA + Acolbifene group, except that the number of cell layers decreased (Table 1; Fig. 2H). In the DHEA + Premarin-treated animals, a thick stratified epithelium of a “mixed” type composed of different ratios of squamous epithelium covered by layers of mucous cells (Table 1; Fig. 2I) was observed from the second to the fifth segment, thus revealing combined estrogenic and androgenic effects.

In this group, three animals displayed mucification over the above-mentioned segments, while in the other two animals, a stratified squamous epithelium was observed in all segments (insert Fig. 2I). When Premarin was combined with DHEA and Acolbifene, the epithelium was similar to that of the DHEA + Acolbifene group, thus indicating a blockade of the estrogen-induced squamous cell proliferation by Acolbifene (Table 1; Fig. 2J).

As illustrated in Fig. 5A, OVX markedly reduced the thickness of the vaginal epithelium (15 ± 1 μm) in comparison with that of the intact group (58 ± 5 μm). When animals of the OVX group received Acolbifene, a similar low value of 16 ± 1 μm was obtained. The epithelium thickness was restored to 76 ± 4 μm in the group of animals which received Premarin, a value significantly higher than that of the intact group, which included animals at different stages of the estrus cycle. When Acolbifene was added to Premarin, the epithelial thickness was reduced to 17 ± 1 μm, thus showing a complete blockade of the effect of the estrogen.

The administration of DHEA to OVX animals increased thickness of the vaginal epithelium to 38 ± 4 μm, while the addition of Acolbifene to DHEA did not change the epithelial thickness which remained at 37 ± 5 μm. When Premarin and DHEA were co-administered to OVX animals, a high thickness of 84 ± 6 μm was observed, a value not significantly different from that of the group of animals which received Premarin alone. Finally, the combination of Premarin, DHEA and Acolbifene resulted in an epithelium of 32 ± 3 μm-thick, a value similar to that of the groups,
which received DHEA alone or DHEA combined with Acolbifene.

3.1.2. Lamina propria

The degree of compactness of collagen fibers in the vagina was categorized as low, moderate and high (Fig. 6). In all the animals, there were relatively few fibrocytes in proportion to the amount of collagen. Careful examination of each animal (Table 1) reveals that, in general, the compactness of the collagen fibers in segment 1 is moderate (except low in rats at estrus and in Premarin-treated OVX animals or high in OVX and DHEA-treated OVX rats), while increasing in compactness in segments 2 and 3 to reach a plateau that generally remains constant until segment 7. Thus, along the longitudinal axis of the vagina in intact rats at estrus, compactness of the collagen fibers was low in segments 1–3 and moderate in segments 4–7. Atrophy was often associated with increased compactness of collagen fibers in the OVX and OVX + Acolbifene groups (Table 1). In Premarin-treated OVX animals, compactness in segment 1 was low
and moderate in segments 2 and 3, while in the other segments, compactness of the collagen fibers increased gradually to become high in segments 6 and 7.

In all segments, except the orifice area of the two estrogen predominant groups as described earlier, compactness of the collagen fibers could be described as moderate in intact rats at proestrus, in OVX animals treated with Premarin + Acolbifene or with Premarin + DHEA. On the other hand, most of the segments displayed highly compact collagen fibers in OVX animals treated with DHEA, DHEA + Acolbifene or DHEA + Premarin + Acolbifene. Light microscope examination of the lamina propria thickness along the vagina generally revealed that it was moderately thick in segment 1 while thinner in segments 2–4. In segments 5–7, the thickness increased progressively to a level similar to segment 1. The corresponding mean values of lamina propria thickness measured in segment 5 (Fig. 5B) indicate that OVX led to a significant decrease in lamina propria thickness (76 ± 2 μm versus 135 ± 28 μm in the intact group) with a non-significant decrease induced by Acolbifene (60 ± 8 μm). The increase observed after Premarin or Premarin + Acolbifene administration in OVX animals remained below the intact group (100 ± 9 and 90 ± 3 μm, respectively, versus 135 ± 28 μm). The administration of DHEA led also to a statistically non-significant increase in lamina propria thickness (96 ± 20 μm), and the addition of Acolbifene led to further increased thickness (136 ± 17 μm), a value similar to that of intact animals. Treatment of OVX animals with Premarin + DHEA significantly increased the thickness (144 ± 14 μm) when compared to DHEA alone. Finally, the animals treated with Premarin + DHEA + Acolbifene displayed a thickness (99 ± 9 μm) similar to that of the group treated with DHEA alone and lower than that of the Premarin + DHEA group, although the difference was not statistically significant.

3.1.3. Muscularis

In all groups, only a few smooth muscle fibers of the muscularis were found in segment 1, along with few irregularly arranged striated muscle fibers. Gradually, the thickness of the muscularis increased, remaining thin until segment 5, where an oblique smooth muscle layer was seen above the longitudinal fibers, thus resulting in a further thickness increase in segments 6 and 7 (Table 1). Measurement of muscularis thickness in segment 5 (Fig. 5C) revealed a value of 69 ± 7 μm in the intact group at estrus while a 46% significant decrease was observed 9 months after OVX (37 ± 2 μm). Acolbifene treatment did not change the muscularis thickness, which remained at 53 ± 3 μm.

On the other hand, Premarin treatment of OVX animals restored a thickness of 77 ± 5 μm, a value similar to that of the intact group while Acolbifene added to Premarin reversed this effect, thus resulting in a thickness of 43 ± 2 μm. DHEA induced a moderate increase in muscularis thickness (50 ± 2 μm), which was slightly decreased by the addition of Acolbifene (41 ± 3 μm). Finally, the combined treatment of Premarin and DHEA resulted in a notable thickness increase (62 ± 3 μm), when compared to animals treated with DHEA alone. When Acolbifene was added to Premarin and DHEA, muscularis thickness decreased (46 ± 5 μm) to a value comparable to DHEA and Acolbifene.

When total vaginal wall thickness was measured (Fig. 5D), the outer layer of connective tissue composing the adventitia was not included. OVX led to a marked (51%) vaginal wall atrophy (128 ± 3 μm versus 262 ± 39 μm in the intact) and the addition of Acolbifene to OVX had no effect (108 ± 8 μm) (Fig. 7A–C). Premarin treatment maintained the total thickness to a value (253 ± 10 μm) similar to that of intact animals while the addition of Acolbifene to Premarin reversed the effect of Premarin (150 ± 4 μm) (Fig. 7D and E, respectively). Total vaginal thickness achieved by DHEA treatment (184 ± 21 μm) was about 25% lower than that of the Premarin alone-treated group (Fig. 7F). On the other hand, the addition of Acolbifene to DHEA led to a non-significant thickness increase (213 ± 20 μm), which became non-significantly different from the intact group (Fig. 7G). Finally, co-administration of Premarin and DHEA to OVX animals markedly increased the thickness (290 ± 13 μm) to a value similar to that of intact animals (Fig. 7H). The addition of Acolbifene to DHEA and Premarin reversed the effect to a value (176 ± 11 μm) not significantly different from DHEA alone (Fig. 7I).

3.2. Vaginal weight

After 9 months of treatment, the changes observed in vaginal weight between the different groups (Fig. 8) generally follow the above-described morphological observations. Indeed, vaginal weight after OVX decreased by about 50% (101 ± 5 mg) compared to the intact group (205 ± 11 mg) while treatment of OVX animals with Acolbifene alone had no effect on vaginal weight. On the other hand, administration of Premarin led to a vaginal weight increase that did not reach the value of intact animals (170 ± 9 mg) while the addition of Acolbifene to Premarin reversed the estrogen-induced weight gain to a value similar to that of OVX animals (96 ± 4 mg). Conversely, when DHEA was given to OVX animals, vaginal weight increased to a value (171 ± 12 mg), similar to that of the Premarin-treated group, and the combination of Acolbifene and DHEA resulted in a decrease in weight (135 ± 9 mg), which remained above that of the OVX group. Finally, co-administration of Premarin and DHEA in OVX animals resulted in a vaginal weight gain (179 ± 10 mg) reaching a value similar to those of the OVX + DHEA and OVX + Premarin groups. The addition of Acolbifene to this combination had no significant effect (194 ± 12 mg).

3.3. Immunohistochemistry of steroid receptors

Examination of the seven vaginal cross-segments from individual animals revealed that, in most groups, the intensity
Fig. 7. Microphotographs of the three vaginal compartments: (E) epithelium, (L) lamina propria and (M) muscularis, at the level of the fifth segment of the rat vagina, with emphasis on the relative muscularis thickness in the different groups. Separation of the three vaginal wall layers with bars is indicated to best estimate the thickness distribution between the different groups: (A) intact; (B) OVX and OVX treated with (C) Acolbifene; (D) Premarin; (E) Premarin + Acolbifene; (F) DHEA; (G) DHEA + Acolbifene; (H) DHEA + Premarin; (I) DHEA + Premarin + Acolbifene. Bar in (I) 50 μm.

Fig. 8. Vaginal weight measured 36 weeks after OVX and treatment of OVX animals with Acolbifene, Premarin and DHEA alone or in combination. Intact animals are added as controls.

Fig. 9. Immunohistochemistry of AR, ERs and PR immunolabeling in the lamina propria and muscularis generally increased from segment 1 to the plateau of segments 5–7. Since segment 5 was previously chosen to compare morphology, the immunostaining results were semi-quantitatively evaluated in this segment, as shown in Fig. 9 and summarized in Table 2.

3.3.1. Androgen receptor

As seen in the upper row of Fig. 9 and summarized in Table 2, the vaginal wall of intact rats at estrus showed a moderate nuclear labeling in the epithelium, with the basal and the three suprabasal layers stained. A moderate number of strongly stained nuclei were seen in the lamina propria while a few weakly stained nuclei were observed in the muscularis. At diestrus, the basal and one suprabasal epithelial layer intensely stained for AR and a large number of nuclei in
Fig. 9. Immunohistochemical localization of AR, ERα and PR in the (E) epithelium, (L) lamina propria and (M) muscularis at the level of the fifth vaginal segment of the different groups. The thin bars indicate the approximate separation between the three vaginal compartments. Bar in lower row (right) 30 μm.
Table 2
Semi-quantitative evaluation of the number and intensity of immunostained nuclei for AR, ER alpha and PR in the epithelium, lamina propria and muscularis in the fifth segment of the rat vagina

<table>
<thead>
<tr>
<th>Group</th>
<th>AR</th>
<th>E</th>
<th>L</th>
<th>M</th>
<th>ERα</th>
<th>E</th>
<th>L</th>
<th>M</th>
<th>PR</th>
<th>E</th>
<th>L</th>
<th>M</th>
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<td>2 +++</td>
<td>1 +</td>
<td>1 +</td>
<td>1 +</td>
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<td>1 +++</td>
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<tr>
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</table>

Sex steroid receptors: AR, androgen receptor; ERα, estrogen receptor alpha; PR, progesterone receptor. Vaginal layers: E, epithelium; L, lamina propria; M, muscularis. Numbers represent the semi-quantitative evaluation of the number of labeled nuclei: 0, none; 1, low; 2, moderate; 3, high. Labeling intensity is indicated as (+) low; (++) moderate; (+++) high.

3.3.2. Estrogen receptor alpha

As presented in Table 2 and the middle row of Fig. 9, in intact rats at estrus, the epithelium revealed few weakly labeled nuclei in the basal cell layer while some weakly stained nuclei were detected in the lamina propria and many weakly stained nuclei were observed in the muscularis. On the other hand, at diestrus, basal and two suprabasal epithelial layers showed many strongly stained nuclei while very few nuclei were moderately stained in the lamina propria and weakly labeled in the muscularis.

The epithelium of OVX animals revealed a moderate number of variably stained nuclei, with weakly to strongly labeled areas and few strongly labeled nuclei in the lamina propria and muscularis. Treatment of OVX animals with Acolbifen eliminated ERα labeling from the three tissue layers. Meanwhile, treatment with Premarin led to strong to moderate staining of the basal cells, while the three suprabasal epithelial layers were weakly stained. Many moderately stained nuclei were found in the lamina propria and muscularis following Premarin. No staining could be detected when the combination of Premarin and Acolbifen was used.

The vaginal epithelium of DHEA-treated animals showed many strongly stained nuclei for ERα in the basal layer and a moderate scattered labeling in the superficial layers. Following DHEA treatment, the lamina propria and muscularis displayed a moderate number and many strongly stained nuclei, respectively. No staining was detected when the combination of DHEA and Acolbifen was used. In animals treated with DHEA and Premarin, the epithelial basal layer was moderately stained while a moderate number of nuclei in the suprabasal layers and some mucous cell nuclei were weakly labeled. Following this treatment, the lamina propria and muscularis showed a moderate and a high number of strongly stained nuclei, respectively. Combining Acolbifen to DHEA and Premarin led to the complete disappearance of ERα staining.

3.3.3. Progesterone receptor

Immunolabeling for PR (Table 2; lower row of Fig. 9) showed many strongly stained nuclei in all tissue compartments of the vagina in intact rats at estrus while such labeling was absent in the epithelium at diestrus, with the exception of cytoplasmic background. A moderate number of moderately...
stained nuclei were seen in the other two vaginal compartments in rats at diestrus. In the vagina of OVX animals and all groups of animals which received Acolbifene, there was no detectable PR labeling. Premarin-treated animals displayed a variable degree of staining with a moderate number of moderately to strongly stained nuclei in the epithelium and lamina propria and a high number of strongly labeled nuclei in the muscularis. The DHEA + Premarin group showed a staining pattern similar to that of Premarin alone, while DHEA-treated animals revealed few moderately stained nuclei only in the muscularis.

4. Discussion

Vaginal dryness or atrophic vaginitis, also referred to as urogenital atrophy, with sexual dysfunction is a common problem in postmenopausal women [27]. The most common symptoms are dryness, burning, pruritus, irritation and dyspareunia, thus leading to decreased libido and quality of life [28].

In the present study, a typical estrogenic effect reflected by a keratinized stratified squamous epithelium was observed in the rat vagina following treatment with the conjugated estrogen Premarin. In a previous study performed in our laboratory, the same morphology was observed following treatment with 17β-estradiol instead of Premarin. Moreover, the increased thickness and PR expression in the muscularis observed after Premarin treatment represent estrogenic effects, similar to the findings in intact animals at estrus. Interestingly, in the uterus of OVX mice treated with the antiestrogen Acolbifene, the atrophic changes were more pronounced in the myometrium [33], thus supporting that, in the absence of estrogen, cell proliferation in the smooth muscle is inhibited in the female rat reproductive tract.

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In the vaginal lamina propria of intact rats at estrus, Premarin-treated rats and, to a lesser extent, Premarin + DHEA-treated animals, low compactness of collagen fibers in the first segments could also be ascribed to an estrogenic effect. These data are in agreement with previous observations in rats and rabbits at estrus and after estradiol treatment following OVX [26,34–36]. Moreover, in postmenopausal women with stress incontinence, a 6-month estrogen treatment has been reported to lead to a significant decrease in total vaginal collagen [37].

In the potential triple combination, the equine estrogen Premarin is aimed at acting in the brain to relieve the vasomotor symptoms. In fact, the benefits of co-administration of the pure selective antiestrogen Acolbifene with an estrogen in order to neutralize the unwanted systemic effects of the estrogen have been well described by Labrie et al. [21].

In the present study, when OVX animals were treated with Acolbifene, the most typical morphological feature induced by the SERM was the appearance of a superficial layer of well-aligned small mucous cells overlying a basal cell layer. This pattern was slightly more pronounced in the Premarin + Acolbifene group and remained preponderant in all groups treated with Acolbifene, including when the SERM was combined with DHEA. Vaginal epithelium mucification has been reported in immature [38] and adult [39] rats under antiestrogen treatment. Although this morphological pattern has been compared to the progesterone-induced mucification [38], the molecular mechanism by which an antiestrogen induces epithelial mucification remains unknown.

The most spectacular effects of Acolbifene in OVX + Premarin rat vagina reside in a weight decrease to the level seen in OVX animals and an important thickness reduction of the epithelial and muscularis layers, as well as decreased total vaginal wall thickness, when compared to intact and Premarin-treated groups (Figs. 5 and 8). Such effects support the presence of a pure antiestrogenic action of Acolbifene on these parameters.

Contrary to its antiestrogenic actions mentioned above, in the rat vaginal orifice (segment 1), Acolbifene surprisingly displays an estrogen-like effect of epithelial proliferation and keratinization. Compared to the thin 4–6 layer-thick epithelium lining the same segment in OVX animals, the 9–11 layers of the cornified Acolbifene-induced epithelium is likely to provide a better resistance, as well as reduction in inflammatory incidence. The latter is supported by the observation of a reduced incidence of inflammatory changes, not only in the first segment but also throughout the vaginal mucosa of Acolbifene-treated OVX rats. At the internal vaginal level, the mucification induced by the SERM is likely to account for the reduced inflammation incidence.

Many beneficial effects of DHEA have been reported in postmenopausal women [5,13]. The morphological changes observed in the rat vagina after DHEA treatment reflect its intracrine conversion into active sex steroids having androgenic and/or estrogenic action through intracrine mechanisms [3]. Those changes comprise marked epithelial mucification, high compactness of delicate, finely woven lamina propria collagen fibers and moderate muscularis thickness increase when compared to OVX animals. The first and the second morphological changes are typical of androgenic effects while the third shows an estrogen-like activity, which is further supported by a concomitant increase in progesterone receptor expression in the muscularis layer.
In addition to the well-described epithelial squamous proliferation and maturation observed under estrogen treatment, it is well known that vaginal epithelial mucification occurs in the O VX rat in response to treatment with testosterone (Testo) or 5α-dihydrotestosterone (DHT) [40]. The vaginal morphology under Testo treatment has been described as "cuboidal to columnar mucified cells exhibiting a pseudostratified appearance at places, superficial vacuolation and occasional cornification in the top layer, with compact lamina propria and atrophic changes in the muscular layer and weight similar to that of intact rats" [26]. Since DHEA is transformed into either or both androgens and estrogens in peripheral tissues, the thick mucified multilayered epithelium observed in the present study after treatment of O VX animals with DHEA, suggests a predominant androgenic effect of mucification in the rat vagina, an effect which could mask any potential co-existing minor estrogenic effect at the epithelial level. A previous study has shown the same mucification effect in the rat vagina and the intravaginal application of DHEA achieved a significant effect at a dose 10× lower than that found to be active following application of DHEA on the dorsal skin [41].

The present data indicate co-existing major androgenic and minor estrogenic actions of DHEA in the rat vagina. Other studies using co-administration of DHEA and the antiandrogen Acolbifene or the antiandrogen FLU, respectively, have demonstrated that the action of DHEA in the rat mammary gland [42], skin sebaceous glands [43] and bone mineral density [44] is almost exclusively androgenic. Nevertheless, the presence of an estrogenic action of DHEA in the rat vagina has been previously demonstrated, either by the formation of a stratified epithelium in the vagina of rats treated with FLU and DHEA (unpublished data), or through induction of vaginal opening and precocious ovulation in immature rats treated with this compound, while DHT, an androgen not aromatizable to estrogens, did not produce such effects [45].

The capacity of rat vaginal tissue to aromatize androgens, especially, Testo, is likely to account for the major part of the estrogenic effect of DHEA in this organ [46]. Androst-5-ene-3β, 17β-diol (5-diol), a DHEA metabolite known to bind the estrogen receptor [47–50], could also contribute to the estrogenic effect [51]. The proposed combination of the antiandrogen Acolbifene with DHEA would thus prevent any unwanted stimulatory effect of 5-diol. In addition, prevention of bone loss would represent an additional benefit of Acolbifene [52].

To the best of our knowledge, no previous study has shown the stimulatory effects of DHEA on the compactness and morphology of the lamina propria’s collagen fibers and, to a lesser extent, on the muscularis. Such actions of DHEA-derived androgens and estrogens could have beneficial effects on vaginal function in postmenopausal women and possibly provide the substrate required for the action of inhibitors of type 5 phosphodiesterase, such as sildenafil or tadalafil, possibly via androgen- or estrogen-induced endothelial nitric oxide synthase (eNOS)-mediated facilitation of vaginal smooth muscle relaxation (reviewed in [53]). It is noteworthy that Acolbifene and DHEA have been found to induce eNOS in human and rat endothelial cells [54,55].

Finally, the combination of Acolbifene, DHEA and Premarin induced mucification of the vaginal epithelium through androgenic and antiestrogenic effects. These effects were reflected by invaginations of multilayered hypertrophied mucous cells in alternance with well-aligned mucous cells. The high compactness of fine collagen fibers found in the lamina propria is indicative of the androgenic action of DHEA, while the significant thickness reduction of the epithelial and muscularis layers, when compared to the Premarin + DHEA group, is indicative of the reversal by Acolbifene of the major and minor estrogenic actions of both Premarin and DHEA in these two vaginal layers, respectively.

While the antiestrogenic action of Acolbifene should protect the uterus and mammary gland against estrogen-induced cancer, and leads, in this study, to the formation of a consistent superficial mucous cell layer lining the rat vaginal lumen, the agonist-like action of Acolbifene gives rise to the unexpected formation of a keratinized squamous stratified epithelium at the vaginal ostium (estrogen-like effect). These two actions of Acolbifene were likely to bring protection against the observed inflammation in the O VX group. The mucifying action of Acolbifene is further increased by the mucification effect of the androgenic component of DHEA, thus potentially leading to an improved vaginal function, which is supported by the stimulatory effect of DHEA on the lamina propria.

The beneficial morphological changes, observed concomitantly with the strong modulation of rat vaginal AR by androgens, suggest that decreased serum levels of DHEA-derived androgens in postmenopausal women could contribute to the decreased vaginal health and eventually to the loss of libido and sexual enjoyment observed in this age group. Decreased serum total Testo, free Testo and DHEA-S were indeed found in women who consulted for decreased sexual desire [56].

The present data show low levels of ERs in the vaginal epithelium and lamina propria of rats at estrus, which are under high estrogen influence, and increased ERs levels in the epithelium of intact rats at diestrus, when estrogen levels are low. In addition, during the estrus cycle, ERs levels fluctuated more in the epithelium than in the lamina propria. These results are in agreement with those of other investigators [57], except that, in the lamina propria, they detected more (nearly 60%) ERs-immunolabeled nuclei throughout the rat estrus cycle than in the present study. Moreover, under the different hormonal treatments, ERs labeling displayed more variations in the number and intensity of stained nuclei in the epithelium than in the other two tissue layers. While in the Premarin, and DHEA + Premarin groups, the presence of estrogens appeared to up-regulate ERs expression, as already demonstrated in the mouse uterus [58], the existence of an ERs up-regulation in the absence or presence of low levels of estrogens was observed in O VX- and DHEA-treated animals,

respectively. As expected, treatment with Acocolbine completely eliminated ERs immunostaining in the whole vaginal wall and co-treatment with Premarin and/or DHEA did not change this response. As discussed earlier, a potential benefi
cial effect of the combination of Acocolbine and DHEA for postmenopausal women is that the binding of the antiestrogen to
ERs would prevent the estrogens produced from aromatiza
tion of DHEA-derived androgens and the metabolite 5-DIOL to bind ERs, thus potentially preventing proliferation of
ERs-sensitive breast cancers cell [59]. Various attempts have been made to solve the problem of vaginal dryness, often linked to dyspareunia and loss of
sexual enjoyment. As an example, local vaginal estrogen preparations are often prescribed to provide relief but the
endometrium may be stimulated by the unopposed estrogen [60]. In order to achieve a more physiological and tissue-
specific HRT, DHEA combined with an estrogen and with
a SERM, such as Acolbifene, which displays antiestrogenic
characteristics and is selective for postmenopausal women. In fact, to bind ERs, thus potentially preventing proliferation of
ERs-sensitive breast cancers cell [59].

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