Regulation of the vitamin D receptor and cornifin beta expression in vaginal epithelium of the rats through vitamin D_3

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The aim of the present study was to determine the respective role of 1,25-dihydroxyvitamin D_3 on vaginal epithelium and 1,25-dihydroxyvitamin D3 receptor expression in ovariectomized rats and vitamin D_3 treated rats. Bilateral ovariectomies were performed in 20 mature, non-pregnant Wistar female rats. All the animals were divided into 2 groups consisting of 10 rats each. Group I served as control. In group II, animals were injected intramuscularly with vitamin D3 (50, 00 IU/kg). Two weeks after the injections, vaginas of animals in group I and group II were removed removed and processed for immunohistochemistry. Epithelial differentiation, 1,25-dihydroxyvitamin D_3 receptor and cornifin β expression were investigated in vaginal epithelium of control group (ovariectomized) and vitamin D_3 treated rats. Vaginal epithelial cells from vitamin D_3 treated animals changed into highly- stratified keratinizing layers. 1,25-dihydroxyvitamin D_3 receptor and cornifin β as a marker of squamous differentiation were present in ovariectomized rats treated with 1,25-dihydroxyvitamin D_3. In contrast, cornifin β and 1,25-dihydroxyvitamin D_3 receptor were absent in all layers of vaginal epithelium in control group. We demonstrated for the first time that 1,25-dihydroxyvitamin D_3 induced proliferation of vaginal epithelium consistent with the cornifin β expression and 1,25-dihydroxyvitamin D_3 up-regulated 1,25-dihydroxyvitamin D3 receptor expression in vaginal epithelium.

Key words: vitamin D, vagina, cornifin β, vitamin D receptor.

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Vagina which is surrounded by squamous, stratified epithelium is under the effect of estrogen hormone. It causes various complaints for women in menopouse, that is to say, in the absence of estrogen hormone. Vaginal dryness or atrophic vaginitis, also referred to as urogenital atrophy, is a common problem for postmenopausal women. The most common symptoms are dryness, burning, pruritus, irritation and dyspareunia, thus leading to decreased libido and quality of life (Notelovitz 2002).

Estrogen treatment in post menopausal women has been shown to increase collagen content, dermal thickness and elasticity. Biophysical properties are also significantly improved for the parameters reflecting hydration and sebum secretion (Castelo-Branco C 2005). However, numerous side effects such as increased incidence of cancer and cardiovascular morbidity limit the use of this treatment (Nohales 2006, Gniadecki 1996, Berger 1988). Unlike the systemic estrogen treatment, which has adverse effects such as bleeding, cancer risk, cardiovascular system problems, local oestrogen treatment has no such side effects. However, many women consider pessaries and creams to be messy, and application times difficult to remember (Suckling J 2003 Cicinelli E 2008). For this reason, to get rid of the menopausal complaints, new alternative treatment methods are needed. Vitamin D (1,25-dihydroksivitamin D3) is a promoter of keratinocyte differentiation and a modulator of keratinocyte proliferation in the epidermis (Bikle 2007). Like the skin, vagina is also surrounded by a stratified squamous epithelium. The hypothesis of if vitamin D can enhance proliferation and differentiation of skin epithelium could it be possible to have the same effect on vaginal epithelium is the base of this study.

1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$, Calcitriol) is a pleiotropic hormone with proliferative, pro-apoptotic and pro-differentiation effects on numerous cell types, in addition to its classical reg-

ulatory action on calcium and phosphate metabolism (Bikle 2007, De Luca 2004). $1,25(OH)_2D_3$ would have important implications for the aetiology and therapy of various diseases, such as cancer, diabetes mellitus and psoriasis (Liu 1996, Holick 2006, Park 2000, Guzey 2002, Pepper 2003, Fischer 2007, Mordan- McCombs 2007, Ginanjar 2007, Arnson 2007, Pittas 2007, Lebwohl 2007, Zhu 2007).

The biological effects of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) are mediated by its nuclear receptor (Petrazzouli 1999). $1,25(OH)_2D_3$ interacts with its intracellular receptor [vitamin D receptor (VDR)] to induce the expression of a variety of genes (Kutuzova 2004, White 2004). These genes are important in the regulation of growth and differentiation of a stratified squamous epithelium. In our earlier study, for the first time the presence of VDR in vaginal epithelium was demonstrated. VDR was preferentially localized in basal and suprabasal layers, where cells undergo proliferation and differentiation. VDR expression in vaginal epithelium changed during the estrus cycle. In ovariectomized rats, VDR was absent from all layers of vaginal epithelium (Yildirim 2004).

Squamous differentiation is a multi-stage process in which each step is characterized by the expression of specific genes, including those encoding various keratins and proteins involved in formation of cross linked envelope. Cornifins or human small proline- rich proteins (SPRR's) are family of cross-linked envelope precursor proteins (Jetten 1992 a, Gijbels 1992, Darwiche 1993, Gibbs 1993). Preliminary studies have indicated that the levels of cornifin expression change with a higher stage of malignancy of the cervical epithelium, and support the idea that cornifin could be a good marker to monitor the stage of malignancy in the female reproductive tract (Jetten 1996 b). In this study cornifin β was used as a marker to determine the squamouse differentiation in overiectomized and vitamin D3 treated vaginal epitehelium.

In this study we searched the answers for two questions. First one is, whether vitamin D has an effect on reshaping the vaginal epithelium and if so, does it create this effect through the vitamin D receptors. The second is whether the vitamin D affects the cornifin β expression. We have found that in the vitamin D applied group, after ovariectomization, the epithelial was reshaped and the expressions of cornifin β and VDR increased.

Materials and Methods

Animals

Twenty adult non-pregnant Wistar female rats were used. They were housed in temperature-controlled rooms (27±10°C) at light/dark 14/10 h regimen. Estrous cyclity was monitored by cytological examination of vaginal smears taken between 08:30 and 09:30 a.m 5 days a week. Only those females that experienced two regular 4-day cycles consisting of 2-day diestrus, 1-day proestrus and 1-day estrus were used. We followed the ethical guidelines for the treatment of laboratory animals.

Tissue collection

Bilateral ovariectomy was performed in all mature non-pregnant Wistar rats. All the animals were divided into 2 groups consisting of 10 rats each. Group I served as control. In group II, animals were injected intramuscularly with vitamin D3 (50, 00 IU/kg). Two weeks after the injections, vaginas of animals in group I and group II were removed under pentobarbital anesthesia and the rats were sacrificed. Tissues were fixed in 10% neutral buffered formalin for 72 h and then embedded in paraffin. Paraffin sections (5 μm) were deparaffinized in xylene and rehydrated through a graded series of ethanol solutions.

Histochemistry

Three sections from each animal were processed by immunocytochemical assay for the expression of VDR and cornifin β. Human epidermis was used as positive control. Omitting the primary antibody performed negative control.

Antibodies and staining procedure for Vitamin D receptor

Endogenous peroxidase activity was blocked by 3% hydrogen peroxidase for 10 minutes and the sections were incubated with saponin to facilitate binding of primary antibody to the antigenic areas. Epitopes were stabilized by application of serum blocking solution (Goat serum, Lot# 20570999, Zymed Laboratories Inc., San Francisco, USA) for 20 min at room temperature. Sections were incubated overnight with VDR primary antibody (Lot# 1039, sc 1009 polyclonal antibody, Santa Cruz Biotechnology) diluted 1:100 in PBS at +4°C. After applying the secondary antibody anti-rabbit Ig, avidin-biotin-complex-peroxidase (ABC, Lot#

20570999, Zymed Laboratories Inc.) was applied to the slides. Diaminobenzidine (DAB, Lot# 10163354, Zymed Laboratories Inc.) was used as chromogen. Afterwards, slides were counterstained with hematoxyline for 1 minute, dehydrated in graded ethanol and mounted in conventional medium.

Antibodies and staining procedure for cornifin β

Endogenous peroxidase activity was blocked in 3% hydrogen peroxidase for 10 minutes and the sections were incubated with saponin to help binding of primer antibody to the antigenic areas. Epitopes were stabilized by application of serum blocking solution (Goat serum, Lot# 20570999, Zymed Laboratories Inc) for 60 min at room temperature. Sections were incubated with 1:1000 diluted cornifin β primer antibody (SQ37A-Ab, Jetten Laboratories) in PBS at room temperature for 60 min. Then secondary antibody anti-rabbit Ig, avidin-biotin-complex-peroxidase (ABC, Lot# 20570999, Zymed Laboratories Inc.) were applied on tissue slides. Diaminobenzidine (DAB, Lot# 10163354, Zymed Laboratories Inc) was used as chromogen. Afterwards, slides were counterstained with hematoxylene for 1 min, dehydrated in graded ethanol and mounted in conventional medium.

Three experienced histologists examined slides for VDR and cornifin β. The intensity of immuno-reaction was semi quantitatively evaluated which was expressed as intensive (+++), moderate (++), weak (+) and negative (-).

Statistical analysis

All data were presented as mean ± standard deviation (S.D.). A computer program (SPSS 12.0 for Windows, SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The data were considered to be non-parametric, therefore, they were analyzed using Mann–Whitney *U*-test. $p<0.05$ were considered to be statistically significant.

Results

Effect of vitamin D_3 on epithelial morphology: The atrophic vaginal epithelium of the ovariectomized rats was only 2–3 cell layers thick (Figure 1A). In response to $1,25(OH)_2D_3$, basal epithelial cells proliferated rapidly, leading to the formation of a highly stratified epithelium (Figure 1B). The suprabasal cells, which were no longer mitotic, underwent a well-characterized differentiative sequence as they moved up through the epithelium; they became enlarged and underwent structural and morphological changes towards cornification, so that the apical layer became keratinized.

Effect of vitamin D_3 on Vitamin D receptor expression

In ovariectomized rats the effect of $1,25(OH)_2D_3$, on VDR expression in rat vaginal epithelium was investigated. Figure 2A and B shows the absence of VDR and cornifin β in ovariectomized rat epithelium before $1,25(OH)_2D_3$, treatment. VDR regulation was tested after 1,25(OH)2D3 injection. The distribution and localization of VDR in vaginal epithelium of rats treated with $1,25(OH)_2D_3$ was shown in Figure 3. Prominent nuclear and cytoplasmic immunostaining of VDR was observed in vaginal epithelium. We found that basal, suprabasal and apical cell layers exhibited a positive VDR reaction, but suprabasal and apical cells of vaginal epihelium demonstrated more diffuse and stronger immunostaining for the VDR compared to basal cells. Nuclei of epithelial cells were positive. A slightly positive immunoreaction was noticed in basal membrane (Figure 3).

Effect of vitamin D on cornifin β expression

In vagina of ovariectomized mice, stratification occured two weeks after $1,25(OH)_2D_3$ injection (Figure 1B). Immunohistochemical staining for cornifin β was very intense in the suprabasal and apical layers (Figure 4). Stronger immunostaining was determined in the cell membrane than cytoplasm in the suprabasal and apical cells. A weak immunohistochemical staining was observed in basal cell and basal membrane. While nuclei of apical cells showed strong immunostaining for cornifin β, no immunostaining was observed in the nuclei of basal cells . Cornified layer was strong negative for cornifin β (Figure 4).

Expression of cornifin β decreased in ovariectomized rat epithelium compared with the vitamin D3 treated rat epithelium layer ($p<0.05$), except basal cells of the epithelial cells ($p>0.05$) (Figure 5). However cornifin β increased in basal membrane in ovariectomized rats. Like cornifin β expression, decrease of the vitamin D receptor was significant in ovariectomized rat epithelium compared with the vitamin D3 treated rat epithelium layer ($p<0.05$) except basal membrane and cornified layer ($p>0.05$) (Figure 6).

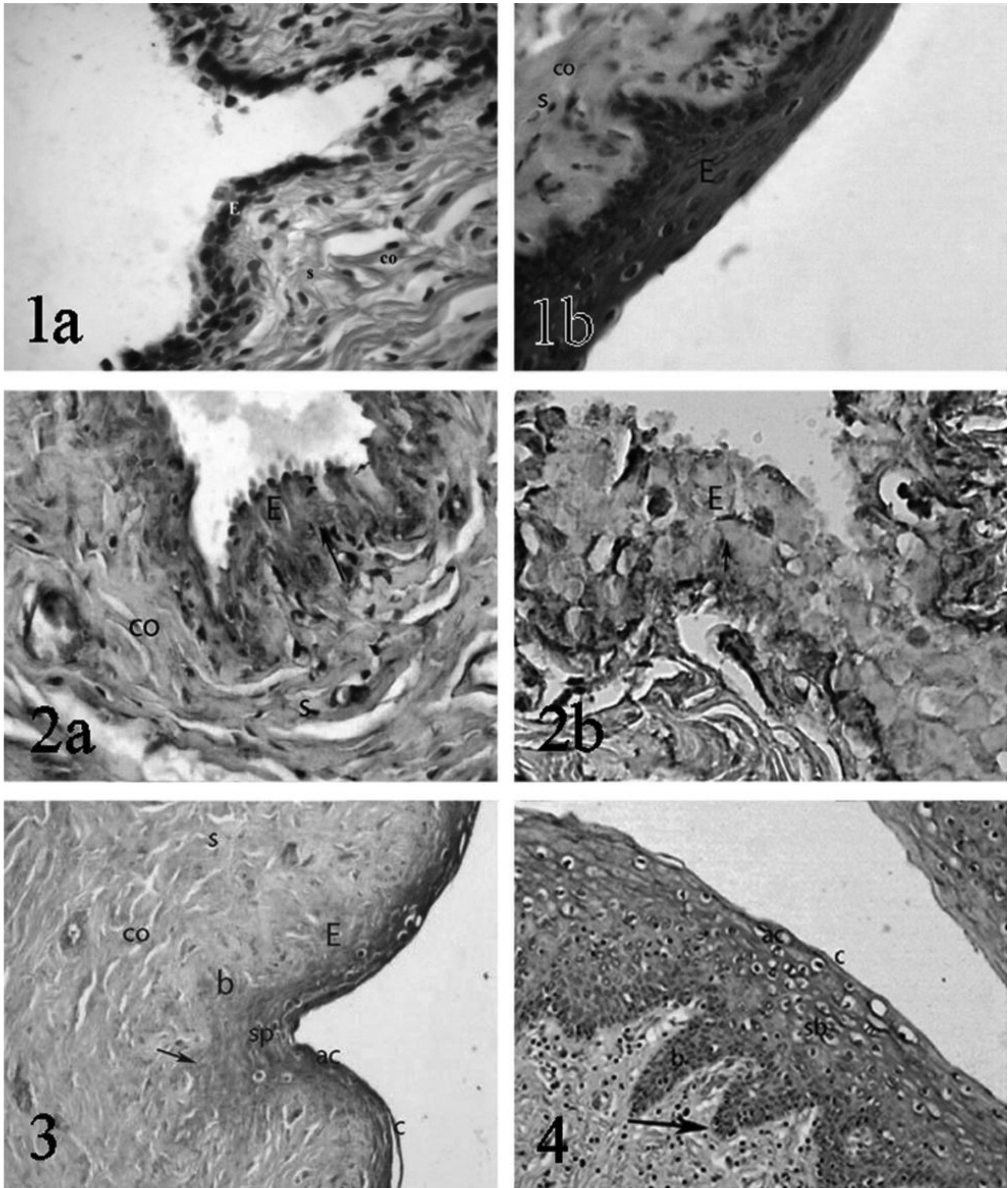

Figure 1. Histological changes in the vaginal epithelium upon treatment with vitamin D3. (A) Ovariectomized vaginal epithelium; (B) vitamin D_3 treated vaginal epithelium. E: Epithelium; arrow: basal membrane; co: collagen fibers; s: stromal cells. Hematoxyline–Eosin, magnification: X400.

Figure 2. Negative immunostaining of VDR (A); and cornifin β (B) in the vaginal epithelium of ovariectomized rats (B). E: Epithelium; arrow, basal membrane; co: collagen fibers; s: stromal cells. Immunoperoxidase, magnification: X 400.

Figure 3. Immunohistochemical detection of VDR in vaginal epithelium treated with vitamin D_3. Positive VDR immunostaining in basal, suprabasal and apical cells. E: Epithelium; arrow: basal membrane; co: collagen fibers; s: stromal cells; b: basal cells; sp: suprabasal cells; ac: apical cells: c: cornified layer. Immunoperoxidase, magnification: X 200.

Figure 4. Immunohistochemical detection of cornifin β in vaginal tissue treated with vitamin D_3. Positive cornifin , immunostaining in suprabasal and apical cells. E: Epithelium; arrow: basal membrane; co: collagen fibers; s: stromal cells; b: basal cells; sp: suprabasal cells; ac: apical cells: c: cornified layer. Immunoperoxidase, magnification: x 200.

Table 1. Localization and distribution vitamin D receptor in vaginal epithelium.

	Cornified layer	*Apical cells*	*Suprabasal cells*	*Basal cells*	*Basal membran*	*Nucleus (apical cell)*	*Nucleus (basal cell)*
Ovariectomized	–	–	–	–	–/+	–	–
Vitamin D Treated	–	+++	+++	++	–/+	+++	++

Table 2. Localization and distribution cornifin β in vaginal epithelium.

	Cornified layer	*Apical cells*	*Suprabasal cells*	*Basal cells*	*Basal membran*	*Nucleus (apical cell)*	*Nucleus (basal cell)*
Ovariectomized	–	–	–	–	+++	–	–
Vitamin D Treated	–/+	+++*	++/+++*	+	–/+	+++	–

** Stronger immunostaining was determined in the cell membrane than cytoplasm in the suprabasal and apical cells*

Table 3. Expression cornifin β in vaginal epithelium.

	Cornified layer	*Apical cells*	*Suprabasal cells*	*Basal cells*	*Basal membran*	*Nucleus (apical cell)*	*Nucleus (basal cell)*
p	0,029	0,00	0,00	0,00	0,00	0,00	1,00

Test is significant if p≤0,05

Table 4. Expression vitamin D receptor in vaginal epithelium.

	Cornified layer	*Apical cells*	*Suprabasal cells*	*Basal cells*	*Basal membran*	*Nucleus (apical cell)*	*Nucleus (basal cell)*
p	1	0,00	0,00	0,00	0,38	0,00	0,00

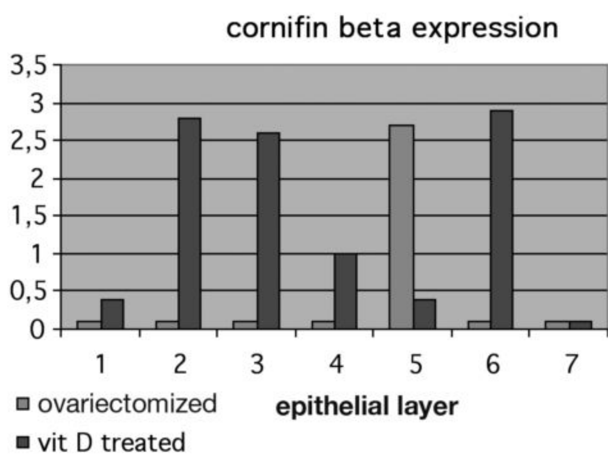

Figure 5. Expression of cornifin β in ovariectomized rats and vitamin D treated group. 1; cornified layer *p<0.05 , 2; apical cells, #p<0.05, 3; suprabasal cells p<0.05, 4; basal cells, p <0.05, 5; basal membran, p<0.05, 6; apical nucleus, p<0.05, 7; basal nucleus, p>0.05

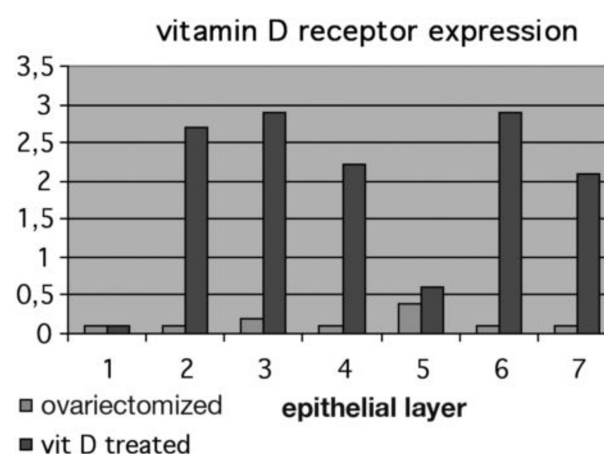

Figure 6. Expression of vitamin D receptor in ovariectomized rats and vitamin D treated group. 1; cornified layer *p<0.05, 2; apical cells, #p<0.05, 3; suprabasal cells p<0.05, 4; basal cells, p<0.05, 5; basal membran, p<0.05, 6; apical nucleus, p<0.05, 7; basal nucleus, p>0.05

Discussion

The use of vitamin D for the therapy of vaginal atrophy can be an emerging new treatment modality. To gain insight into the effect of $1,25(OH)_2D_3$ on vaginal epithelization we used ovariectomized rats with vaginal tissue deprived of functional estrogen. And also we used cornifin β as a marker of vaginal epithelial differentiation. The other aim of the present study was to determine the respective role of $1,25(OH)_2D_3$ on VDR and cornifin β expression in vaginal epithelium.

Menopause is the lasting cessation of menstruation because of the loss of follicular activity. There comes some changes in menopausal woman: pubic hair grays and becomes sparse, the labia majora lose subcutaneous fat, and the labia minora, vestibule, and vaginal mucosa atrophy (Jones 1983, Erickson 1972). Menopausal and post-menopausal atrophic vulvovaginitis is an almost common condition. The decrease in vaginal secretions reduces lubrication and increases coital discomfort. Thinned tissue is more easily irritated and may be more vulnerable to infection. The prevalence of colonization by enteric organisms associated with urinary tract infections increases due to the rise of vaginal pH (Fischer 1998). In addition to these physiologically induced changes, certain vulvar dermatoses, such as lichen sclerosus, are most common post-menopausal women (Kamarashev 1997).

In the absence of estradiol, vaginal epithelium was shown to be atrophic, and consisted of 2–3 layers of squamous epithelial cells as we demonstrated experimentally in this study.

Atrophic vaginitis can be treated with estrogen therapy. Notably, estrogen is also considered to increase the risk of developing breast cancer. It improved susceptibility to estradiol (Wadia 2007), decreased apoptosis, and increased the number of progesterone receptor-positive epithelial cells (Mun?oz-de-Toro 2007). There is a large group of epidemiological and experimental evidence that estrogens are strongly implicated in the aetiology of some carcinomas requiring oestrogen for continued growth and progression (Henderson 1990, Henderson 1988). Nowadays, as an alternative to systemic estrogen treatment, local estrogen treatment is being applied. Of course, Replens, a non-hormonal alternative treatment to oestrogen replacement treatment, overcomes the symptoms caused by vaginal atrophy (Van der Laak JA. 2002). Although the local estrogen treatment does not have adverse effects such as bleeding, cancer risk, cardiovascular system problems, women do not prefer it due to its difficulties in the application (Suckling J 2003, Cicinelli E 2008).

$1,25(OH)_2D_3$ actions on target tissues regulate: protein kinase cascades leading to cell proliferation, differentiation and apoptosis. Protein kinase cascades have been known as mitogen-activated protein kinase (MAPK) signaling cascades. MAPKs regulate cellular responses through the phosphorylation of other kinases, cytoplasmic and membrane proteins and transcription factors. The ERK pathway is a major determinant in the control of cell growth, differentiation and survival (Johansen 2003, Eckert 2002).

The biological effects of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) are mediated by its nuclear receptor (Petrazzouli 1999). They are expressed in multiple tissues within the body such as liver, kidney, thyroid, adrenal, gastrointestinal tract, breast and skin (Eckert 2002). Vitamin D3 and its receptors form a complex that binds to the vitamin D3 response element of genes, and either positively or negatively affects transcription of some genes (Johansen 2003).

Our results showed that VDR and cornifin β epitopes are not present in vaginal epithelium of ovariectomized rats, which is not the case in the presence of estradiol. Exagenous $1,25(OH)_2D_3$ treatment of overioctomized rats has been induced proliferation and thickening and to increase the expression of vitamin D receptor and cornifin β in the vaginal epithelium with flattened cells that accumulated in the superficial layers. Both VDR and cornifin β-receptor have been identified in vaginal epithelium treated vitamin D_3 while they were neagative in overioctomized vaginal epithelium. VDR mainly localized in basal, suprabasal and apical cell layer implicates the role of VDR with cellular proliferation and differentiation. A slightly positive immunohistochemical staining was observed in basal cell layers although we observed strong expression for cornifin β in suprabasal and apical cell layer. Therefore, this made us think that cornifin β has an effective role in the differentiation of vaginal epithelium. These results are the first to be shown in vivo up-regulation of the VDR expression in vaginal epithelium in response to vitamin D_3. The demonstration of induced VDR expression by $1,25(OH)_2D_3$ raised a question concerning the role

of VDR in vaginal differentiation and proliferation. It is likely, that the regulation of proliferation and differentiation of the vaginal epithelium and cornifin β expression by vitamin D_3 and vaginal epithelial cells may be mediated by VDR receptors.

In conclusion, to our knowledge, in vaginal epithelium, this is the first study showing the proliferative effect of $1,25(OH)_2D_3$ and VDR up-regulation by vitamin D_3. The mechanism of $1,25(OH)_2D_3$ induced epithelial interactions may have clinical implications. Estrogen is routinely used for vaginal atrophy, but estrogen treatment is within the initiation and progression of neoplasias of estrogen target organs as mammary gland, endometrium, vagina, and cervix (Redeuilh 2002, Ito 2007, Nair 2005, Deligeoroglou 2003, Salazar 2001, Yue 1998 Beresford 1997, Brisson 1994, Brinton 1993, Jick 1993a, Jick 1993b, Whitehead 1981). In this regard, vitamin D potentially may be a new safe therapeutical agent for vaginal atrophy. Of course the mechanisms underlying vaginal epithelium differentiation and VDR up-regulation by $1,25(OH)_2D_3$ remains to be further evaluated.

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