

Comparable Vitamin D3 Metabolism in the Endometrium of Patients With Recurrent Spontaneous Abortion and Fertile Controls

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SUMMARY

Vitamin D exerts important roles during pregnancy, and its deficiency may be associated with several pregnancy complications, including pregnancy loss, yet no data are available for molecules involved in vitamin D metabolism in patients with unexplained recurrent spontaneous abortion. In this study, we investigated possible difference in endometrial expression of vitamin D3 receptor (*VDR*), 1 α -hydroxylase (*CYP27B1*), and 24-hydroxylase (*CYP24A1*) in women with recurrent spontaneous abortion ($n=8$) and healthy controls ($n=8$). Gene expression of *VDR*, *CYP27B1*, and *CYP24A1* was determined by real-time PCR, while *VDR* and *CYP27B1* proteins were localized by immunohistochemistry and their abundance was validated by Western blot. We found that both patient and control groups expressed comparable levels of endometrial *VDR*, *CYP27B1*, and *CYP24A1* transcripts. In line with the gene-expression results, *CYP27B1* and different isoforms of *VDR* protein were present at the same abundance in the endometria of both groups. No significant alteration in *VDR* and *CYP27B1* immunoreactivity pattern was found in the endometrium of patients compared to fertile controls, however. The results of the present study, therefore, do not support the hypothesis of differential expression of key molecules involved in vitamin D3 metabolism in the endometrium of recurrent spontaneous abortion patients and fertile controls.

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INTRODUCTION

1,25-dihydroxyvitamin D3 (1,25(OH)2D3 or calcitriol) is the major regulator of calcium and phosphate homeostasis (Harrison, 1964). In renal proximal tubules, the active form

Abbreviations: 1,25(OH)2D3, 1,25-dihydroxyvitamin D3 or calcitriol; 25(OH)D, 25-hydroxy vitamin D; *CYP24A1*, 1,25-dihydroxyvitamin D3 24-hydroxylase; *CYP27B1*, 25-hydroxyvitamin D3 1 α -hydroxylase; RSA, recurrent spontaneous abortion; *VDR*, vitamin D receptor

of vitamin D3 (1,25-dihydroxyvitamin D3) is produced from 25(OH)D3 by 25-hydroxyvitamin D3 1 α -hydroxylase (1 α -OHase or CYP27B1) (Brown et al., 1999). Within the regulatory circuit, 1,25(OH) 2D3 and 25(OH)D3 are converted to their inactive forms by CYP24A1, a mitochondrial cytochrome P450 enzyme (Henry, 2001).

The active form of vitamin D3 affects gene expression in target cells via the intracellular vitamin D receptor (VDR) (Haussler et al., 1998). In immune cells, for example, vitamin D3 exerts many immunoregulatory actions (van Etten et al., 2008), including inhibition of dendritic cell maturation, induction of the T-helper-cell-2 cytokine profile (Griffin et al., 2000; Penna and Adorini, 2000; Adorini et al., 2004; van Etten and Mathieu, 2005), and differentiation of regulatory T cells (Gregori et al., 2001) – all of which consistent with a possible therapeutic use in autoimmune diseases (Adorini, 2002) and management of transplantation rejection (Sugiyama et al., 2004).

Recent studies highlight important roles for vitamin D3 in the course of pregnancy (Zehnder et al., 2002; Vigano et al., 2006). During pregnancy, maternal circulating levels of vitamin D3 are increased (Kovacs and Kronenberg, 1997). Expression of vitamin D3 receptor by epithelial and stromal cells in mouse (Zarnani et al., 2010) and human (Vigano et al., 2006) endometrium and its increased levels in pregnancy (Shahbazi et al., 2011) imply the importance of vitamin D3 in maintenance of normal pregnancy. This hormone participates in endometrial decidualization, increases the abundance of HOXA10 (an essential element for implantation), and has important regulatory functions in placental development (Evans et al., 2004). Recently, an association between vitamin D and uterine receptivity has been reported (Vanni et al., 2014). For example, vitamin D could increase endometrial thickness in infertile women with polycystic ovary syndrome undergoing intrauterine insemination (Lerchbaum and Rabe, 2014). Vitamin D3 deficiency may also be associated with several pregnancy complications such as pre-eclampsia (Tabesh et al., 2013) and recurrent abortion (Ota et al., 2014). Finally, given the immunomodulatory activity of 1,25(OH) 2D3 (Di Rosa et al., 2011), vitamin D3 has been proposed to have beneficial effects in recurrent spontaneous abortion (RSA) patients (Bubanovic, 2004).

Molecular evidence supporting the activity of vitamin D3 signaling in the uterus is extensive. CYP27B1 is present in the endometrium (Vigano et al., 2006) and placental trophoblasts (Weisman et al., 1979). VDR expression was shown in human placenta, decidua (Zehnder et al., 2002) and endometrium (Vigano et al., 2006). Similar localization (Vigano et al., 2006; Agic et al., 2007; Becker et al., 2007) and abundance of VDR (Vienonen et al., 2004) and CYP27B1 transcripts (Vigano et al., 2006) have been reported in the endometrium and endometrial stromal cells of human proliferative- and secretory-phase uteri. Furthermore, the human endometrium is able to express CYP24A1 either constitutively (Agic et al., 2007) or after stimulation with 1,25(OH) 2D3 (Vigano et al., 2006). Thus, vitamin D3 signaling likely participates in the normal function of the uterus and placenta during pre- and post-implantation periods.

Considering this supportive role of 1,25(OH) 2D3 in maintenance of pregnancy (Bubanovic, 2004; Hyppönen, 2011), we evaluated the endometrial expression of *VDR*, *CYP27B1*, and *CYP24A1* in women with unexplained RSA and healthy controls. We hypothesized that altered endometrial expression of aforesaid markers could contribute to the spontaneous abortion.

RESULTS

Age of Participants With and Without RSA

Eight unexplained RSA patients (median age: 30.5, interquartile range: 26.25–34.5, range: 24–36) and eight fertile controls (median age: 31.5, interquartile range: 29.5–33.75, range: 28–35) of similar age participated in this study ($P = 0.63$). The median numbers of miscarriages in RSA participants was 3 (IQR: 3–4, range: 2–4).

Real-Time PCR Quantification of *VDR*, *CYP27B1*, and *CYP24A1* Gene Expression

Quantitative real-time PCR was used to compare the gene expression of *VDR*, *CYP27B1*, and *CYP24A1* in RSA patients and fertile controls. No significant differences were found in relative transcript abundance of these genes between the two groups (Fig. 1). Negative controls always showed no amplification.

Western Blot Analysis of *VDR* and *CYP27B1*

A single band of about 56 kDa, corresponding to the predicted molecular weight of CYP27B1, and different bands with molecular weights of about 28, 42, 48, 75, and 105 kDa for VDR were detected (Fig. 2A). No signal was detected in the negative-control sample, implying the specificity of these bands for both proteins of interest.

For each sample, the actin-normalized CYP27B1 and VDR abundances were compared, and revealed no differences between the RSA or control groups. This was true for CYP27B1 and each independent VDR band (not shown) or total VDR (Fig. 2B).

Immunohistochemical Investigation of *CYP27B1* and *VDR*

Immunohistochemical analysis of endometrial biopsies revealed a diffuse distribution of CYP27B1 in the cytoplasm of both epithelial and stromal endometrial cells (Fig. 3A,C). Scattered CYP27B1 immunoreactivity was also evident in some non-stromal immune cells, and was generally weak in endothelial cells. VDR, on the other hand, mainly localized to the nuclei of epithelial cells, stromal cells, and some immune cells; we also observed weak cytoplasmic expression in these cell populations (Fig. 3B,D). Endothelial cells of both groups showed intense staining for VDR. No differences were observed between endometrial samples of RSA and control groups. No antibody-reactive signal was detected in negative-reagent controls (Fig. 3E,F) or in mouse liver (Fig. 3G).

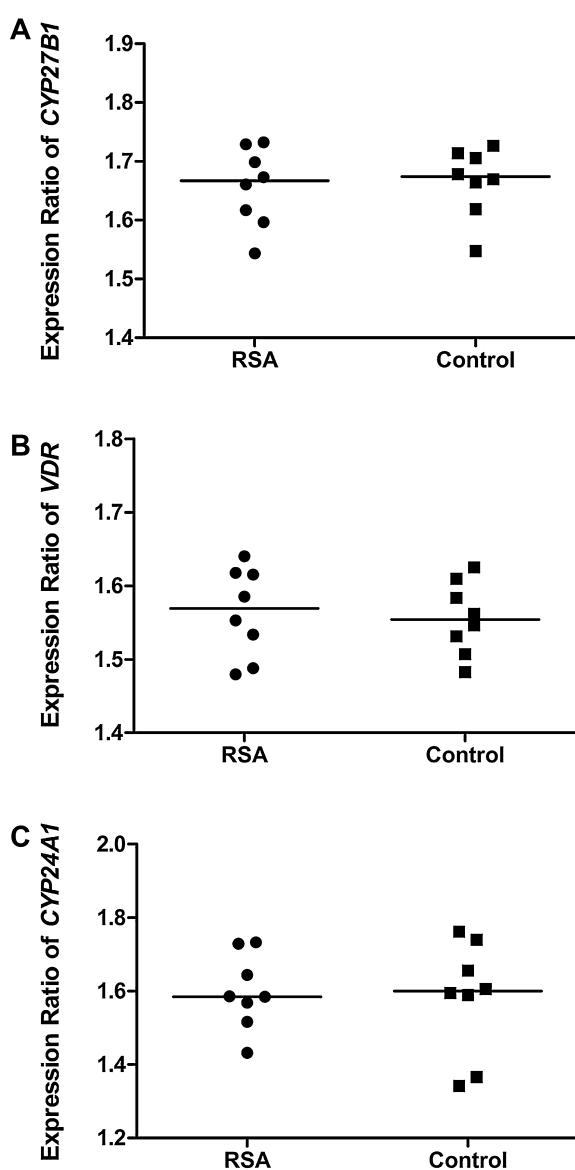


Figure 1. Real-time PCR evaluation of relative endometrial *CYP27B1*, *VDR*, and *CYP24A1* transcripts in RSA ($n=8$) and control ($n=8$) groups. No significant differences between RSA and controls were observed for (A) *CYP27B1* ($P=0.79$), (B) *VDR* ($P=0.72$), or (C) *CYP24A1* ($P=0.64$) transcript abundance. The horizontal lines show the median of the data.

DISCUSSION

The beneficial effects reported for vitamin D₃ in fertility and the establishment and maintenance of pregnancy (Zehnder et al., 2002; Bubanovic, 2004; Viganò et al., 2006; Hyppönen, 2011) have made this hormone a focus in reproduction science. In line with previous studies (Viganò et al., 2006; Agic et al., 2007), we observed

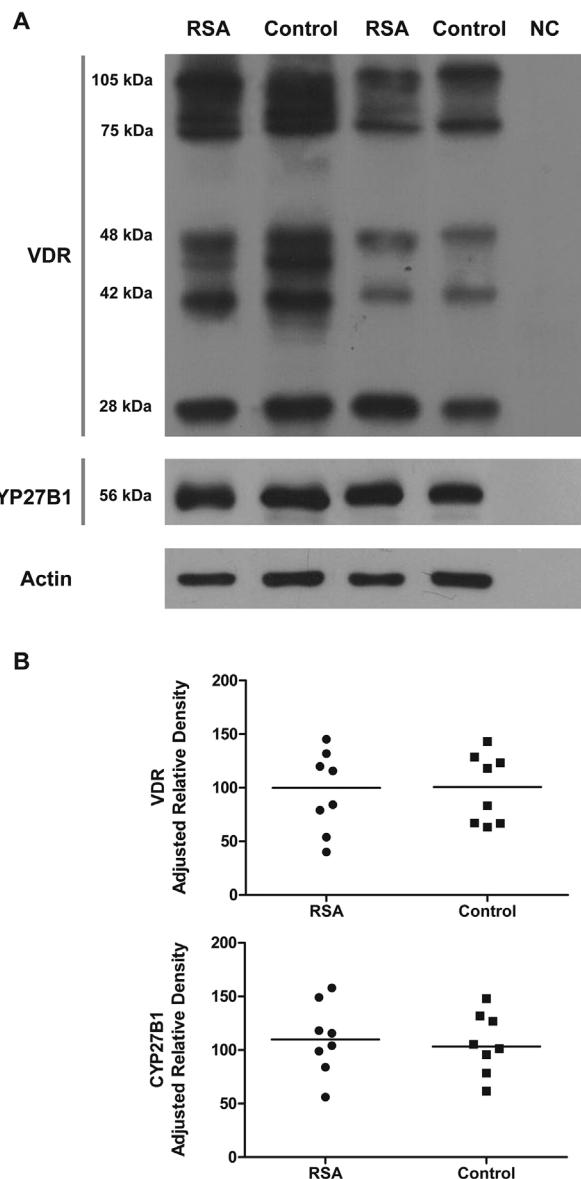


Figure 2. Western blot analysis of *CYP27B1* and *VDR* protein expression. In both RSA ($n=8$) and controls ($n=8$), different *VDR* bands with molecular weights of about 28, 42, 48, 75, and 105 kDa (A, upper blot), and a single band of about 56 kDa corresponding to *CYP27B1* (A, middle blot) were detected. Denitometric analysis did not show any significant differences in total *VDR* ($P=0.95$) or *CYP27B1* ($P=0.79$) abundance relative to actin between RSA and control groups; the horizontal lines show the median of the data (B). NC, negative control (pre-absorbed antibodies).

the endometrial expression of *CYP27B1*, *VDR*, and *CYP24A1*. No difference in their transcript or protein abundance was seen between RSA patients and controls, however. Also, no statistical difference was observed in serum levels of vitamin D₃ between two groups as well (data not shown). Although the relatively small sample size may be one potential limitation of this study, we believe that the data are valid based on the stringent

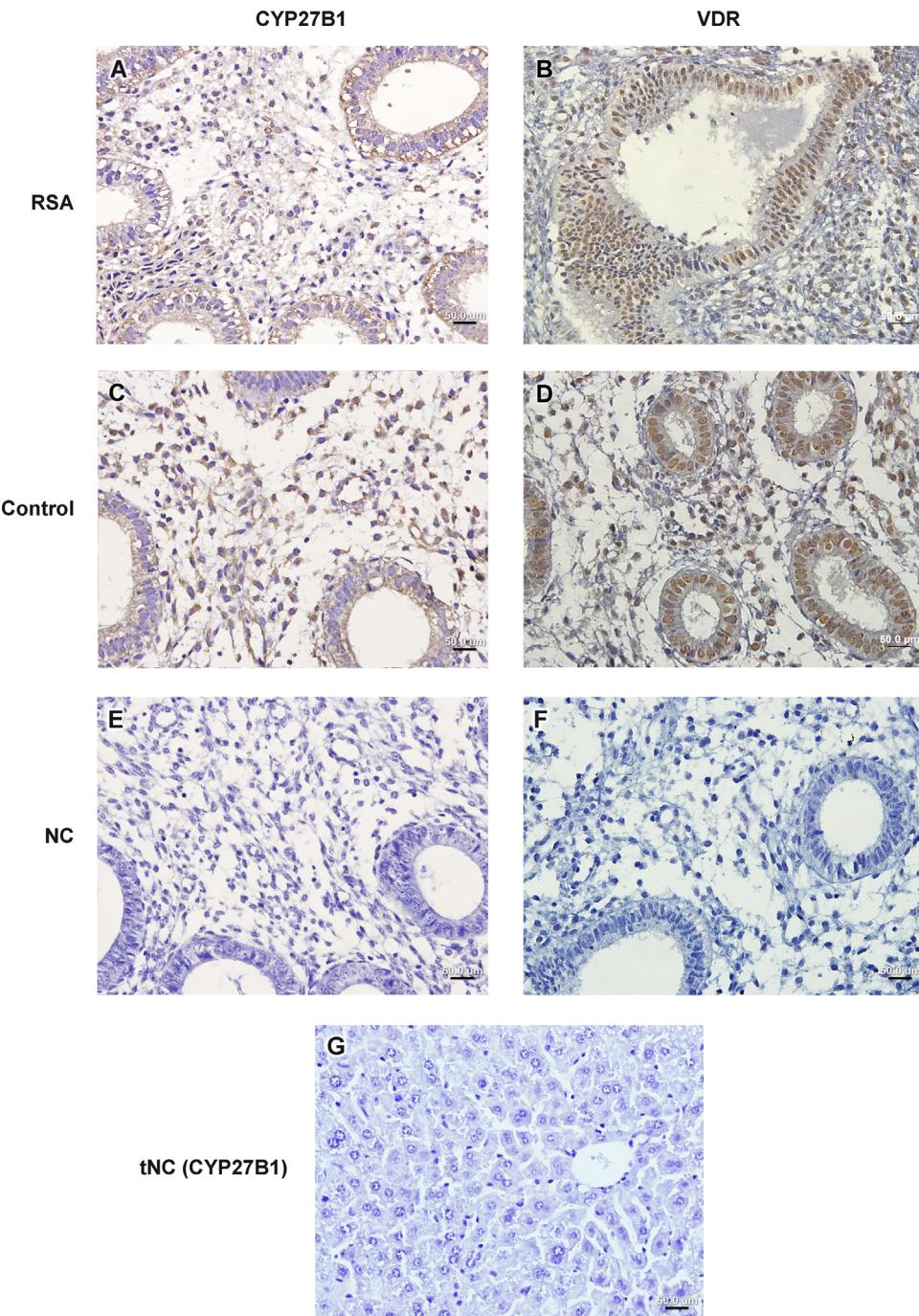


Figure 3. Immunohistochemical evaluation of CYP27B1 and VDR expression in endometrial tissues of RSA ($n = 8$) and control ($n = 8$) groups. Diffuse distribution of CYP27B1 (A, C) was revealed in cytoplasm of both epithelial and stromal cells of RSA and control endometria. VDR (B, D) was mainly localized to the nuclei of epithelial and stromal cells, with some cytoplasmic distribution. Negative-reagent controls (NC) for CYP27B1 (E) and VDR (F) always lacked signal. Mouse liver was used as negative tissue control (tNC) for CYP27B1 staining (G). Scale bar, 50 μ m.

inclusion and exclusion criteria used for patient selection as well as confirmation of results by three different read out systems at both gene and protein levels. Considering the absence of previous reports, we are unable to compare our results with the findings of others.

Of note, we observed several VDR-specific bands of about 28, 42, 48, 75, and 105 kDa – which may partly correspond to the 10 transcript variants reported for the human *VDRA* gene locus (Crofts et al., 1998). Alternative splicing of *VDR* upstream exons produces two further

distinct proteins, VDRB1 and VDRB2, with 50 and 23 amino acids N-terminal extensions, respectively (Sunn et al., 2001). Known VDR isoforms reported in the National Center for Biotechnology Information (NCBI, National Institutes of Health, United States of America) database have masses of about 11, 12, 28, 44, 48, and 54 kDa. The anti-VDR antibody we used in this study recognizes a specific sequence present in all the reported variants, except for the 11- and 12-kDa isoforms. Therefore, the ladder of distinct bands we observed in this study could be related to different VDR isoforms, which is consistent with reports that both VDRB1 and VDRA can be produced at the same time (Crofts et al., 1998; Sunn et al., 2001). The higher molecular weight bands (75 and 105 kDa), on the other hand, may be attributed to dimerization, post-transcriptional modifications, or covalent binding to other proteins such ubiquitin (Nangia et al., 1998; Li et al., 1999).

One impetus for the different VDR isoforms observed is the distinct function of each isoform in different subcellular compartments (Nibbelink et al., 2007), possibly with different interacting partners (Crofts et al., 1998). With this in mind, we investigated the subcellular localization of VDR in endometrial tissues. In both RSA and control groups, VDR was found in the nucleus and cytoplasm of endometrial epithelial and stromal cells; no clear differences in the abundance within each compartment or pattern were observed. Considering our anti-VDR antibody recognized several isoforms of VDR, we cannot use these immunohistochemical results to assess if the subcellular localization of specific isoforms differed between RSA and control women.

We also observed a single, 56-kDa CYP27B1 protein in Western blots of endometrial tissues, as reported previously (Vigano et al., 2006). By immunohistochemical analysis, this protein had diffuse expression in cytoplasm of both epithelial and stromal cells. No significant difference in CYP27B1 expression pattern was found between RSA and control groups. These findings are consistent with our previous study showing that whole endometrial cells and endometrial stromal cells from RSA and fertile women converted 25(OH)D₃ to 1,25(OH)₂D₃ at comparable levels *in vitro* (Tavakoli et al., 2011). Collectively, these data indicate a similar activity for CYP27B1 in the endometria of both groups.

Some important cytokines are involved in the course of pregnancy (Saito et al., 2010), whose production can be affected by vitamin D (Griffin et al., 2000; Penna and Adorini, 2000; Gregori et al., 2001; Adorini et al., 2004; van and Mathieu, 2005). We recently reported that whole endometrial and endometrial stromal cells of RSA and control groups produce the same levels of IL-6, IL-8, IL-10, and IFN- γ after treatment with 1,25(OH)₂D₃ (Tavakoli et al., 2011). Comparable VDR expression and responsiveness to 1,25(OH)₂D₃ in endometrial cells could, therefore, be interpreted as RSA and normal women having the same quantity and function of this receptor.

In summary, we found comparable endometrial expression of VDR and the enzymes involved in vitamin D metabolism in RSA patients and healthy controls, confirming our

previous findings of similar hormone-dependent cytokine production by endometrial cells from these groups (Tavakoli et al., 2011). Confirmation of the results presented here using larger cohorts, as well as the investigation of other key components involved with vitamin D₃ signaling—including calcium, parathyroid hormone, and vitamin D-binding protein—are needed. Nevertheless, our findings do not minimize the beneficial effect of vitamin D₃ in the course of pregnancy.

MATERIALS AND METHODS

Subjects

This study was approved by Institutional Review Board and the Ethics Committee for Medical Research of the Avicenna Research Institute (No: 30/51/7915). Written consent was received from all participants.

Eight women who were 24–36 years old, with two or more unexplained spontaneous abortions (based on following criteria) before the 20th week of gestation and referred to Avicenna Infertility Clinic, were enrolled in the study. Participant exclusion criteria included: abnormal parental karyotypes; irregular menstrual cycles; anatomic and endocrinologic disorders; relevant infectious diseases (Toxoplasma, Rubella, Cytomegalovirus, and syphilis); thrombophilic state (abnormal protein C, protein S, anti-thrombin III, lupus anti-coagulant, and activated protein C resistance, mutation in factor V [Leiden], prothrombin [G20210A], plasminogen activator inhibitor 1 [675 4G/5G] or methylenetetrahydrofolate reductase [C677T and A1298C], and presence of anti-phospholipid and anti-cardiolipin antibodies); abnormal anti-thyroid, anti-nuclear, and anti-double-stranded-DNA antibody titers; and male-factor infertility. Endometrial samples were taken at the secretory phase during diagnostic hysteroscopy—as a recommended procedure for detection of anatomical uterine defects, adhesions, and fibroids (Berek, 2012)—for evaluation of endometrial expression of VDR, CYP27B1, and CYP24A1.

The control group consisted of eight women who were 28–35 years old, had at least one healthy live birth and disclosed no history of preeclampsia, abortion, stillbirth, or infertility. Endometrial samples were obtained at the secretory phase from these women, who were referred to the gynecologic clinic for hysteroscopic tubal ligation and had no evidence of pathology in their pathologic evaluations.

The patients and control women enrolled in the current study were the same women we examined in our previous work (Tavakoli et al., 2011). Considering seasonal variation in vitamin D levels, sample collection from patients and controls was seasonally matched for comparison. None of the participants in the study used hormones or vitamin D for at least 3 months before enrollment in this study.

Sample Collection

For endometrial samples obtained through biopsy cuvette, a small fragment was fixed in 10% formalin for 48 hr for immunohistochemical staining. Another fraction was

homogenized in lysis buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES]-KOH [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol [DTT], 1 mM phenyl-methanesulfonyl fluoride [PMSF], and 1% (v/v) protease inhibitor cocktail, then subsequently centrifuged at 1,000g, 4°C for 20 min. Supernatants were collected, and their protein concentration was determined by Bradford assay. Clarified supernatants were stored at -80°C for Western blot analysis. The remaining fraction of each endometrial tissue was transferred to microtubes containing RNA-Bee solution (Nordic Biosite, Täby, Sweden), and homogenized by Pellet Pestel for RNA extraction.

Evaluation of *VDR*, *CYP27B1*, and *CYP24A1* Gene Expression by Real-Time PCR

Total tissue RNA was extracted according to the standard method (Zarnani et al., 2010). The quality and concentration of isolated RNA were assessed spectrophotometrically using a Picodrop (Picopetol-UK) and agarose gel electrophoresis. Samples with acceptable RNA integrity and purity (A260/280>1.8) were used for cDNA synthesis.

One microgram of total RNA was solubilized in DEPC-treated water (total volume of 10 µL), denatured for 5 min at 65°C, and placed on ice immediately for cDNA synthesis. Denatured RNA was mixed with 5X reaction buffer (Fermentase, Vilnius, Lithuania), 1 mM dNTP mix (Roche, Mannheim, Germany), 1 mM random hexamers (Cybergene, Stockholm, Sweden), and 20 IU/mL reverse transcriptase M-MULV (Fermentase, Vilnius, Lithuania) and was incubated at 42°C for 60 min in a thermocycler (Eppendorf AG, Hamburg, Germany). Prepared cDNA was aliquoted, and stored at -20°C.

Quantitative real-time PCR was performed in clear MicroAmp Optical 8-tube strips with MicroAmp Optical 8-cap strips (AB Applied Biosystem, USA) with the specific primers and probes (Operon, Germany) for *ACTB* (Kreuzer et al., 1999), *VDR*, *CYP27B1*, and *CYP24A1* (Table 1) mixed with 10 µL 2X Premix Takara buffer (Takara Bio, Japan), 0.4 µL 50X ROX (Takara Bio, Japan), and 100 ng cDNA in a final volume of 20 µL. *ACTB* was used as the reference gene. In negative controls (no-template controls), deionized water was used instead of cDNA. Amplifications were performed on an ABI 7500 (Applied Biosystems) in duplicate using the following program: 10 min at 95°C, followed by 50 cycles of 5 sec at 95°C and 35 sec at 60°C. Baseline optical data were adjusted, exported, and analyzed with LinRegPCR.11.0 software (Ramakers et al., 2003; Ruijter et al., 2009). The quantification cycle (Cq) of samples and mean PCR efficiency for each pair of primers was calculated using this software. REST Software (REST-RG - version 3) was used to analyze genes expression ratios (Pfaffl et al., 2002).

Western Blot Analysis for *VDR* and *CYP27B1*

Western blot was performed using previously described method (Mahmoudi et al., 2013). Briefly, tissue lysates were resolved by SDS-PAGE (10 µg of total protein per lane) under reducing (for *CYP27B1*) or non-reducing (for *VDR*) conditions, and transferred to polyvinylidene difluoride (PVDF) membrane (Roche). After blocking the non-specific binding sites with 10% skim milk in phosphate-buffered saline (PBS) containing 0.05% Tween (PBST), overnight at 4°C, membranes were incubated at room

TABLE 1. Sequence and Specifications of *CYP27B1*, *VDR*, *CYP24A1*, and *ACTB* Primers and Probes

Gene symbol	Sequence	Sequence accession number	Amplicon length	Splice variants	Location	Amplification efficiency
<i>CYP27B1</i>	F: 5'-GTC AAG GAA GTG CTA AGA CTG TA	NM_000785.3	114		Exons 6–7 junction	1.95
	R: 5'-ACA CAG AGT GAC CAG CGT ATT TTT				Exons 7–8 junction	
	P: 5'-[6~FAM]CCT GGA AAT TCT CGT GTC CCA GAC AAA G[TAMRA~6~FAM]				Exon 7	
<i>VDR</i>	F: 5'-GAC CTA CGA CCC CAC CTA CTC	NM_000376.2	178	variant 1	Exon 5	1.943
	R: 5'-GGA CGA GTC CAT CAT GTC TGA AG	NM_001017535.1		variant 2	Exons 6–7 junction	
	P: 5'-[6~FAM]CAA CTC CCA GAC ACA CTC CCA GCT TCT CT[TAMRA~6~FAM]	NM_001017536.1		variant 3	Exon 6	
<i>CYP24A1</i>	F: 5'-GAC AAC AAA ATC AAT GAG GTC TTG	NM_000782.4	132	variant 1	Exons 3–4 juntoin	1.96
	R: 5'-CAA CAC GAG GCA GAT ACT TTC AA	NM_001128915.1		variant 2	Exons 4–5 junction	
	P: 5'-[6~FAM]AGA ATA GAT GAG CTC TGT GAT GAA AGA GGC[TAMRA~6~FAM]				Exon 4	
<i>ACTB</i>	F: 5'-AGC CTC GCC TTT GCC GA	NM_001101.3	174		Exon 1	1.926
	R: 5'-CTG GTG CCT GGG GCG				Exon 2	
	P: 5'-[6~FAM]CCG CCG CCC GTC CAC ACC CGC C[TAMRA~6~FAM]				Exon 1	

F, forward; R, reverse; P, probe.

temperature for 1 hr with 3 µg/mL rabbit anti-VDR or anti-CYP27B1 antibodies (Avicenna Research Institute, Tehran, Iran), diluted in 3% skim milk in PBST. After washing with PBST (4 × 15 min), membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (Sigma, MO) (1:8,000). After washing as above, signals were detected with an enhanced chemiluminescence (ECL) detection kit (GE Healthcare, Uppsala, Sweden), according the manufacturer's instruction. In negative control lanes, primary antibodies were pre-absorbed with 150-fold excess of immunizing peptides.

Membranes were re-probed with rabbit anti-actin (1:1,000) and horseradish peroxidase-conjugated goat anti-rabbit antibody (1:4,000) to control for protein loading. Digitalization was performed with a CanoScan LiDE 90 scanner (Canon, Vietnam), and the linearity of the measurements was checked by titration of loading material concentration in Western blot experiment. Quantification was performed using ImageJ 1.48 (Mac OS X version, <http://rsb.info.nih.gov/nih-image/>). Integrated optical density for each lane and each band was measured using a region as wide as one-third of the lane width, centered within the lane. Baseline subtraction was done for each lane to correct the background. Calculated optical densities per band were normalized to actin, and data were expressed as adjusted-relative density. Based on the presence of different isoforms of VDR by Western blotting, these quantifications were performed for total and individual VDR protein bands.

Localization of VDR and CYP27B1 by Immunohistochemistry

Immunohistochemistry was performed using the protocol we published previously, with minor modifications (Mahmoudi et al., 2013). Briefly, paraffin-embedded, formalin-fixed tissues were cut into 2-µm sections, dewaxed, and rehydrated. Antigen retrieval was processed in 0.01 M sodium citrate buffer (pH = 6.0) at 95°C for 20 min in a water bath. Slides were then washed three times in 0.5% bovine serum albumin (BSA)-Tris-buffered saline (TBS) [pH 7.4]. Non-specific binding sites and endogenous biotin activity were blocked with 5% normal sheep serum for 30 min followed by avidin/biotin blocking solutions (Dako, Glostrup, Denmark) for 20 min. After washing, sections were incubated with 3 µg/mL rabbit anti-VDR or anti-CYP27B1 antibodies at room temperature for 90 min, followed by washing and treatment with 3% H₂O₂-methanol for 20 min to block endogenous peroxidase activity. After rinsing as above, slides were sequentially incubated with biotinylated sheep anti-rabbit immunoglobulin (Avicenna Research Institute, Tehran, Iran) (2 µg/mL) for 45 min and streptavidin-horsradish peroxidase (Biosource, Camarillo, CA) (1:500) for 30 min. After rinsing, 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Roche) was added to slides as the chromogen. Slides were then counterstained and mounted. Anti-CYP27B1 antibody used in this study had specificity towards the cognate molecule in both human and mouse, so mouse liver was used as a negative tissue-

control for this antibody. Considering the widespread expression of VDR, no negative tissue control was used for its respective antibody. In negative-reagent control sections, the primary antibodies were pre-adsorbed with 50-fold excess of immunizing peptides. BX51 microscope and DP70 CCD camera (Olympus, Japan) were used for digital imaging.

Statistical Analysis

Real-time PCR data was analyzed using the Pair-Wise Fixed Reallocation Randomization Test by REST Software (REST-RG - version 3), with 2,000 iterations, to determine the significant difference between groups. Non-parametric (Mann-Whitney) test was used to compare the women's age and to analyze Western blot densities. Among the eight RSA patients that we analyzed for VDR, CYP27B1, and CYP24A1 expression, only one patient had had two successive abortions; the rest had three or more spontaneous abortions. In this regard, statistical analysis between these two subgroups was not possible. *P* < 0.05 was considered statistically significant.

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REFERENCES

- Adorini L. 2002. Immunomodulatory effects of vitamin D receptor ligands in autoimmune diseases. *Int Immunopharmacol* 2:1017–1028.
- Adorini L, Penna G, Giarratana N, Roncari A, Amuchastegui S, Daniel KC, Uskokovic M. 2004. Dendritic cells as key targets for immunomodulation by Vitamin D receptor ligands. *J Steroid Biochem Mol Biol* 89-90:437–441.
- Agic A, Xu H, Altgassen C, Noack F, Wolfler MM, Diedrich K, Friedrich M, Taylor RN, Hornung D. 2007. Relative expression of 1,25-dihydroxyvitamin D3 receptor, vitamin D 1 alpha-hydroxylase, vitamin D 24-hydroxylase, and vitamin D 25-hydroxylase in endometriosis and gynecologic cancers. *Reprod Sci* 14:486–497.
- Becker S, Cordes T, Diesing D, Diedrich K, Friedrich M. 2007. Expression of 25 hydroxyvitamin D3-1alpha-hydroxylase in human endometrial tissue. *J Steroid Biochem Mol Biol* 103: 771–775.
- Belkacemi L, Bédard I, Simoneau L, Lafond J. 2005. Calcium channels, transporters and exchangers in placenta: A review. *Cell Calcium* 37:1–8.
- Berek JS. 2012. Berek & Novak's gynecology. Philadelphia: Lippincott Williams & Wilkins. p 1213.
- Brown AJ, Dusso A, Slatopolsky E. 1999. Vitamin D. *Am J Physiol* 277:F157–F175.

- Bubanovic I. 2004. 1alpha,25-dihydroxy-vitamin-D3 as new immunotherapy in treatment of recurrent spontaneous abortion. *Med Hypotheses* 63:250–253.
- Crofts LA, Hancock MS, Morrison NA, Eisman JA. 1998. Multiple promoters direct the tissue-specific expression of novel N-terminal variant human vitamin D receptor gene transcripts. *Proc Natl Acad Sci USA* 95:10529–10534.
- De-Regil LM, Palacios C, Ansary A, Kulier R, Peña-Rosas JP. 2012. Vitamin D supplementation for women during pregnancy. *Cochrane Database Syst Rev* DOI: 10.1002/14651858.CD008873.pub2
- Di Rosa M, Malaguarnera M, Nicoletti F, Malaguarnera L. 2011. Vitamin D3: A helpful immuno-modulator. *Immunology* 134: 123–139.
- Evans KN, Bulmer JN, Kilby MD, Hewison M. 2004. Vitamin D and placental-decidua function. *J Soc Gynecol Investig* 11:263–271.
- Gharesi-Fard B, Zolghadri J, Kamali-Sarvestani E. 2014. Alteration in the expression of proteins in unexplained recurrent pregnancy loss compared with the normal placenta. *J Reprod Dev* 60:261–267.
- Gregori S, Casorati M, Amuchastegui S, Smiroldo S, Davalli AM, Adorini L. 2001. Regulatory T cells induced by 1 alpha,25-dihydroxyvitamin D3 and mycophenolate mofetil treatment mediate transplantation tolerance. *J Immunol* 167:1945–1953.
- Griffin MD, Lutz WH, Phan VA, Bachman LA, McKean DJ, Kumar R. 2000. Potent inhibition of dendritic cell differentiation and maturation by vitamin D analogs. *Biochem Biophys Res Commun* 270:701–708.
- Grundmann M, von Versen-Höynck F. 2011. Vitamin D—Roles in women's reproductive health. *Reprod Biol Endocrinol* 9:146. DOI: 10.1186/1477-7827-9-146
- Harrison MT. 1964. Interrelationships of vitamin D and parathyroid hormone in calcium homeostasis. *Postgrad Med J* 40: 497–505.
- Haussler MR, Whitfield GK, Haussler CA, Hsieh JC, Thompson PD, Selznick SH, Dominguez CE, Jurutka PW. 1998. The nuclear vitamin D receptor: Biological and molecular regulatory properties revealed. *J Bone Miner Res* 13:325–349.
- Henry HL. 2001. The 25[OH]D[3]/1alpha,25[OH][2]D(3)-24R-hydroxylase: A catabolic or biosynthetic enzyme. *Steroids* 66: 391–398.
- Hickie JP, Lavigne DM, Woodward WD. 1983. Reduced fecundity of vitamin D deficient rats. *Comp Biochem Physiol A Comp Physiol* 74:923–925.
- Hyppönen E. 2011. Preventing vitamin D deficiency in pregnancy: Importance for the mother and child. *Ann Nutr Metab* 59:28–31.
- Johnson LE, DeLuca HF. 2001. Vitamin D receptor null mutant mice fed high levels of calcium are fertile. *J Nutr* 131: 1787–1791.
- Johnson LE, DeLuca HF. 2002. Reproductive defects are corrected in vitamin d-deficient female rats fed a high calcium, phosphorus and lactose diet. *J Nutr* 132:2270–2273.
- Kinuta K, Tanaka H, Moriwake T, Aya K, Kato S, Seino Y. 2000. Vitamin D is an important factor in estrogen biosynthesis of both female and male gonads. *Endocrinology* 141:1317–1324.
- Kovacs CS, Kronenberg HM. 1997. Maternal-fetal calcium and bone metabolism during pregnancy, puerperium, and lactation. *Endocr Rev* 18:832–872.
- Kreuzer KA, Lass U, Landt O, Nitsche A, Laser J, Ellerbrok H, Pauli G, Huhn D, Schmidt CA. 1999. Highly sensitive and specific fluorescence reverse transcription-PCR assay for the pseudo-gene-free detection of beta-actin transcripts as quantitative reference. *Clin Chem* 45:297–300.
- Kwiecinski GG, Petrie GI, DeLuca HF. 1989a. Vitamin D is necessary for reproductive functions of the male rat. *J Nutr* 119:741–744.
- Kwiecinski GG, Petrie GI, DeLuca HF. 1989b. 1,25-Dihydroxyvitamin D3 restores fertility of vitamin D-deficient female rats. *Am J Physiol* 256:E483–E487.
- Lapillonne A. 2010. Vitamin D deficiency during pregnancy may impair maternal and fetal outcomes. *Med Hypotheses* 74:71–75.
- Lerchbaum E, Rabe T. 2014. Vitamin D and female fertility. *Curr Opin Obstet Gynecol* 26:145–150.
- Li XY, Boudjelal M, Xiao JH, Peng ZH, Asuru A, Kang S, Fisher GJ, Voorhees JJ. 1999. 1,25-Dihydroxyvitamin D3 increases nuclear vitamin D3 receptors by blocking ubiquitin/proteasome-mediated degradation in human skin. *Mol Endocrinol* 13:1686–1694.
- Mahmoudi AR, Zarnani AH, Jeddi- Tehrani, Katouzian L, Tavakoli M, Soltanghoraei H, Mirzadegan E. 2013. Distribution of vitamin D receptor and 1 α -hydroxylase in male mouse reproductive tract. *Reprod Sci* 20:426–436.
- Nangia AK, Butcher JL, Konety BR, Vietmeier BN, Getzenberg RH. 1998. Association of vitamin D receptors with the nuclear matrix of human and rat genitourinary tissues. *J Steroid Biochem Mol Biol* 66:241–246.
- Nassar N, Halligan GH, Roberts CL, Morris JM, Ashton AW. 2011. Systematic review of first-trimester vitamin D normative levels and outcomes of pregnancy. *Am J Obstet Gynecol* 205:e1–e7. DOI: 10.1016/j.ajog.2011.03.058
- Nibbelink KA, Tishkoff DX, Hershey SD, Rahman A, Simpson RU. 2007. 1,25(OH) 2-vitamin D3 actions on cell proliferation, size, gene expression, and receptor localization, in the HL-1 cardiac myocyte. *J Steroid Biochem Mol Biol* 103:533–537.
- Ota K, Dambaeva S, Han AR, Beaman K, Gilman-Sachs A, Kwak-Kim J. 2014. Vitamin D deficiency may be a risk factor for recurrent pregnancy losses by increasing cellular immunity and autoimmunity. *Hum Reprod* 29:208–219.
- Penna G, Adorini L. 2000. 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. *J Immunol* 164:2405–2411.

- Pfaffl MW, Horgan GW, Dempfle L. 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 30:e36.
- Ramakers C, Ruijter JM, Deprez RH, Moorman AF. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* 339:62–66.
- Ruijter JM, Ramakers C, Hoogaars WM, Karlen Y, Bakker O, van den Hoff MJ, Moorman AF. 2009. Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res* 37:e45. DOI: 10.1093/nar/gkp045
- Saito S, Nakashima A, Shima T, Ito M. 2010. Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy. *Am J Reprod Immunol* 63:601–610.
- Shahbazi M, Jeddi-Tehrani M, Zareie M, Salek-Moghaddam A, Akhondi MM, Bahmanpoor M, Sadeghi MR, Zarnani AH. 2011. Expression profiling of vitamin D receptor in placenta, decidua and ovary of pregnant mice. *Placenta* 32:657–664.
- Sugiyama T, Shigetomi M, Ihara K, Ohno T, Muramatsu K, Tanaka H, Kawai S, Kawano H. 2004. Not only vitamin D but also statin may be a good choice in the management of patients undergoing transplantation. *Transplantation* 77:635–636.
- Sunn KL, Cock TA, Crofts LA, Eisman JA, Gardiner EM. 2001. Novel N-terminal variant of human VDR. *Mol Endocrinol* 15:1599–1609.
- Tabesh M, Salehi-Abargouei A, Tabesh M, Esmaillzadeh A. 2013. Maternal vitamin D status and risk of pre-eclampsia: A systematic review and meta-analysis. *J Clin Endocrinol Metab* 98:3165–3173.
- Tavakoli M, Jeddi-Tehrani M, Salek-Moghaddam A, Rajaei S, Mohammadzadeh A, Sheikhhasani S, Kazemi-Sefat GE, Zarnani AH. 2011. Effects of 1,25(OH)₂ vitamin D₃ on cytokine production by endometrial cells of women with recurrent spontaneous abortion. *Fertil Steril* 96:751–757.
- van Etten E, Mathieu C. 2005. Immunoregulation by 1,25-dihydroxyvitamin D₃: Basic concepts. *J Steroid Biochem Mol Biol* 97:93–101.
- van Etten E, Stoffels K, Gysemans C, Mathieu C, Overbergh L. 2008. Regulation of vitamin D homeostasis: Implications for the immune system. *Nutr Rev* 66:125S–134S.
- Vanni VS, Vigano' P, Somigliana E, Papaleo E, Paffoni A, Pagliardini L, Candiani M. 2014. Vitamin D and assisted reproduction technologies: current concepts. *Reprod Biol Endocrinol* 12:47. DOI:10.1186/1477-7827-12-47
- Vienonen A, Miettinen S, Bläuer M, Martikainen PM, Tomás E, Heinonen PK, Ylikomi T. 2004. Expression of nuclear receptors and cofactors in human endometrium and myometrium. *J Soc Gynecol Investig* 11:104–112.
- Viganò P, Lattuada D, Mangioni S, Ermellino L, Vignali M, Caporizzo E, Panina-Bordignon P, Besozzi M, Di Blasio AM. 2006. Cycling and early pregnant endometrium as a site of regulated expression of the vitamin D system. *J Mol Endocrinol* 36:415–424.
- Weisman Y, Harell A, Edelstein S, David M, Spirer Z, Golander A. 1979. 1 alpha, 25-Dihydroxyvitamin D₃ and 24,25-dihydroxyvitamin D₃ in vitro synthesis by human decidua and placenta. *Nature* 281:317–319.
- Yousefzadeh P, Shapses SA, Wang X. 2014. Vitamin D binding protein impact on 25-Hydroxyvitamin D levels under different physiologic and pathologic conditions. *Int J Endocrinol* 2014:981581. DOI: 10.1155/2014/981581
- Zehnder D, Evans KN, Kilby MD, Bulmer JN, Innes BA, Stewart PM, Hewison M. 2002. The ontogeny of 25-hydroxyvitamin D(3) 1alpha-hydroxylase expression in human placenta and decidua. *Am J Pathol* 161:105–114.
- Zarnani AH, Shahbazi M, Salek-Moghaddam A, Zareie M, Tavakoli M, Ghasemi J, Rezania S, Moravej A, Torkabadi E, Rabbani H, Jeddi-Tehrani M. 2010. Vitamin D₃ receptor is expressed in the endometrium of cycling mice throughout the estrous cycle. *Fertil Steril* 93:2738–2743.
- Zhang RJ, Zou LB, Zhang D, Tan YJ, Wang TT, Liu AX, Qu F, Meng Y, Ding GL, Lu YC, Lv PP, Sheng JZ, Huang HF. 2012. Functional expression of large-conductance calcium-activated potassium channels in human endometrium: A novel mechanism involved in endometrial receptivity and embryo implantation. *J Clin Endocrinol Metab* 97:543–553.