Decreased expression of the vitamin D receptor in women with recurrent pregnancy loss

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ABSTRACT

The multiple functions of vitamin D_3 have stimulated interest in the role that this vitamin may play during pregnancy. The present study investigated the expression of the vitamin D receptor (VDR) in women during the first trimester of pregnancy in order to determine whether VDR is associated with recurrent pregnancy loss (RPL). Forty women at 7—10 weeks gestation with RPL and 40 women of similar gestational age with a healthy pregnancy were recruited. VDR mRNA and protein in chorionic villi and decidua were evaluated by immunohistochemistry, confocal laser scanning microscopy (CLSM), western blot, and quantitative real-time polymerase chain reaction. The serum levels of VDR were measured by an enzyme-linked immunosorbent assay. Women with RPL had a significantly weaker expression of VDR mRNA in villi and decidual tissues compared with the control women (both $p < 0.0001$). Western blot analysis showed an approximately 46% decrease in VDR expression in villi and a 52% decrease in decidua in the RPL vs. the controls. Serum VDR levels were also significantly lower in the RPL group than in the control group ($p = 0.003$). Compared with the controls, immunohistochemical and CLSM analysis revealed significantly lower VDR expression in villous cytotrophoblasts and stromal cells, as well as in decidual glandular epithelial and stromal cells (all $p < 0.05$). In conclusion, these observations show that women with RPL have lower levels of VDR expression in chorionic villi, decidua and serum compared with normal pregnant women, suggesting that decreased VDR expression in the first trimester pregnancy may be associated with RPL.

1. Introduction

Several pathogenic mechanisms associated with recurrent pregnancy loss (RPL) have previously been described, including chromosomal anomalies, congenital uterine anomalies, acquired and inherited thrombophilia, endocrine problems, infections, autoimmune disorders, and male factors. Furthermore, up to 50% of cases of RPL have no clearly identifiable etiology [1]. Immunological mechanisms have been proposed to explain some cases of RPL. Women with RPL exhibit what is considered a generalized exaggerated inflammatory immune response during pregnancy and show signs of a disruption in the tolerance of autoantigens and fetal antigens [2].

It is well established that 1, 25 dihydroxy vitamin D_3 ($1,25(OH)_2D_3$), a pleiotropic steroid hormone, has classic effects on bone metabolism and mineral homeostasis. However, the discovery that multiple functions of vitamin D_3 are important for growth and development, including immune system development [3], has stimulated interest in the role that this vitamin may play during pregnancy. Several studies have suggested that vitamin D carries out important functions in a large number of immune-mediated responses [4—8]. Vitamin D_3 inhibits adaptive immunity and cell proliferation and simultaneously promotes innate immunity and stimulates cellular differentiation [4]. Th17 cells, characterized by IL-17 production, play a critical role in the pathogenesis of autoimmune diseases and are inhibited by $1,25(OH)_2D_3$ in vitro [5].

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Vitamin D_3 deficiency or insufficiency is associated with an increased prevalence of autoimmune diseases, such as rheumatoid arthritis [6], systemic lupus erythematosus (SLE) [7], and multiple sclerosis [8]. Vitamin D_3 may also have potential utility in the treatment of asthma by suppressing TNF-α expression [9]. Studies have shown that vitamin D_3 acts as a potent modulator of both innate and acquired immune responses and is also an immuno-regulatory hormone with beneficial effects on T-helper 1 (Th1) cell-mediated inflammatory diseases.

Vitamin D_3 exerts its effects by binding to a single vitamin D receptor (VDR) in the cell nucleus, which is present in multiple tissues [10]. The presence of VDR in human placenta, decidua, and endometrium [10–12] suggests that the cells of these tissues are targets of vitamin D_3 action. However, the level of VDR expression in the decidua and chorionic villi in women with RPL has received very limited attention. Thus, the purpose of our study was to test the hypothesis that VDR expression is decreased in the fetal-maternal interface in women with RPL. In this study, we investigated VDR expression in the chorionic villous, decidual tissues and serum of women with RPL and normal pregnant women in the first trimester of pregnancy.

2. Materials and methods

2.1. Subjects

In this study, research subjects were recruited from the First Affiliated Hospital of Xi'an Jiaotong University, Shaanxi, China, between October 2013 and October 2014. Forty women (aged 25–32 years) with an intact gestational sac and no visible cardiac activity in the embryo were diagnosed with a pregnancy loss by trans-vaginal ultrasound (RPL group). The average gestational age was 64.8 ± 7.9 days (range, 7–10 weeks). In addition, these women had a history of at least two consecutive pregnancy losses at 7–10 weeks gestation without any prior normal pregnancies. Those women who were diagnosed with uterine anomalies; chromosomal abnormalities; thyroid dysfunction; infections with rubella, toxoplasma, cytomegalovirus, and herpes virus; hypertension; diabetes mellitus; and autoimmune disorders were excluded. The control group consisted of forty women (aged 24–32 years) with viable pregnancies between 7 and 10 weeks gestation (63.7 ± 9.4 days) who were gestationally matched to the study group and who underwent voluntary pregnancy termination. The control group had a history of one or more prior normal pregnancies, no history of pregnancy loss and were generally in good health. Women in the control and RPL groups did not differ significantly in average maternal and average gestational age.

The study was approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University and was conducted according to Declaration of Helsinki principles. An informed consent was obtained from all participants.

2.2. Human chorionic villus, decidua, and serum analysis

Chorionic villous and decidual tissues were obtained during the surgical evacuation of uterus in women with RPL within the first 24 h after diagnosis or elective termination of normal uncompli-cated pregnancy. Additionally, a blood sample (3 mL) was collected into 5-mL sterile plain tubes without anticoagulanton the same day in 40 women with RPL and 40 women in the control group. For RNA and protein extraction, a total of 40 villous and decidual samples, including 20 RPL and 20 control samples, were collected and snap-frozen in liquid nitrogen ($-80\ ^{\circ}C$). For immunohistochemistry and confocal laser scanning microscopy (CLSM), 60 villous and decidual samples (30 RPL and 30 control samples) were routinely processed

and frozen at $-70\ ^{\circ}C$.

2.3. Streptavidin-peroxidase immunohistochemistry for VDR

VDR expression in decidua and chorionic villi was analyzed using a streptavidin-peroxidase immunohistochemistry kit (Zymed Laboratories, Inc., San Francisco, CA, USA) in accordance with the manufacturer's instructions. Briefly, frozen sections were incubated with 30% hydrogen peroxide (1:10) to block endogenous peroxi-dase activity for 5–10 min at room temperature and then washed in distilled water. The sections were then incubated in normal goat serum blocking solution, followed by anti-VDR monoclonal anti-body (1:400; Abcam Inc., Cambridge, MA) for 2 h at 37 $^{\circ}C$. After rinsing with phosphate-buffered saline (PBS) (pH 7.4) for 15 min, the biotinylated goat anti-mouse IgG was added to sections for 30 min at 37 $^{\circ}C$, followed by horseradish peroxidase-conjugated avidin for 30 min at 37 $^{\circ}C$. Staining was developed using dia-minobenzidine, followed by counterstaining with hematoxylin and washing with cold running water (DAB-Stock Stain box; Boster Biological Technology, Ltd., Wuhan, China). The primary antibody was omitted in the negative controls. Digital images were acquired using a section microscope scanner (Leica MP SCN400, German). The software was used to semi-automatically estimate the volume of immunopositive cells within a tissue sample. In the final quan-tification, the average gray-scale value of five fields in each slide was estimated. To further exclude operator bias, observations were performed on coded samples in a blinded manner following the same procedure.

2.4. Confocal laser scanning microscopy for VDR

The fluorescent intensity detection of VDR immunostaining was performed using previously described methods [13]. Briefly, air-dried cryosections immersed in hydrogen peroxide were incu-bated in normal goat serum blocking solution, followed by anti-VDR monoclonal antibody (1:500; Abcam Inc., Cambridge, MA) overnight at 4 $^{\circ}C$. After rinsing with PBS (pH 7.4) for 15 min, the sections were exposed to fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G for 2 h at 37 $^{\circ}C$, and their nuclei were counterstained with 4$'$,6-diamidino-2-phenylindole (DAPI, 1:5000) for 15 min at room temperature. The fluorescence signal from VDR immunostaining was observed and quantified using CLSM. The images were analyzed by IMAGE PLUS software (Leica, Microsystems, Wetzlar, Germany), and the mean fluores-cence intensity within a specific population was calculated. Fluo-rophores were excited using a 488-nm argonlaser beam, and fluorescence emission was assessed at 543 nm.

2.5. Western blot for VDR

Samples of villi and decidual tissues (200 mg) were treated with 500 μL of cold lysis buffer containing 2 μL of protease inhibitor mix. Lysates were subsequently centrifuged at 12,000 g for 20 min at 4 $^{\circ}C$; the supernatant was collected; and the protein concentration was determined using a commercial protein assay kit (BCA Protein Assay Kit, Beijing Tiangen Co., China). The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) mem-branes (Millipore, USA). For VDR detection, after briefly washing with TBST (25 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.1% Tween 20), the membranes were blocked with 5% skim milk for 2 h at room temperature. The membranes were incubated overnight at 4 $^{\circ}C$ with anti-VDR monoclonal antibody (Abcam Inc., Cambridge, MA) diluted in 1000 mL of TBST. After washing with TBST, the mem-branes were incubated for 2 h at 37 $^{\circ}C$ with peroxidase-conjugated

goat anti-rabbit IgG. For the protein load control, anti-β-actin rabbit monoclonal antibodies (Beijing Biosynthesis Biotec. CO., LTD., China) were used. Anti-rabbit IgG secondary antibody was used at a 1:5000 dilution. Bound antibodies were visualized by chemiluminescence. Results were expressed as the signal intensity of VDR bands with respect to β-actin as the loading control.

2.6. Quantitative real-time PCR assay of VDR mRNA

Real-time RT-quantitative polymerase chain reaction (RT-qPCR) analysis was performed on total RNA isolated from snap-frozen villi and decidual tissues. Briefly, total RNA was isolated from villi and decidual tissues with the Mini BEST Universal RNA Extraction Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. RNA content was quantified by spectrophotometry. For each sample, 1–5 μg of RNA was reverse-transcribed in a volume of 10 μL using PrimeScript™ RTMaster Mix (TaKaRa, Dalian, China). Analysis of gene expression was performed using specific primers for the VDR target gene and β-actin reference genes. The primer sequences for VDR were as follows: forward 5′-CTCCTGCTCA-GATCACTGTATCAC-3′, reverse 5′-CTGGGACAGCTCTAGGGTCA-3′; β-actin: forward 5′- TGGCACCCAGCACAATGAA-3′, reverse 5′-CTA-AGTCATAGTCCGCCTAGAAGCA-3′. The SYBR Green method was performed, and a reaction mixture of the SYBR@ Premix Ex TaqTMII (Tli RNaseH Plus) (TaKaRa, Dalian, China) was prepared. The PCR was performed using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, California, USA). The PCR conditions were as follows: 30 s at 95 °C, followed by 39 cycles at 95 °C for 5 s and 60 °C for 30 s. The results were analyzed using the comparative $\triangle\triangle$Ct method. For each experimental sample, $2^{-\triangle\triangle Ct}$ was calculated, and the data were reported as relative expression levels.

2.7. Measurement of serum VDR

After centrifugation for 10 min at 3000 rpm at room temperature, the serum was stored at −20 °C until assayed. Serum levels of VDR were measured with a commercially available enzyme-immunoassay kit for VDR (Cusabio Biotech Co., LTD., Wuhan, China) according to the manufacturer's instructions. All measurements were performed in duplicate. Biological samples were blinded prior to the analyses. The soluble serum VDR levels are expressed in picograms per milliliter.

2.8. Statistical analysis

Statistical analysis was performed using SPSS-PC + software (SPSS Inc., Chicago, IL, USA). Fluorescence intensity, gray-scale values, and serum levels are expressed as the mean ± SEM. Statistical significance was determined using Student's t-test. A P < 0.05 was considered significant.

3. Results

3.1. VDR mRNA expression

The chorionic villi and decidual tissues obtained from the RPL and control groups were evaluated for VDR mRNA expression by real-time RT-qPCR. The VDR mRNA was detected in all the villi and decidual samples. VDR mRNA expression in villi (0.31 ± 0.04) and decidual tissues (0.34 ± 0.02) was significantly decreased in the RPL group compared with the control group (1.09 ± 0.10; 1.01 ± 0.09; $t = 7.07$, $t = 7.25$, both P < 0.0001). It showed a decrease of approximately 52% and 66% in VDR mRNA expression in chorionic villi and decidua in the RPL group compared with the control group, respectively (Fig. 1A). The ACTB (β-actin) mRNA was used as an endogenous control gene.

3.2. VDR protein expression: western blot

Western blot analysis was conducted to assess the total amount of VDR protein in the chorionic villi and decidua of pregnant women in the RPL and control groups. Through Western blot analysis, the 48-kD VDR protein was detected in samples of chorionic villi and decidua. Quantification of VDR expression revealed that VDR protein levels were decreased in chorionic villi and decidua from pregnant women with RPL compared with the control group. Densitometric analysis of Western blots showed a decrease of approximately 46% in VDR expression in chorionic villi and a 52% decrease in VDR expression in decidua in the RPL group compared with the control group (Fig. 1B and Table 1). VDR protein levels were normalized to β-actin (Fig. 1C).

3.3. VDR protein expression: immunohistochemical and CLSM

VDR protein was detected in all tissue samples. The chorionic villous and decidual cells exhibited specific VDR immunoreactivity in their nuclei with some scattered patterns of reactivity in their cytoplasm. In the decidua, VDR staining was observed in the stromal cells, glandular epithelial cells, and vascular endothelial cells. In the chorionic villi, VDR expression was located on the stromal cells, syncytiotrophoblast cells, cytotrophoblast cells, and vascular endothelial cells. Furthermore, immunohistochemical analysis of the decidua revealed a significantly weaker VDR expression in decidual stromal and glandular epithelial nuclei in the RPL group compared with the control group (nucleoplasmic ratio: 0.76 ± 0.15 vs. 0.86 ± 0.12, $t = 3.230$, $P = 0.002$; 0.64 ± 0.11 vs. 0.70 ± 0.13, $t = 2.209$, $P = 0.030$). Additionally, weak expression was observed in the decidual vascular endothelial cells in the RPL group, but the expression was not significantly different from that in the control group. In the chorionic villi, compared with the control group, cytotrophoblasts and stromal nuclei in the RPL group expressed lower levels of VDR protein (nucleoplasmic ratio: 0.70 ± 0.09 vs. 0.76 ± 0.11, $t = 2.655$, $P = 0.009$; 0.81 ± 0.12 vs. 0.89 ± 0.10, $t = 2.913$, $P = 0.005$). Other cells, including vascular endothelial and syncytiotrophoblast cells, did not show significant differences in VDR expression between the RPL and control groups (Table 2 and Fig. 2).

To confirm the immunohistochemistry results, we performed immunofluorescence staining for VDR in the chorionic villi and decidua to evaluate the relative localization and nuclear translocation of VDR and analyze the fluorescence intensity using CLSM. We found similar localization and consistent nuclear expression intensity of VDR in four types of villous cells and three types of decidual cells (Fig. 3).

3.4. The serum levels of VDR

Serum levels of VDR (57.55 ± 3.81 pg/mL) were significantly lower in the RPL group (87.07 ± 8.65 pg/mL) compared with the control group ($t = 3.12$, $P = 0.003$). The mean serum VDR level in the RPL group reduced approximately 34% compared with the control group (Fig. 1D).

4. Discussion

In this study, the chorionic villous and decidual tissues from first trimester pregnancies co-expressed VDR mRNA and protein. The results of real-time RT-qPCR analysis were consistent with those of the Western blot analysis; specifically, the expression levels of VDR were reduced in the chorionic villi and decidua in the RPL group compared with the controls. Based on the immunohistochemistry
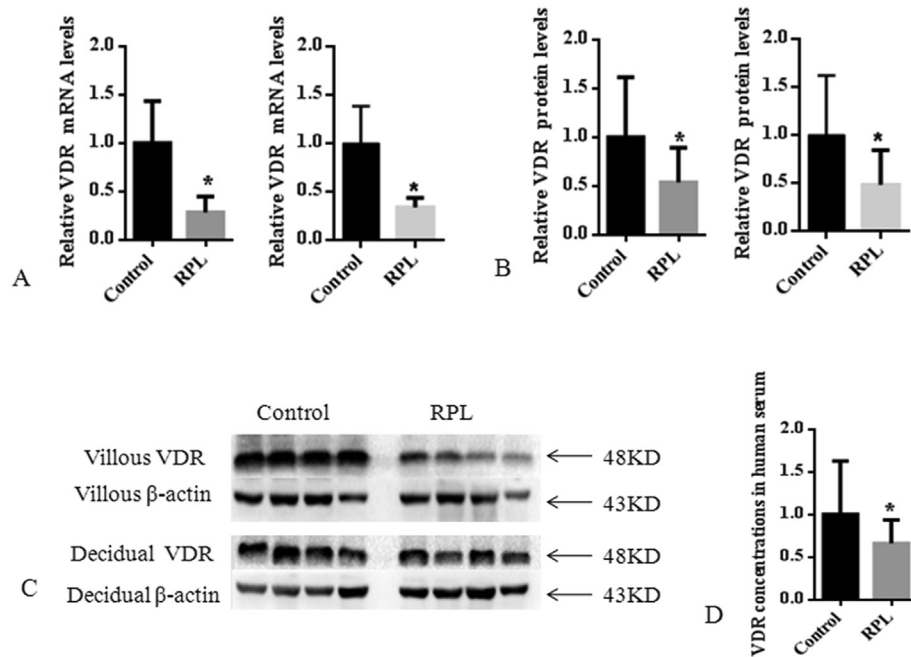
Fig. 1. VDR expression in chorionic villous, decidual tissues, and serum. (A) RT-qPCR analysis of VDR mRNA in villous and decidual tissues obtained from control pregnant women and RPL during the first trimester of pregnancy. VDR relative expressions in chorionic villous and decidual tissues from RPL were lower than that from controls. Left: chorionic villus; Right: decidua. *: both $P < 0.0001$. (B) VDR protein expression in chorionic villous and decidual tissues from RPL were significantly decreased compared to controls. Left: chorionic villus; Right: decidua. *: $P = 0.006$ and 0.003. (C) Western blots profile of total homogenate of villous and decidual tissues. Four representative cases of early pregnancy from RPL and controls were shown. Detection of β-actin was used for protein load control. (D) Mean serum VDR level in RPL was lower than that in controls. *: $P = 0.003$.

and CLSM results, in the first trimester of human pregnancy, VDR is expressed in villous cells, including trophoblasts, villous stromal cells, and vascular endothelial cells, as well as in decidual cells, including stromal, glandular epithelial, and vascular endothelial cells. Furthermore, VDR expression was mostly decreased in cytotrophoblasts, decidual glandular epithelial cells, and villous and decidual stromal cells. These results provide evidence for disrupted VDR metabolic homeostasis in early pregnancy loss. Several recent studies have reported that trophoblast cells are the principal cells expressing VDR in human term placentas [12,14]. VDR protein expression has also been previously demonstrated in isolated cultured human extravillous trophoblasts (EVT) from first trimester placentas [15]. In all of the studies, VDR expression has been mainly addressed in the term placenta or cultured human trophoblasts. However, to our knowledge, this is the first study reporting the expression and distribution of VDR in human chorionic villi and decidua during the first trimester of pregnancy in vivo. Furthermore, this is the first report of a correlation between VDR expression and RPL. The presence of VDR in both villi and decidua indicates that vitamin D metabolism and localized autocrine or paracrine regulatory signaling occurs in the fetal-maternal interface in early human pregnancy.

The effects of alterations in VDR expression in the fetal-maternal interface of women with RPL are unclear. Previous published studies have shown several biological effects of $25(OH)_2D_3$ in the placenta [16]. For example, $1,25(OH)_2D_3$ regulates the expression of

HCG, estradiol, and progesterone [17,18]. It also promotes innate immune responses in fetal trophoblasts [19] and regulates both acquired and innate immune responses in maternal decidua [20] and placental inflammation [21,22]. Together, these observations suggest that a function of vitamin D during pregnancy is to support normal immune responses. Locally synthesized $1,25(OH)_2D_3$ also contributes to the successful engraftment and growth of the maternal-fetal unit [23]. $1,25(OH)_2D_3$ up-regulates the expression of HOXA10 in primary human endometrial stromal cells [24], which is directly involved in the regional development of uterine decidualization and embryo implantationby controlling downstream target genes. Thus, $1,25(OH)_2D_3$ may contribute to successful embryo implantation [25,26]. Collectively, these data suggest that $1,25(OH)_2D_3$ aids embryo implantation, controls the secretion of multiple placental hormones, limits the production of proinflammatory cytokines, and maintains successful pregnancies. It is well known that the biological function of $1,25(OH)_2D_3$ is mediated through binding to VDR on cells. Based on the broad distribution of VDR in different cell types in first trimester villi and decidua, vitamin D_3 signaling appears to be a basic requirement for

Table 1
Comparison of VDR protein expression in chorionic villus and decidua between the RPL group and control group.

	RPL (n = 20)	Control (n = 20)	t	p value
Chorionic villus	0.77 ± 0.50	1.42 ± 0.87	2.917	0.006
Decidua	0.54 ± 0.41	1.12 ± 0.70	3.242	0.003

Table 2
Comparison of VDR expression in different cell types of the chorionic villus and decidua between the RPL group and control group.

	RPL (n = 30)	Control (n = 30)	t	p value
Chorionic villus				
Syncytiotrophoblast cells	0.66 ± 0.10	0.69 ± 0.12	1.119	0.266
Cytotrophoblast cells	0.70 ± 0.09	0.76 ± 0.11	2.655	0.009
Stromal cells	0.81 ± 0.12	0.89 ± 0.10	2.913	0.005
Vessel endothelial cells	0.86 ± 0.11	0.90 ± 0.11	0.588	0.558
Decidua				
Glandularepithelial cells	0.64 ± 0.11	0.70 ± 0.13	2.209	0.030
Stromal cells	0.76 ± 0.15	0.86 ± 0.12	3.230	0.002
Vessel endothelial cells	0.80 ± 0.12	0.83 ± 0.10	0.996	0.332
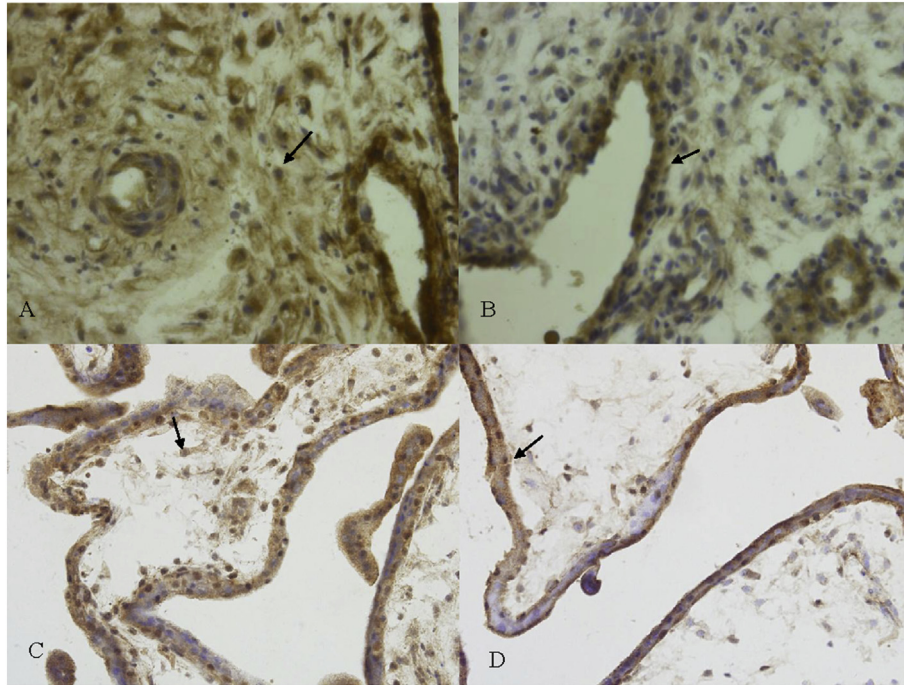
Fig. 2. Immunohistochemistry for VDR: A, control decidual tissue, B, decidual tissue with RPL, C, control villous tissue, and D, villous tissue with RPL. The arrows show the immunostaining cells. (Scale bar, 73 μm).

most cells in the fetal-maternal interface. In the placental tissue of pregnant women with $25(OH)D_3$ deficiencies, VDR expression was downregulated [27]. $1,25(OH)_2D_3$ regulates the expression and downstream signaling of VDR by binding to VDR [21,28]. In this study, it was interesting that, compared with a normal early pregnancy, VDR expression was significantly decreased in the villi and decidual tissues of women with RPL. This finding emphasizes the role of VDR in conception and survival of a pregnancy. In view of the well-recognized functions of $1,25 (OH)_2D_3$, the proper VDR expression at the fetal-maternal interface may contribute to the
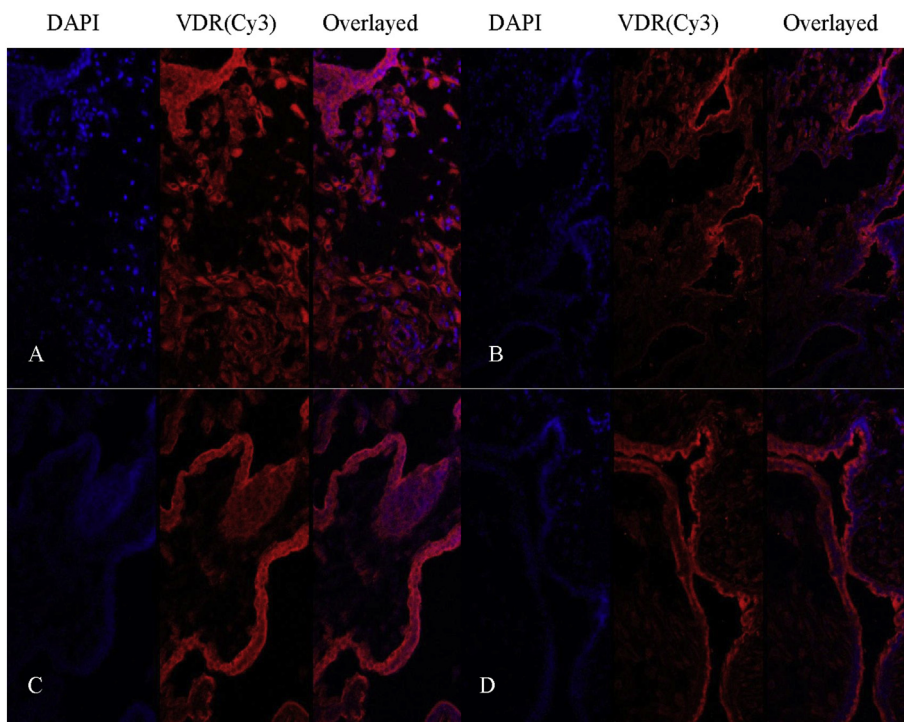
Fig. 3. CLSM for VDR: A, control decidual tissue, B, decidual tissue with RPL, C, control villous tissue, and D, villous tissue with RPL. Red color indicates positive staining for VDR protein; Blue color indicates nuclear. CLSM × 400.

establishment and maintenance of the fetal-placental unit and favorable immune environment during pregnancy.

We also found that serum VDR levels were decreased in women with RPL compared with the control group during early stages of pregnancy. In another report, we found that 25(OH)D levels in serum were lower in early spontaneous abortion than those in normal early pregnant women [29]. These results suggest that serum 25(OH)D and VDR levels may predict the level of VDR expression at the fetal-maternal interface. Considering that vitamin D insufficiency is common in spontaneous early abortion [30,31] and that few foods contain vitamin D, as well as vitamin D_3 immunomodulatory effect [11], the measurement of serum 25(OH)D level by a reliable assay was recommended in women with RPL. Vitamin D supplementation should be explored further as a possible therapeutic option to decrease risk of spontaneous early abortion.

In the present work, we focused only on VDR expression level at the fetal-maternal interface in the first trimester pregnancy during women with RPL and normal pregnant women. Although it is expected that proper VDR expression plays critical roles in trophoblast and stromal cell's function during the first trimester pregnancy, the consequences of reduced VDR expression in RPL are not known. It is not clear whether reduced VDR expression is associated with local immune tolerance of the fetal-maternal interface or altered downstream target gene activity in villous and decidual tissues in RPL. Further study of cellular and molecular regulation of VDR signaling in villous and decidual tissues during the first trimester pregnancy answers these questions.

In summary, VDR is expressed in the villous and decidual cells of pregnant women during the first trimester of pregnancy. In addition, women with RPL have a lower level of VDR expression in chorionic villi, decidua, and serum compared with normal pregnant women, suggesting that decreased VDR expression during the first trimester of pregnancy may be associated with RPL. The above phenomenon is consistent with the role of vitamin D_3 in the immunological adjustments that occur during pregnancy. Considering the complexity network of immunoregulation at the fetal-maternal interface, potential beneficial effects of vitamin D_3 in patients with RPL should be investigated in clinical practice.

Conflict of interests

The authors declare that there is no conflict of interest.

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