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Altered decidual and placental catabolism of vitamin D may contribute to the aetiology of spontaneous miscarriage

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ABSTRACT

Introduction: Vitamin D catabolizing enzymes, along with vitamin D receptor (VDR) and vitamin D binding protein (DBP) are expressed in the decidua and placenta during pregnancy and capable of synthesizing active vitamin D. Vitamin D plays roles in immunoregulation and trophoblast invasion, key features of a successful pregnancy. Epidemiological data suggests that vitamin D deficiency is associated with both spontaneous and recurrent miscarriage but few studies have investigated the expression of the key vitamin D catabolizing enzymes in miscarriage.

Methods: Placenta and decidua were collected after termination of apparently normal pregnancies (controls, n = 22) or spontaneous miscarriage (n = 20). Immunohistochemical staining, Western Blot and qRT-PCR were performed for CYP27B1, CYP24A1, CYP2R1, VDR and DBP (not qRT-PCR). HTR-8/SVneo cells were cultured in $CoCL_2$ (hypoxic mimetic) or LPS (bacterial infection mimetic) for 24 h, RNA extracted and qRT-PCR performed for CYP27B1, CYP24A1, CYP2R1 and VDR.

Results: In spontaneous miscarriage, placental and decidual expression of CYP27B1 was reduced, while expression of CYP24A1, VDR and DBP was increased. When a trophoblast cell line was treated with $CoCL_2$ expression of CYP27B1 was increased and CYP24A1 was reduced, while LPS induced expression of VDR.

Discussion: This is the first report of altered utero-placental vitamin D catabolism in spontaneous miscarriage. It is becoming accepted that women who are undergoing assisted reproductive technologies should ensure they have sufficient vitamin D levels prior to pregnancy, these data support that all women should ensure they are vitamin D replete before planning to get pregnant.

1. Introduction

Spontaneous miscarriage is a common complication of early pregnancy and is classified as early (less than 12 weeks) or late (12–21 weeks) miscarriage based on gestational age. The aetiology of spontaneous miscarriage is multifactorial with several known causes including chromosomal anomalies, abnormal uterine anatomy, infection, endosecretory factors, autoimmune disorders, thrombophilias, environmental factors and abnormal placentation (trophoblast invasion and/or spiral artery remodeling) [1]. However, the cause of many miscarriages remains unknown.

Vitamin D was first recognized for its roles in the regulation of calcium metabolism and bone health, however it has also been shown to play roles in regulating cell proliferation, differentiation; and apoptosis [2]. By exposure to ultraviolet light (270–300) nm, 7-dehydrocholesterol is transformed into vitamin D in the skin. In the human body, the native forms of vitamin D do not have biological activity and need to be converted to active metabolites, a process that occurs via several cytochrome P450 (CYP) enzymes. In the liver vitamin D undergoes hydroxylation by 25-hydroxylase (CYP2R1) to create 25 hydroxyvitamin D (25OHD), the main circulating form of vitamin D, which is then transported to the kidney where it undergoes further hydroxylation by

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1α-hydroxylase (CYP27B1) to produce 1,25$(OH)_2$D3, which is the major bioactive form of the hormone. When in excess, both 25OHD and 1,25$(OH)_2$D3 can be degraded by CYP24A1 to 24,25$(OH)_2$D and excreted by the kidney [3]. Vitamin D and its metabolites are transported by its carrier protein, vitamin D binding protein (DBP). The active form, 1,25$(OH)_2$D3, interacts with the vitamin D receptor (VDR), which is expressed in most tissues and cells, facilitating a wide range of biological actions [4]. Due to the rapid turnover of 1,25$(OH)_2$D3, it is not suitable as a biomarker for vitamin D status, therefore serum 25OHD levels are commonly used to indicate vitamin D status [5].

The maternal-fetal interface is one of the few extra-renal tissues, capable of metabolizing both 25OHD and 1,25$(OH)_2$D3 by expressing related enzymes in both the placenta and decidua [6,7]. In the first- and second-trimester placenta and decidua, VDR and CYP27B1 are strongly expressed and there is evidence for local conversion of 25OHD into 1,25$(OH)_2$D3 [8,9] that can play autocrine and paracrine roles, including the potential to influence immune cells (such as macrophages, uterine natural killer cells, dendritic cells and T-lymphocytes) to maintain homeostasis [9–11], and regulate extravillous trophoblast cell (EVT) invasion [12]. Abnormal placentation due to shallow invasion of EVT and insufficient spiral artery remodeling can cause important pregnancy disorders, and vitamin D deficiency is associated with increasing the risk for the same gestational complications including preeclampsia (PE) [13], miscarriage [14], fetal growth restriction (FGR) and small for gestational age babies (SGA) [15].

While it is becoming increasingly clear that vitamin D may play a role in the establishment and maintenance of a healthy pregnancy, little is known about the expression of the key metabolizing enzymes, VDR and DBP in the placenta and decidua in spontaneous miscarriage. Therefore, the aim of this study was to determine mRNA and protein expression levels of vitamin D metabolizing enzymes (CYP2R1, CYP24A1, CYP27B1, DBP and VDR), as well as to identify their location in placenta and decidual tissues in spontaneous miscarriage patients and controls. In addition, to determine whether any differences observed were causative of the miscarriage or a response to hyperoxia or infection, two of the major causes of miscarriage, the expression of CYP2R1, CYP24A1, CYP27B1 and VDR by HTR-8/SVneo trophoblast cells were examined after cobalt chloride or lipopolysaccharide treatment.

2. Materials and methods

2.1. Subjects and sample collections

This was a case-control study. Placenta and decidua samples were obtained from 20 pregnant women with unexplained spontaneous miscarriage between 5 and 7 weeks gestation (determined by ultrasound) and 22 pregnant women undergoing elective surgical termination of apparently uncomplicated pregnancies between 5 and 7 weeks gestation in Guangzhou Women and Children's Medical Center (GWCMC). The karyotype of the embryos was not determined. Blood samples were taken prior to surgery and were processed within 3 h; serum was stored at −80 °C until required for analysis. Placental and decidua samples were separated and washed thoroughly with sterile saline. Samples intended for RNA extraction were placed in RNALater (Invitrogen, California, USA); samples for immunohistochemistry (IHC) were fixed with 10% formalin and embedded in paraffin; samples for protein extraction were snap-frozen in dry-ice and transferred to −80 °C freezer until required for analysis.

2.2. Measurement of serum 25OHD

Whole blood samples were taken just prior to elective surgical termination of pregnancy or surgical removal of retained products of conception after miscarriage and kept at 4 °C until centrifuged (15 min at 3000 g) and serum stored at −80 °C until required for analysis. Total serum 25-hydroxyvitamin D (25OHD2 plus 25OHD3) was determined using enzyme immunoassay (Immuno Diagnostic System, AC-57SF1, Boldon, UK) in the Virus Laboratory, Guangzhou Institute of Pediatrics, GWCMC. The inter- and intra-assay coefficients of variation were both below 5%.

2.3. Immunohistochemistry evaluation of CYP2R1, CYP27B1, CYP24A1, DBP and VDR

The localization of CYP2R1, CYP27B1, CYP24A1, DBP and VDR was examined by immunostaining of paraffin-embedded tissue sections. A standard immunohistochemistry staining procedure was used [16]. Briefly, sections were deparaffinized in xylene and rehydrated through a series of ethanol/water baths. Sections were pre-treated using heat mediated antigen retrieval with sodium citrate buffer (pH6.0) and pressure cooked for 1 min, then 3% hydrogen peroxide in methanol was used to quench the endogenous peroxidase activity. After blocking with primary antibody diluent with background reducing components (Dako, Agilent, California, USA), tissue sections were incubated with the primary antibodies: anti-CYP2R1 (1:100 dilution; ab137634, Abcam, Cambridge, UK), anti-CYP27B1 (1:100 dilution; ab206655, Abcam), anti-CYP24A1 (1:100 dilution; ab203308, Abcam), anti-VDBP (1:100 dilution; ab81307, Abcam), or anti-VDR (1:100 dilution; ab134826, Abcam) at 4 °C overnight and then biotinylate-conjugated secondary antibody HRP rabbit/mouse (Dako) at room temperature for 1 h. Stained slides were counterstained with Gill's formulation hematoxylin. Tissue sections stained with isotype IgG or without primary antibody served as negative controls. Slides stained with the same antibody were all stained at the same time.

2.4. Quantitative real-time PCR analysis of CYP2R1, CYP27B1, CYP24A1 and VDR

All RNA samples were extracted using E.Z.N.A. total extraction kit with RNase-free DNase Set (Omega Bio-tek, USA). Extracted RNA (800ng/reaction) was synthesized to cDNA by reverse transcription using Superscript III (InVitrogen) and a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, California, USA). After synthesis, the cDNA was diluted 1:2 prior to use in qPCR. The RT-PCR primers for CYP2R1, CYP27B1, CYP24A1, VDR and GAPDH were designed and synthesized by Beijing Genomics Institute (BGI, China). The primers for CYP2R1 were: forward, 5′-CTG CCA TTT ATC GGC AAC ATCT-3'; reverse, 5′-GGA AGG CAT GGT CTG TCT GC-3'. The primers for CYP27B1 were: forward, 5′-CAC CCG ACA CGG AGA CCTT-3'; reverse, 5′-TCA ACA GCG TGG ACA CAA ACA-3'. The primers for CYP24A1 were: forward, 5′- GCT TAC GCC GAG TGT ACC AT-3'; reverse, 5′-GCA TGA GCA CTG TTC CTT TGG-3'. The primers for VDR were: forward, 5′-ACA TCG GCA TGA TGA AGGA-3'; reverse, 5′-TTC CGC TTC AGG ATC ATC TC-3'. The primers for GAPDH were: forward, 5′-TCA CCA TCT TCC AGG AGC GA-3'; reverse, 5′-TGG ACT CCA CGA CGT ACT CA-3'. qRT-PCR was performed on a 7300 ABI thermal cycler (Applied Biosystems, USA) under standard conditions using SYBR green master mix (Takara, Japan.). To differentiate specific from nonspecific products and primer dimers, melting curve analysis was performed after amplification. The results were analyzed using the comparative ΔΔCt method and 2-ΔΔCt (fold change) was calculated.

2.5. Western blot analysis of CYP2R1, CYP27B1, CYP24A1, DBP and VDR

Total tissue protein was extracted from snap-frozen placental and decidua tissue using RIPA buffer containing 1% protease inhibitor cocktail and 1% phenylmethanesulfonyl fluoride (PMSF) (Beyotime, Shanghai, China). Prior to separation by 10% SDS-PAGE, protein lysates (Beyotime) were mixed with 5X SDS-PAGE loading buffer (Beyotime) and denatured at 95 °C for 5 min. Proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes (0.45 mm; Millipore,

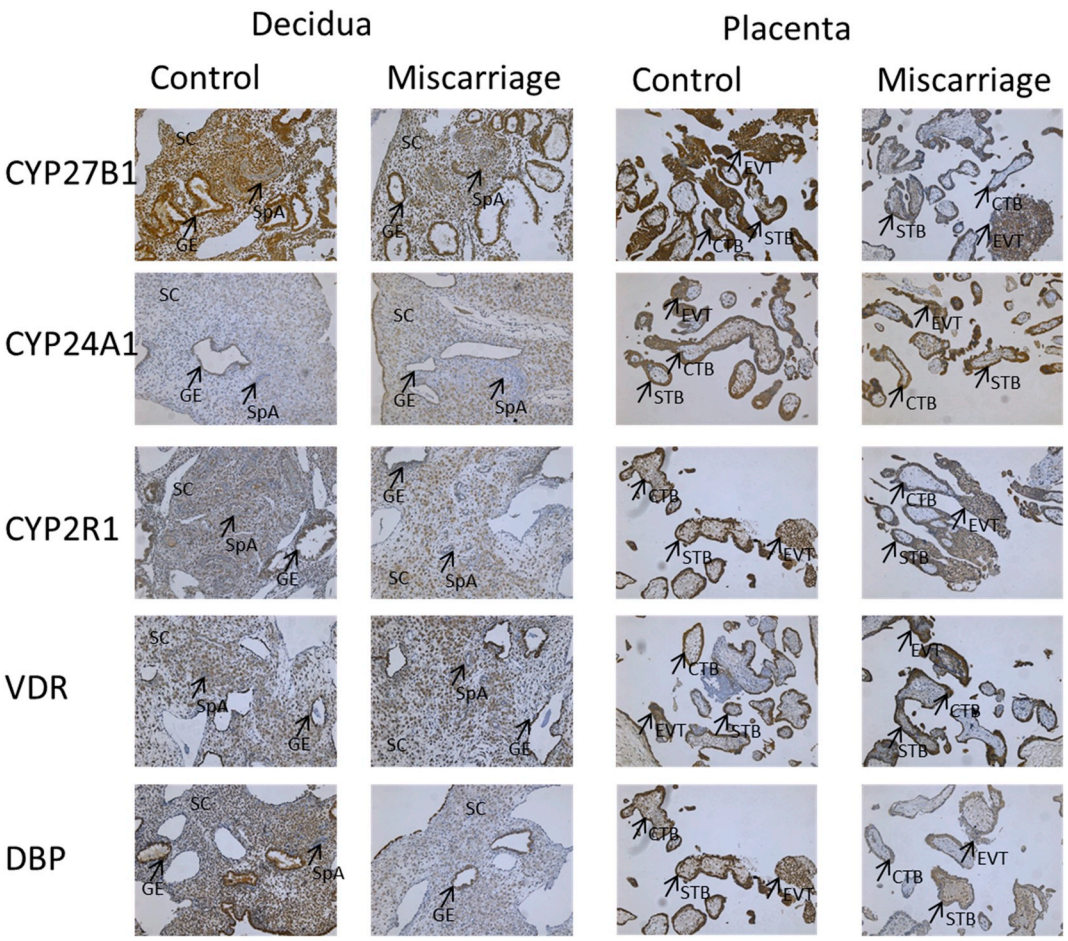

Fig. 1. Representative photomicrographs of immunohistochemical staining for 25-hydroxylase (CYP2R1), 25-hydroxylase (CYP27B1), vitamin D binding protein (DBP), 24-hydroxylase (CYP24A1), and vitamin D receptor (VDR) in decidua and placenta from normal control and spontaneous miscarriage pregnancies. Original magnification X200. SC = stromal cells, GE = glandular epithelium, SpA = spiral artery, EVT = extravillous trophoblast, STB = syncytiotrophoblast, CTB = villous cytotrophoblast.

Massachusetts, USA) and blocked in blocking buffer (Beyotime) and incubated overnight at 4 °C with rabbit anti-CYP2R1 (1:100 dilution; ab137634, Abcam), anti-CYP27B1 (1:1000 dilution; ab206655, Abcam), anti-CYP24A1 (1:500 dilution; ab203308, Abcam), anti-VDBP (1:1000 dilution; ab81307, Abcam), or anti-VDR (1:500 dilution; ab134826, Abcam). Protein targets were detected with anti-rabbit IgG (1:5000 dilution; R&D Systems, California, USA) and quantified by direct infrared fluorescence using the Li-Cor Odyssey infrared imaging system that allows for simultaneous detection of the target and reference proteins labeled with different infrared dyes. After incubating in stripping buffer (Beyotime) blots were probed with mouse anti-GAPDH (1:5000 dilution; AM1020b, Abgent, California, USA) to ensure equal loading. Scans were performed at 700 nm (GAPDH) and 800 nm (CYP2R1, CYP27B1, CYP24A1, DBP and VDR) with the Odyssey instrument. The ratio of CYP2R1, CYP27B1, CYP24A1, DBP and VDR to GAPDH was calculated for each sample.

2.6. Hypoxia and LPS induction of trophoblast-like cells

HTR-8/SVneo trophoblast like cells were obtained from American Tissue Culture Collection (ATCC) and maintained in RPMI1640 complete medium supplemented with 10% fetal bovine serum (Gibco, New York, USA). HTR-8/SVneo cells were treated with 200 ng/ml and 500 ng/ml $CoCl_2$ (Sangon Biotech, Shanghai, China) for 24 h to induce hypoxia. HTR-8/SVneo cells were treated with 100 ng/ml, 250 ng/ml and 500 ng/ml LPS (Proteintech, Chicago, USA) to create an inflammatory environment. RNA extracts were prepared and expression of VDR, CYP24A1, CYP27B1 and CYP2R1 determined by qRT-PCR analysis as above.

2.7. Statistical analysis

Data are presented as mean ± SEM. Statistical significance was determined using Statview by one-way ANOVA with Fisher's post hoc test or Student's *t*-test as appropriate. $P < 0.05$ was considered significant.

3. Results

3.1. Serum levels of 25OHD in women with spontaneous miscarriage

Total levels of 25OHD were measured in the serum of women with spontaneous miscarriage and gestational age matched controls. There was no difference in the mean level of serum 25OHD, although both groups were considered to be vitamin D insufficient (<50 nmol/L) (control: 48.6 ± 10.1 nmol/L; miscarriage: 49.6 ± 12.1 nmol/L, mean ± SD).

3.2. Localization of VDR, DBP, CYP27B1, CYP24A1, CYP2R1 in placenta and decidua

Immunohistochemical staining of placental and decidual sections

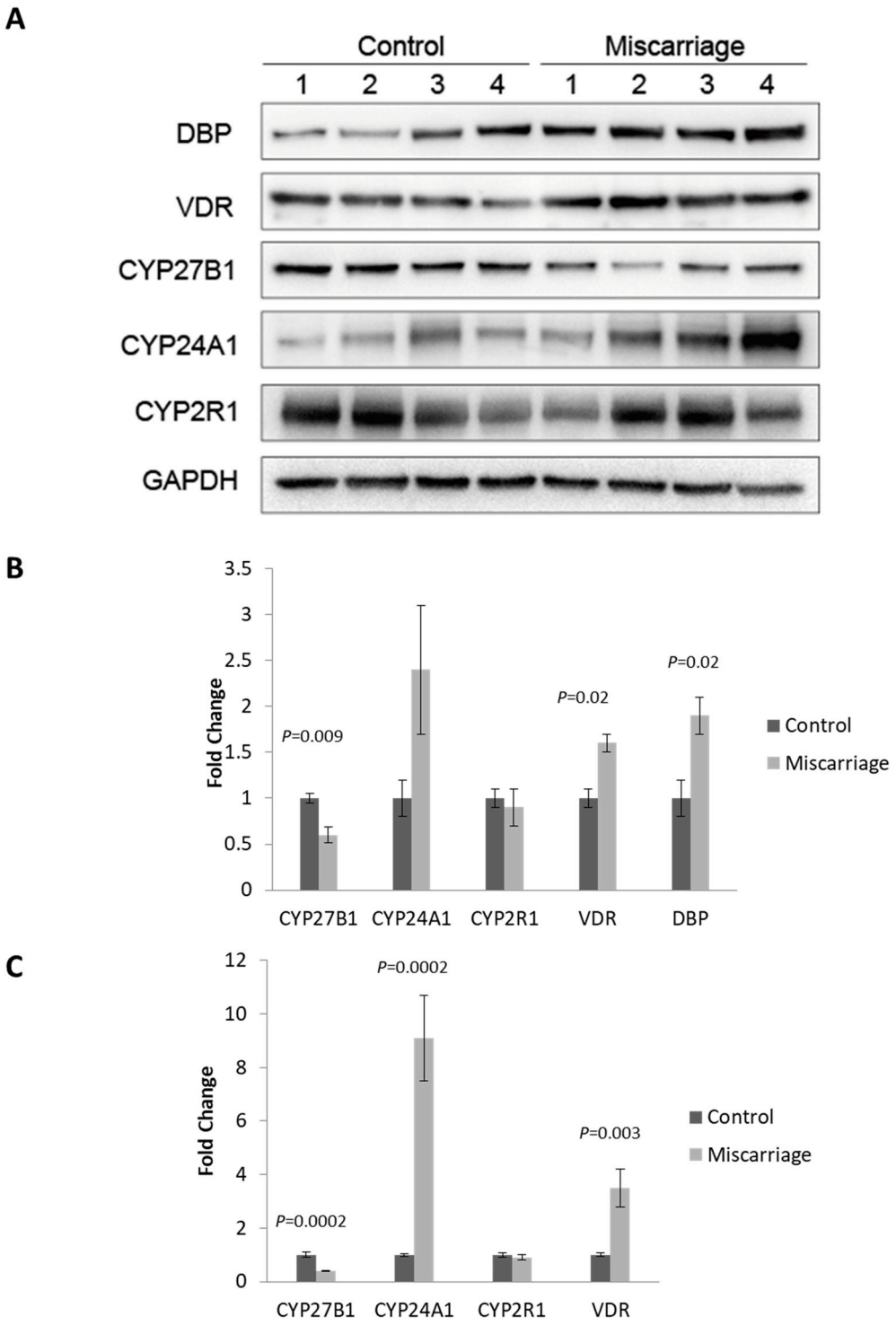

Fig. 2. Expression of VDR, DBP, CYP27B1, CYP24A1 and CYP2R1 in decidua of normal controls and spontaneous miscarriage pregnancies as determined by Western Blot **(A, B)** and qRT-PCR **(C)**. **(A)** Representative Western Blot, n = 4 each group. **(B)** Quantitative densitometry of Western Blots, n = 16 each group. **(C)** qRT-PCR, n = 16 each group.

from controls (n = 16) and spontaneous miscarriage (n = 16) was used to determine the localization of CYP2R1, CYP27B1, DBP, CYP24A1, and VDR (representative images are shown in Fig. 1). In decidua CYP27B1, DBP, VDR, and CYP24A1 were strongly expressed in glandular epithelium, with weak expression in the stroma. CYP2R1 was strongly expressed in both stromal and glandular epithelial cells. In placenta, strong expression of DBP, VDR, CYP24A1, CYP27B1 and CYP2R1 was observed in syncytiotrophoblast, cytotrophoblast, and extravillous trophoblast while only weak immunostaining was observed in the villous stroma.

3.3. Altered expression of DBP, VDR, CYP27B1, CYP24A1, and CYP2R1 in spontaneous miscarriage decidua and placenta

The expression of placental and decidual DBP, VDR, CYP27B1, CYP24A1, and CYP2R1 in spontaneous miscarriage and normal controls

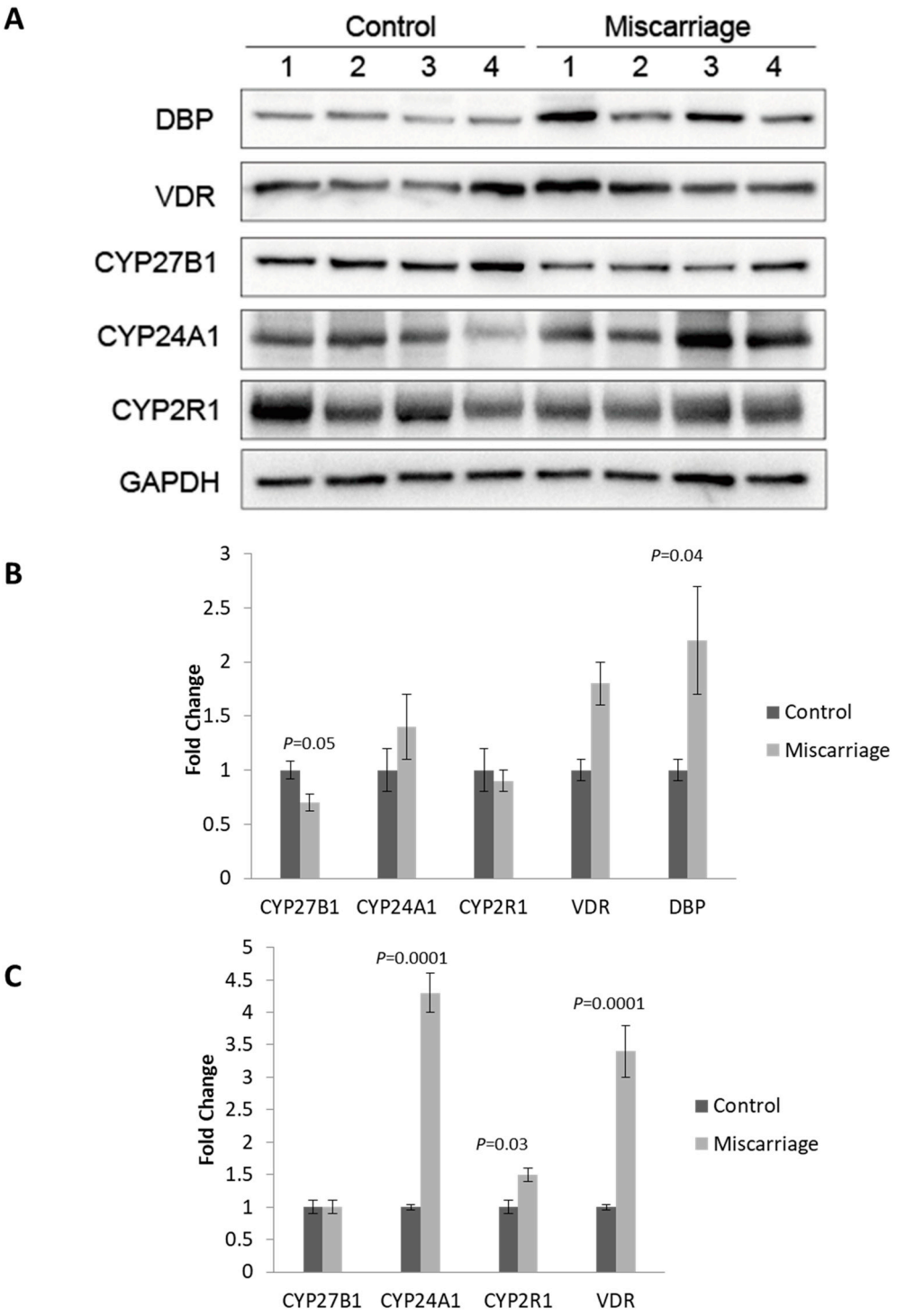

Fig. 3. Expression of VDR, DBP, CYP27B1, CYP24A1 and CYP2R1 in placenta of normal controls and spontaneous miscarriage pregnancies as determined by Western Blot **(A, B)** and qRT-PCR **(C)**. **(A)** Representative Western Blot, n = 4 each group. **(B)** Quantitative densitometry of Western Blots, n = 16 each group. **(C)** qRT-PCR, n = 16 each group.

at the mRNA and protein level were determined by qRT-PCR and Western blot, respectively. Compared with decidua from control women, the total protein expression of DBP ($P = 0.02$) and VDR ($P = 0.02$) was increased and expression of CYP27B1 ($P = 0.009$) decreased in the decidua of women with a miscarriage (Fig. 2A and B). mRNA levels of VDR ($P = 0.003$) and CYP24A1 ($P = 0.0002$) were increased, and mRNA levels of CYP27B1 ($P = 0.0002$) decreased in the decidua of women with miscarriage compared to controls (Fig. 2C). Due to primer instability levels of DBP mRNA were not able to be measured, despite trialing several pairs of primers. In the placenta of women who had a miscarriage, protein levels of DBP ($P = 0.04$) were increased and levels of CYP27B1 ($P = 0.05$) decreased compared to control women (Fig. 3A and B). mRNA levels of VDR ($P = 0.0001$), CYP24A1 ($P = 0.0001$) and CYP2R1 ($P = 0.03$) were all increased in the placenta of women with miscarriage compared with controls (Fig. 3C).

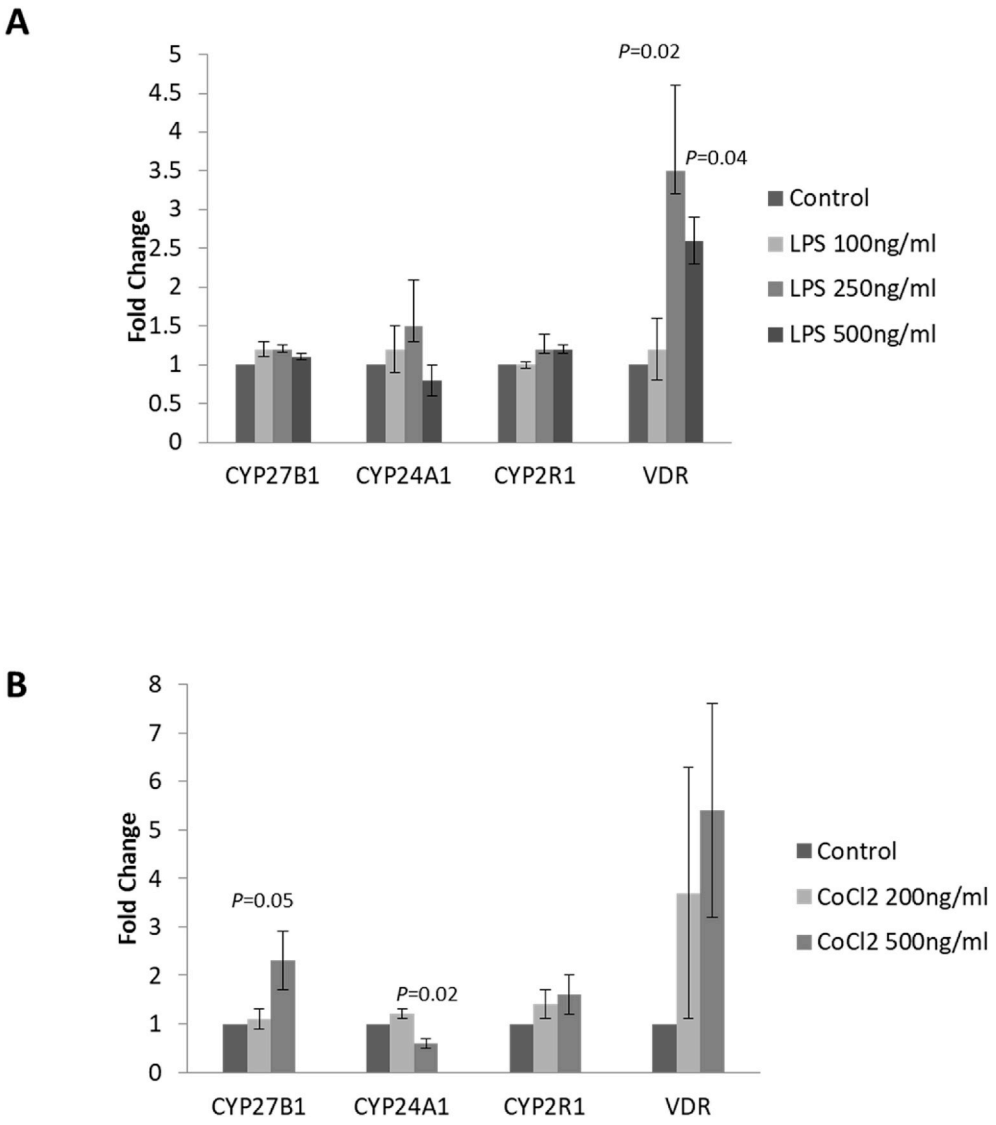

Fig. 4. Expression of VDR, CYP27B1, CYP24A1 and CYP2R1 in HTR-8/SVneo trophoblast like cells after treatment with **(A)** LPS or **(B)** $CoCl_2$ as determined by qRT-PCR, n = 3 each group.

3.4. Regulation of expression of VDR, CYP27B1, CYP24A1, and CYP2R1 in trophoblast-like cells

The trophoblast-like cell line HTR-8/SVneo was cultured in either LPS or $CoCL_2$ to mimic infection and hypoxia respectively, two key triggers of spontaneous miscarriage. In response to LPS, mRNA levels of VDR were increased (250 ng/ml $P = 0.02$, 500 ng/ml $P = 0.04$; Fig. 4A). In response to $CoCl_2$, mRNA levels of CYP27B1 ($P = 0.05$) were increased and levels of CYP24A1 ($P = 0.02$) decreased (Fig. 4B).

4. Discussion

In the current study we demonstrate that in the decidua and placenta after spontaneous miscarriage there is a potential decrease in the local levels of active vitamin D, $1,25(OH)_2D$ due to decreased expression of CYP27B1 and increased expression of CYP24A1.

Spontaneous miscarriage is one of the most common gynecological problems. A few previous studies have investigated the association between vitamin D and spontaneous miscarriage, although these have generally focused on circulating levels of vitamin D. A recent meta-analysis suggested that there is an association between very low vitamin D levels (<20 ng/ml) and incidence of miscarriage, though the association is not strong [17]. In the current study there was no difference in serum 25OHD concentrations between groups, but both the control and miscarriage groups had clinically low levels of vitamin D. More studies have focused on the association between vitamin D and recurrent miscarriage where there is greater evidence for a role for vitamin D deficiency in this condition [18]. In contrast to our findings, in recurrent miscarriage a decrease in both placental and decidual expression of VDR has been reported [11,19]. It is not clear why there is such a difference between recurrent and spontaneous miscarriage, but may reflect the difference in aetiology between the two conditions where one is more endometrial in origin (recurrent miscarriage) and the other a consequence of sequelae occurring during the pregnancy (spontaneous miscarriage). There are conflicting reports on the expression of CYP27B1 in recurrent miscarriage placenta and decidua, with one suggesting a decrease and another no difference [11,20]. The study of Wang et al. [20] also contained a spontaneous miscarriage group, however no comparisons to control samples were performed for expression levels of CYP27B1, their data suggests that CYP27B1 was also decreased in these samples.

Vitamin D has been proposed to play several roles in early pregnancy, including immunomodulatory effects and regulation of extravillous trophoblast cell invasion. Treatment of both decidual cells and

trophoblast with vitamin D increases secretion of the antibacterial proteins cathelicidin and β-defensins [21,22]. Therefore, a decrease in local levels of active vitamin D in miscarriage as proposed from the findings of the current study may contribute to the aetiology of miscarriage by reducing levels of antibacterial proteins making the pregnancy more susceptible to infection. Vitamin D has also been proposed to play roles in both innate and acquired immunity, with direct effects on macrophages, uterine natural killer cells and T cells [10]. Of particular note is that vitamin D can induce T cell differentiation into regulatory T cells (Tregs), the key immune cell at the maternal-fetal interface for promotion of immune tolerance. Low numbers of Tregs have been reported in women with spontaneous miscarriage [23]. The regulation of induction of Treg cells is complex, however it is easy to speculate that decreased local levels of vitamin D would lead to reduced numbers of Tregs compromising the immunological balance within the uterus and potentially causing immune rejection of the developing fetus. Trophoblast cells express the VDR and not only respond to vitamin D by increasing synthesis of antibacterial proteins but also by increasing their invasive capacity [12]. Invasion of extravillous trophoblast cells into the maternal uterus and subsequent remodeling of the uterine spiral arteries are key features of early pregnancy, their dysregulation is associated with late but not early miscarriage [24,25]. Therefore, it is not clear to what extent disruption in trophoblast invasion might contribute to early miscarriage.

Embryo and placental development occurs in a relatively hypoxic environment [26], whereas a relatively hyperoxic environment has been shown in women undergoing both spontaneous and recurrent miscarriage [27]. Intrauterine infection has also been associated with the aetiology of spontaneous miscarriage, though this may be a consequence of fetal demise [28]. To determine whether the changes in expression levels of the vitamin D catabolizing enzymes and VDR were due to changes in oxygen tension or infection trophoblast cells were treated with either $CoCl_2$ (a hypoxia mimic) or LPS (a gram negative bacterial membrane protein). LPS induced trophoblast expression of VDR while $CoCl_2$ increased CYP27B1 and decreased CYP24A1 expression. Conversely, if you consider $CoCl_2$ treatment to mimic the normal oxygen environment for the developing placenta, then the control culture conditions (20% oxygen) would be considered hypoxic, where levels of CYP27B1 are lower and CYP24A1 are higher mimicking the results observed in the miscarriage samples in the current study. These data suggest that the altered levels of vitamin D catabolizing enzymes and VDR may be a consequence of intrauterine infection and hyperoxia and therefore late in the aetiology of miscarriage. Further work is required to determine the sequence of events by which decreased local levels of vitamin D, infection and hyperoxia contribute to the aetiology of early spontaneous miscarriage.

In conclusion, to the best of our knowledge this is the first study investigating expression of vitamin catabolizing enzymes, DBP, and VDR in the placenta and decidua after spontaneous miscarriage. We have demonstrated that in women with spontaneous miscarriage there was no difference in circulating levels of vitamin D. The intrauterine levels of vitamin D are likely reduced due to reduced expression of CYP27B1 leading to lower levels of active vitamin D, and increased expression of CYP24A1 leading to increased degradation of the active vitamin D that is present.

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Authors roles

HH performed the experiments, performed data analysis, and wrote the manuscript; JYZ collected samples, performed experiments and performed data analysis; DC collected samples and performed experiments; FD collected samples; AN Morse collected samples and edited the manuscript; PH collected samples and designed the study; GEL designed the study and edited the manuscript. All authors approved the final version of the manuscript.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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