Heliyon 10 (2024) e27280

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Study on the immunomodulatory mechanism of vitamin D in patients with unexplained recurrent spontaneous abortion

Panyu Yang [a], Fenjian Lu [b,*]

[a] Department of Laboratory Medicine, Sichuan Jinxin Xinan Women's and Children's Hospital, Chengdu, China
[b] Center for Reproductive Medicine, The Third People's Hospital of Chengdu, Chengdu, China

ARTICLE INFO

keywords:
Recurrent miscarriage
Vitamin D
Th1/Th2 cytokines
Lymphocyte subsets
Flow cytometry

ABSTRACT

Background: To investigate the mechanism of vitamin D level on the regulation of peripheral blood lymphocyte subsets and serum Th1/Th2 cytokines in patients with unexplained recurrent spontaneous abortion (URSA).

Methods: Eighty female patients with URSA attending Sichuan Jinxin Xinan Women's and Children's Hospital from January 2020 to May 2021 were selected as the study group, and 30 age-matched women with a history of healthy deliveries were chosen as the control group, and peripheral blood lymphocyte subpopulations and serum Th1/Th2 cytokines of people with different levels of vitamin D were detected in the study group by flow cytometry, respectively. The results of immune factors before and after supplementation were analyzed in 40 of these patients with low vitamin D levels. The results of lymphoid subpopulations and Th1/Th2 cytokines in 19 patients with normal pregnancy before and after vitamin D supplementation and after normal pregnancy were also analyzed comparatively.

Results: (1) Serum 25(OH)D in the study group was lower than in the control group; peripheral blood Th cells, B cells and NK cells in the study group were higher than in the control group; IL-2, TNF-α, IFN-γ and IL-6 in the study group were higher than in the control group, while IL-4 and IL-10 in the study group were lower than in the control group ($P < 0.05$). (2) Th cells, B cells and NK cells of URSA patients in the vitamin D low level group were higher than those in the vitamin D normal group; serum cytokines IL-2, TNF-α and IFN-γ of patients in the vitamin D low level group were higher than those in the vitamin D normal group ($P < 0.05$); (3) Th cells, B cells and NK cells in URSA patients after vitamin D supplementation were lower than before vitamin D supplementation; serum cytokines IL-2, TNF-α and IFN-γ after vitamin D supplementation were lower than before vitamin D supplementation, IL-4 and IL-10 after vitamin D supplementation were higher than before vitamin D supplementation ($P < 0.05$), and there was no significant difference in IL-6 before and after vitamin D supplementation. (4) Th cells, B cells and NK cells in patients with normal pregnancy after vitamin D supplementation and after pregnancy were lower than those before vitamin D supplementation; serum cytokines IL-2, TNF-α and IFN-γ after vitamin D supplementation and after pregnancy were lower than those before vitamin D supplementation, and serum cytokines IL-4 and IL-10 after vitamin D supplementation and after pregnancy were higher than those before vitamin D supplementation, TNF -α, IFN-γ after pregnancy were lower than after vitamin D supplementation ($P < 0.05$), IL-6 was not significantly different before and after vitamin D supplementation and after pregnancy.

* Corresponding author.
E-mail addresses: 1255286391@qq.com (P. Yang), 578666493@qq.com (F. Lu).

https://doi.org/10.1016/j.heliyon.2024.e27280
Received 10 December 2023; Received in revised form 27 February 2024; Accepted 27 February 2024
Available online 9 March 2024

Conclusion: Vitamin D deficiency rate was high in URSA patients. Th、B、NK cells and IL-2, TNF-α, IFN-γ, IL-6 cytokines were high, while IL-6 and IL-10 were low in URSA patients. IL-2, TNF-α, IFN-γ cytokines and Th, B, NK cells were increased in vitamin D deficient URSA patients, and Vitamin D deficiency may be an important cause or aggravating factor of immune dysfunction in URSA patients. Vitamin D has an immunomodulatory effect on URSA patients, promoting successful pregnancy by down-regulating peripheral blood Th, B, and NK cells and IL-2, TNF-α, and IFN-γ cytokines, while up-regulating IL-4 and IL-10.

1. Introduction

In a normal pregnancy, maternal-fetal interactions produce tolerance so that the embryo is not rejected by the maternal immune system [1]. Successful pregnancy requires a delicate balance of maternal immune system activity and embryonic immune regulation [2]. Disruption of various regulatory factors in the maternal immune system during pregnancy may lead to miscarriage [3]. The American Society for Reproductive Medicine (ASRM) defines recurrent spontaneous abortion (RSA) as two or more consecutive spontaneous abortions with the same sexual partner, and the incidence of RSA in women of childbearing age is about 1%–5%, and it is a rather difficult problem faced by obstetricians and gynecologists as well as patients at present [4]. The etiology of RSA is multifactorial and complex, and in addition to the identifiable causes of RSA, which include hormonal, anatomical, autoimmune, chromosomal, and infectious factors, about 50% of the cases are still unexplained [5].

Unexplained recurrent spontaneous abortion (URSA) is to some extent thought to be due to maternal immune rejection of the fetus [6]. Vitamin D is a steroid hormone and 25-hydroxyvitamin D [25(OH)D] is the main circulating form of vitamin D [7], and recent revised guidelines of the North American Society of Endocrinology define vitamin D deficiency as blood levels of less than 20 ng/ml and vitamin D insufficiency as having a 25(OH)D level of 20–30 ng/ml [8]. Vitamin D is mainly known for its regulation of calcium and phosphate absorption from the gastrointestinal tract and mineralization in the skeletal system [9]. In addition to this, vitamin D is an overall regulator of immune, reproductive and other systems, including the regulation of maternal-fetal immune imbalance disease leading to RSA [10,11]. Vitamin D deficiency during pregnancy is associated with several adverse pregnancy outcomes, such as preeclampsia (PE), gestational diabetes mellitus (GDM), and recurrent spontaneous abortion (RSA) [12,13]. Clinical studies have found reduced expression of vitamin D and its receptor at the maternal-fetal interface in RSA women, which may increase the risk of miscarriage [14]. Adequate preconception vitamin D levels may increase the likelihood of successful reproductive treatment, and diagnosis and treatment of vitamin D deficiency in women planning assisted reproductive therapy may be beneficial in optimizing pregnancy outcomes [15].

The series of studies described above suggest that vitamin D is an important immunomodulatory factor that may influence pregnancy outcomes by modulating immune function during pregnancy, but the mechanisms of vitamin D immunomodulation in vivo have not been fully clarified. In this study, we investigated the differences in peripheral blood lymphoid subpopulations and serum Th1/Th2 cytokines in different 25(OH)D level groups by flow technique. At the same time, vitamin D supplementation was performed in patients with insufficient 25(OH)D, comparing the levels of lymphocyte subpopulations and cytokines in the body before and after vitamin D supplementation. We also retrospectively analyzed the results of peripheral blood lymphocyte subpopulations and serum cytokines of 19 normal pregnancies in 28 URSA patients supplemented with vitamin D. We explored the mechanism of the immune-regulating effect of vitamin D on URSA patients, which can provide a basis for clinical diagnosis and prevention, and also provide a therapeutic solution to improve the IVF-ET pregnancy rate of URSA patients with immune dysfunction.

2. Materials and methods

2.1. Study population

Study group: 80 female patients with recurrent miscarriage attending Sichuan Jinxin Sinan Women's and Children's Hospital from January 2020 to May 2021 were selected for the study; Inclusion criteria: (1) history of RSA (two or more consecutive spontaneous abortions before 20 weeks of gestation); (2) no previous history of targeted treatment for RSA; (3) normal chromosomal karyotypes of both husband and wife; (4) normal pelvic ultrasound to exclude organic lesions of the reproductive tract; (5) Secretions from the cervix were negative for chlamydia, mycoplasma, and gonococcus to rule out external genital tract infection; (6) The woman has normal hormone levels and no history of endocrine disease; (7) Normal coagulation without thrombotic tendency or hypercoagulable state; (8) Regular menstrual cycle, no endocrine or immunomodulators or vitamin D in the last 3 months; (9) The male partner's routine semen test is normal and negative for chlamydia, mycoplasma and gonococcus. The study group was divided into 26 cases in the vitamin D normal group (25(OH)D $\geq$ 30 ng/ml) and 54 cases in the vitamin D low level group (25(OH)D $<$ 30 ng/ml) based on serum 25(OH)D level. Normal control group: 30 women with a history of healthy deliveries from January 2020–May 2021. In the study group, 40 URSA patients with low levels of Vitamin D were supplemented with Vitamin D. 12 of the 40 patients supplemented with Vitamin D had missing pregnancy outcomes or test records, and 19 of the remaining 28 patients had normal pregnancies.

2.2. Instruments and reagents

Vitamin D: Fully automated chemiluminescence immunoassay system I3000, kits purchased from Mike's Bio Co, acridine ester labeled 25(OH)D antibody.

Lymphocyte subpopulation and absolute counting: BD Facscanto II flow cytometer, Lymphocyte subpopulation fluorescent monoclonal antibody kit and TS Cell-Count absolute counting microsphere kit were purchased from Beijing Tongsheng Times Biotechnology Co. The fluorescent antibodies were labeled as follows: PerCP-Cy5.5 labeled CD45 antibody; FITC labeled CD3 antibody; APC labeled CD19 antibody; PE-Cy7 labeled CD4 antibody; APC-Cy7 labeled CD8 antibody; PE labeled CD16 antibody and CD56 antibody.

Cytokines: BD Facscanto II flow cytometer, human Th1/Th2 subpopulation detection kit (flow fluorescence method) was purchased from Hangzhou Sage Biotechnology Co. Antibodies were labeled as follows: capture microsphere antibodies to six different cytokines were fluorescently labeled with different APC intensities, and the fluorescence intensities of the APC-labeled microspheres were from strong to weak for IL-2, IL-4, IL-6, IL-10,TNF-α, and IFN-γ, respectively. PE fluorescently labeled secondary antibodies.

2.3. Experimental procedure

Lymphocyte subsets and cytokines before Vitamin D supplementation were collected during the luteal phase. Lymphocyte subsets and cytokines after Vitamin D supplementation were collected during the luteal phase 1–3 months after Vitamin D supplementation. Lymphocyte subsets and cytokines after normal pregnancy were collected in early pregnancy.

Vitamin D:Serum was separated in yellow-tipped tubes and tested within 24 h. The immunoassay is performed by magnetic particle chemiluminescence technique following the principle of competitive method. The analytical buffer (containing dissociative agent), acridine ester-labeled antibody and samples were mixed and incubated, the 25-hydroxyvitamin D in the samples was separated from the binding protein under the action of dissociative agent and combined with acridine ester-labeled antibody, and then the magnetic particles encapsulated with 25-hydroxyvitamin D were added, the 25-hydroxyvitamin D in the samples competed with the 25-hydroxyvitamin D encapsulated in the magnetic particles for acridine ester-labeled antibody together. The sample is washed to remove unbound material, a luminescent substrate is added and a chemiluminescent reaction occurs, and the relative luminescence value (RLU) is measured, which is inversely proportional to the concentration of 25-hydroxyvitamin D in the sample.

Lymphocyte subpopulations: Anticoagulated whole blood was drawn from a purple-tipped tube for lymphatic subgroups and stored at room temperature for testing within 24 h. Detection steps (1) Draw 50 μL of mixed EDTA anticoagulated whole blood and add it to the bottom of the absolute count microsphere tube. (2) Add 20 μL of fluorescent antibody reagent. (3) Vortex well and mix for about 10 s, and react for 20 min at room temperature away from light. (4) Add 450 μL of hemolytic agent to the tube and react for 10 min at room temperature away from light. (5) Add 450 μL of Phosphate Buffered Saline (PBS) to the tube and test on the machine. (6) Results processing: set the gate with CD45/SSC.

Cytokines: Separate serum with yellow-tipped tubes, keep refrigerated at 2–8 °C, and assay within 48 h. The principle of cytokine detection is flow CBA (Multiple Protein Quantification Assay) technology. (1) Take the kit capture microsphere mixture (A), vortex for 3–5 s, take n*25 μL (n is the number of samples) in a test tube, centrifuge at 200 g for 5 min, then carefully aspirate the supernatant with a pipette gun and discard it, and then take an equal amount of microsphere buffer (H) to resuspend the microspheres. After vortexing and shaking for 3–5 s, incubate for 30 min away from light; (2) Add 25 μL of microsphere after shaking and mixing to tubes separately; (3) Add 25 μL of the sample to be detected. (4) Finally, 25 μL of fluorescence detection reagent (C) was added. (5) All the sample tubes were shaken and mixed well, and incubated for 2.5 h at room temperature and away from light; (6) 1 ml of PBS solution was added to each tube for washing, centrifuged at 200 g for 5 min, and the supernatant was discarded. (7) Add 100 μL of PBS solution to each tube, resuspend with shaking and put on the machine. (8) Results processing: FSC/SSC set up to capture the microsphere gate, PE/APC show the concentration of different cytokines.

2.4. Statistical analysis

The data were processed using the software Rstudio, and characteristics between the two groups was expressed as mean ± standard deviation (x ± s), and independent samples *t*-test and paired *t*-test were used. The difference was considered statistically significant at $P < 0.05$.

Table 1
Comparison of baseline and 25(OH)D in study and control groups.

groups	study group (n = 80)	Control group (n = 30)	P value
Age	33.06 ± 5.84	31.63 ± 3.49	0.12
BMI	22.37 ± 3.75	22.78 ± 2.79	0.53
Menstrual cycles (days)	29.41 ± 4.74	29.53 ± 4.12	0.90
25(OH)D (ng/ml)	22.69 ± 6.74	34.43 ± 3.31	<0.05*
Proportion of 25(OH)D deficiencies (%)	67.5	16.7	

*P < 0.05, statistically significant.

3. Results

3.1. General clinical characteristics in the study

There were no statistically significant differences in age, body mass index (BMI), number of miscarriages, and menstrual cycle between the study group and the control group, and between the group with normal levels of Vitamin D and the group with low levels of Vitamin D in the study group (Table 1 and Table 2, $P > 0.05$).

3.2. 25(OH)D levels and immune factor status in peripheral blood of URSA patients

Vitamin D deficiency as blood levels of less than 20 ng/ml and vitamin D insufficiency as having a 25(OH)D level of 20–30 ng/ml, normal vitamin D as blood levels of more than 30 ng/ml [8]. The serum 25(OH)D level in the study group was lower than that in the control group, and the difference was statistically significant (Table 1, $P < 0.05$), and the proportion of serum 25(OH)D insufficiency in the study group was higher than that in the control group.

Th cells ($CD3^+CD4^+$), B cells ($CD19^+$) and NK cells ($CD3^-CD16^+CD56^+$) in the study group were higher than in the control group, with a statistically significant difference (Table 3, $P < 0.05$), whereas the differences between the two groups were not statistically significant for T cells ($CD3^+$) and Tc cells ($CD3^+CD8^+$); serum Th1 cytokines IL-2, TNF-α, IFN-γ, and Th2 cytokines IL-6 in the study group were higher than in the control group, with a statistically significant difference, while Th2 cytokines IL-4 and IL-10 in the study group were lower than in the control group, with a statistically significant difference (Table 4, $P < 0.05$).

3.3. Peripheral blood immune cells and serum cytokines in URSA patients with different 25(OH)D levels

Th cells, B cells, and NK cells in RSA patients in the low 25(OH)D group were higher than in the normal 25(OH)D group, and the difference was statistically significant (Table 5, $P < 0.05$), whereas T cells ($CD3^+$) and Tc cells ($CD3^+CD8^+$) had no statistically significant difference between the two groups. The levels of serum Th1 cytokines IL-2, TNF-α and IFN-γ in the low 25(OH)D group were higher than in the normal 25(OH)D group, and the difference was statistically significant (Table 6, $P < 0.05$); whereas the differences of IL-4, IL-6 and IL-10 were not statistically significant between the two groups.

3.4. Alterations in peripheral blood immune cells and serum cytokines before and after vitamin D supplementation in URSA patients

Th cells, B cells, and NK cells of the 40 patients supplemented with vitamin D were lower than the levels before supplementation, and the difference was statistically significant (Table 7, $P < 0.05$). And the difference between T and Tc cells before and after Vitamin D supplementation was not statistically significant. Meanwhile, the levels of serum Th1-type cytokines IL-2, TNF-α and IFN-γ after vitamin D supplementation were lower than those before supplementation, and the levels of Th2-type cytokines IL-4 and IL-10 were higher than those before supplementation, with a statistically significant difference (Table 8, $P < 0.05$), while the difference of IL-6 before and after vitamin D supplementation was not statistically significant.

3.5. Alterations in peripheral blood immune cells and serum cytokines before and after vitamin D supplementation and after normal pregnancy in URSA patients

Th cells, B cells and NK cells in patients with normal pregnancy after Vitamin D supplementation and after pregnancy were lower than the pre-supplementation levels, and the difference was statistically significant (Table 9, $P < 0.05$), whereas the differences between after vitamin D supplementation and after pregnancy were not statistically significant. The differences in T and Tc cells in normal pregnant patients were not statistically significant between before vitamin D supplementation, after vitamin D supplementation and after pregnancy.

IL-2, TNF-α and IFN-γ after vitamin D supplementation and after pregnancy were lower than the levels before vitamin D supplementation, and the differences were statistically significant, TNF-α and IFN-γ after pregnancy were lower than after vitamin D supplementation, and the differences were statistically significant, whereas the differences in IL-2 was no statistically significant between after pregnancy and after vitamin D supplementation; IL-10 and IL-4 after vitamin D supplementation and after pregnancy were higher than the levels before vitamin D supplementation, and the differences were statistically significant (Table 10, $P < 0.05$), whereas differences in IL-4 and IL-10 were no statistically significant between after pregnancy compared with those after vitamin D

Table 2
Baseline comparison of groups with different 25(OH)D levels.

groups	normal group (n = 26)	low level group (n = 54)	P value
Age	32.42 ± 6.62	33.37 ± 5.47	0.53
BMI	22.23 ± 3.89	22.43 ± 3.71	0.83
Number of abortions	2.38 ± 0.57	2.65 ± 0.76	0.09
Menstrual cycles (days)	30.15 ± 4.31	29.06 ± 4.93	0.31

*$P < 0.05$, statistically significant.

Table 3
Comparison of the proportions and absolute counts of peripheral blood lymphoid subpopulations in the study and control groups.

groups	$CD3^+$(%)	$CD3^+$ (cells/ul)	$CD3^+CD4^+$(%)	$CD3^+CD4^+$(cells/ul)	$CD3^+CD8^+$ (%)	$CD3^+CD8^+$(cells/ul)	$CD19^+$ (%)	$CD19^+$ (cells/ul)	$CD16^+CD56^+$(%)	$CD16^+CD56^+$ (cells/ul)
study group (n = 80)	61.23 ± 5.71	995.21 ± 105.02	37.88 ± 5.58	883.56 ± 124.08	19.97 ± 2.11	450.33 ± 98.09	18.52 ± 3.98	329.13 ± 70.88	19.31 ± 4.91	300.79 ± 109.78
Control group (n = 30)	61.39 ± 5.76	992.55 ± 74.05	32.68 ± 4.02	726.24 ± 37.66	19.96 ± 2.27	442.29 ± 106.19	15.55 ± 2.58	242.95 ± 45.20	15.28 ± 2.87	164.36 ± 34.32
P value	0.89	0.88	<0.05*	<0.05*	0.98	0.72	<0.05*	<0.05*	<0.05*	<0.05*

*$P < 0.05$, statistically significant.

Table 4
Comparison of serum Th1/Th2 cytokines between study and control groups (pg/ml).

groups	IL-2	TNF-α	IFN-γ	IL-4	IL-6	IL-10
study group (n = 80)	6.75 ± 4.65	12.16 ± 8.69	12.18 ± 8.61	1.35 ± 0.66	14.82 ± 3.74	2.64 ± 1.44
Control group (n = 30)	1.28 ± 0.53	1.32 ± 0.67	1.59 ± 0.76	12.26 ± 0.54	3.63 ± 1.30	12.19 ± 1.57
P value	<0.05*	<0.05*	<0.05*	<0.05*	<0.05*	<0.05*

*P < 0.05, statistically significant.

supplementation, and the differences in IL-6 were no statistically significant between before vitamin D supplementation, after vitamin D supplementation, and after pregnancy.

4. Discussion

4.1. Relationship between vitamin D and miscarriage

Research suggests that VD may play an immunomodulatory role to promote implantation and successful pregnancy [16,17]. However, during pregnancy, vitamin D levels tend to decline from the body's optimal requirements for a number of reasons [18], which may lead to maternal vitamin D insufficiency or deficiency and unfavorable pregnancy outcomes [19]. The rate of vitamin D deficiency in patients with RSA is quite high compared to patients with normal pregnancies [10,20].30.8%–51.5% of RSA women have vitamin D insufficiency (20–30 ng/ml), and 13.1%–16.6% of RSA women have vitamin D deficiency (<20 ng/ml) [10,21]. Women with vitamin D insufficiency or deficiency have significantly higher rates of miscarriage than women with normal vitamin levels [21]. The results of this study showed that the vitamin D level in URSA was lower than that of normal control group ($p < 0.05$). The rate of vitamin D insufficiency in URSA group and control group was 67.5% and 16.7%, respectively, which suggests that there is a high rate of vitamin D insufficiency or deficiency in patients with URSA, which may be an association between RSA, and the findings of the present study are in consistent with those of previous studies.

4.2. Modulation of lymphoid subpopulations by vitamin D in RSA patients

VD may play an important role in the regulation of the percentage and function of peripheral blood lymphocyte subpopulations in patients with RSA, inducing changes favorable for successful pregnancy [10,21]. $CD4^+$T cells mediate cellular immunity, and an increase in their rate enhances maternal cellular immunity, leading to increased maternal immune rejection of the embryo, and therefore pregnancy cannot be maintained. $CD8^+$T cells can simultaneously inhibit humoral immunity mediated by B cells and type IV hypersensitivity and immunoproliferative responses mediated by $CD4^+$T cells, ensuring that embryos hemizygous antigens are not rejected [22].1,25 (OH)2D3 may inhibit the number and function of $CD4^+$ cells as well as modulate the inflammatory response by binding to the VDR [23,24]. The results of this study showed that Th cells in the study group were higher than in the control group, higher in the group with low levels of vitamin D than in the group with normal vitamin D in URSA patients, and lower in the patients after vitamin D supplementation than before vitamin D supplementation. Th cells in patients with normal pregnancy after vitamin D supplementation and after pregnancy were lower than pre-supplementation levels. The results of the present study are consistent with the above findings that patients with URSA, especially those with low levels of vitamin D, have higher Th cells, and that vitamin D has the effect of reducing peripheral blood Th cells, thereby decreasing maternal rejection of the embryo and enhancing maternal immune tolerance to the embryo. In addition, the present study further suggests that maintaining low levels of Th cells before pregnancy and during early pregnancy is beneficial to the successful pregnancy.

$CD19^+$ lymphocytes indicate the state of the body's humoral immune function. Vitamin D insufficiency or deficiency increases the risk of miscarriage by promoting cellular and autoimmunity, which is characterized by an increase in $CD19^+$ B cells and autoantibodies [7,21]. Vitamin D decreases B cells in patients with RSA, thereby reducing immunoglobulin production [10,23]. The results of the present study are consistent with those of the above studies, but the present study, based on the above studies, by doing its own comparison of vitamin D-deficient patients before and after vitamin D supplementation and after pregnancy, further confirms that vitamin D may be beneficial in improving the poor pregnancy outcome of URSA patients by down-regulating the B-lymphocytes to reduce autoantibody production in vivo and to promote the maintenance of the embryo in the mother's womb.

The NK cell surface-specific leukocyte differentiation antigens CD56 and CD16 are their specific markers. $CD56^+CD16^+$ NK cells are the major cells mediating ADCC (antibody-dependent cytotoxicity of NK cells) with immune rejection killing and cytotoxicity, which damages and kills embryos through cytokine alterations. Studies have shown that a high percentage of NK cells is an indicator of poor prognosis in patients with RSA [25,26]. NK cell levels are elevated in vitamin D-deficient RSA patients [7,21]. In vitro experiments have demonstrated that VD3 reduces NK cytotoxicity [10,21]. In this experiment, $CD3^-CD16^+CD56^+$ was used to label NK cells, and the results of the study showed that NK cells in the study group were higher than in the control group, and NK cells were high in the study group, especially in URSA patients with low vitamin D levels. The URSA patients with low levels of vitamin D were compared with themselves before and after vitamin D supplementation, pregnant patients were compared with themselves before and after vitamin D supplementation and after pregnancy, and it was found that the NK cells in URSA patients after vitamin D supplementation and in pregnant patients after vitamin D supplementation and after pregnancy were lower than the levels of NK cells before vitamin D supplementation ($P < 0.05$). This study confirms that vitamin D reduces peripheral blood NK cells and decreases maternal rejection

Table 5
Comparison of the proportions and absolute counts of peripheral blood lymphoid subpopulations in different 25(OH)D level groups.

groups	$CD3^+$(%)	$CD3^+$ (cells/ul)	$CD3^+CD4^+$(%)	$CD3^+CD4^+$(cells/ul)	$CD3^+CD8^+$ (%)	$CD3^+CD8^+$(cells/ul)	$CD19^+$ (%)	$CD19^+$ (cells/ul)	$CD16^+CD56^+$(%)	$CD16^+CD56^+$ (cells/ul)
normal group (n = 26)	62.72 ± 5.78	992.95 ± 99.86	33.43 ± 4.01	726.76 ± 46.76	20.04 ± 1.6	432.04 ± 84.71	15.34 ± 2.43	252.55 ± 36.40	14.92 ± 2.97	157.34 ± 35.35
low level group (n = 54)	60.51 ± 5.59	996.30 ± 108.31	40.02 ± 4.95	959.07 ± 63.61	19.94 ± 2.33	459.13 ± 103.50	20.05 ± 3.67	366.00 ± 51.01	21.42 ± 4.22	367.21 ± 54.49
P value	0.11	0.89	<0.05*	<0.05*	0.81	0.22	<0.05*	<0.05*	<0.05*	<0.05*

*$P < 0.05$, statistically significant.

Table 6
Comparison of serum Th1/Th2 cytokines in different 25(OH)D level groups (pg/ml).

groups	IL-2	TNF-α	IFN-γ	IL-4	IL-6	IL-10
normal group (n = 26)	1.04 ± 0.56	1.23 ± 0.61	1.58 ± 0.77	1.23 ± 0.73	14.99 ± 4.30	2.36 ± 1.48
low level group (n = 54)	9.51 ± 2.89	17.42 ± 5.06	17.29 ± 5.33	1.40 ± 0.62	14.74 ± 3.48	2.78 ± 1.41
P value	<0.05*	<0.05*	<0.05*	0.32	0.79	0.24

*P < 0.05, statistically significant.

and killing of embryos, thereby promoting successful pregnancy in URSA patients.

4.3. Regulation of serum Th1/Th2 cytokines by vitamin D in RSA patients

Modern reproductive immunology suggests that the dynamic balance of Th1/Th2 type cytokines has a crucial role in successful pregnancy [27,28]. Pro-inflammatory cytokines such as IL-2, TNF-α and IFN-γ secreted by Th1 cells can regulate cell proliferation and differentiation and provide energy metabolism, providing a favorable environment for fetal growth and development, but excessive Th1 cytokines can cause immune rejection and thus increase the difficulty of placental implantation, which may be detrimental to the outcome of the pregnancy [29]. Th2 cells secrete IL-4, IL-6, IL-10 and other Anti-inflammatory cytokines, which can reduce the rejection between mother and fetus and increase the adaptability and compatibility between mother and fetus, thus favoring the success of pregnancy [30]. Vitamin D promotes the transition from a Th1 to a Th2 cell phenotype by decreasing the production of INF-γ, IL-2, and TNF-αand increasing the production of cytokines such as IL- 4,6,10 [10,31,32], but these studies investigated the effects of vitamin D on cytokines from an in vitro perspective, and were not combined with in vivo serum cytokine assays.

In this study, we applied the flow CBA technique to investigate the in vivo regulatory effects of vitamin D on Th1/Th2 cytokines, and the results showed that serum IL-2, TNF-α, IFN-γin the study group were higher than in the control group, and IL-4、IL-10 in the study group were lower than in the control group, and that serum IL-2, TNF-α, and IFN-γ in the low vitamin D group were higher than in the normal vitamin D group; in addition, serum cytokine IL-2, TNF-α, and IFN-γ levels in the URSA patients after vitamin D supplementation, the pregnant patients after vitamin D supplementation, and after pregnancy were lower than before vitamin D supplementation ($P < 0.05$), whereas IL-4 and IL-10 after supplementation and after pregnancy were higher than the pre-supplementation levels ($P < 0.05$). And it was also found that serum TNF-αand IFN-γafter pregnancy were lower than after vitamin D supplementation in pregnant patients. And the differences in IL-6 in URSA patients before vitamin D supplementation, after vitamin D supplementation, and after pregnancy were not statistically significant. The results of IL-2, TNF-α, IFN-γ, IL-4, and IL-10 in the present study are consistent with the results of the above studies, suggesting that Vitamin D does have the effect of down-regulating the Th1-type cytokines IL-2, TNF-α, and IFN-γ and up-regulating the Th2-type cytokines IL-4 and IL-10, thereby maintaining the development of pregnancy. Lower levels of TNF-α and IFN-γ in early pregnancy in this study were more favorable for successful pregnancy.

In our study, IL-6 in the study group was higher than in the control group, but it was not statistically different between the group with low levels of Vitamin D and the group with normal levels of Vitamin D, and it was not statistically different before and after Vitamin D supplementation, and after pregnancy. This is where the conflicting performance of IL-6 in our study comes into play. This is also inconsistent with the results of the above studies. Whether IL-6 is favorable or unfavorable to pregnancy is currently controversial. IL-6 is a multifunctional cytokine that plays an important role in acute and chronic inflammation and autoimmunity [33]. IL-6 induces B-cell differentiation, promotes T-cell proliferation, and mediates acute inflammatory responses, and it plays an important role in resisting infection by external pathogens and in maintaining the stability of the internal environment of the organism, and its aberrant expression and function are involved in the development of inflammation, tumors, and other diseases [34]. Although IL-6 is an anti-inflammatory cytokine, it has been shown that low-level IL-6 status plays an important role in the maintenance of normal pregnancy, whereas plasma and localized maternal-fetal interface IL-6 expression is elevated in patients with URSA [5,35]. This suggests that the immune role of IL-6 in RSA patients is not unilateral, but may have multifaceted effects, and that differences in the consistency and antagonism of the results of the various effects may lead to different pregnancy outcomes. This may be the cause of the contradictions in our results. There was no significant change in IL-6 before and after Vitamin D supplementation in this study, and we hypothesized that it may be that vitamin D has no modulatory effect on IL-6 or that the multiple modulatory effects on IL-6 are balanced with each other, resulting in a statistically non-significant difference, given the small sample size of the present study, the specific modulatory effect of vitamin D on IL-6 remains to be investigated in more detail in a larger sample.

4.4. Discussion of the strengths and weaknesses of this study

This study is an in vivo experiment. It compares changes in immune status before and after vitamin D supplementation and after pregnancy in URSA patients with low levels of vitamin D, excluding the effect of individual differences and more fully confirming the specific regulatory mechanisms of vitamin D on lymphoid subpopulations and Th1/Th2 cytokines. In addition, the regulatory effects of vitamin D on lymphoid subpopulations and cytokines were also explored, and in particular, both proportional and absolute counts of lymphocyte subpopulations were investigated, and the results of both were compatible. Therefore, this study is more comprehensive and provides a reliable basis for clinical diagnosis and treatment. However, there are two shortcomings in this study, firstly, the sample size is small. It is hoped that more scholars will do research on the immunomodulatory effect of Vitamin D on URSA with a larger

Table 7
Comparison of peripheral blood lymphatic subpopulation proportions and absolute count before and after vitamin D supplementation in URSA patients with low vitamin D levels.

groups	$CD3^+$(%)	$CD3^+$ (cells/ul)	$CD3^+CD4^+$(%)	$CD3^+CD4^+$(cells/ul)	$CD3^+CD8^+$(%)	$CD3^+CD8^+$(cells/ul)	$CD19^+$ (%)	$CD19^+$ (cells/ul)	$CD16^+CD56^+$ (%)	$CD16^+CD56^+$ (cells/ul)
Before Supplementation (n = 40)	60.19 ± 5.72	987.08 ± 94.84	39.93 ± 5.15	963.64 ± 66.35	19.58 ± 2.26	459.89 ± 92.85	20.39 ± 3.87	365.80 ± 50.75	21.44 ± 4.19	364.62 ± 54.62
After supplementation (n = 40)	59.16 ± 5.51	984.88 ± 89.12	33.81 ± 3.55	767.67 ± 77.43	19.89 ± 3.21	457.71 ± 76.49	16.24 ± 1.69	267.68 ± 38.90	16.33 ± 3.00	174.64 ± 45.71
P value	0.10	0.89	<0.05*	<0.05*	0.47	0.78	<0.05*	<0.05*	<0.05*	<0.05*

*$P < 0.05$, statistically significant.

Table 8
Comparison of serum Th1/Th2 cytokines before and after vitamin D supplementation in URSA patients with low levels of vitamin D (pg/ml).

groups	IL-2	TNF-α	IFN-γ	IL-4	IL-6	IL-10
Before supplementation (n = 40)	9.98 ± 2.64	16.96 ± 5.12	16.75 ± 5.30	1.39 ± 0.63	14.81 ± 3.79	2.69 ± 1.32
After supplementation (n = 40)	2.08 ± 1.12	3.90 ± 3.04	4.46 ± 3.27	9.46 ± 3.49	13.93 ± 2.63	9.62 ± 2.58
P value	<0.05*	<0.05*	<0.05*	<0.05*	0.15	<0.05*

*P < 0.05, statistically significant.

Table 9
Comparison of the proportions and absolute count levels of peripheral blood lymphoid subpopulations before, after Vitamin D supplementation and after pregnancy in URSA patients with normal pregnancies.

groups	CD3+ (%)	$CD3^+$ (cells/ul)	$CD3^+CD4+$ (%)	$CD3^+CD4+$ (cells/ul)	$CD3^+CD8^+$ (%)	$CD3^+CD8+$ (cells/ul)	$CD19^+$ (%)	$CD19^+$ (cells/ul)	CD16 + CD56+ (%)	CD16 + CD56+ (cells/ul)
Before Supplementation (n = 19)	61.21 ± 5.08	975.51 ± 81.24	39.92 ± 4.23	964.21 ± 71.50	20.34 ± 2.03	439.13 ± 96.89	19.95 ± 3.84	351.32 ± 50.19	21.36 ± 4.84	364.13 ± 62.33
After supplementation (n = 19)	60.48 ± 4.58	983.78 ± 77.34	33.23 ± 2.78	767.82 ± 69.46	21.36 ± 2.41	439.89 ± 72.01	15.97 ± 1.47	249.74 ± 35.39	15.64 ± 2.95	168.66 ± 36.09
after pregnancy (n = 19)	59.46 ± 4.28	967.61 ± 73.34	32.45 ± 3.26	779.39 ± 71.26	21.21 ± 3.16	430.76 ± 48.99	16.32 ± 1.97	249.25 ± 31.47	15.32 ± 1.66	167.60 ± 27.34
P1 value	0.44	0.71	<0.05*	<0.05*	0.07	0.96	<0.05*	<0.05*	<0.05*	<0.05*
P2 value	0.14	0.76	<0.05*	<0.05*	0.24	0.66	<0.05*	<0.05*	<0.05*	<0.05*
P3 value	0.34	0.46	0.26	0.35	0.85	0.43	0.47	0.95	0.54	0.84

Note: P1 values are comparisons between before and after vitamin D supplementation. p2 values are comparisons between before vitamin D supplementation and after normal pregnancy. P3 values are comparisons between after vitamin D supplementation and after normal pregnancy.
*P < 0.05, statistically difference.

Table 10
Comparison of peripheral blood Th1/Th2 cytokines before, after Vitamin D supplementation and after pregnancy in URSA patients with normal pregnancies (pg/ml).

groups	IL-2	TNF-α	IFN-γ	IL-4	IL-6	IL-10
Before Supplementation (n = 19)	10.18 ± 3.04	16.13 ± 5.15	16.90 ± 6.86	1.52 ± 0.57	14.37 ± 3.70	2.94 ± 1.56
After supplementation (n = 19)	1.98 ± 1.07	4.29 ± 3.55	5.30 ± 3.88	8.62 ± 2.84	13.22 ± 2.57	8.99 ± 2.47
after pregnancy (n = 19)	2.43 ± 0.90	2.75 ± 1.78	2.88 ± 1.85	9.08 ± 2.27	13.05 ± 4.07	9.34 ± 3.02
P1 value	<0.05*	<0.05*	<0.05*	<0.05*	0.19	<0.05*
P2 value	<0.05*	<0.05*	<0.05*	<0.05*	0.17	<0.05*
P3 value	0.17	0.04*	<0.05*	0.40	0.77	0.54

Note: P1 values are comparisons between before and after vitamin D supplementation. p2 values are comparisons between before vitamin D supplementation and after normal pregnancy.P3 values are comparisons between after vitamin D supplementation and after normal pregnancy.
*P < 0.05, statistically difference.

sample size. Secondly, although a comparison of patients with normal pregnancies was made and it was found that 19 out of 28 patients supplemented with Vitamin D had normal pregnancies and the rate of successful pregnancy after Vitamin D supplementation was 67.8%, there was a lack of control of the pregnancy rate of URSA patients not supplemented with Vitamin D. Therefore, it could not adequately reflect the role of Vitamin D in assisting pregnancy in URSA, nor could it fully demonstrate that Vitamin D status in patients with URSA reduces the risk of further miscarriage and improve the outcome of assisted reproductive technology, thus increasing the pregnancy rate of patients, so more researchers need to follow up to do further high-quality randomized controlled trials on the relationship between Vitamin D supplementation and reproductive outcomes in URSA patients.

Ethical statement

This study was approved by the Research Ethics Committee of Sichuan Jinxin Xinan Women's and Children's Hospital (2023-045). The informed content was waived. The study conformed to the Declaration of Helsinki.

Data availability statement

All data will be made available upon reasonable request to and with the approved consent of the corresponding author, and will be shared in accordance with the standards of ethical policies regulating data sharing in human subjects.

CRediT authorship contribution statement

Panyu Yang: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Fenjian Lu:** Supervision, Methodology, Investigation.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors did not use generative AI or AI-assisted technologies in the preparation of this manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] L.B. Andersen, J.S. Jørgensen, T.K. Jensen, et al., Vitamin D insufficiency is associated with increased risk of first-trimester miscarriage in the Odense Child Cohort, Am. J. Clin. Nutr. 102 (3) (2015) 633–638, https://doi.org/10.3945/ajcn.114.103655.
[2] Y. Zhang, Z. Liu, M. Tian, et al., The altered PD-1/PD-L1 pathway delivers the 'one-two punch' effects to promote the Treg/Th17 imbalance in pre-eclampsia, Cell. Mol. Immunol. 15 (7) (2018) 710–723, https://doi.org/10.1038/cmi.2017.70.
[3] K. Sharif, Y. Sharif, A. Watad, et al., Vitamin D, autoimmunity and recurrent pregnancy loss: more than an association, Am. J. Reprod. Immunol. 80 (3) (2018) e12991, https://doi.org/10.1111/aji.12991.
[4] Practice Committee of the American Society for Reproductive Medicine, Definitions of infertility and recurrent pregnancy loss: a committee opinion, Fertil. Steril. 99 (1) (2013) 63, https://doi.org/10.1016/j.fertnstert.2012.09.023.
[5] B. Saifi, S.A. Rezaee, N. Tajik, et al., Th17 cells and related cytokines in unexplained recurrent spontaneous miscarriage at the implantation window, Reprod. Biomed. Online 29 (4) (2014) 481–489, https://doi.org/10.1016/j.rbmo.2014.06.008.
[6] W.J. Wang, C.F. Hao, Yi-Lin, et al., Increased prevalence of T helper 17 (Th17) cells in peripheral blood and decidua in unexplained recurrent spontaneous abortion patients, J. Reprod. Immunol. 84 (2) (2010) 164–170, https://doi.org/10.1016/j.jri.2009.12.003.
[7] D.R. Gonçalves, A. Braga, J. Braga, A. Marinho, Recurrent pregnancy loss and vitamin D: a review of the literature, Am. J. Reprod. Immunol. 80 (5) (2018) e13022, https://doi.org/10.1111/aji.13022.
[8] M. Molazadeh, H. Karimzadeh, M.R. Azizi, Prevalence and clinical significance of antinuclear antibodies in Iranian women with unexplained recurrent miscarriage, Iran. J. Reproductive Med. 12 (3) (2014) 221–226.
[9] A.L. Ponsonby, R.M. Lucas, S. Lewis, J. Halliday, Vitamin D status during pregnancy and aspects of offspring health, Nutrients 2 (3) (2010) 389–407, https://doi.org/10.3390/nu2030389.
[10] K. Ota, S. Dambaeva, A.R. Han, K. Beaman, A. Gilman-Sachs, J. Kwak-Kim, Vitamin D deficiency may be a risk factor for recurrent pregnancy losses by increasing cellular immunity and autoimmunity, Hum. Reprod. 29 (2) (2014) 208–219, https://doi.org/10.1093/humrep/det424.
[11] W. Dankers, E.M. Colin, J.P. van Hamburg, E. Lubberts, Vitamin D in autoimmunity: molecular mechanisms and therapeutic potential, Front. Immunol. 7 (2017) 697, https://doi.org/10.3389/fimmu.2016.00697. Published 2017 Jan 20.
[12] J. Kwak-Kim, A. Skariah, L. Wu, D. Salazar, N. Sung, K. Ota, Humoral and cellular autoimmunity in women with recurrent pregnancy losses and repeated implantation failures: a possible role of vitamin D, Autoimmun. Rev. 15 (10) (2016) 943–947, https://doi.org/10.1016/j.autrev.2016.07.015.
[13] S. Shahgheibi, F. Farhadifar, B. Pouya, The effect of vitamin D supplementation on gestational diabetes in high-risk women: results from a randomized placebo-controlled trial, J. Res. Med. Sci. 21 (2) (2016), https://doi.org/10.4103/1735-1995.175148. Published 2016 Jan 28.
[14] N. Li, H.M. Wu, F. Hang, Y.S. Zhang, M.J. Li, Women with recurrent spontaneous abortion have decreased 25(OH) vitamin D and VDR at the fetal-maternal interface, Braz. J. Med. Biol. Res. 50 (11) (2017) e6527, https://doi.org/10.1590/1414-431X20176527. Published 2017 Sep. 12.
[15] J. Chu, I. Gallos, A. Tobias, B. Tan, A. Eapen, A. Coomarasamy, Vitamin D and assisted reproductive treatment outcome: a systematic review and meta-analysis, Hum. Reprod. 33 (1) (2018) 65–80, https://doi.org/10.1093/humrep/dex326.
[16] N. Li, H.M. Wu, F. Hang, Y.S. Zhang, M.J. Li, Women with recurrent spontaneous abortion have decreased 25(OH) vitamin D and VDR at the fetal-maternal interface, Braz. J. Med. Biol. Res. 50 (11) (2017) e6527, https://doi.org/10.1590/1414-431X20176527. Published 2017 Sep. 12.
[17] X. Yan, L. Wang, C. Yan, et al., Decreased expression of the vitamin D receptor in women with recurrent pregnancy loss, Arch. Biochem. Biophys. 606 (2016) 128–133, https://doi.org/10.1016/j.abb.2016.07.021.
[18] H.T. Christesen, C. Elvander, R.F. Lamont, J.S. Jørgensen, The impact of vitamin D in pregnancy on extraskeletal health in children: a systematic review, Acta Obstet. Gynecol. Scand. 91 (12) (2012) 1368–1380, https://doi.org/10.1111/aogs.12006.
[19] A. Olmos-Ortiz, E. Avila, M. Durand-Carbajal, L. Díaz, Regulation of calcitriol biosynthesis and activity: focus on gestational vitamin D deficiency and adverse pregnancy outcomes, Nutrients 7 (1) (2015) 443–480, https://doi.org/10.3390/nu7010443. Published 2015 Jan 9.
[20] J. Ji, H. Zhai, H. Zhou, S. Song, G. Mor, A. Liao, The role and mechanism of vitamin D-mediated regulation of Treg/Th17 balance in recurrent pregnancy loss, Am. J. Reprod. Immunol. 81 (6) (2019) e13112, https://doi.org/10.1111/aji.13112.
[21] X. Chen, B. Yin, R.C. Lian, et al., Modulatory effects of vitamin D on peripheral cellular immunity in patients with recurrent miscarriage, Am. J. Reprod. Immunol. 76 (6) (2016) 432–438, https://doi.org/10.1111/aji.12585.
[22] O.B. Christiansen, H.S. Nielsen, B. Pedersen, Active or passive immunization in unexplained recurrent miscarriage, J. Reprod. Immunol. 62 (1–2) (2004) 41–52, https://doi.org/10.1016/j.jri.2003.09.003.
[23] D.L. Kamen, V. Tangpricha, Vitamin D and molecular actions on the immune system: modulation of innate and autoimmunity, J. Mol. Med. (Berl.) 88 (5) (2010) 441–450, https://doi.org/10.1007/s00109-010-0590-9.
[24] B.W. Hollis, C.L. Wagner, Vitamin D supplementation during pregnancy: improvements in birth outcomes and complications through direct genomic alteration, Mol. Cell. Endocrinol. 453 (2017) 113–130, https://doi.org/10.1016/j.mce.2017.01.039.
[25] S.K. Lee, B.J. Na, J.Y. Kim, et al., Determination of clinical cellular immune markers in women with recurrent pregnancy loss, Am. J. Reprod. Immunol. 70 (5) (2013) 398–411, https://doi.org/10.1111/aji.12137.
[26] R.J. Kuon, K. Vomstein, M. Weber, et al., The "killer cell story" in recurrent miscarriage: association between activated peripheral lymphocytes and uterine natural killer cells, J. Reprod. Immunol. 119 (2017) 9–14, https://doi.org/10.1016/j.jri.2016.11.002.
[27] U.R. Markert, D.M. Morales-Prieto, J.S. Fitzgerald, Understanding the link between the IL-6 cytokine family and pregnancy: implications for future therapeutics, Expet Rev. Clin. Immunol. 7 (5) (2011) 603–609, https://doi.org/10.1586/eci.11.60.
[28] R. Raghupathy, J. Kalinka, Cytokine imbalance in pregnancy complications and its modulation, Front. Biosci. 13 (2008) 985–994, https://doi.org/10.2741/2737. Published 2008 Jan 1.
[29] Y. Gao, P.L. Wang, Increased CD56(+) NK cells and enhanced Th1 responses in human unexplained recurrent spontaneous abortion, Genet. Mol. Res. 14 (4) (2015) 18103–18109, https://doi.org/10.4238/2015.December.22.36. Published 2015 Dec 22.

[30] L. Zhu, H. Chen, M. Liu, et al., Treg/Th17 cell imbalance and IL-6 profile in patients with unexplained recurrent spontaneous abortion, Reprod. Sci. 24 (6) (2017) 882–890, https://doi.org/10.1177/1933719116670517.
[31] J.A. Tamblyn, M. Hewison, C.L. Wagner, J.N. Bulmer, M.D. Kilby, Immunological role of vitamin D at the maternal-fetal interface, J. Endocrinol. 224 (3) (2015) R107–R121, https://doi.org/10.1530/JOE-14-0642.
[32] I. Mayan, R. Somech, A. Lev, A.H. Cohen, N.W. Constantini, G. Dubnov-Raz, Thymus activity, vitamin D, and respiratory infections in adolescent swimmers, Isr. Med. Assoc. J. 17 (9) (2015) 571–575.
[33] C.A. Hunter, S.A. Jones, IL-6 as a keystone cytokine in health and disease [published correction appears in Nat Immunol. 2017 Oct 18;18(11):1271], Nat. Immunol. 16 (5) (2015) 448–457, https://doi.org/10.1038/ni.3153.
[34] M. von Wolff, S. Stieger, K. Lumpp, J. Bücking, T. Strowitzki, C.J. Thaler, Endometrial interleukin-6 in vitro is not regulated directly by female steroid hormones, but by pro-inflammatory cytokines and hypoxia, Mol. Hum. Reprod. 8 (12) (2002) 1096–1102, https://doi.org/10.1093/molehr/8.12.1096.
[35] P.Y. Liang, L.H. Diao, C.Y. Huang, et al., The pro-inflammatory and anti-inflammatory cytokine profile in peripheral blood of women with recurrent implantation failure, Reprod. Biomed. Online 31 (6) (2015) 823–826, https://doi.org/10.1016/j.rbmo.2015.08.009.