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ORIGINAL RESEARCH

Vitamin D Supplementation Selectively Affects Peripheral Lymphocyte Subsets in Infertile Women

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Background: This study aimed to explore the effect of vitamin D supplementation on lymphocyte subsets in infertile women.

Methods: The study involved a total of 247 patients who suffered recurrent embryo implantation failure (RIF) or recurrent spontaneous abortion (RSA) between January and December 2019 in the Reproductive Medicine Center of Children's Hospital of Shanxi and Women Health Center of Shanxi. The differences in the vitamin D and lymphocyte subsets of the two diseases, the correlation between the 25-hydroxy vitamin D and lymphocyte subsets, the changes in the lymphocyte subsets after vitamin D supplementation and the impact on pregnancy outcome were analysed.

Results: The prevalence of vitamin D deficiency was 77.33% (191/247). After vitamin D supplementation, there were no significant differences in helper T cells (Th), cytotoxic T cells (Tc), Th/Tc and natural killer cells (NK) ($p > 0.05$), but there were significant differences in leukocyte differentiation antigens 3+ (CD3+), natural killer T cells (NKT), B lymphocytes and vitamin D ($p < 0.05$). Before vitamin D supplementation, the difference in the composition ratio of different vitamin D levels between the RIF and RSA groups was not statistically significant. At different vitamin D levels, there were no significant differences in CD3+, Th, Tc, Th/Tc, NK or B lymphocyte ($p > 0.05$), but there was a significant difference in NKT ($p < 0.05$).

Conclusion: Vitamin D supplementation can reduce the level of NKT in infertile women, which may be of benefit to the pregnancy outcome of couples who experience RIF.

Keywords: recurrent embryo implantation failure, infertility, lymphocyte subsets, recurrent spontaneous abortion, vitamin D

Introduction

The incidence rate of infertility is currently increasing year by year,[1] and in-vitro fertilisation embryo transfer (IVF-ET) is generally used to achieve a patient's fertility wishes.[2] However, IVF-ET and its success depend on multiple factors. Recurrent miscarriages and recurrent embryo implantation failure (RIF) can be the result of genetic factors, anatomical factors, endocrine abnormalities, infection, prethrombotic state and immune disorders, with the latter accounting for 50–60% of all the aetiology.[2] During successful pregnancy, the immune system plays an important role, and studies suggest that some 50% of unexplained infertility is due to immune system problems. Various immune cells are involved in the development of immune disorders, but it is believed that lymphocyte subsets are highly important in disease development, Lymphoid cells, including monocytes and natural killer (NK) cells, play a central role in this process. These cells are not only involved in the engulfment of the implanting embryo, ensuring successful implantation, but they

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are also critical for sculpting the vasculature that will provide the blood supply to the placenta. The infiltration of NK cells in the endometrium increases during early pregnancy, and they regulate endometrial receptivity and angiogenesis by secreting cytokines and growth factors, such as interleukin-15 (IL-15) and vascular endothelial growth factor (VEGF). Additionally, monocytes also play a key role in immune regulation and angiogenesis during early implantation. Therefore, understanding how vitamin D affects the function of these critical immune cells is essential for optimizing treatment strategies for infertility.[3] If lymphocyte subsets can reflect endometrial receptivity, they may provide theoretical evidence for the analysis of the causes of recurrent spontaneous abortion (RSA) and RIF.

Vitamin D is an essential vitamin and, in a normal human body, 80–90% of vitamin D is synthesised by the body after exposure to sunlight; it can also be ingested through diet.[4] However, due to their lifestyle, many people do not have enough sunlight exposure,[5] which leads to an inability to synthesise enough vitamin D in the body, and this problem can affect women of reproductive age.[6] Since vitamin D has immunomodulatory properties, a vitamin D deficiency can lead to abnormalities in the immune system.[7] In addition to its regulatory role in bone development, recent studies have suggested that vitamin D deficiency or insufficiency may be associated with pregnancy loss in women.[8] This is because it can lead to fertility disorders and other diseases, and it may, therefore, also impact the success rate of IVF-ET.[9] Since vitamin D and lymphocyte subsets are both associated with the immune system, RSA and RIF, there may be an unknown association between vitamin D and lymphocyte subsets and the RSA and RIF that are common manifestations of IVF-ET failure. In addition, localized immune responses within the uterine endometrium are crucial for the success of embryo implantation. Although our study focuses on cells in the bloodstream, our introduction needs to provide a clearer summary of the existing literature, especially regarding the role of immune cells in the uterine endometrium. The research by Braun et al[10] provides compelling evidence that NK and T cell subtypes in the endometrium are associated with recurrent pregnancy loss and recurrent implantation failure. These findings underscore the potential importance of specific immune cell subsets within the uterine endometrial microenvironment for pregnancy success. At present, there is no clear understanding as to whether the causes and mechanisms of the two manifestations are the same. This study sets out to evaluate the effect of vitamin D supplementation on lymphocyte subsets to provide evidence for its potential values in the clinical treatment of RSA and RIA.

Materials and Methods

Study Design and Participants

This study was approved by the Ethics Committee of the Children's Hospital of Shanxi and Women Health Center of Shanxi, (IRB-KY-2017), and a total of 247 patients with RIF or RSA participated in it. The inclusion criteria were as follows: 1) women with RIF or RSA; 2) women aged >20 years; and 3) women who had lymphocyte subset tests for aetiological screening. The exclusion criteria were as follows: patients with endocrine disorders, prethrombotic state, abnormal reproductive anatomy, infection or other obvious factors related to the two diseases. The sample size was determined as follows: through previous research literature and meta-analysis, α, β and effective treatment rates were assumed, and the sample size was calculated using the software PASS 15.1 (IBM, NY, US). All included patients were informed and gave consent prior to the study. The patients were divided into two groups: the RIF group and the RSA group. The inclusion criteria for the RSA patients were those with two or more consecutive pregnancy losses before 20 weeks of gestation,[11] and the inclusion criteria for the patients with RIF were failure to achieve pregnancy after 2–6 cycles of IVF.[12] The patients were also divided into groups according to serum 25-hydroxy vitamin D (25[OH]D) levels; patients with 50 nmol/L ≤ vitamin D < 75 nmol/L were classified as the vitamin D-insufficiency group, those with vitamin D < 50 nmol/L were classified as the vitamin D-deficiency group and those with vitamin D ≥ 75 nmol/L were classified as the vitamin D-sufficiency group.

Outcomes

This study divided the patients into a high-level group and a low-level group with vitamin D 50 nmol/L as the boundary.[13] All patients were required to fast before blood sampling. Then, 3 mL of peripheral venous blood were

placed in a coagulation-promoting tube and subsequently centrifuged at 3000 r/min for 5 min to determine the 25(OH)D content using an automated immunoassay analyser (Modular Analytics E170, Roche Diagnostics, Mannheim, Germany).

We performed immunophenotyping of immune cells in the peripheral blood of patients using flow cytometry. In the flow cytometry analysis, the values we obtained are expressed as the "percent of cells counted". To gain a more comprehensive understanding of the distribution of immune cells, we recognized the need to convert these percentages into absolute cell counts per milliliter of blood.

Flow cytometry: Immune cells in the patient's serum were immunophenotyped using flow cytometry. A monoclonal antibody combination was used for cell staining: PC5-CD3+/FITC-CD4+/PE-CD8+, FITC-CD3+/PE-CD16+CD56+/PC5-CD19+, PC7-CD45+ were obtained from the Immunotech Beckman Coulter Company (Beckman Coulter Company, Paris Nord, France). Flow cytometry (PC 500 MPL, Beckmann Coulter, Miami, FL, USA) was used to analyse the results. In our study, to accurately identify and quantify lymphocyte subsets in peripheral blood, we employed a detailed gating strategy for flow cytometry. We initially used FSC (forward scatter) and SSC (side scatter) parameters to exclude cell debris and non-cellular events, followed by the fluorescence signals from monoclonal antibody markers to distinguish different lymphocyte subsets (Figure 1). We utilized a panel of antibodies including CD3, CD4, CD8, CD19, CD56, and CD16, which allowed us to differentiate Th(CD3+CD4+), Tc(CD3+CD8+), B(CD3-CD19+), NK(CD3-CD16+CD56+), NKT(CD3+CD16+CD56+) cells. To further subset these populations, we also used additional markers such as CD45 to exclude the interference of monocytes. We optimized the performance of the flow cytometer by setting specific voltages and compensation to ensure the accuracy and reproducibility of the data. Moreover, we conducted multiple experiments to validate the consistency of the gating strategy, ensuring the reliability of our experimental results. We believe that this rigorous gating strategy is crucial for accurately assessing the impact of vitamin D on lymphocyte subsets. To enhance the transparency and reproducibility of our study, we also documented the number of cells analyzed for each sample. For instance, we performed immunophenotyping on a fixed number of 50,000 cells per patient, which helps standardize our experimental procedures and ensures consistency in our data.

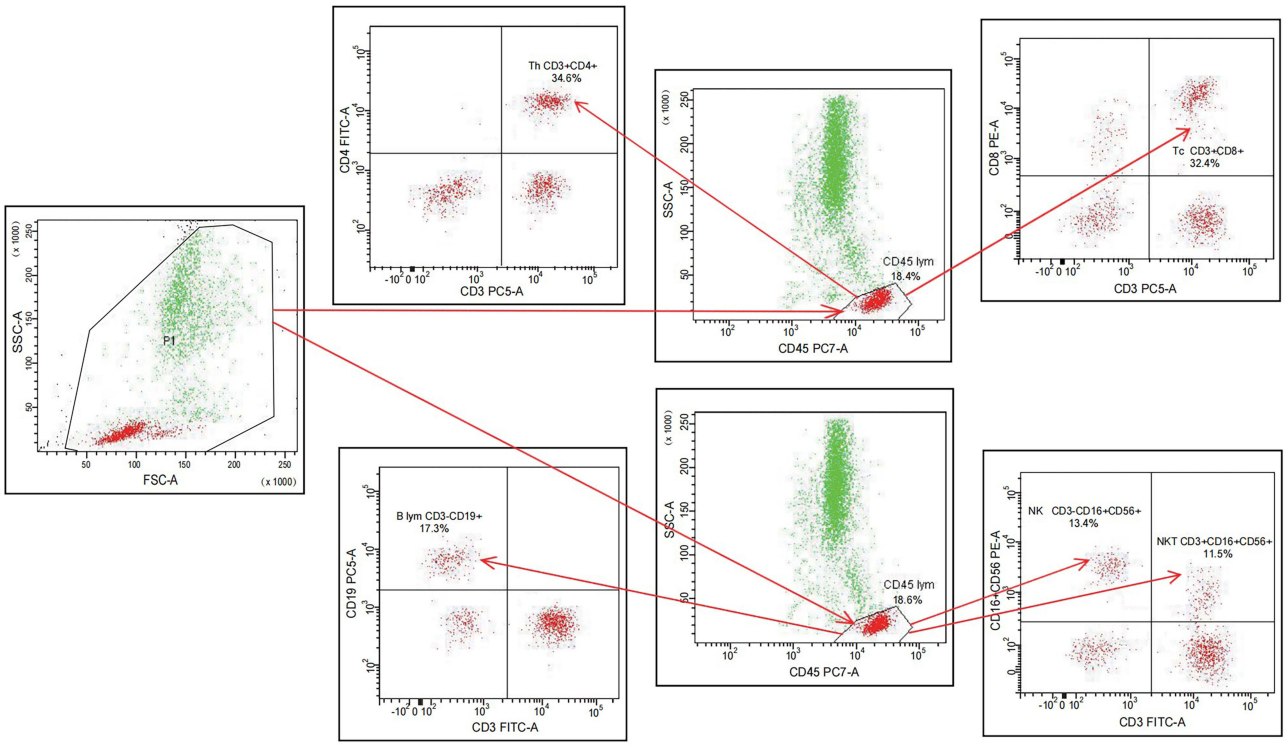

Figure 1 Representative flow cytometry plots and our gating strategy.

Intervention measurement

Patients with vitamin D deficiency (25[OH]D < 50 nmol/L) were given a vitamin D supplementation of 400 IU twice a day (BID), followed by a review of the patients' vitamin D and lymphocyte subsets 1–2 months later. The patients with insufficient vitamin D (50 nmol/L ≤ 25[OH]D < 75 nmol/L) were advised to increase the time spent on outdoor activities and increase the intake of vitamin D-rich foods. Those with an adequate level of vitamin D (25[OH]D ≥ 75 nmol/L) were told to continue to follow their current diet and lifestyle. The pregnancy outcomes of patients were followed up for 1 year. Patients without RIF who underwent embryo transfer during the same period as the control group were selected for pregnancy outcomes analysis of the RIF group after vitamin D supplementation.

Statistical analysis

In the statistical analysis, the statistical analysis was performed using SPSS version 24.0 (IBM, NY, US). The Spearman rank correlation coefficient was used for correlation analysis, and pregnancy outcome was analysed using a chi-squared test. The continuous data are shown as mean ± standard deviation or median (interquartile range). Non-parametric tests were used for data with a non-normal distribution. The *t*-test was used to assess the differences in age and vitamin D level before supplementation between the two diseases, and a Mann–Whitney *U*-test was used to compare the lymphocyte subsets of the two disease groups and their different vitamin D levels. A value of $p < 0.05$ was considered significant.

Results

Vitamin D Deficiency in the Pregnancy Loss/Implantation Failure Population

Figure 1 showing representative flow cytometry plots and our gating strategy. Lymphocytes were gated by CD45/SSC, and then the proportion of lymphocyte subtypes was analyzed according to fluorescent markers. The 247 patients who participated in the study were aged 25–40 years, with an average age of 30.74 ± 3.64 years. Their mean serum 25(OH)D value was 39.20 ± 14.75 nmol/L. There were 100 cases of RSA, and the mean serum 25(OH)D value was 37.80 ± 14.40 nmol/L. There were 147 cases of RIF, and the mean serum 25(OH)D value was 40.15 ± 14.95 nmol/L. There were 191 (77.33%) patients with vitamin D deficiency, 52 (21.05%) with vitamin D insufficiency and 4 (1.62%) with vitamin D sufficiency (Table 1). There was not a statistically significant difference in the composition ratio of different vitamin D levels between the RIF and RSA groups.

Effect of Vitamin D Supplementation on Lymphocyte Subsets

The 191 cases of vitamin D deficiency were given vitamin D supplementation of 400 IU BID, and the lymphocyte subset levels before and after vitamin D supplementation are shown in Table 2. After vitamin D supplementation, the plasma vitamin D level was significantly increased. There were significant changes in leukocyte differentiation antigens 3+ (CD3 +), ($p = 0.007$), natural killer T cells (NKT) ($p < 0.001$) and B lymphocytes ($p < 0.001$), but no significant differences in helper T cells (Th), cytotoxic T cells (Tc), Th/Tc or natural killer cells (NK).

Table 1 Basic Information of Two Groups

Group	Age	25 (OH) D (nmol/L)	Vitamin D deficiency	Vitamin D insufficient	Vitamin D sufficient
RSA(n=100) RIF(n=147)	30.62±3.74 30.82±3.58	37.80±14.40 40.15±14.95	81(81.00%) 110(74.83%)	18(18.00%) 34(23.13%)	1(1.00%) 3(2.04%)
T value P value	0.426 0.671	1.236 0.218	2.248 0.325		

Abbreviations: RIF, Repeated implantation failure; RSA, recurrent spontaneous abortion.

Table 2 The Changes in Lymphocyte Subsets Levels Before and After Vitamin D Supplementation. Values are Presented with Median (Interquartile Range)

	CD^{3+}	Th	Tc	Th/ Tc	NK	NKT	B lymphocyte	25(OH)D
Before	72.00(8.90)	38.60(8.90)	28.00(8.40)	1.39(0.68)	14.10(8.60)	2.90(4.00)	11.60(5.20)	37.43(19.63)
After	70.20(9.20)	38.90(8.40)	26.80(7.70)	1.40(0.53)	14.90(8.80)	1.90(3.50)	12.80(5.70)	49.16(18.25)
Z value	−2.676	−0.208	−1.588	−0.606	−1.513	−4.354	−3.605	−10.792
P value	0.007	0.835	0.112	0.544	0.130	0.000	0.000	0.000

Note: CD3+: leukocyte differentiation antigens 3+.
Abbreviations: NK, natural killer cell; NKT, Natural killer T cell; Tc, cytotoxic T cell; Th, helper T cell.

The Differences Between the Two Groups in Vitamin D and Lymphocyte Subsets

There were 100 patients with RSA and 147 patients with RIF in this study, and there was no statistically significant difference in the composition ratio of different 25(OH)D levels of the RSA and RIF groups ($p > 0.05$). Furthermore, the lymphocyte subsets of the two groups were not significantly different ($p > 0.05$) (Table 3).

Correlation Between Vitamin D and Lymphocyte Subsets

The results of the correlation analysis of the serum 25(OH)D levels and lymphocyte subsets before treatment are shown in Table 4. The NKT levels correlated negatively with the serum 25(OH)D levels ($p < 0.05$), but the other lymphocyte subsets did not correlate with the serum 25(OH)D levels. The differences in the lymphocyte subsets of the two groups before treatment are shown in Table 5. The NKT levels in the 25(OH)D high-level group were significantly different ($p < 0.05$), but the serum CD3+, Th, Tc, Th/Tc and the NK and B lymphocyte levels were not significantly different ($p > 0.05$).

The Impact of Vitamin D Supplementation on Pregnancy Outcome

There were 147 patients in the RIF group, and in 138 of them, RIF performed the embryo transfer, and there were 62 clinical pregnancies and 14 miscarriages. As a control, 6310 patients without RIF who underwent conventional frozen embryo transfer were selected, and the pregnancy outcome was analysed. The clinical pregnancy rate and spontaneous abortion rate of the RIF group and the conventional frozen embryo transfer group were not significantly different ($p > 0.05$). However, the spontaneous abortion rate of the RIF group was higher than that of the conventional frozen embryo transfer group (22.58% vs 15.05%). In the RSA group, 24 patients (24%) became pregnant after follicle monitoring, 8 of them (33.33%) spontaneously aborted and 16 delivered a baby (66.67%). The spontaneous abortion rate of the RSA group was higher than that of the RIF group (33.33% vs 22.58%).

Discussion

In this study, the deficiency rate of vitamin D was 77.33% and the insufficiency rate of vitamin D was 21.05%, which corresponds to the rates in previous studies.[14] In this study, the mean value of 25(OH)D in the RIF group was higher than that in the RSA group (40.15 nmol/L vs 37.80 nmol/L, respectively), and there was no statistical difference in the level of 25(OH)D in the two groups, which may be related to the overlap in aetiology and similar pathogenesis between the two groups. After vitamin D supplementation, the expression of CD3+, NKT and B lymphocytes was significantly different, but the expression of Th, Tc, Th/Tc and NK lymphocytes was not significantly different across all 247 patients. The median of CD3+, Tc and NKT decreased, and the median of Th, Th/Tc, NK and B lymphocytes increased. Interquad spacing decreased, except for CD3+, NK and B cells, suggesting that vitamin D affects the composition of lymphocyte subsets, and these changes have a positive effect on the body's immune regulation.[15] The next step in this research is to expand the sample size and study the cytokine level or the absolute lymphocyte count. After vitamin D supplementation, there was not a significant difference in the clinical pregnancy rate and spontaneous abortion rate between the RIF group and the conventional frozen embryo transfer group, which indicated patients with RIF have similar pregnancy outcomes

Table 3 Analysis of lymphocyte subsets with RSA and RIF groups

Group		CD^{3+}	Th	Tc	Th/ Tc	NK	NKT	B lymphocyte	25(OH)D
Before treatment	RSA (n=100)	70.40 (10.13)	38.10 (9.57)	28.45 (8.38)	1.35 (0.64)	15.10 (8.60)	3.20 (3.75)	11.65 (5.40)	36.63 (18.71)
	RIF (n=147)	72.60 (8.50)	38.80 (8.70)	27.70 (8.30)	1.43 (0.67)	13.80 (8.90)	2.50 (4.00)	11.60 (5.10)	38.01 (21.04)
Z		-0.393	-0.891	-0.930	-1.276	-0.042	-1.605	-0.258	-1.258
P		0.694	0.373	0.352	0.202	0.967	0.108	0.797	0.208
After treatment	RSA (n=100)	70.10 (9.80)	39.80 (8.32)	26.50 (7.30)	1.45 (0.52)	16.10 (8.80)	2.00 (2.70)	13.60 (6.50)	49.57 (19.87)
	RIF (n=147)	70.30 (9.10)	37.60 (8.00)	27.10 (8.40)	1.39 (0.55)	14.90 (8.90)	1.80 (4.00)	12.40 (5.50)	49.16 (16.99)
Z		-0.817	-1.120	-0.744	-1.159	-0.073	-1.224	-1.270	-0.088
P		0.414	0.263	0.457	0.246	0.942	0.221	0.204	0.930

Note: CD3+: leukocyte differentiation antigens 3+.

Abbreviations: NK, natural killer cell; NKT, Natural killer T cell; RIF, Repeated implantation failure; RSA, recurrent spontaneous abortion; Th, helper T cell; Tc, cytotoxic T cell.

Table 4 Correlation Analysis Between 25(OH)D and Lymphocyte Subsets

25(OH)D	CD^{3+}	Th	Tc	Th/ Tc	NK	NKT	B lymphocyte
Spearman R	0.041	0.084	−0.084	0.109	0.048	−0.201	−0.057
P value	0.519	0.188	0.186	0.086	0.452	0.001	0.372

Note: CD^{3+}: leukocyte differentiation antigens 3+.
Abbreviations: NK, natural killer cell; NKT, Natural killer T cell; Tc, cytotoxic T cell; Th, helper T cell.

Table 5 Analysis of Lymphocyte Subsets with Different 25(OH)D Level

	CD^{3+}	Th	Tc	Th/ Tc	NK	NKT	B lymphocyte
High level group	71.25(7.95)	39.75(8.80)	27.30(7.57)	1.51(0.60)	16.45(9.75)	1.60(4.20)	11.60(5.15)
Low level group	72.00(9.50)	38.40(9.20)	28.50(8.50)	1.35(0.66)	13.80(8.20)	3.10(3.60)	11.70(5.30)
Z value	−0.208	−1.075	−1.456	−1.762	−1.212	−2.487	−0.709
P value	0.835	0.282	0.145	0.078	0.225	0.013	0.478

Note: CD3+: leukocyte differentiation antigens 3+; High level group: serum 25-(OH) D ≥50 nmol/L; Low level group: serum 25-(OH) D <50 nmol/L.
Abbreviations: NK, natural killer cell; NKT, Natural killer T cell; Tc, cytotoxic T cell; Th, helper T cell.

to general embryo transfer patients. However, the spontaneous abortion rate of the RIF group was higher than that of the conventional frozen embryo transfer group. Further studies will explore more immune indicators.

Vitamin D is important in immune system function.[16,17] In T cell-mediated cellular immunity, CD3+ is a surface marker of T cells and a sign of the mature differentiation of T lymphocytes. The normal function of lymphocyte subsets and the activation of T lymphocytes are also closely related to vitamin D,[18] and CD4+ cells are mainly Th cells, which can be differentiated into Th1 and Th2 cells. The Th1 cells mainly secrete interleukin 2 (IL-2), IL-12, interferon-γ and tumour necrosis factor-α, which are involved in cellular immunity and graft rejection, while Th2 cells primarily secrete IL-4, IL-5, IL-10 and IL-13, which are required for humoral immunity.[19,20] During the peri-implantation period, an appropriate amount of Th1 factor is conducive to embryo implantation. After pregnancy, the balance of Th1/Th2 is shifted to Th2, which is conducive to the continuation of the pregnancy. The characteristic surface antigen of Tc is CD8+, which is involved in maternal–foetal immune regulation. Although Tc has a cytotoxic effect, it almost disappears in the endometrial endocrine stage. At the same time, it participates in immune regulation and maternal–foetal interface immune balance by secreting its own and Th2-type cytokines.[21]

NKT cells have garnered attention for their bridging role between innate and adaptive immune responses, modulating the immune environment through the secretion of cytokines such as IL-4 and IFN-γ. Additionally, NKT cells also play a crucial role in pregnancy, potentially modulating embryo implantation by affecting the immune microenvironment of the endometrium.[22]

In the Chinese diet, primary sources of vitamin D include fatty fish (such as salmon and mackerel), egg yolks, milk, and fortified foods. However, due to cultural preferences and lifestyle choices, many individuals in China may avoid excessive sun exposure, which could limit the synthesis of vitamin D in the body.[23] This aversion to sun exposure, coupled with possible dietary restrictions, may be potential causes of vitamin D deficiency among the Chinese population. This study showed that vitamin D supplementation could influence CD3+, NKT and B lymphocytes, and there were significant differences in NKT between the different vitamin D levels, which was consistent with previous research.[22] Vitamin D can increase the activity of NK and NKT.[23,24] Most mature NK cells express CD2+, CD16+ and CD56+, but an abnormal number and function of CD3− can lead to spontaneous abortion.[25] In this study, vitamin D was negatively correlated with NKT. However, 25(OH)D did not correlate with NK and CD3+; Given the relatively low numbers of NKT cells in circulation and tissues, this finding is particularly intriguing. The role of NKT cells in immune regulation is well recognized, and they are capable of releasing signalling cytokines that influence immune responses. Specifically, within the uterine endometrium, NKT cells may play a crucial role during embryo implantation. For instance, a study by Lobo et al[26] emphasizes the importance of NKT cells in modulating the immune microenvironment

of the endometrium. Additionally, research by Lu et al[27] indicates that NKT cells influence the receptivity of the endometrium through the cytokines they produce. These findings resonate with our results, suggesting that vitamin D may affect pregnancy outcomes by modulating NKT cells. Thus, it is necessary to further investigate their internal relationship from the perspective of cytokine level or absolute lymphocyte count, and the authors intend to further study the impact of lymphocyte subsets on diseases and their clinical significance in the future.

In this study, we assessed the impact of vitamin D on lymphocyte subsets in infertile women through immunophenotyping of blood samples. However, it is important to recognize that the phenotype of immune cells in blood samples may not fully reflect the status of immune cells in tissues. Immune cells in tissues, such as leukocytes in the uterine endometrium, may exhibit different phenotypes and functions due to the influence of the local microenvironment. For instance, immune cells in the endometrium may differ from those in the bloodstream due to the effects of local hormone levels, cytokines, and growth factors. Therefore, while our study provides important insights into the effects of vitamin D on immune cells in the bloodstream, these findings may not necessarily be directly extrapolated to immune cells in tissues. In addition, we assessed the impact of vitamin D by analyzing lymphocyte subsets in the bloodstream. However, it is important to note that lymphocytes in circulation represent only about 2% of the total lymphocyte pool and are often immature and less differentiated cells that traffic between different locations. In contrast, lymphocytes in tissues, such as those in the uterine endometrium, may have more specific phenotypes and functions, playing key roles in local immune responses and tissue-specific processes. Therefore, while our study provides important insights into the effects of vitamin D on lymphocytes in the bloodstream, these findings may not fully reflect the actions of vitamin D at the tissue level. Finally, the inability to provide a quantitative count of lymphocytes per mL of blood due to not performing a Complete Blood Count (CBC).

To gain a more comprehensive understanding of the impact of vitamin D on tissue immune cells, future studies could consider conducting immunophenotyping analyses on tissue samples. Additionally, advanced imaging techniques and single-cell sequencing technologies could be utilized to explore the differences between immune cells in blood and tissues. These approaches will help reveal the mechanisms of action of vitamin D in various immune cell subsets and provide more precise targets for the clinical treatment of infertility. Future studies should consider a variety of methods, including tissue samples. This may involve conducting immunophenotyping analyses on endometrial biopsy samples and utilizing advanced imaging techniques and single-cell sequencing technologies to explore the differences between immune cells in blood and tissues. Through these approaches, we can more accurately assess the impact of vitamin D on tissue immune cells and provide more precise targets for the treatment of infertility.

In conclusion, vitamin D supplementation was beneficial to pregnancy outcomes in couples who experienced RIF. Lymphocyte subsets serve as an important means of analysing immune status, particularly in women who experience RIF and RSA. Attention should be paid to the level of vitamin D in these people, and timely supplementation should be given to those lacking in vitamin D since it can be conducive to the establishment and maintenance of a later pregnancy.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article.

Ethics Approval and Consent to Participate

This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Children's Hospital of Shanxi and Women Health Center of Shanxi. Written informed consent was obtained from all participants.

Consent for Publication

The manuscript is not submitted for publication or consideration elsewhere.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests in this work.

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