A novel update on vitamin D in recurrent pregnancy loss (Review)

HAOYU ZHAO[1*], XIAOQI WEI[2*] and XIUHUA YANG[2]

Departments of [1]Hepatobiliary Surgery and Organ Transplant and [2]Obstetrics, The First Hospital of China Medical University, Shenyang, Liaoning 110001, P.R. China

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Abstract. Recurrent pregnancy loss (RPL) is usually characterized as ≥3 miscarriages before 20 weeks of gestation. Patients with RPL may have autoimmune abnormalities or alloimmune problems. Vitamin D has a major function on the mechanism of immunomodulation at the maternal-fetal interface. However, whether vitamin D can be used as an effective method to treat patients with RPL requires investigation. It has been reported that vitamin D could prevent the occurrence of antiphospholipid syndrome (APS) by reducing the expression levels of anti-β2 glycoprotein and tissue factor in RPL cases with APS. In addition, there is an opposite relationship between vitamin D and thyroid peroxidase antibody levels in autoimmune thyroid disease cases with RPL. Vitamin D changes the ratio of T helper (Th) 1/Th2 and regulatory T cell/Th17 to a certain extent, as well as affects the activity of natural killer cells and the production of cytokines to reduce the incidence of RPL. The objective of the current review was to address the research progress of vitamin D in RPL in recent years, which could facilitate the use of vitamin D treatment to enhance the pregnancy outcome of RPL. Collectively, it was suggested that vitamin D may be used as an important and effective immunotherapeutic agent for patients with RPL.

Correspondence to: Dr Xiuhua Yang, Department of Obstetrics, The First Hospital of China Medical University, 155 Nanjing North Street, Shenyang, Liaoning 110001, P.R. China
E-mail: 57470040@qq.com

*Contributed equally

Abbreviations: RPL, recurrent pregnancy loss; APAs, antiphospholipid antibodies; ANAs, antinuclear antibodies; NK cells, natural killer cells; VDR, vitamin D receptor; VDD, vitamin D deficiency; VDL, low vitamin D; APS, antiphospholipid syndrome; TF, tissue factor; VDN, normal vitamin D; AITD, autoimmune thyroid disease; anti-TPO, thyroid peroxidase antibodies; VDI, vitamin D insufficient; Treg, regulatory T cell; G-CSF, granulocyte colony-stimulating factor

Key words: vitamin D, RPL, autoimmunity, cellular immunity, NK cells

Contents

1. Introduction

Recurrent pregnancy loss (RPL) is usually characterized as ≥3 miscarriages before 20 weeks of gestation, but an increasing number of researchers describe it as ≥2 spontaneous abortions (1). The etiology of RPL is multifactorial (2), including chromosomal abnormalities, genetic issues (3), congenital uterine defects, acquired or hereditary thrombotic diseases, endocrine issues, infections, autoimmune diseases and male factors (4). However, ~50% of patients with RPL do not have a definite etiology (5). Patients with RPL may also have autoimmune abnormalities or alloimmune problems (2). The former mainly includes antiphospholipid antibodies (APAs) (6), antithyroid antibodies (7) and antinuclear antibodies (ANAs) (8,9), while the latter mainly refers to cellular immune problems, such as increased natural killer (NK) cells (10-12) or decreased inhibitory T cells (13). Therefore, the success of pregnancy depends to a large degree on the development of an appropriate immune response (14).

It has been suggested that in patients, RPL, is largely associated with immune abnormalities (14), thereby contributing to the development and use of different forms of immunomodulatory therapies (1). Prednisone has been reported to help women who have multiple consecutive abortions by reducing inflammation and inhibiting the function of multiples types of immune cells, including T cells (15,16). Moreover, plaquenil can be useful in the treatment of RPL as a drug with comprehensive protection identified during pregnancy (17). The therapeutic role of plaquenil is associated with pharmacological properties, such as antithrombotic activity, vascular defense, immunomodulation, improved glucose resistance, hypolipidemic

activity and anti-infection function (17). Several studies have revealed that intravenous immunoglobulin modulated immune dysfunction and contributed to positive pregnancy outcomes in women with RPL, although controversial results have been reported (18-23).

In humans, vitamin D is involved in the metabolism of numerous elements, such as calcium and phosphorus (24). Vitamin D is a crucial modulator of essential biological effects, such as immune function and hormone secretion via the vitamin D receptor (VDR) (25). Vitamin D affects the innate and acquired immune response (26), as well as exerts an inhibitory function on the adaptive immune system (27). In comparison, it inhibits T helper (Th)1 cytokines (such as IFN-γ), and promotes the response of Th2 by both downregulating IFN-γ and upregulating IL-4 (26). As VDR is expressed in the placenta, and it has been suggested that vitamin D has a major function on the mechanism of immunomodulation at the maternal-fetal interface (28). In the clinical setting, multiple patients with RPL have vitamin D deficiency (VDD) (29). As a result, these women have impaired cellular immune systems, including elevated peripheral NK levels, NK cytotoxicity and higher Th1/Th2 ratios (30-32). Moreover, women with low vitamin D (VDL) levels are more susceptible to autoimmune defects (29). For example, the decreased expression of vitamin D is associated with a higher occurrence of APAs, ANAs, anti-single stranded DNA and thyroid peroxidase antibodies (anti-TPO) in patients with RPL (27).

Few foods contain vitamin D (33), and vitamin D supplementation could be applied as a natural therapy to minimize the risk of spontaneous early abortion (4). However, excessive vitamin D intake can have adverse effects (34). For example, excessive vitamin D molecules can affect the production of oocytes and the quality of embryos due to their anti-estrogen effect (34). It is also important to periodically measure vitamin D levels and change the therapy during pregnancy (4). In clinical trials, doctors may neglect vitamin D intake partly due to the lack of knowledge of the mechanism of vitamin D in RPL (4). The objective of the present review was to address the research progress of vitamin D in RPL in recent years, which could facilitate the use of vitamin D treatment to enhance the pregnancy outcome of RPL. In total, two independent researchers searched for articles in PUBMED with the following medical subject heading: 'Vitamin D', 'RPL', 'recurrent miscarriages', 'autoimmunity', 'cellular immunity' or '1,25-dihydroxy vitamin D'. All articles were published in English between January 1995 and August 2020. Review manuscripts and letters were excluded.

2. Vitamin D and antiphospholipid syndrome (APS) in RPL

As an autoimmune disease, APS is closely associated with adverse obstetrical outcomes (32,35). APS is a systemic autoimmune disease characterized by thrombosis (36). It has been well documented that the increased susceptibility to the thrombosis of blood vessels in APS may lead to microvascular thrombus in the placenta, as well as the reduction of blood flow at the maternal-fetal interface (6,37). A significant crosstalk between inflammation and coagulation involves the complement system and tissue factor (TF), and both mice and humans experience APS-related pregnancy complications (38-40).

As aforementioned, complement activation serves a critical function in adverse outcomes of pregnancy, including RPL with APS in both mice and humans (41-46). Vitamin D can enhance the level of complement inhibitor CD55 in human monocytes (47). Moreover, the related inhibitory effect of complement activation can prevent preterm birth observed in APS (47). In patients with APS, vitamin D exerts a suppressive effect on anti-β2 glycoprotein expression, thus decreasing the risk of thrombosis (48,49). Furthermore, as shown in *in vitro* studies, APA-induced TF expression was suppressed by vitamin D (36,48). Another study reported that vitamin D regulated TF in vascular smooth muscle cells (50). In addition, abnormal TF/protease activated receptor 2 signaling was considered to be involved in the pathogenesis of pregnancy-associated complications, which included abortions in an APS murine model (38). The frequency of APS antibodies in women with RPL is 15-20% (51). Researchers have confirmed that VDD in APS women with RPL was more common relative to normal controls (49.5 vs. 30%; $P<0.05$) (48). Additionally, women with VDD were found to have enhanced levels of several autoantibodies, including APAs (29). The prevalence of total APA in women with RPL was substantially increased in VDL relative to a normal vitamin D (VDN) group (39.7 vs. 22.9%; $P<0.05$; odds ratio = 2.22; 95% CI, 1.0-4.7) (29). In brief, VDD is more common in RPL cases with APS. Moreover, an increased percentage of patients with RPL and VDD are at risk of autoimmune abnormalities, including APS (29).

3. Vitamin D and autoimmune thyroid disease (AITD) in RPL

Accumulating evidence has suggested that thyroid autoimmunity is the cause of miscarriage during pregnancy (52,53). An increased morbidity of VDD was observed among patients with AITDs, in particular, Hashimoto's thyroiditis (54). Although the mechanisms underlying the relation between vitamin D and AITDs are not fully understood, the causes may be associated with anti-inflammatory and immunomodulatory functions (55). An opposite relationship between vitamin D and anti-TPO levels has been identified in women with AITD (56). Decreased levels of anti-TPO result in lower incidence of preterm birth and reduced rates of pregnancy loss (57). Antithyroid antibodies are more prevalent in patients with VDD compared with those with high levels of vitamin D (43 vs. 17%, respectively; $P<0.001$) (54). Women with abnormal thyroid autoantibodies also had lower levels of vitamin D compared with healthy controls (54). Lower thyroid stimulating hormone levels are directly associated with a higher vitamin D level (54). Ozkan *et al* (58) revealed that 25(OH)-D in follicular fluid could independently predict successful pregnancy during the *in vitro* fertilization cycle. For women whose vitamin D levels in follicular fluid were <10 ng/ml, the presence of clinical pregnancy reduced significantly, while for patients

whose vitamin D levels in follicular fluid increased by 1 ng/ml, the rate of clinical pregnancy was enhanced by 6% (58). Moreover, vitamin D can activate HOX genes, including HOXA10, which is essential for the process of implantation (59).

4. Vitamin D and Th1/Th2 ratio in RPL

Vitamin D suppresses the proliferation of Th1 cells and restricts the secretion of cytokines, including IFN-γ, IL-2 and TNF-α (29). Moreover, vitamin D postpones subsequent antigen presentation and the accumulation of T lymphocytes by suppressing the transcription of IFN-γ, which is the main positive response symbol for antigen-presenting cells (60). Vitamin D also prevents the activation and spread of IL-2, which is the autocrine growth factor for T lymphocytes (61). By decreasing the synthesis of IL-2 and IFN-γ and inducing the polarization of $CD4^+$ T lymphocytes to a Th2 reaction, as well as finally reducing the autoimmune response, vitamin D increases the secretion of Th2 cytokines (62), including IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (63,64). In a trial with mice, there was an increased accumulation of Th2 cells and higher counts of cytokines (IL-4, IL-5 and IL-10) when $1,25(OH)_2D_3$ was administered (65). The $1,25(OH)_2D_3$-related functions on the augment of Th2 cells were mainly regulated via IL-4 (62). The phenomenon could also be confirmed in the mouse model (66).

Excessive inflammatory response and lower levels of vitamin D are found in women with RPL (67). As observed in a previous study, patients with VDD showed an notable higher percentage of TNF-α-secreting Th cells relative to individuals with normal vitamin D levels (35.1±10.2 vs. 28.3±4.8%; $P<0.05$), whereas the difference between vitamin D insufficient group (VDI) and VDN was negligible (2). In women with elevated vitamin D concentrations, the mean serum TNF-α was substantially lower compared with those with low vitamin D expression (0.79±0.11 vs. 1.22±0.11 pg/ml; P=0.02) (68).

In a retrospective cross-sectional analysis conducted by Ota *et al* (29), it was observed that external vitamin D promoted Th2 polarization in a dose-dependent manner. In total, 70 women with VDN and 63 women with VDL were analyzed in this previous study (29). T cell cultures were supplied with 100 nM vitamin D for 16 h, and it was found that the TNF-α/IL-10 expressed $CD3^+/CD4^+$ cell ratio was substantially decreased (31.3±9.4) relative to the controls (40.4±11.3; $P<0.05$) (29). However, when 10 nM vitamin D was applied to T cells, no statistically significant difference was observed relative to the controls (29). After treatment with 100 nM vitamin D, the IFN-γ/IL-10 expressed $CD3^+/CD4^+$ cell ratio was also notably decreased compared with the controls (12.1±4.0 vs. 14.8±4.6; $P<0.05$) (29), whereas there was no significant difference after adding 10 nM vitamin D compared with controls (29). During this study (29), no significant difference was found in the Th1/Th2 cytokine-expressed $CD3^+/CD4^+$ Th cell ratio among VDL cases with RPL compared with VDN cases with RPL. Since it has been reported that only the treatment of 100 nM vitamin D_3 can affect the Th1/Th2 shift *in vitro*, the difference in average vitamin D levels between VDL and VDN for the study may not be sufficient to change the ratio of Th1/Th2 *in vivo* (29). There is a relatively small difference in immunological biomarkers of normal vitamin D cases compared with low vitamin D cases in this study, and they may not have a strong clinical correlation with vitamin D levels.

Other studies have suggested that vitamin D could exert a vital influence on preventing excessive adaptive immune response among patients with RPL (2,29,69). In one study, women with RPL were classified into three groups based on vitamin D level in serum: VDN, VDI and VDD groups (2). The definition of VDD was a level of 25(OH)D at <20 ng/ml, and VDI was defined as levels between 20-30 ng/ml, according to previously published literature (70). In 35 VDI cases, 0.5 μg/day $1,25(OH)_2D$ was applied for 2 months, and two patients with VDD were treated with the same amount of $1,25(OH)_2D$. There was a substantial decrease in the proportion of TNF-α-producing Th cells following treatment compared with that observed before treatment, whereas there was no obvious difference in the proportion of IFN-γ-producing Th cells between pre- and post-treatment (2). However, other research regarding $CD4^+$ T cells in human has indicated that $1,25(OH)_2D$ could decrease both the production of IFN-γ and the numbers of $IFN\text{-}\gamma^+$ $CD4^+$ T cells (71,72).

5. Vitamin D and regulatory T cell (Treg)/Th17 ratio in RPL

Decreased Treg cells and increased Th17 cells serve a vital role in the pathogenesis of RPL (73). Due to VDD in pregnancy, the activity of Treg cells is disrupted (74). It has been reported that population of Treg cells was lower (maternal, 0.2±0.01; cord, 0.63±0.03) in $25(OH)D_3$ deficient pregnant women (≤19 ng/ml; n=80) compared with insufficient (20-29 ng/ml; n=55; maternal, 0.34±0.01; cord, 1.05±0.04) and sufficient (≥30 ng/ml; n=18; maternal, 0.45±0.02; cord, 1.75±0.02) pregnant women ($P<0.05$) (74). When compared with insufficient and sufficient vitamin D levels, TGF-β and IL-10 levels were decreased in the $25(OH)D_3$ deficient pregnancy group (74). Other studies have revealed that 1,25 vitamin D treatment significantly augmented the percentage of Tregs from baseline in the patients with RPL compared with the same percentage in the control group (69,75,76).

A double-blind placebo-controlled analysis identified that vitamin D_3 reduced the frequency of Th17, as well as decreased the Th17/Treg ratio in peripheral blood of women with RPL (77). In total, 44 women with RPL were involved in this research and were randomly assigned to the treatment [n=22; one 300,000 IU dose of intramuscular vitamin D_3 and Lymphocyte Immune Therapy (LIT)] and control (n=22; one dose of packaged inert placebo and LIT) groups (77). The findings revealed that the average proportion of Th17 cells and Th17/Treg ratio was significantly decrease in the treatment group (average proportion of Th17; 0.93 vs. 0.43%; P=0.001; ratio of Th17/Treg, 0.48 vs. 0.12; P=0.001) and the control group (mean percentage of Th17, 0.92 vs. 0.65%; P=0.001; ratio of Th17/Treg, 0.32 vs. 0.17; P=0.001) when compared with those before therapy (77). The decline in the number of Th17 cell displayed a substantial increase in the treatment group

Table I. Studies investigating vitamin D and RPL.

Authors (year) (Ref.)	Research objective	Research type	Number of experimental group vs. control group	Type of samples	Vitamin D supplementation	Conclusions
Tavakoli *et al* (2011) (33)	To assess the influence of 1,25 $(OH)_2D_3$ on cytokines.	Case control study (*in vitro*)	n=8 women with RPL vs. n=8 healthy women	Endometrium	10^{-7} M 1,25$(OH)_2D_3$ for 6 h	Vitamin D_3 lowered IFN-γ/IL-10 ratio and reduced IL-6, TGF-β and IL-10 production.
Ibrahim *et al* (2013) (72)	To evaluate the role of vitamin D_3 in prevention of RPL.	Randomized controlled trial (*in vitro*)	40 pregnant women with RPL, n=20 (study group) vs. n=20 (control group)	Peripheral blood	0.25 mcg vitamin D_3 given twice daily after pregnancy was documented till delivery.	Vitamin D_3 supplementation resulted in a lower risk of pregnancy loss among women with RPL.
Ota *et al* (2014) (29)	To investigate the relationship between autoimmune or cellular immunity and vitamin D.	Retrospective cross sectional study (*in vitro*)	133 females with RPL: VDN (n=70) vs. VDL (n=63)	Peripheral blood	10 and 100 nM vitamin D_3	VDD was associated with autoimmune or cellular immune abnormalities in RPL.
Ota *et al* (2015) (27)	To study the influence of vitamin D upon NK cells.	Case-control study (*in vitro*)	18 women with RPL vs. 16 healthy women	Peripheral blood	10 and 100 nM 1,25$(OH)_2D_3$	1,25 $(OH)_2D_3$ has immunomodulatory influence upon NK cell cytotoxicity, cytokine production, the process of degranulation and TLR4 expression.
Rafiee *et al* (2015) (77)	To research the impact of vitamin D_3 upon Th17 and Treg cells.	A double-blind placebo-controlled study (*in vivo*)	44 women with RPL: n=22 (experimental group) vs. n=22 (control group)	Peripheral blood	300,000 IU vitamin D_3	Vitamin D_3 decreased the number of Th17 cells and the ratio of Th17/Treg in RPL.
Chen *et al* (2016) (2)	To study the function of vitamin D on cellular immunity in RPL.	Prospective study (*in vivo*)	99 women with RPL: VDN (n=35) vs. VDI (n=51) vs. VDD (n=13).	Peripheral blood	1,25$(OH)_2D$ 0.5 μg/day for 2 months	Abnormal cellular immune reactions were shown in RPL cases with low vitamin D levels.
Samimi *et al* (2017) (79)	To examine the influence of vitamin D supplementation on RPL.	A double-blind randomized and controlled clinical trial (*in vivo*)	77 pregnant women with RPL: n=39 (experimental group) vs. n=38 (control group)	Peripheral blood	400 IU/day	Vitamin D_3 leads to decreased IL-23 and lower morbidity of abortion among patients with RPL.

Table I. Continued.

Authors (year) (Ref.)	Research objective	Research type	Number of experimental group vs. control group	Type of samples	Vitamin D supplementation	Conclusions
Ji *et al* (2019) (76)	To identify the relationship between vitamin D and Treg/Th17.	Clinical trial (*in vivo*) and a case control study (*in vitro*)	Patients with RPL (n=107) vs. healthy pregnant women (n=48)	Peripheral blood	*In vivo*: 2,000 IU/day for 2 months; *In vitro*: 1, 10 and 100 nmol/l for 4.5 days	The Treg/Th17 imbalance observed in patients with RPL can be restored by vitamin D supplementation.
Abdollahi *et al* (2020) (75)	To study the function of $1,25(OH)_2D_3$ on Tregs and Th17.	Case control study	Non-pregnant women with RPL (n=20) vs. healthy non-pregnant women (n=20)	Peripheral blood	$1,25(OH)_2D_3$ 50 nM for 16 h	$1,25\ (OH)_2D_3$ supplementation substantially enhanced the proportion of Treg cells in patients with RPL.

VDN, vitamin D normal; VDI, vitamin D insufficient; VDD, vitamin D deficiency; VDL, vitamin D low; TLR4, Toll like receptor 4.; Treg, regulatory T; Th, T helper; RPL, recurrent pregnant loss; NK, natural killer.

compared with the control group relative to the baseline values, and the decline in the ratio of Th17/Treg showed a significant enhancement in the treatment group when compared with the controls (77).

Further research revealed an association between the ratio of Treg/Th17 and vitamin D, as well as the impact on the balance between Treg and Th17, among patients with RPL who were given vitamin D (76). This study found that the expression of vitamin D had a strong association with the proportion of Treg cells, a negative association with the proportion of Th17 cells and a positive association with the ratio of Treg to Th17 in the RPL group (76). Moreover, the results were reversed in the control group (76). It has also been revealed that, relative to the control decidual tissues, 25(OH) D was significantly decreased among patients with RPL (78). Furthermore, correlation analysis indicated that there was a significantly negative correlation between 25(OH) D and IL-23. On the contrary, IL-23 increased the expression of IL-17 (78). Vitamin D can also be a beneficial medication by decreasing the amount of IL-23 (78). In addition, a study analyzing the impact of women with RPL given vitamin D found that vitamin D could decrease not only serum IL-23 but also the frequency of miscarriage (79).

6. Vitamin D and peripheral NK cells in RPL

Increased NK cells in the peripheral blood can lead to RPL (80). A previous retrospective cross-sectional study has reported that VDD may regulate cell immunity (29). Moreover, VDD may serve regulatory roles in peripheral NK cells (29). Significant differences have been identified in $CD56^+$ NK cell levels from the peripheral blood between VDL (n=63) and VDN (n=70) cases with RPL ($P<0.05$) (29). In VDD (n=22), a significant increase of peripheral NK cell cytotoxicity has been indicated compared with that in the VDN group (n=70) (29). After the vitamin D supplement, the cytotoxicity of NK cells from peripheral blood was significantly inhibited in a dose-dependent manner, when compared with the controls (29). Another study suggested that $1,25(OH)_2D_3$ exerted an immunomodulatory impact on NK cell cytotoxicity, the secretion of cytokines, including Toll-like receptor 4 expression, and the degranulation process in RPL peripheral blood samples (27). In women with RPL, $1,25(OH)_2D_3$ supplementation substantially decreased peripheral NK cell cytotoxicity compared with that of the vehicle group which was given reconstitution agent ethanol ($P<0.01$) (27). Previous research has also observed a substantial rise in peripheral NK cytotoxicity between VDI (n=51) and VDN (n=35) cases (2). Moreover, peripheral NK cell cytotoxicity was significantly decreased after $1,25(OH)_2D_3$ treatment compared with the levels prior to treatment (2). However, no obvious distinction was found in the percentage of $CD3^-CD56^+$ NK cells among three groups, VDN, VDI and VDD, before and after treatment (2). Therefore, it can be concluded that the effect of vitamin D on the count or cytotoxicity of NK cells tends to be independent (2). Taken together, a sufficient amount of vitamin D was found to be critical in regulating NK cell cytotoxicity in peripheral blood from patients with RPL.

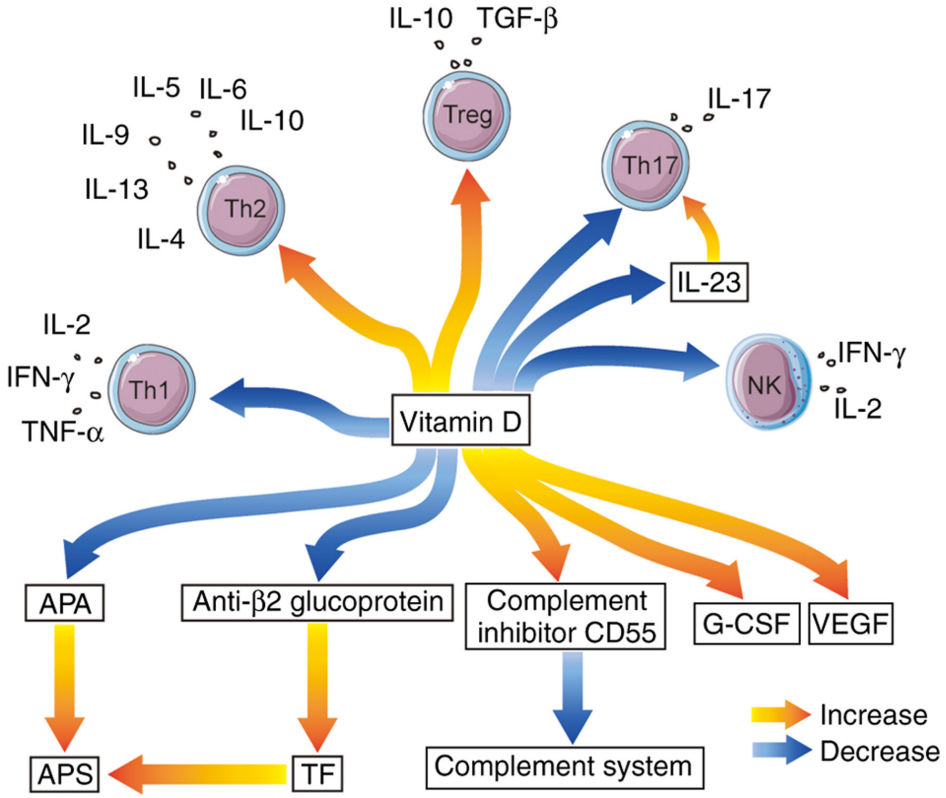

Figure 1. Immune impact of vitamin D on RPL. Vitamin D suppresses cytokines produced by Th1 cells and promotes cytokines secreted by Th2 cells. Moreover, vitamin D increases the function of Treg cells, whereas it reduces the number of Th17 cells, which secrete IL-17. Vitamin D is negatively correlated with IL-23, while IL-23 is positively associated with IL-17. Peripheral NK cell activation and its cytotoxic actions are inhibited by vitamin D. Vitamin D inhibits peripheral NK cytotoxicity by suppressing IFN-γ and IL-2. VEGF and G-CSF production are stimulated by vitamin D. Vitamin D promotes the proliferation of the complement inhibitor CD55 and inhibits anti-β2 glycoprotein. By inhibiting TF and APAs, vitamin D can prevent the occurrence of APS in RPL. RPL, recurrent pregnant loss; G-CSF, granulocyte colony-stimulating factor; TF, tissue factor; APS, antiphospholipid syndrome; APAs, antiphospholipid antibodies; Treg, regulatory T; Th, T helper; NK, natural killer.

7. Vitamin D and cytokines in RPL

It has been reported that the levels of VEGF and granulocyte colony-stimulating factor (G-CSF) can be induced by vitamin D in NK cells (29). These two factors may induce weak angiogenesis and complications of pregnancy; for instance, in RPL, NK cells decrease the production of VEGF at the maternal fetal interface (29). The receptor of G-CSF exists in the trophoblast cells. It has been identified that G-CSF exerts significant impacts on autocrine and paracrine activities in both the decidua and placenta, and increased G-CSF markedly decreased the risk of pregnancy loss in a human trial (81). Vitamin D could also be a possible therapeutic alternative to avoid RPL by increasing the levels of VEGF and G-CSF.

8. Conclusions

In conclusion, vitamin D can significantly affect both autoimmunity and cellular immunity in RPL based on previously published studies (Table I; Fig. 1). Vitamin D is proposed to be available as a potential therapy for RPL. For patients with RPL or high risk factors, appropriate vitamin D supplement could be given, and the serological level of vitamin D should be detected regularly to obtain favorable maternal and fetal outcomes. In the future, a larger multicenter, prospective controlled study with a larger sample size is required.

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Authors' contributions

HZ and XW wrote the manuscript, and XY revised this manuscript. All authors read and approved the final version.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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