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Effect of Vitamin D Supplementation on Unexplained Recurrent Spontaneous Abortion: A Double-Blind Randomized Controlled Trial

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Abstract

Background: Recurrent spontaneous abortion is a serious problem in pregnancy, which affects between 2 and 5% of women of reproductive age. This study was designed to examine the effect of vitamin D supplementation on the occurrence of unexplained recurrent spontaneous abortion (URSA).

Methods: A double-blind randomized and controlled clinical trial was performed on 80 patients with URSA. They were treated with vaginal progesterone (400 IU/day) after confirmation of pregnancy and received vitamin D and placebo in two intervention (n=40) and control groups (n=40), respectively. The incidence of abortion and the serum levels of IL-23 were examined within 20 weeks of gestation.

Results: The levels of vitamin D3 prior to the start of the study were equal to 11.65±3.76 ng/ml and 11.53±2.39 ng/ml (p=0.86) in the intervention and control groups, respectively, which were decreased to 13.21±3.47 ng/ml and 11.08±2.76 ng/ml (p=0.004) at the end of the study, when the mean serum levels of IL-23 were equal to 18.4±3.78 pg/ml and 23.16±4.74 pg/ml in the two groups (p <0.004). The frequency of abortion in the control and intervention groups was equal to 5 (12.8%) and 13 (34.2%), respectively (p=0.03, OR=3.53, 95% CI= 1.12-11.2).

Conclusions: Vitamin D3 leads to decreased serum levels of IL-23 and incidence of abortion among women with URSA.

Keywords: spontaneous abortion, Vitamin D, Interleukin 23

1. Introduction

Recurrent spontaneous abortion (RSA) refers to at least two consecutive or three non-consecutive pregnancy losses affecting between 2 and 5% of women of reproductive age before 20 weeks of gestation (Abdel-Razik et al., 2014). This not only causes significant physical and mental problems in families, but also a heavy economic burden on families and health systems (Gao et al., 2015; Vaiman et al., 2015). Although many causes (uterine abnormalities, endocrine disorders, genetic disorders, coagulation disorders and environmental factors, such as chemicals, and psychological factors) have been proposed as an etiology of RSA, the exact cause for much of RSA, named unexplained recurrent spontaneous abortion (URSA), remains unknown (Galamb et al., 2015; Ke, 2014; Ali et al., 2014; Cao et al., 2014).

Various immunological disorders have been observed in women with URSA, such as antiphospholipid antibody antibodies, antinuclear antibody (ANA), antithyroglobulin antibody and increased natural killer (NK) cell (Ticconi et al., 2010; Motak-Pochrzest et al., 2013; Youan et al., 2015). Animal studies have found evidence of a link between successful pregnancy and the predominant levels of anti-inflammatory cytokines relative to inflammatory cytokines. In human studies, patients with recurrent abortion have been found to have high levels of inflammatory cytokines and low levels of anti-inflammatory cytokines (Demirturk et al., 2014; Saifi et al., 2014; Bahadori et al., 2014; Potdar et al., 2005).

T helper 17 (Th17) cells are a subset of T cells that play an important role in the pathogenesis of many autoimmune and inflammatory diseases and in the immunological rejection of non-self cells (Blander et al., 2012; Zepp et al., 2011). Th17 can produce various inflammatory cytokines such as IL-17T, IL-22 and tumor necrosis

factor alpha (TNF-α) (Qu et al., 2013). The regulatory Th17 cells have been known to play an important role in the onset and persistence of pregnancy. Their deregulated regulation creates problems for the pregnancy. In the studies, the increased blood levels of Th17 cells and the induced cytokines have inevitably been seen in women who have an abortion (Nakashima et al., 2010; Najafi et al., 2014). IL-23 is a cytokine, essential for the proliferation and differentiation of Th17 cells. The IL-23/Th17/IL-17 immune axis is well known for its role in the development of many immunological diseases (Toussirot, 2012).

In addition to the main immunomodulatory function, vitamin D3 (along with its compounds) plays an important role in regulating the immune system (Imazeki et al., 2006). A deficiency of the vitamin has been observed in many autoimmune diseases (Iruretagoyena et al., 2015; Ma et al., 2015). Studies have shown that vitamin D3 is effective in regulating the activity of Th17 cells and the secretion levels of the induced cytokines (Zhang et al., 2014; Wen et al., 2015). This can reduce the immunological events leading to URSA, with an effect on the IL-23/Th17/IL-17 axis. This study examined the effect of vitamin D3 supplementation on the occurrence of URSA and the serum level of IL-23 (as a stimulating factor) in Th17 cells.

2. Patients and Methods

2.1 Subjects

In a randomized clinical trial, 80 women aged 18 to 35 years were examined from November 2013 to March 2015 at the Shabihkhani Maternity Hospital in Kashan, Iran - a public, specialty and subspecialty hospital with 100 beds in the maternity ward and neonatal intensive care units (NICU), which serves as a maternity referral hospital in the area.

As no specific studies have been performed to evaluate the effect of vitamin D3 supplementation on the level of IL-23, the sample size was calculated based on the results of a study on women with recurrent abortion, which was performed to evaluate the effect of vitamin D3 on serum levels of interferon gamma (INF-ɤ). It was reported as 41.8±29.8 pg/ml in women treated with vitamin D3 and 65.6±32.5 pg/ml in women in the control group (Ibrahim et al., 2013). The sample size in each group was calculated equal to 27 for a power of 80% and a type one error of 5% according to the following formula.

$$n = \frac{\left(z_{1-\frac{\alpha}{2}} + z_{1-\beta}\right)^2 \left(\sigma_1^2 + \sigma_2^2\right)}{\left(\mu_1 - \mu_2\right)^2}$$

Considering the facilities available in this study, 40 patients were studied in each group with a probability of falling of 20%.

The study focused on women with recurrent abortion (two consecutive or at least three non-consecutive miscarriages) who had been referred to the Clinic of Gynecology and Obstetrics of Shabihkhani Hospital for pre-pregnancy checkups. The convenient sampling method was used to select all eligible patients referred from the start of the study until the completion of the required sample size. Inclusion criteria were: age of 18-35 years, past miscarriages of unknown cause and pregnancy by a spouse in all past and present pregnancies. Exclusion criteria included the development of any diseases and abnormalities related to the uterus, the development of any kind of immunological diseases, the incidence of endocrine disorders, family history of genetic disorders, coagulation and anticoagulation disorders, the employment in the chemical-related industry, sensitivity to various forms of progesterone, and a history of allergy to vitamin D3. From a total of 108 patients who were invited to participate in the study, 28 patients were excluded from the study based on the exclusion criteria. All patients involved in the study and their husbands provided written informed consent after receiving information on how to perform the study.

Then, the physical examination of all patients was performed after a history was taken by a gynecologist. Demographic and clinical data were recorded in the questionnaire, including age, education level, economic status, medical history, obstetric history and examination results. Venous blood samples (10 ml) were taken from patients and then sent to the reference laboratory of Kashan University of Medical Sciences to check PT, PTT, INR, TSH, anticardiolipin, lupus anticoagulant, prolactin, protein S, C, factor V Leiden, beta 2-glycoprotein antibody and serum level of vitamin D3. To check for abnormalities in the uterus, all patients were also examined by HSG, which was then interpreted and reported by an expert radiologist.

All couples had a karyotype test to assess chromosomal abnormalities and the results of laboratory and radiological studies were recorded in the patient questionnaire. All participants in the study received antenatal care and were given folic acid and ferrous sulfate at least one month prior to pregnancy, under the supervision of

a gynecologist. They were checked by monitoring serum β-hCG level levels and abdominal ultrasound until the confirmation of pregnancy, after which the mothers were divided into two groups of intervention and control using permuted block randomization with twenty blocks of size four. Only the person responsible for the distribution of drugs knew how the patients were allocated to the treatment groups. The others involved (patients and person responsible for data collection) did not.

2.2 Interventions

Both groups received standard treatment with vaginal progesterone (Behvarzan, Iran) at a dose of 400 mg per day. In addition to the standard treatment, the intervention group received a pill of Vitamin D3 400 IU/day (NatureMade®_USA), whilst the control group was given tablets that were similar to vitamin D3, with no active ingredient as a placebo. The serum levels of vitamin D3 were evaluated in the tenth and twentieth weeks to prevent any possible poisoning. If so, the patient was excluded from the study.

2.3 Measurement of Outcomes

The outcomes of the intervention were evaluated in two ways:

Pregnancy loss was considered as abortion and failure of treatment at any time from the beginning of the study to the twentieth week of pregnancy. As another outcome of the intervention, serum levels of IL-23 were measured at the start of the study and at the time of abortion in pregnant women with pregnancy loss (and/or in the twentieth week in other women) by ELISA using the Ready-SET-Go ELISA (eBioscience).

2.4 Ethical Considerations

All ethical principles were respected in accordance with the resolution 196/96 on the research involving human subjects. The ethics committee of Kashan University of Medical Sciences approved the study protocol with the code No. P/29/5/1/2059 and supervised all the stages. This study was also recorded in the Iranian Registry of Clinical Trials (IRCT2013100114853N1).

2.5 Statistical Analysis

The data obtained from the study was analyzed using SPSS version 18. Categorical results were reported as relative frequency and continuous results as mean±SD. In this study, the distribution of the data was examined using the Kolmogorov-Smirnov test that used Chi square tests, independent t-test and logistic regression for data analysis. The significant level of P was considered less than 0.05.

3. Results

Three out of eighty patients (one in the intervention group and two in the control group) were excluded due to unwillingness to participate and 77 patients were finally evaluated in the final analysis. The average age of the patients was equal to 26.1±4.28 and 26.29±4.43 years in the intervention and control groups, respectively (p=0.85). Demographic and clinical characteristics of the study are shown in Table 1.

Table 1. Baseline Characteristics

Variables	Groups		P value
	Intervention (n=39)	Control (n=38)	
Age[a]	26.1±4.28	26.29±4.43	0.85
Education[b]			0.14
Primary School	7(17.9)	4(10.5)	
Middle School	15(38.5)	8(21.1)	
High School	5(12.8)	11(28.9)	
Academic Degree	12(30.8)	15(39.5)	
Economic State[b]			0.11
Low	17(43.6)	8(21.1)	
Middle	9(23.1)	12(31.6)	
High	13(33.3)	18(47.6)	

Gravidity[b]			0.39
3	13(33.3)	8(21.1)	
4	12(30.8)	17(44.7)	
5	13(33.3)	12(31.6)	
6	1(2.6)	0(0)	
7	0(0)	1(2.6)	
Abortion[b]			0.26
2	21(53.8)	18(47.4)	
3	16(41.0)	20(52.6)	
4	2(5.2)	0(0)	

[a] mean±Standard Deviation

[b] The values presented as No.(%)

The serum level of vitamin D3, before the start of the study, was equal to 11.65±3.76 ng/ml in the intervention group and 11.53±2.39 ng/ml in the control group (p=0.86). At the end of the study, it was increased to 13.21±3.47 ng/ml and 11.08±2.76 ng/ml, respectively (p=0.004). Before the start of the study, the serum level of IL-23 was equal to 20.69±3.01 pg/ml in the intervention group and 21.52±4.37 pg/ml in the control group (p=0.33). This was found to be on average 18.4±3.78 pg/ml and 23.16±4.74 pg/ml at the end of the study in the intervention and control groups, respectively (p <0.001). The serum levels of vitamin D3 and IL-23 showed an inverse relationship between the two variables (p=0.004, R=-0.33).

Until the end of the study, 5 (12.8%) and 13 (34.2%) spontaneous abortions occurred in total in the intervention and control groups, respectively (p=0.03, OR=3.53, 95% CI= 1.12-11.2). In the logistic regression analysis, the effect of vitamin D3 and the incidence of abortion is not statistically significant when applying the confounding factors of age, gravidity, number of previous abortions and serum levels of IL-23 (p=0.28, OR=0.37, 95% CI= 0:06 to 2:26). However, the relationship between the serum level of IL-23 and the incidence of abortion remains significant (p <0.001, OR=1.63, 95% CI= 1.26-2.11). According to this model, vitamin D3 can be concluded to have an effect on abortion through the causal pathway of IL, although more conclusions can be drawn by examining other biological confounders.

4. Discussion

This study was designed to evaluate the effect of vitamin D3 in preventing unexplained recurrent spontaneous abortion (URSA) and the role of inflammatory cytokines IL-23. Results of the study showed that the administration of vitamin D3 in women with URSA can reduce the level of IL-23 and subsequently the incidence of abortion. In addition, the serum level of IL-23 was found to decrease when the serum level of vitamin D3 increased.

The immune system plays an important role in many physiological and pathological events in the female reproductive system such as menstruation, dysmenorrhea, endometriosis, uterine leiomyomas and adverse pregnancy outcomes (Nishida et al., 2011; Beric et al., 2014; Kralickova et al., 2015; Regal et al., 2015). Different cells, cytokines and proteins might contribute to folliculogenesis, implantation and pregnancy, meaning the fetus can be rejected and aborted as a semi-allograft due to disruption of immunomodulation (Kwak-Kim et al., 2014). Th17 is a highly pathogenic subset of CD4 + T cells that can secrete various cytokines that trigger or exacerbate inflammatory diseases, autoimmune diseases and transplant rejection in humans (Harrington et al., 2005; Park et al., 2005). Th17 function is regulated by interleukin IL-17, which induces the expression of proinflammatory cytokines and chemokines and leads to the infiltration and destruction of tissue (Li et al., 2006; Tang et al., 2001). IL-23 secreted by activated type-1 macrophages and dendritic cells causes Th17 cell maintenance and development as well as the increased secretion of IL-17 (Iwakura et al., 2006). Previous studies have found high levels of IL-23 and Th17 in the peripheral blood and placenta of pregnant women with URSA (Wang et al., 2010). Vitamin D3 is able to reduce the occurrence of URSA through an effect on different parts of the IL-23/IL-17 axis.

Dendritic cells are a heterogeneous family of cells of hematopoietic origin and part of the cellular immune system, which are known as highly specialized antigen-presenting cells (Breton et al., 2015). Dendritic cells can

induce their effects on the immune system by direct cell-cell contact or by secretion of multiple cytokines including IL-1, IL-6, IL-12 (to which IL-23 belongs), tumor necrosis factor-α and interfron-α/β (Blanco et al., 2008). High serum and cervical levels have been observed for some of the cytokines (such as IL-6 and IL-23) in women with URSA (Galazios et al., 2011; Hattori et al., 2007). Vitamin D3 inhibits the differentiation and maturation of dendritic cells by the vitamin D receptor (VDR) (Barragan et al., 2015). In human and animal studies, vitamin D3 has been found to be able to induce the production of myeloid dendritic cells with tolerogenic phenotype (Adorini et al., 2003).

Not only can it reduce the proliferation and differentiation of TH17 cells through the impact on dendritic cells and the reduced levels of IL-23, but it also has direct effects on the cells. Studies have revealed that vitamin D3 supplementation in animal models can reduce the number of TH17 cells and the level of IL-17 production, as compared to the placebo group (Chang et al., 2010; Chang et al., 2010).

Although the exact mechanism underlying the effect of vitamin D3 on TH17 cells remains only partially understood, studies have shown the inhibition of cells through VDR, the repression of gene transcription of IL-17 by blocking the nuclear factor of activated T cells (NFAT), recruitment of histone deacetylase, and induction of programmed cell death (Chang et al., 2010; Joshi et al., 2011; Nashold et al., 2013).

Macrophages are another group of immune cells in the decidual, which can account for about 20-30% of immune cell populations in deciduas. They are similar in functional nature to Th 1 and Th 2 cells, although being able to differentiate into M1 and M2 subtypes. Whereas M1 macrophages have a high capacity to produce proinflammatory cytokines (such as IL-12, IL-23 and TNF) and inflammation, M2 macrophages can alter the inflammatory response to provide the conditions for remodeling and tissue repair (piccinni, 2003; Cupurdija et al., 2004). Although there is not enough information about the ratio of M1/M2 macrophages in the peripheral blood and decidua of women with URSA, an imbalance in favor of the M1 cells seems to provide the conditions for abortion (Hutter et al., 2013; Zhang et al., 2015). An in vitro study showed that vitamin D3 can increase the population of M2 macrophages and convert M1 to M2 cells (Zhang et al., 2015). The effect of vitamin D3 on macrophages (and thus a decrease in IL-23 production) may partially explain the decline in the incidence of URSA in response to vitamin D3, which was observed in this study. Further studies may be necessary for a proper understanding of how vitamin D3 reduces the amount of URSA; for example, the study of all components of the immune system (including cell and humoral immunity, and complement system).

Although the study was intended to be the most accurate, it was constrained by limitations. First, it was not possible to perform the study on a larger population due to the limitations imposed by the ethics committee of Kashan University of Medical Sciences to use the minimum possible sample in clinical trials. In addition, the single supplementation of vitamin D3 (without progesterone prescription) to determine the net effect of vitamin D3 was impossible because of ethical considerations. Technical limitations also prevented us from evaluating all immunological factors in the blood and decidua.

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Competing Interests Statement

The authors declare that there is no conflict of interests regarding the publication of this paper.

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