Evaluating Vitamin D and foxp3 mRNA levels in women with recurrent spontaneous abortion

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ABSTRACT

Objective: This current survey investigated the role of the Forkhead 3 box protein (foxp3) gene and serum vitamin D levels in women with recurrent spontaneous abortion (RSA).

Methods: The mRNA level of the foxp3 gene in peripheral blood was evaluated in women with a history of RSA (N=40) and in controls (N=40) via quantitative polymerase chain reaction. We employed the enzyme-linked immunosorbent assay to assess the serum levels of 1,25-dihydroxyvitamin D3 (1,25(OH)2 D) in both groups. The Mann-Whitney U test and Pearson’s correlation coefficient were used to statistically compare study groups between and within themselves, respectively.

Results: Although mRNA levels of foxp3 were higher in women with RSA than in controls, we observed no significant change in mRNA levels of foxp3 between the two groups (p=0.16). An important positive correlation was observed between foxp3 mRNA levels and 1,25(OH)2 D in controls (p=0.003). In contrast, the correlation between foxp3 expression and 1,25(OH)2 D was not significant in the case group (p=0.14). Serum vitamin D levels were lower in women with RSA than in controls (p<0.001).

Conclusions: Our findings demonstrated that 1,25Vitamin D3 along with other molecules might help prevent RSA by providing for an anti-inflammatory state not necessarily through foxp3 expression or T cell differentiation.

Keywords: recurrent spontaneous abortion, 1,25-dihydroxyvitamin D3, Forkhead 3 box protein

INTRODUCTION

Recurrent spontaneous abortion (RSA) refers to the recurrent loss of a fetus (bodyweight <1000 g) occurring before the 20th week of pregnancy in more than two occasions with the same sexual partner (Practice Committee of the American Society for Reproductive Medicine, 2020). About 1-5% of all women of reproductive age suffer from RSA (Kim et al., 2004; Pildner von Steinburg & Schneider, 2009). Early pregnancy failure is a prevalent issue accounting for 15% to 20% of known clinical pregnancy cases with the same sexual partner (Practice Committee of the American Society for Reproductive Medicine, 2020).

Our findings demonstrated that 1,25Vitamin D3 along with other molecules might help prevent RSA by providing for an anti-inflammatory state not necessarily through foxp3 expression or T cell differentiation.

Keywords: recurrent spontaneous abortion, 1,25-dihydroxyvitamin D3, Forkhead 3 box protein

Vitamin D is a secosteroid hormone with a fundamental role in bone metabolism and mineral homeostasis as well as immune system modulation (Tamblyn et al., 2015; Lagishetty et al., 2011). Evidence indicates that Vitamin D may be involved in the pathogenesis of RSA (Triggianese et al., 2016). Its deficiency has a remarkable impact on pregnancy outcomes and has been linked to RSA, low birth weight, poor growth, fragile ossification, and increased risk of autoimmune disorders (Tabesh et al., 2013). Indeed, several pregnancy complications including gestational diabetes, preeclampsia, maternal infection, cesarean section, and preterm labor have been considered to result from vitamin deficiency (Tabesh et al., 2013; Theodoratou et al., 2014). The extra 1- alpha-hydroxylase activity released from the decidua, placenta, and both fetal and maternal kidneys increase the metabolism of vitamin D during pregnancy (Sanz-Salvador et al., 2015). Vitamin D affects bone metabolism more than its classic counterparts (Christakos et al., 2016; Baek et al., 2010). There is a growing trend of reusing vitamin D to reduce the regulation of pathological immune responses in patients with autoimmune or inflammatory diseases. Higher levels of vitamin D may trigger different anti-inflammatory functions, which contain the function of T regulatory cells (Tregs) and/or increase their numbers. Other small molecules such as nia-cin, short-chain fatty acids, and vitamin A may gain Tregs (Sakaki et al., 2005; Wacker & Holick, 2013). High levels of 1,25-dihydroxyvitamin D3 (1,25(OH)2 D) may affect the lineage-specific foxp3 transcription factor involved in the function and creation of Tregs (Joshi et al., 2011; Jeffery et al., 2009). High levels of 1,25(OH)2D have been related to anti-inflammatory lymphoid polarization containing large amounts of Tregs (Dimeloe et al., 2010).

Forkhead 3 box protein (foxp3) is a nuclear transcription factor required to induce immunosuppressive activity (Hu et al., 2019). The role and expression of foxp3 in tumorigenesis is conflicting. Expression of foxp3 in tumor cells plays an important role in tumorigenesis (Zuo et al., 2007a; b). Selective expression of foxp3 in human trophoblasts may be related to the multiplication and aggressive behavior of trophoblasts (Dimeloe et al., 2010). Foxp3 is known as the marker of regulatory T cells (Treg), CD4 + CD25 + and is a major determinant of immune function. Due to the strong immunosuppressive effect of foxp3 in Treg cells, foxp3 in trophoblasts may have immunosuppressive impacts analogous to those seen in Treg cells, and the function of Treg cells may be a new mechanism of maternal and embryonic endurance (Hu et al., 2019; Niu et al., 2011). As shown by the role of foxp3 expression in tumor cells and tumorigenesis, foxp3 may play an analogous role in trophoblasts.

However, to our knowledge, there are no reports on serum vitamin D levels and foxp3 expression patterns in women with a history of RSA. Therefore, the main goal of this prospective study was to investigate the serum concentration of vitamin D and foxp3 mRNA levels in women with a history of recurrent miscarriage compared to healthy fertile women with at least one successful pregnancy and live birth.
MATERIAL AND METHODS

Study population and sample collection
This case-control study was based on blood samples taken from women referred to the Yazd Institute of Reproductive Sciences, Iran. The Ethics Review Board of the Science and Arts University of Yazd approved this study. Informed consent was obtained from all participants included in the study. The procedures involving humans performed during the course of the study met the ethical standards of our Institution and/or the National Research Committee and the requirements set out in the 1964 Helsinki Declaration and later amendments made thereto.

The control group consisted of 40 pregnant women without a history of miscarriage and at least one successful pregnancy. The case group included 40 women with a history of two or more cases of RSA (Maesawa et al., 2015). The groups were matched for age. A gynecologist monitored the women in the case group. No male factor etiologies, anatomical or endocrinal complications were allowed in the study; patients had normal parental chromosomal karyotypes, lack of anti-phospholipid antibodies (IgG and IgM classes), no thrombophilia, TORCH syndrome negativity, anti-cardiolipin antibodies (IgG, IgA, and IgM classes) were considered as inclusion criteria of two groups. Samples of 5 ml of peripheral blood were taken from each study participant; half of the samples were used in gene expression evaluation and half in vitamin D level testing.

Enzyme-linked immunosorbent assay
The 2.5-ml blood samples of each participant were separated after clotting and serum was removed. Enzyme-linked immunosorbent assay (ELISA) test kits were used to specify the serum concentrations of 1,25(OH)2D in each group. The detection limit of 1,25(OH)2D was 1.2 ng/ml.

RNA extraction and cDNA synthesis
Two ml of intravenous blood samples taken from the study population were transferred to tubes containing ethylenediaminetetraacetic acid. Then, the total RNA of each sample was extracted using the easy cDNA Synthesis kit (parstous biotechnology, Iran). The purity and concentration of extracted RNAs were determined using a spectrophotometer in the absorbance of the A260 / A280 ratio and 260 nm, respectively. (PhotoBiometer, Eppendorf, Germany). All complementary DNA (cDNA) was synthesized from 1μg RNA with the Revert Aid First Strand cDNA Synthesis Kit (parstous biotechnology, Iran). The cDNA product was kept at -20°C until use.

Gene expression assessment
Quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine the mRNA levels of foxp3 in case and control groups. GAPDH was considered as a reference gene for the normalization of foxp3 expression levels. The primer sequences used in this study are presented in Table 1. Master Mix Green with high ROX™ (Ambicon) was utilized for PCR reaction in a StepOne system (Applied Biosystems, CA, USA). Each PCR run was performed in a final volume of 20 µL containing, cDNA (2 μL), forward primer (1 μL), reverse primer (1 μL), the master mix (10 μL), and 6 µL nucleotide-free water. All run methods consisted of one cycle of holding stage (10 min at 95°C), followed by 40 cycles in the amplification stage at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. A melting curve stage was run after the cycling stage in the range of 60°C to 95°C to verify amplicon specificity. The relative expression level of each gene was analyzed using the 2-∆∆Ct method.

Statistical Analysis
SPSS 20 (Chicago, IL, USA) was used in statistical analysis. The Mann-Whitney U test was applied to compare the variables between the case and control groups. Pearson’s correlation coefficient was used to measure the correlation between gene expression and vitamin D levels in each group. The data was presented as mean ± standard deviation and p<0.05 was deemed significant.

RESULTS
A total of 40 cases and 40 healthy controls were included in this study. The demographic features of the two study groups are summarized in Table 2. As mentioned before, women in the case group were matched in regards to age with any significant differences than those in the controls (p>0.05). The mRNA levels of foxp3 were higher in women with a history of RSA than in controls; however, there was no significant fold change in foxp3 mRNA levels between patients in the case and control groups (p=0.16, Mean ± SEM: 16.87±13.11 vs. 5.26±1.39, respectively) (Figure 1). There was a significant direct correlation between foxp3 expression and 1,25(OH)2D levels was not significant in the case group (p=0.14, R=-0.25) (Table 3). 1,25(OH)2D levels were significantly lower in the serum samples of women with a history of RSA compared to controls (p<0.001, Mean ± SEM: 36.84±3.97 versus 21.39±1.94 respectively) (Figure 2).

| Table 1. Primer sequences used for the real-time polymerase chain reaction. |
|-----------------|-----------------|-----------------|
| Gene            | sequence (5’−3’) | Accession No.   |
| Foxxp3          | F: ACC TGG AAG AAC GCC ATC | NM_014009.4    |
|                 | R: TGT TCG TCC ATC CTC CT T C |               |
| GAPDH           | F:AAA TCA AGT GGG GGC ATG CTG | NM_001357943.2 |
|                 | R:GCA GAG ATG ATG ACC CT T TT |               |
| PCR product (bp)| 192              | 118             |

PCR, polymerase chain reaction; F, forward; R, reverse; Foxp3, forkhead box P3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

| Table 2. Demographic data of the study groups. |
|-----------------|-----------------|-----------------|
| Groups          | Age (Year)      | Number of miscarriages | Successful pregnancies |
| Case (N=40)     | 31±3.78         | 4.0±0.9           | 0                           |
| Control (N=40)  | 31.06±3.43      | 0                | 2.12±0.93                    |

Data are presented as mean ± S.D.
Case; Recurrent spontaneous abortion, Control; Healthy fertile women
Figure 1. Comparison of foxp3 gene expression in cells obtained from blood samples of women with a history of recurrent spontaneous abortion (case) and healthy fertile women (controls). There was no significant fold change in foxp3 mRNA levels between case and control groups (p value=0.16, Mean ± SEM: 16.87±13.11 versus 5.26±1.39, respectively).

Figure 2. Comparison of vitamin D in serum from blood samples of women with a history of recurrent spontaneous abortion (case) and healthy fertile women (controls). There was a significantly lower level of vitamin D in the serum samples of case group subjects compared to controls (p value ≤0.001, Mean ± SEM: 21.39±1.94 versus 36.84±3.97, respectively).

DISCUSSION

The cause of RSA, a negative pregnancy outcome that occurs in women around the world, is related to endocrine abnormalities, thrombophilic disorders, genetic factors, and anatomical abnormalities. However, the causes of most cases of RSA are unknown and may relate to immune factors (Wolf et al., 2005).

Poor vitamin D levels have been associated with pregnancy loss, recurrent implantation failure, and pregnancy-related disorders such as preeclampsia (Schröder-Heu-rich et al., 2020). This study found significantly lower concentrations of vitamin D in the blood serum samples of patients with a history of RSA compared to controls. Li et al. (2017) assessed the concentration of vitamin D and the expression of vitamin D receptors in the decidual tissues of women with a history of RSA (N=30) and women in early pregnancy undergoing elective abortion as controls (N=30). As seen in our study, individuals with a history of RSA had significantly lower levels of vitamin D and its receptor in deciduous tissues compared to controls, indicating that vitamin D has a role in the production of inflammatory cytokines and possibly in the etiology of RSA (Li et al., 2017). Sharif et al. (2018) assessed the relationship between vitamin D, recurrent pregnancy loss, and autoimmunity. According to the authors, during pregnancy, fetal endurance can occur through a complex interaction of various regulatory factors in the innate and adaptive immune system, which can result in miscarriage. While deficiency has been associated with pregnancy failure, vitamin D may modulate the immune response of the fetal interface and help create a more conducive environment for a successful pregnancy. The authors suggested a significant role for adequate vitamin D supplementation in preventing recurrent miscarriage (Sharif et al., 2018).

Two reviews conducted by Gonçalves et al. (2018) and Zhang et al. (2017) concluded that vitamin D might be essential in the immune system, promoting implantation and successful pregnancy. Also, women with a history of RSA with low levels of vitamin D appear to have more autoimmune and cellular immune abnormalities.

Lack of Vitamin D suggests that pre-pregnancy vitamin D supplementation in women may affect glucose-induced tumor necrosis factor receptors such as foxp3 and glucocorticoid receptors protect against RSA (Abdollahi et al., 2020a). One research showed that foxp3 was also expressed in human trophoblasts in addition to Treg cells and abnormal expression of foxp3 in trophoblasts may relate to RPL (Hu et al., 2019). Hu et al. (2019) used RT-qPCR to study the foxp3 expression profile in the trophoblasts of women with recurrent pregnancy loss. They concluded that foxp3 is expressed in trophoblasts and is reduced in recurrent pregnancies. Thus, regulation of foxp3 may

Table 3. Correlation between Vitamin D and FOXP3 expression in case and control groups.

| Groups | Variable | FOXP3 expression |
|--------|----------|------------------|
| Case   | Vitamin D| R=-0.25, P=0.14, CI=-0.53--0.08 |
| Control| Vitamin D| R=0.49, P=0.003, CI= 0.18-0.7 |

Foxp3, forkhead box P3. Pearson’s correlation coefficient was used.
help maintain maternal tolerance and fetal growth (Hu et al., 2019). Selective expression of foxp3 in human trophoblasts suggests that foxp3 expression may be associated with aggressive behavior of trophoblasts and proliferation. Abdollahi et al. (2020b) reported an increase in foxp3 gene expression in the peripheral blood mononuclear cells (PBMCs) of women with unexplained recurrent pregnancy loss (URPL) in the presence of vitamin D3 compared to untreated PBMCs. In contrast, there was no significant change in the control group regarding foxp3 gene expression in the PBMCs in the presence of 1,25Vitamin D3 compared to untreated PBMCs. They also showed that foxp3 gene expression decreased significantly in PBMCs in women with URPL compared to controls (Abdollahi et al., 2020b). Unlike the abovementioned studies, in this study, the level of foxp3 mRNA was increased in the blood of women with a history of RSA compared to controls, albeit not significantly. Also, we detected no significant direct correlation between 1,25 Vitamin D3 concentrations and foxp3 expression in the case group. Despite the enhanced foxp3 expression in CD4+ T cells by 1,25(OH)2D and the capacity of this vitamin to regulate gene expression via direct binding to the genes, it is unknown whether 1,25(OH)2D3 may directly induce foxp3 gene expression without involving other molecules (Kang et al., 2012). Based on the findings of this study, 1,25(OH)2D3 may, along with other molecules, alter foxp3 expression (Kang et al., 2012).

In vitro cultures of human CD4+CD25+ T cells and plain putative T cells, 1,25(OH)2D3 was reported to increase the frequency of activation-induced foxp3+ T cells, inflammation and the allergic response-The role of glucocorticoids and Vitamin D. J Steroid Biochem Mol Biol. 2010;120:86-95. PMID: 20227496 DOI: 10.1016/j.jsbmb.2010.02.029

In conclusion, our findings demonstrated that 1,25Vitamin D3 along with other molecules might act to prevent RSA by providing an anti-inflammatory state not necessarily through foxp3 expression and T cell differentiation.

Author contributions

Contribution of each author made to the manuscript included: (1) Concept and design: F.D.T, S.M.M., F.Z., F.F., (2) Administrative support: F.F., F.Z, (3) Provision of study materials or patients: F.D.T., S.M.M., F.F., F.Z., (4) Data collection and treatment: F.F., F.Z., F.D.T., (5) Data analysis and interpretation: S.M.M., F.D.T., F.F., (6) Manuscript writing: All authors.

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CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

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