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Poor ovarian response is associated with serum vitamin D levels and pro-inflammatory immune responses in women undergoing in-vitro fertilization

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ABSTRACT

Poor ovarian response (POR[1]) limits the success of infertility treatment modality. In this study, we aim to investigate if POR is associated with serum 25(OH) vitamin D (VD[2]) levels and pro-inflammatory immune responses in infertile women with a history of in-vitro fertilization and embryo transfer failures. A retrospective cross-sectional study included 157 women with IVF failures. Study patients were divided into four groups based on serum 25(OH)VD level and ovarian responses during the most recent IVF cycle; low VD (LVD[3]) with POR, LVD with normal ovarian response (NOR[4]), normal VD (NVD[5]) with POR, and NVD with NOR. Serum 25(OH)VD level, cellular- and auto-immunity, and metabolic parameters, including homocysteine and plasminogen activator inhibitor-1 were investigated. Peripheral blood $CD56^+$ NK cell levels (%) and NK cytotoxicity were significantly higher in POR-LVD when compared to the other groups ($P < 0.05$, respectively). CD19 + B and CD19+/5+ B-1 cell levels were significantly higher in women with POR-LVD as compared with those of NOR-LVD and POR-NVD ($P < 0.05$, respectively). TNF-α/IL-10 producing Th1/Th2 cell ratio of POR-LVD was significantly higher than those of POR-NVD and NOR-NVD ($P < 0.05$ respectively). Peripheral blood homocysteine level of POR-LVD was significantly higher than those of NOR-LVD and POR-NVD ($P < 0.05$ respectively). We conclude that assessment of cellular and autoimmune abnormalities and metabolic factors, such as homocysteine should be considered in women with POR and LVD. VD and folic acid supplementation may be explored further as a possible therapeutic option for POR with immune and metabolic etiologies.

1. Introduction

Vitamin D (VD[6]) is involved in calcium and phosphorus metabolism, ovarian function, and immune regulation (D'ambrosio et al., 1998; Bouillon and Suda, 2014; Irani and Merhi, 2014). VD deficiency is widespread in reproductive-age women (20%–90%) in North America (Holick et al., 2011). VD plays a significant role in human reproduction and various maternal and fetal complications, such as an increased risk of gestation diabetes, recurrent pregnancy losses (RPL[7]), preeclampsia and fetal growth restriction have been reported to be associated with VD deficiency (Rosen et al., 2012; Ota et al., 2014). In addition, VD regulates many functions of the reproductive organs in both men and

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[1] POR, poor ovarian response
[2] VD, vitamin D
[3] LVD, low vitamin D
[4] NOR, normal ovarian response
[5] NVD, normal vitamin D
[6] VD, vitamin D
[7] RPL, recurrent pregnancy losses

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women. VD status has been associated with in-vitro fertilization (IVF[8]) outcome, clinical characteristics of the polycystic ovarian syndrome (PCOS[9]) and endometriosis, semen quality, sperm count, sperm motility and morphology (Grundmann and von Versen-Hoynck, 2011; Lerchbaum and Obermayer-Pietsch, 2012). These observations provide new understandings for the multifaceted underlying pathology of infertility.

Previously, we reported the increased prevalence of VD deficiency in RPL patients (45%) (Ota et al., 2014). In women with VD deficiency, the prevalence of autoantibodies such as antiphospholipid antibody (APA[10]), anti-thyroperoxidase (TPO[11]) antibody, anti-nuclear antibody (ANA[12]), and anti-ssDNA antibody was significantly higher as compared with that of RPL women with normal VD levels (Ota et al., 2014). VD suppresses proliferation of T helper 1 (Th1[13]) cells and limits Th1 type cytokine production, including IFN-γ and TNF-α, while inducing the production of Th2 type cytokines, such as IL-10 and IL-4. In addition, VD regulates peripheral blood NK and Th1 cell immune responses in women with RPL (Ota et al., 2015). A large number of women with RPL have VD deficiency, which has immunological implications in RPL (Ota et al., 2014). Women with repeated implantation failures (RIF[14]) after IVF and embryo transfer (ET[15]) have significantly higher NK cell levels and cytotoxicity, and dysregulated cytokine production patterns as compared to those of controls (Santillan et al., 2015). When considering these facts, VD deficiency may be keenly associated with underlying immunopathology of reproductive failures.

Poor ovarian response (POR[16]) is characterized by a decreased production of follicles/oocytes after controlled ovarian hyperstimulation (COH[17]) in IVF treatment. Compared with normal responders, patients with POR exhibit decreased fertilization rates and lower embryo quality. Additionally, women with POR have high IVF cycle cancellation rates and diminished pregnancy rates, which reduce the overall IVF success rate and decrease cost-effectiveness (Oudendijk et al., 2012). However, the underlying pathology of POR in reproductive age women has not been elucidated yet. So far, 4 of 7 studies on serum 25(OH)VD reported the significant association with ovarian reserve markers. In addition, a recent study demonstrated that serum 25(OH)VD levels after VD supplementation significantly correlated with anti-Müllerian hormone (AMH[18]) levels (Naderi et al., 2018). Hence, VD effect on folliculogenesis should be thoroughly explored in the future.

Autoimmune activities and pro-inflammatory immune responses may impair the ovarian response. The AMH levels of systemic lupus erythematosus (SLE[19]) patients were significantly lower than that of the control group and negatively correlated with erythrocyte sedimentation rate and disease activity (Gao et al., 2018). Follicular maturation was paralleled with a decreased number of cytotoxic NK cells, suggesting cytotoxic NK cells may have deleterious effects on follicular maturation (Fainaru et al., 2011). Previously, we reported increased Th1/Th2 cell ratios in women with RIF (Kwak-Kim et al., 2003). However, the association between POR and inflammatory immune responses, i.e., dominant Th1 over Th2 immune response, has not been studied. In this study, we intend on exploring if the peripheral blood pro-inflammatory immune responses are associated with POR and serum 25(OH)VD levels in infertile women with IVF failures.

2. Materials and methods

2.1. Study population

The retrospective cross-sectional study included women with IVF failures (n = 157) aged 25 to 40 years, who were registered sequentially at the Reproductive Medicine and Immunology, Chicago Medical School, Rosalind Franklin University of Medicine and Science from January 2015 to December 2016. Forty-nine women had no pregnancy history (G = 0), and 108 women had 1 or more pregnancy losses before 10 weeks gestation. The study was approved by the Institutional Review Board, Rosalind Franklin University of Medicine and Science and informed consent process was waved. Study patients were divided into four groups determined by the serum 25(OH)VD level and the ovarian response during the recent IVF cycle. In this study, study populations were divided into normal (≥30 ng/ml) and low VD (< 30 ng/ml) levels, and then based on ovarian responses, subdivided them into a normal and POR. POR, specifically as non-age related POR, was defined as 3 or fewer oocytes retrieved during the recent stimulation cycle and abnormal ovarian reserve test including antral follicle count or AMH following Bologna criteria (Ferraretti et al., 2011). Finally, patients were allocated into four groups: POR with low 25(OH)VD level (POR-LVD, n = 18), POR with normal 25(OH)VD (POR-NVD, n = 40), normal ovarian response (NOR) with low 25(OH)VD (NOR-LVD, n = 30), and NOR with normal 25(OH)VD (NOR-NVD, n = 69). Age, obstetrical and infertility histories, anthropometric and metabolic variables) including homocysteine (HCY) and plasminogen activator inhibitor-1 (PAI-1) were analyzed.

2.2. Laboratory

2.2.1. Autoantibodies

Venous blood was drawn during the early follicular phase in women without any treatment. 25(OH)VD level was assessed using the liquid chromatography/tandem mass spectrometry method at the reference laboratory (Hedman et al., 2014). APA was tested by ELISA as previously reported (Gilman-Sachs et al., 1991) including IgG and IgM autoantibodies to cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, phosphatidylglycerol, and phosphatidic acid (Gilman-Sachs et al., 1991). ANA was measured by indirect immunofluorescence using a commercially available kit (Immunoconcepts, Sacramento, CA, USA). Anti-dsDNA, anti-ssDNA, anti-histone, anti-Scl-70, anti-thyroglobulin, and anti-TPO antibodies were tested by ELISA using commercially available kits (Inova Diagnostic, San Diego, CA, USA).

2.2.2. Peripheral blood immunophenotype

Immunophenotype assay was performed using mAb against CD45-PC5, CD3-FITC, CD56-PE, or CD45-PC5 and CD19-FITC (Beckman Coulter, Fullerton, CA, USA) as previously reported (Ota et al., 2014). An FC500 flow cytometer was utilized to analyze the prepared sample using CXP software (Beckman Coulter). Lymphocytes were gated based on side scatter characteristics and CD45 expression, as previously reported (Ota et al., 2014). Within lymphocytes, $CD3^+$ T cells, $CD56^+$ NK cells, $CD19^+$ B cells, and $CD19^+/CD5^+$ B-1 cells were analyzed.

2.2.3. NK cell cytotoxicity (NKC[20]) assay

NKC was measured by using flow cytometry as previously described (Gilman-Sachs et al., 1999). Briefly, PBMCs were co-cultured with the human erythromyelocytic leukemia cell line, K562 (ATTC, Manassas, VA, USA) which was prestained with PKH67 (PKH67 fluorescent cell

[8] IVF, in-vitro fertilization
[9] PCOS, polycystic ovarian syndrome
[10] APA, antiphospholipid antibody
[11] TPO, thyroperoxidase
[12] ANA, antinuclear antibody
[13] Th1, T helper 1
[14] RIF, repeated implantation failure
[15] ET, embryo transfer
[16] POR, poor ovarian response
[17] COH, controlled ovarian hyperstimulation
[18] AMH, anti- Müllerian hormone
[19] SLE, systemic lupus erythematosus
[20] NKC, NK cell cytotoxicity

linker kit, Sigma). NKC was measured at effector to target cell (E:T[21]) ratios of 50:1, 25:1, and 12.5:1. Effector and target cells were in 200 ml of RPMI-1640 culture medium (Gibco, Grand Island, NY, USA) with 10% heat-inactivated fetal calf serum (Gemini Bio-Products, Inc., Woodland, CA, USA) for 2 h. Propidium iodide (PI,[22] Sigma) 10 mg/ml was added to the assay tubes, and flow cytometric analysis was made using FC500 flow cytometer. PI incorporated and non-incorporated K562 cells were analyzed to determine the % killed cells. The spontaneous K562 cell death rate was measured and subtracted before the calculation of NK cytotoxicity.

2.2.4. Analysis of Th1/Th2 cell ratio

The flow cytometric assay was performed as previously described (Kwak-Kim et al., 2003). PBMCs (100 µl) were incubated for 16 h at 37 °C and 5% CO2 with phorbol myristate acetate (PMA, 25 ng/ml), ionomycin (1 mM)(both from Sigma) and GolgiPlug (0.7 ml) per 1 ml of cell suspension, containing Brefeldin A (BD Biosciences, San Jose, CA, USA). After the incubation and washing, anti-CD45-PC5, anti-CD3-ECD, and anti-CD8-FITC were utilized to label the cells (Beckman Coulter). Since PMA and ionomycin down-regulate CD4, CD4 cells were detected by negative selection. To fix and permeabilize cells, Cytofix/Cytoperm and Perm/Wash buffer (BD Pharmingen, Franklin Lakes, NJ, USA) were used according to manufacturer's instructions. PE-conjugated mAbs against TNF-α, IFN-γ, or IL-10 (BD Immunosciences, San Jose, CA, USA) were utilized to stain cells. An FC500 flow cytometer (Beckman Coulter) was utilized to analyze the samples. Th1/Th2 cells ratios were calculated by dividing the percent of TNF-α or IFN-γ expressing $CD3^+CD4^+$ Th cells by the percent of IL-10 expressing $CD3^+CD4^+$ T cells.

2.2.5. Statistical analysis

Statistical analysis was made using SPSS Statistics version 20.0 (2011, SPSS, Inc., Chicago, IL, USA). For the continuous variables, ANOVA and post-hoc analysis by Fisher's Least Significant Difference were applied to compare the means among the study groups. For the categorical variables, logistic regression analysis and contingency tables were used to calculate odds ratios (ORs[23]). Values were expressed as mean ± SD, for continuous variables or percent of the total for categorical variables. A significant P-value was set as less than 0.05.

3. Results

3.1. Patient characteristics and prevalence of low VD

The mean ages of POR-LVD (36.5 ± 4.3) were significantly older than those of the NOR-LVD (29.3 ± 3.2, P < 0.01) and NOR-NVD (28.9 ± 4.1, P < 0.05) and (Table 1). The mean ages of POR-NVD (37.8 ± 3.5) were significantly older than those of the NOR-LVD (29.3 ± 3.2, P < 0.01) and NOR-NVD (28.9 ± 4.1, P < 0.01). The number of gravidities was significantly higher in women with POR-LVD group (2.9 ± 3.0) as compared with those of NOR-NVD (1.8 ± 1.8), NOR-LVD (1.7 ± 1.8) and POR-NVD (1.7 ± 1.8) (P < 0.05 respectively). The body mass index (BMI[24]) of POR-LVD (31.0 ± 5.7) was significantly higher as compared with those of POR-NVD (24.4 ± 4.6, P < 0.005) and NOR-NVD (25.7 ± 4.9, P < 0.005). The AMH of POR-LVD (0.9 ± 0.7) was significantly lower as compare with those of NOR-LVD (2.4 ± 0.9, P < 0.01). The AMH of NOR-LVD were significantly higher as compare with those of POR-NVD (1.0 ± 0.9, P < 0.005) and NOR-NVD (1.6 ± 1.2, P < 0.05). There were no differences in blood drawing seasons and racial distributions between the four groups.

In 157 women with IVF failure, 109 (69.4%) had normal VD level (≥ 30 ng/ml), and 48 (30.6%) had low 25(OH)VD level (< 30 ng/ml). The mean (± SD) of the serum 25(OH)VD level in NOR-NVD was 43.2 ± 12.2 (ng/ml), whereas NOR-LVD was 23.1 ± 4.7 (ng/ml). In women with POR-NVD, serum 25(OH)VD levels were 44.1 ± 12.1 (ng/ml), and in women with POR-LVD, it was 23.3 ± 4.1 (ng/ml) (Table 1).

3.2. Prevalence of autoantibodies

In women with NOR, the prevalence of total APA was significantly higher in NOR-LVD than that of NOR-NVD (50% versus 29%, P < 0.05, OR 2.45, 95% CI 1.0–6.0) (Table 2). Nevertheless, the prevalence of autoantibodies to phospholipid epitopes, including cardiolipin and anionic phospholipids were not different. The prevalence of ANA, anti-thyroglobulin, and TPO antibodies was not different between the two groups.

In women with POR, the prevalence of total APA was significantly higher in POR-LVD than POR-NVD (61.1% versus 25.0%, P < 0.05, OR 4.71, 95% CI 1.4–15.5) (Table 3). The prevalence of IgG and IgM anti-anionic phospholipids were significantly higher in POR-LVD (44.4%, 38.9%) as compared to that of the POR-NVD (17.5%, 12.5%) (P < 0.05 respectively). The prevalence of autoantibodies to nuclear antigens, including anti-dsDNA, anti-ssDNA, anti-histone, and anti-Scl70 was not different between the two groups. The prevalence of anti-thyroglobulin and TPO antibodies was not different between the two groups.

3.3. Peripheral blood immune-phenotypes

Peripheral blood $CD56^+$ NK cell levels (%) were significantly different among four study groups (P = 0.000). The $CD56^+$ NK cell proportion in women of the POR-LVD (15.5 ± 2.7%) were significantly higher than those of NOR-LVD (9.2 ± 4.5), POR-NVD (8.7 ± 4.2) and NOR-NVD (9.0 ± 4.2) (P < 0.005, respectively) (Fig. 1A). The $CD19^+$ B cell proportion was significantly higher in women of POR-LVD (13.1 ± 3.9) as compared with those of NOR-LVD (10.9 ± 3.9, P < 0.05), POR-NVD (9.5 ± 3.4, P < 0.005) and NOR-NVD (10.6 ± 3.2, P < 0.01) (Fig. 1A). The proportion of $CD19^+/CD5^+$ B-1 cells were significantly higher in women with POR-LVD (11.7 ± 5.3) as compared to NOR-LVD (6.7 ± 3.3) and POR-NVD (7.5 ± 4.6) (P < 0.05 respectively). There was no difference in B1 cell level between POR-LVD and NOR-NVD (9.3 ± 8.5) (Fig. 1A).

3.4. NK cytotoxicity

NKC at effector-to-target cell ratios (E:T) of 50:1, 25:1 and 12.5:1 are significantly different between the 4 study groups (P = 0.017, 0.005 and 0.018 respectively). NKC at E:T of 50:1 of POR-LVD (27.7 ± 5.6%) was significantly higher than those of NOR-LVD (22.1 ± 6.2, P < 0.01), POR-NVD (21.9 ± 5.5, P < 0.005) and NOR-NVD (23.1 ± 6.8, P < 0.05) (Fig. 1B). NKC at E:T of 25:1 of POR-LVD (20.9 ± 6.1) was significantly higher than those of NOR-LVD (14.9 ± 3.1, P < 0.005) and POR-NVD (16.3 ± 5.0, P < 0.05). There was no difference in NKC between POR-LVD and NOR-NVD (18.0 ± 6.2). NKC at E:T of 12.5:1 of POR-LVD (14.5 ± 4.9) was significantly higher than that of NOR-LVD (10.2 ± 2.7, P < 0.01). Again, NKC at E:T ratio of 12.5:1 of NOR-LVD was significantly lower than that of NOR-NVD (13.0 ± 4.9, P < 0.05).

3.5. Intracellular cytokine for T helper cell (Th1/Th2 cell ratios)

The TNF-α/IL-10 producing Th1/Th2 cell ratios were significantly different among the study groups (P = 0.029). The TNF-α/IL-10 ratio was significantly higher in women within POR-LVD (39.9 ± 3.5) as

[21] E:T, effector to target
[22] PI, propidium iodide
[23] ORs, odds ratios
[24] BMI, body mass index

Table 1
Characteristics of women undergoing IVF cycles based on ovarian response and vitamin D levels; normal ovarian response with normal vitamin D level (NOR-NVD), normal ovarian response with low vitamin D level (NOR-LVD), poor ovarian responses with normal vitamin D level (POR-NVD) and poor ovarian response with low vitamin D level (POR-LVD). Data are mean ± SD unless stated otherwise. P < 0.05 was considered to be statistically significant.

Characteristics	POR-LVD (n = 18)	NOR-LVD (n = 30)	POR-NVD (n = 40)	NOR-NVD (n = 69)	P-value[+]
Age (years)	36.5 ± 4.3[a**, c*]	29.3 ± 3.2[d**]	37.8 ± 3.5[f**]	28.9 ± 4.1	< 0.01
Gravidity	2.9 ± 3.0[a*, b*, c*]	1.7 ± 1.8	1.7 ± 1.8	1.8 ± 1.8	NS
Parity	0.4 ± 0.9	0.1 ± 0.3	0.1 ± 0.4	0.1 ± 0.3	NS
BMI (kg/m^2)	31.0 ± 5.7[b***,c***]	27.9 ± 6.8	24.4 ± 4.6	25.7 ± 4.9	< 0.001
AMH (ng/ml)	0.9 ± 0.7[a**]	2.4 ± 0.9[d**,e*]	1.0 ± 0.9	1.6 ± 1.2	< 0.01
Vitamin D level (ng/ml)	23.3 ± 4.1[b***,c***]	23.1 ± 4.7[d***,e***]	44.1 ± 12.1	43.2 ± 12.2	< 0.001
Season for blood test					NS
Summer, n (%)	5 (27.8)	7 (23.3)	11 (27.5)	12 (17.4)	NS
Winter, n (%)	9 (50)	13 (43.3)	17 (42.5)	29 (42.1)	NS
Other time, n (%)	4 (22.2)	10 (33.3)	12 (30)	28 (40.6)	NS
Race					NS
Caucasian, n (%)	12 (66.7)	20 (66.7)	27 (67.5)	53 (76.8)	NS
Hispanic, n (%)	4 (22.2)	5 (16.7)	6 (15)	10 (14.5)	NS
Asian, n (%)	2 (11.1)	5 (16.7)	7 (17.5)	6 (8.6)	NS

[+]P values between 4 groups by ANOVA.
[a]POR-LVD as compare with NOR-LVD.
[b]POR-LVD as compare with POR-NVD.
[c]POR-LVD as compare with NOR-NVD.
[d]NOR-LVD as compare with POR-NVD.
[e]NOR-LVD as compare with NOR-NVD.
[f]POR-NVD as compare with NOR-NVD.
[*]P < 0.05.
[**]P < 0.01.
[***]P < 0.005.

Table 2
Prevalence of antiphospholipid antibody (APA), anti-nuclear antibody (ANA), anti-thyroid antibody and other non-organ-specific autoantibodies in women with normal ovarian responses (NOR) during the IVF cycle with normal vitamin D level (NVD) or low vitamin D level (LVD).

Autoantibodies	NOR-NVD (≥30 ng/ml) (n = 69) (%)	NOR-LVD (< 30 ng/ml) (n = 30)	Odds ratio (95% CI)	P-value
APA (IgG, IgM)[a]	20 (29.0)	15 (50.0)	2.45 (1.0–6.0)	P < 0.05
Anti-cardiolipin				
IgG	9 (13.0)	3 (10.0)	0.74 (0.18–3.0)	NS
IgM	7 (10.1)	4 (13.3)	1.36 (0.4–5.1)	NS
Anti-anionic phospholipids[b]				
IgG	17 (24.6)	7 (23.3)	0.93 (0.3–2.6)	NS
IgM	16 (23.2)	7 (23.3)	1.59 (0.6–4.2)	NS
Anti-nuclear antibody	14 (20.3)	7 (23.3)	1.20 (0.4–3.3)	NS
Anti-nuclear antigen antibody	12(17.4)	6(20.0)	1.15(0.5–2.8)	NS
Anti-dsDNA	2 (2.9)	3 (10.0)	3.72 (0.6-23.5)	NS
Anti-ssDNA	8 (11.6)	2 (6.7)	0.54 (0.1–2.7)	NS
Anti-histone	11 (15.9)	3 (10.0)	0.59 (0.2–2.3)	NS
Anti-Scl70	0	0	–	NS
Anti-thyroid antibody				
Anti-thyroglobulin	8 (11.6)	1 (3.3)	0.26 (0.0–2.2)	NS
Thyroperoxidase antibody	16 (23.2)	6 (20.0)	0.83 (0.3–2.4)	NS

[a] Any IgG or IgM antibodies to phospholipids.
[b] Antibodies to anionic phospholipids including antibodies to phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid, phosphatidylglycerol, and phosphatidylserine.

compared with those of POR-NVD (34.5 ± 8.0) and NOR-NVD (34.6 ± 5.0) (P < 0.01 respectively).

The ratios of IFN-γ/IL-10 producing Th1/Th2 cells among four study groups were significantly different (P = 0.007). The IFN-γ/IL-10 ratio was significantly higher in POR-LVD (21.4 ± 4.2) as compared to those of POR-NVD (18.0 ± 5.2, P < 0.05) and NOR-NVD (18.4 ± 5.2, P < 0.05) (Fig. 1C). The IFN-γ/IL-10 ratio of NOR-LVD (21.1 ± 5.6) was significantly higher that those of POR-NVD (P < 0.01) and NOR-NVD (P < 0.05).

3.6. Homocysteine and plasminogen activator inhibitor-1

The level of HCY was significantly higher in women within POR-LVD (8.8 ± 1.3) as compared with those of NOR-LVD (7.4 ± 1.6, P < 0.005), POR-NVD (7.1 ± 1.6, P < 0.005) and NOR-NVD (7.7 ± 1.7, P < 0.05) (Fig. 2). The PAI-1 level was significantly higher in women within POR-LVD (17.8 ± 9.0) as compared with those of NOR-NVD (12.7 ± 7.6, P < 0.05). There were no differences in PAI-1 level among POR-LVD (17.8 ± 9.0), POR-NVD (15.9 ± 11.8), and NOR-LVD (15.8 ± 9.3).

4. Discussion

In this study of women with IVF failures, we found strong evidence of associations between POR, LVD and proinflammatory immune responses, such as increased NK cell levels and NKC, increased Th1/Th2 cell ratios, and a presence of autoimmunity particularly APA. In addition, POR and LVD were associated with metabolic dysregulation of serum homocysteine and PAI-1.

During IVF treatment, POR was reported to be correlated with old

Table 3
Prevalence of antiphospholipid antibody (APA), anti-nuclear antibody (ANA), anti-thyroid antibody and other non-organ-specific autoantibodies in women with poor ovarian responses (POR) during the IVF cycle with normal vitamin D level (NVD) or low vitamin D level (LVD).

Autoantibodies	POR-NVD (≥30 ng/ml) (n = 40) (%)	POR-LVD (< 30 ng/ml) (n = 18) (%)	Odds ratio (95% CI)	P-value
APA (IgG, IgM)[a]	10 (25)	11 (61.1)	4.71 (1.4–15.5)	P < 0.05
Anti-cardiolipin antibody				
IgG	2 (5.0)	1 (5.6)	1.14 (0.1–13.5)	NS
IgM	1 (2.5)	2 (11.1)	4.88 (0.4–57.6)	NS
Anti-anionic phospholipids[b]				
IgG	7 (17.5)	8 (44.4)	3.77 (1.1–13.0)	P < 0.05
IgM	5 (12.5)	7 (38.9)	4.45 (1.2–16.9)	P < 0.05
Anti-nuclear antibody	12 (30.0)	2 (11.1)	0.29 (0.1–1.5)	NS
Anti-nuclear antigen antibody	11(27.5)	2(11.1)	0.40(0.1–1.6)	NS
Anti-dsDNA	1 (2.5)	0	–	–
Anti-ssDNA	5 (12.5)	1 (5.6)	0.41 (0.0–3.8)	NS
Anti-histone	3 (7.5)	1 (5.6)	0.72 (0.1–7.5)	NS
Anti-Scl70	0	0	–	–
Anti-thyroid antibody				
Anti-thyroglobulin	5 (12.5)	2 (11.1)	0.87 (0.2–5.0)	NS
Thyroperoxidase antibody	11 (27.5)	4 (22.2)	0.75 (0.2–2.8)	NS

[a] Any IgG or IgM antibodies to phospholipids.
[b] Antibodies to anionic phospholipids including antibodies to phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid, phosphatidylglycerol, and phosphatidylserine.

age. In this study, women with POR had a significantly higher age than women with NOR regardless of VD levels. However, BMI was higher in women with both POR and LVD as compared with others. Indeed, BMI was reported to inversely correlate with serum 25(OH)VD, regardless of season or age (Wortsman et al., 2000; Delle Monache et al., 2018). In a recent meta-analysis, the ovarian reserve marker, AMH, is significantly lower in obese than non-obese women, and BMI is negatively correlated with AMH (Moslehi et al., 2018). Hence, VD deficiency may be related to POR directly and indirectly via high BMI levels. Patients with obesity and type II DM exhibit increased numbers and activation of NK cells in the circulation or adipose tissue than controls (Henegar et al., 2008), although contradictory studies have been reported (Lynch et al., 2009). In addition, cytotoxic activity of the circulating NK cells is decreased in obese human (Viel et al., 2017) and homozygous for KIR haplotype A has a greater risk of type II DM (Romero et al., 2008). In this study, POR-LVD group had higher BMI and increased NK cell numbers and cytotoxicities. To rule out a possibility of obesity being a confounding factor for NK cell pathology, subgroup analysis was made. There were no differences in NK cell levels ($P > 0.05$) and cytotoxicity ($P > 0.05$) between women with high BMI (> 25) and normal BMI ($19 < BMI < 25$) in this study (Supplemental Table 1).

VD deficiency has been related to increased risks of ovulatory dysfunction, POR in polycystic ovarian syndrome, RPL, pre-eclampsia, intrauterine growth restriction, and gestational diabetes mellitus (Irani and Merhi, 2014). Recently, we reported that a high proportion of women undergoing the first IVF cycle had VD deficiency (50%) (Wu et al., 2018). In these women, VD deficiency was associated with fewer mature oocytes, blastocyst formation rate, and peripheral blood cellular immune activation, including increased NK and B cell proportions and Th/Tc cell ratios. Interestingly, VD had a statistically significant indirect effect on the overall number of mature eggs through Th/Tc cell ratios (36–40%), but not on blastocyst formation rate regardless of stimulation protocol (Wu et al., 2018). In this study, around 31% of IVF failure patients had VD deficiency, which was lower than that of RPL patients (Ota et al., 2014). The prevalence of VD deficiency and the mean serum levels of NOR and POR were not different. However, in women with LVD, $CD56^+$, $CD19^+$, and $CD19^+/CD5^+$ cell levels, NK cytotoxicity and the prevalence of APA were significantly higher in women with POR as compared with those of NOR. Therefore, POR is directly associated with inflammatory-immune and autoimmune conditions as well. Since VD was reported to regulate immune responses, LVD level may indirectly affect POR via inflammatory immune responses. To test this hypothesis, the mediation analysis was performed to find if there is a significant indirect effect of VD level on the ovarian response via inflammatory immune factors. In this study, there was no significant indirect effect of VD on the ovarian response via inflammatory immune factors. Therefore, inflammatory immune condition and VD independently affect POR. AMH was positively correlated with 25(OH)VD levels, and adequate VD supplementation was able to suppress the seasonal changes of serum AMH levels (Irani and Merhi, 2014). In addition, VD acts as immunosuppressant as well as an anti-inflammatory vitamin (Ota et al., 2014, 2015), which may have protective effects on ovarian reserve from immunological attack. Therefore, VD effect on ovulation may be via its direct physiological role on folliculogenesis and the regulatory effect on inflammatory immune responses. In this study, however, the indirect effect of VD on inflammatory immune response was insignificant. Therefore, VD treatment may improve ovarian response in the POR cases directly or indirectly. VD derivatives and VD receptor agonists which provide higher immunoregulatory capacity with less toxicity may shed light on the immunoregulatory treatment of the POR (Penna et al., 2007).

Women with a history of RPL or IVF failures often demonstrate autoantibodies, including APA, ANA, and anti-thyroid antibodies (Kwak-Kim et al., 2016). Antiphospholipid antibody syndrome (APS) patients have a high prevalence of VD deficiency (Riancho-Zarrabeitia et al., 2018), which is often associated with clinically defined thrombotic events in these patients (Agmon-Levin et al., 2011). Besides, women suffering from primary APS had reduced ovarian reserve, with prevalence higher than 50% (Yamakami et al., 2014). Antibodies directed to pre-ovulatory follicles but not to corpus luteum-specific antigens may influence ovarian reserve as well (Yamakami et al., 2014). Previously, we reported women with VD deficiency had an increased prevalence of autoantibodies including APA, anti-TPO antibody, ANA and anti-ssDNA antibody compared to those of RPL patient with normal VD levels (Agmon-Levin et al., 2011; Ota et al., 2014). In this study, women with LVD, regardless of ovarian response status had a significantly higher prevalence of total APA as compared with those of NVD. Women with NOR-LVD had 2.45 times higher risk of having APA than those with NOR-NVD. Women with POR-LVD had 4.71 times higher risk of having APA than those with POR-NVD. Notably, women with LVD have 3.77- and 4.45-times higher risks to develop IgG and IgM anti-anionic phospholipids antibodies, respectively, than those of women with NVD. None of the women with POR and APA had clinical evidence of ovarian thrombosis, which is consistent with the findings reported from primary APS patients (Andre et al., 2004). Thyroid autoantibodies have been associated with decreased ovarian reserve. The

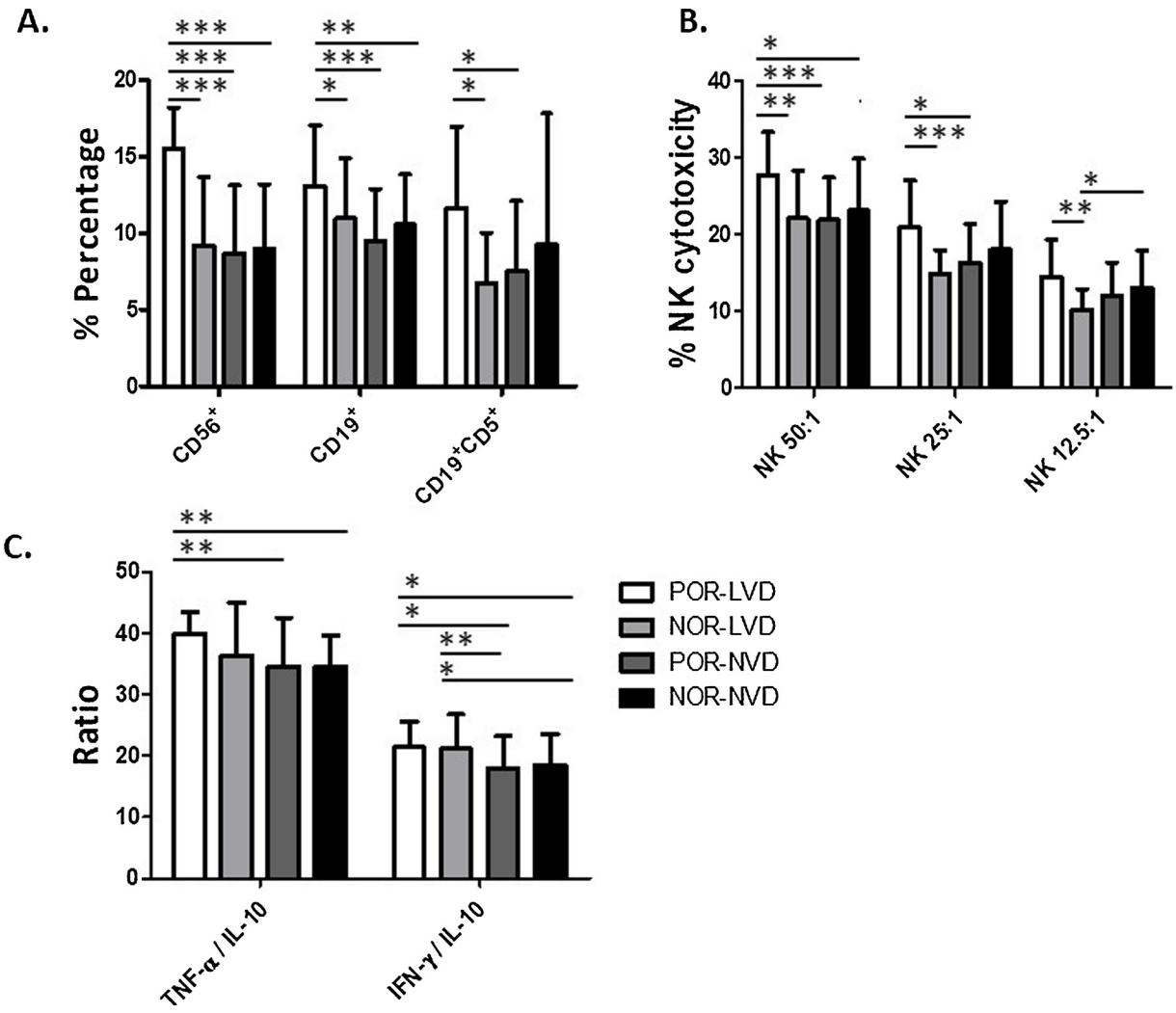

Fig. 1. Peripheral blood immunophenotype including $CD56^+$ natural killer (NK), $CD19^+$ B and $CD19^+/CD5^+$ B1 cells, NK cytotoxicity and Th1/Th2 ratios of women with poor ovarian responses with low vitamin D (POR-LVD, n = 18), normal ovarian response with low vitamin D (NOR-LVD, n = 30), poor ovarian responses with normal vitamin D (POR-NVD, n = 40) and normal ovarian responses with normal vitamin D (NOR-NVD, n = 69) are plotted.

(A) Peripheral blood $CD56^+$ NK cell levels (%) were significantly different between study groups (P = 0.000). Women with POR-LVD had a significantly higher proportion of $CD56^+$ NK cells than NOR-LVD, POR-NVD, and NOR-NVD ($P < 0.005$, respectively); $CD19^+$ B cell levels were significantly different between four study groups (P = 0.005). Women with POR-LVD had a significantly higher proportion of $CD19^+$ B cells than NOR-LVD ($P < 0.05$), POR-NVD ($P < 0.005$) and NOR-NVD ($P < 0.01$); $CD19^+/CD5^+$ B1 cell levels were significantly different between the study groups ($P < 0.05$). Women with POR-LVD had a significantly higher proportion of $CD19^+/CD5^+$ B-1 cells than NOR-LVD and POR-NVD ($P < 0.05$, respectively). ***$P < 0.005$; **$P < 0.01$; *$P < 0.05$.

(B) NK cytotoxicity at effector-to-target cell ratios (E:T) of 50:1, 25:1 and 12.5:1 are significantly different between the 4 study groups (P = 0.017, 0.005 and 0.018 respectively). NK cytotoxicity at E:T ratio of 50:1 of POR-LVD was significantly higher than those of NOR-LVD ($P < 0.01$), POR-NVD ($P < 0.005$) and NOR-NVD ($P < 0.05$); NK cytotoxicity at E:T of 25:1 of POR-LVD was significantly higher than those of NOR-LVD ($P < 0.005$) and POR-NVD ($P < 0.05$); NK cytotoxicity at E:T of 12.5:1 of POR-LVD group was significantly higher than those of NOR-LVD ($P < 0.01$), and NOR-LVD had significantly lower NK cytotoxicity as compared with those of NOR-LVD ($P < 0.05$). ***$P < 0.005$; **$P < 0.01$; *$P < 0.05$.

(C) T helper (Th)1/Th2 cell ratios were significantly different between the four groups (P = 0.029). The TNF-α/IL-10 producing Th cell ratios were significantly higher in women with POR-LVD as compared with those of POR-NVD and NOR-NVD ($P < 0.01$, respectively). The ratio of IFN-γ/IL-10 among four study groups were significantly different (P = 0.007). The IFN-γ/IL-10 producing Th cell ratios were significantly higher in women with POR-LVD as compared with those of POR-NVD and NOR-NVD ($P < 0.05$ respectively). NOR-LVD had significantly higher IFN-γ/IL-10 producing Th cell ratios than POR-NVD ($P < 0.01$) and NOR-NVD ($P < 0.05$). ***$P < 0.005$; **$P < 0.01$; *$P < 0.05$.

prevalence of autoimmune thyroid disease was significantly higher in women with early ovarian aging as compared with age-related POR, and presence of anti-TPO antibody was a risk factor for poor cycle outcome (Beydilli Nacak et al., 2018). However, in our study, either thyroid autoimmunity or ANA was not associated with POR. Immune effectors including monocytes, macrophages, dendritic cells, activated T and B cell express vitamin D receptors and secrete 1-a-OHase and 24 hydroxylase which convert vitamin D into the active form, $1,25(OH)_2D$ (Kamen and Tangpricha, 2010). Vitamin D has immunomodulatory effects on B cells and immunoglobulin production (Lemire et al., 1984), and interrupts B cell differentiation in-vitro (Chen et al., 2007). Vitamin D deficiency is associated with increased susceptibility to deregulated immune system and autoimmunity. In this study, LVD group had elevated peripheral blood B cell levels than others. This finding was consistent with our previous report of women with RPL, in which RPL women with low VD had significantly increased peripheral blood B and NK cells than those of normal VD (Ota et al., 2014).

VD regulate NK cell cytotoxicity, cytokine secretion, and degranulation process as well as TLR4 expression (Ota et al., 2014, 2015). Additionally, VD promote Th2 cells while suppressing Th1 responses. In

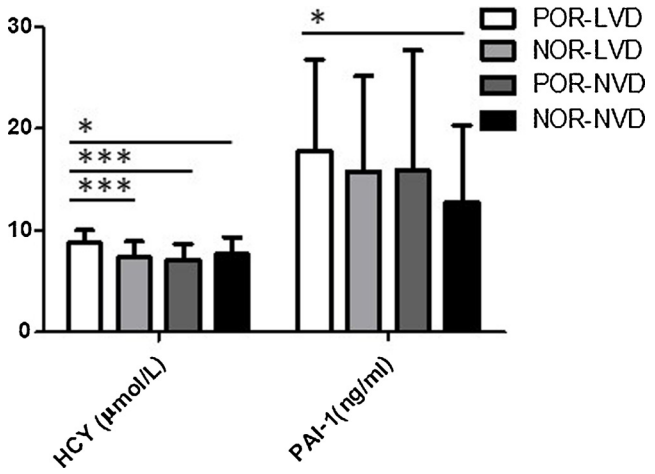

Fig. 2. The serum levels of homocysteine (HCY) and plasminogen activator inhibitor-1 (PAI-1) in four groups of women with poor ovarian responses with low vitamin D (POR-LVD, n = 18), normal ovarian response with low vitamin D (NOR-LVD, n = 30), poor ovarian responses with normal vitamin D (POR-NVD, n = 40) and normal ovarian responses with normal vitamin D (NOR-NVD, n = 69).

(A) HCY level was significantly higher in women with POR-LVD as compared with those of NOR-LVD ($P < 0.005$), POR-NVD ($P < 0.005$) and NOR-NVD ($P < 0.05$).

(B) PAI-1 level was significantly higher in women with POR-LVD as compared with that of NOR-NVD ($P < 0.05$). ***$P < 0.005$; **$P < 0.01$; *$P < 0.05$.

this study, women with POR-LVD, NK, B, and B1 cell populations were significantly higher as compared with those of NOR. NK cytotoxicity of POR-LVD was significantly higher as compared with those of NOR. Increased NK cell cytotoxicity has been reported in women with RPL and IVF failures (Karami et al., 2012; Lee et al., 2016); however, its association with POR has not been elucidated well. $1,25(OH)_2D3$ was reported to decrease NK cytotoxicity and NK-cell degranulation via reduction of NKp30 and NKp44 expression. In addition, $1,25(OH)_2D3$ significantly reduced the expression of TLR4 on NK cell and LPS-induced proinflammatory cytokine production derived from NK cells (Ota et al., 2015). Increased follicular fluid cytotoxic $CD56^{dim}CD16^+$ NK cells were reported in women with unexplained infertility as compared with those of tubal or male factor infertility (Lukassen et al., 2003), and it was correlated positively to day 3 FSH levels and negatively to the number of eggs retrieved. It was suggested that both good ovarian reserve and the response to gonadotropins inversely correlated with the presence of cytotoxic NK cells (Fainaru et al., 2010).

In this study, we report that a presence of inflammatory responses and autoimmunity is associated with POR, which is consistent with previous reports (Merhi, 2014; Beydilli Nacak et al., 2018). TNF-α/IL-10 and IFN-γ/IL-10 producing Th cell ratios were significantly higher in POR-LVD as compared with those of POR-NVD and NOR-NVD. Therefore, the presence of pro-inflammatory immune responses has strong evidence of association with LVD and POR. VD also participates in regulating B cell proliferation and function during a successful pregnancy (Chen et al., 2007). In women with POR-LVD, $CD19^+$ cell levels were significantly higher than those of NOR-LVD, POR-NVD and NOR-NVD and $CD19^+/CD5^+$ cell levels were significantly higher than those of NOR-LVD and POR-NVD. Therefore, B and B-1 cell expansions are associated with both POR and LVD levels. A possible synergetic mechanism of ovarian factors related to POR and low VD level on B and B-1 cell expansion should be explored further.

Folic acid and vitamin B12 deficiencies result in homocysteinemia, which is associated with a variety of adverse pregnancy outcomes, such as RPL, pregnancy-induced hypertension, and abruptio placentae (Steegers-Theunissen et al., 1992; Nelen et al., 2000; Hague, 2003). Additionally, in women with folate deficiency, COH often resulted in lower oocyte quality, lower pregnancy rates, and impaired ovarian function (Forges et al., 2007). Mono-follicular fluid (MFF) from better embryo quality had high cobalamin while MFF from poor embryo quality had high homocysteine (Boxmeer et al., 2009). Interestingly, homocysteinemia is associated with proinflammatory responses in $CD14^+$ $CD16^+$ monocytes and insulin resistance in patients with PCOS (Yang et al., 2016). In our study, homocysteine levels were significantly elevated in women with POR-LVD who had proinflammatory immune responses, which is in line with previous reports. In addition, women with POR-LVD had higher serum PAI-1 level as compared with that of NOR-NVD. Low concentration of 25(OH)VD was reported to be associated with increased PAI-1 and tissue factor pathway inhibitor. Moreover, insufficient 25(OH)VD is related to the proinflammatory condition and greater concentrations of homocysteine (Blondon et al., 2016). In this study, women with POR and LVD had high homocysteine and PAI-1 level, and proinflammatory conditions. Hence, complex metabolic and immunological mechanisms are linked to ovarian dysfunction such as POR. The normal range of homocysteine is less than 10.4 μmol/l, and PAI-1 is the 4–43 ng/ml. The mean of each study group for PAI-1 and homocysteine were in the normal ranges of each testing. It is possible that current cut-off values for general population do not detect women with POR and LVD. New cut-off values and normal ranges for these parameters in reproductive women are needed.

Our study should be interpreted, given its limitations. Since it is a retrospective cross-sectional study, possible patient selection and information bias should be considered, and a cause and effect relationship cannot be inferred. Additionally, 25(OH)VD concentrations were not measured during the POR cycle, and the 25(OH)VD level may not reflect the actual biological effect of VD. The sample size may be limited for the statistical significance of our findings for some biomarkers. Strengths of our study include that it is the first comprehensive combined analysis of metabolic and proinflammatory biomarkers in relationship to ovarian responses, and the study includes the precise measure of peripheral blood autoimmunity, immune effectors and their functions of women with IVF failures.

5. Conclusions

In conclusion, we found that women with POR-LVD had a significantly increased risk for proinflammatory immune responses, homocysteinemia, and increased PAI-1 levels. These findings shed light to explore an underlying pathology of POR and develop a possible therapeutic modality for POR. Whether these possible mechanisms may increase risks for POR and premature ovarian failures, remain to be elucidated.

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Declaration of Competing Interest

No author has any conflict of interest due to relationships with commercial/corporate interest.

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LW developed study design, worked on data acquisition and analysis, and drafted the article. JAV and AS worked on the acquisition of data. MDSG and NS worked on data acquisition and article preparation. SD and AGS revised the manuscript for important intellectual content. JKK supervised the whole project including study design, data analysis and interpretation, and the finalizing the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jri.2019.102617.

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