Journal of Reproductive Immunology 133 (2019) 18–26

Contents lists available at ScienceDirect

Journal of Reproductive Immunology

journal homepage: www.elsevier.com/locate/jri

Metabolic syndrome mediates inflammatory and oxidative stress responses in patients with recurrent pregnancy loss

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ARTICLE INFO

Keywords:
Recurrent pregnancy loss
Metabolic syndrome
Oxidative stress
Inflammation

ABSTRACT

Recurrent pregnancy loss (RPL) is defined as three or more consecutive pregnancy losses prior to the 20th week of gestation. Exaggerated maternal immune response and oxidative stress status have been proposed as one of the main underlying mechanisms for RPL. The aim of this study was to evaluate the role of inflammatory pathway and oxidative stress imbalance in RPL patients with or without metabolic syndrome (MetS). 21 and 28 RPL patients with (RPL-MS) and without (RPL-NMS) metabolic syndrome were enrolled in this clinical study. 42 healthy women also were considered as the control group. The levels of IL1β, IL6, IL17, TNFα, CCL2, CXCL8 were evaluated by ELISA method. Additionally, the oxidative stress biomarkers including TAS, TOS, NO, CAT, SOD, AOPP, MPO were analyzed by spectrophotometry. The expression levels of IL1β, IL6, IL17, TNFα, CCL2, CXCL8, NFκB, AP1, miR-21, miR-146-a, miR-223 were also assessed by real time PCR. The frequency of Th17 and T-reg cells was also measured by flow cytometry. Significant increase in the expression levels of IL1β, IL6, IL17, TNFα, CCL2, CXCL8, NFκB, AP1 and miR-21 was observed in RPL-MS patients. Furthermore, significant decreased expression levels of FoxP3, miR-146-a and miR-223 was also observed in RPL-MS group. The levels of IL1β, IL6, IL17, TNFα, CCL2, CXCL8, NO, MPO and TOS were found to be higher in RPL-MS group compared to the RPL-NMS and healthy controls. In contrast, the level of CAT and SOD in RPL-MS patients was decreased. The frequency of Th17 and Treg cells was also higher and lower in RPL-MS patients compared to the other groups, respectively. Our results support the concept that subclinical inflammatory state, oxidative stress and metabolic syndrome play a crucial role in the etiopathogenesis of RPL assisting clinicians for pregnancy consequences prediction.

Abbreviation: AOPP, advanced oxidation protein products; AP1, activator protein1; APS, antiphospholipid antibody syndrome; CAT, catalase; cDNA, complementary DNA; CTLA4, cytotoxic T-lymphocyte-associated protein 4; CCL2, chemokine (C-C motif) ligand 2; CXCL8, (C-X-C motif) ligand 8; ELISA, enzyme linked immunosorbent assay; FCS, fetal calf serum; FoxP3, fork head winged helix transcription factor; HDL-C, high-density lipoprotein cholesterol; HIF-1α, hypoxia inducible factor 1 α; IFNγ, interferon γ; MetS, metabolic syndrome; miRNA, micro RNA; MPO, myeloperoxidase; MS, metabolic syndrome; NO, nitric oxide; NFκB, nuclear factor κB; NMS, none metabolic syndrome; OS, oxidative stress; PBMC, peripheral blood mononuclear cell; PBS, phosphate buffered saline; PMA, phorbol myristate acetate; PMN, polymorpho nuclear; RM, recurrent miscarriage; ROS, reactive oxygen species; RPL, recurrent pregnancy loss; SOD, superoxide dismutase; TAS, total antioxidant status; TOS, total oxidant status; TGFβ, transforming growth factor β; TH17, T helper 17; TNFα, tumor necrosis factorα; TOS, total oxidant status; Treg, T regulatory

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https://doi.org/10.1016/j.jri.2019.05.001
Received 4 February 2019; Received in revised form 9 March 2019; Accepted 7 May 2019

1. Introduction

Recurrent pregnancy loss (RPL) is a common complication occurred in approximately 15% of all clinically recognized pregnancies resulting in pregnancy failure. RPL is defined when 3 or more successive pregnancy losses are happened prior to 20 week from the last menstrual period, which affects about 5% of women (Medicine, 2013). Although, different studies have been conducted to understand the etiology of this phenomenon, only limited factors have been suggested to be associated with this reproductive disorder. Studies focusing on RPL have examined the anatomic, antiphospholipid syndrome, genetics, age, thrombophilias, autoimmunity, infection, sperm quality, and lifestyle issues factors (Carp, 2014). However, oxidative stress (OS) factors are recently considered as the other potential causes of idiopathic RPL (Gupta et al., 2007). Such inflammatory alterations are indispensable for the different processes of successful embryonic implantation, such as trophoblast invasion, angiogenesis, and placental growth. Although, an uncontrolled and continuous inflammatory responses during pregnancy can damage antenatal and postnatal development, placental growth and even maternal health (Kwak-Kim et al., 2009). OS is one of the substantial factors affecting the pathophysiology of pregnancies. OS is defined as an imbalanced condition between reactive oxygen species (ROS) production and the defensive mechanism offered by antioxidants. Even though, the etiology of RPL in 50% of the cases is unknown, several studies have illustrated the adverse effects of OS during pregnancy complications (Benjamin et al., 2012; Seet et al., 2010). In the present study, total oxidant status (TOS), total antioxidant status (TAS), and the advanced oxidation protein products (AOPPs) were examined instead of separate measurement of oxidant and antioxidants molecules as oxidative stress markers. Metabolic syndrome (MetS) is another complication which could negatively affect pregnancy outcomes. MetS is identified by cluster of conditions such as raised blood pressure, atherogenic dyslipidemia, insulin resistance, glucose intolerance, prothrombotic state, abdominal obesity and proinflammatory state (Expert Panel on Detection, 2001). In principal, the primary mechanisms of the pathophysiology of MetS complications are elevated free radicals and common inflammatory stress situations. The redox imbalance and risk factors that participate in cellular impairment and contribute towards the development of pro-oxidative milieu, lead to biomolecules injuries, which are extremely reactive in nature and can boost tissue and cell dysfunction and eventually result in metabolic diseases (Matsuda and Shimomura, 2014). Available proofs demonstrate that elevation in systemic oxidative stress is closely affiliated with MetS (Reuter et al., 2010; Furukawa et al., 2017). This study aimed to evaluate the oxidative stress-related biomarkers in RPL patients with or without metabolic syndrome.

2. Materials & methods

2.1. Study population

Twenty-eight RPL-none metabolic syndrome (RPL-NMS) and twenty-one RPL- with metabolic syndrome (RPL-MS) patients were recruited in this study. In parallel, 42 healthy women in reproductive age were assigned as the control group. All of the participants have signed the informed consent and enrolled in this study at Al Zahra hospital of Tabriz University of Medical Science from February 2017 to November 2017. The study was approved by the Research Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.REC. 1396.943). Inclusion criteria were willingness to join to this study and being able to give informed consent; age between 18 and 41 with three or more miscarriages before 20th week of gestation. Anatomical abnormalities, infectious, endocrine, or genetic etiologies were also considered as exclusion criteria. The presence of at least three criteria including waist circumference > 88 cm, hypertriacyl glyceridemia ≥150 mg/dL; low levels of high-density lipoprotein cholesterol (HDL-C) < 50 mg/dL; high blood pressure ≥130/85 mm Hg; high fasting blood glucose ≥110 mg/dL were considered for MetS diagnosis. The patients had no chronic or acute inflammatory disease, and used no steroids, anti-inflammatory, or antioxidant medications (Table1). Also, the RPL-MS patients were not taken any medication for their metabolic syndrome disease.

2.2. Separation of peripheral blood mononuclear cells

10 mL of peripheral blood was taken in the luteal phase of menstrual cycle and collected in heparinized tubes. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll. After adding Ficoll (lymphosep) (Biosera, UK) with the density of 1.077 g/mL to the cells and centrifugation process (25 min, 450 g), cells were washed twice with phosphate buffered saline (PBS) (Sigma, Germany). About 5×10^6 cells were cultured in 5 mL of a medium containing 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin and 200 mM L-glutamine. Subsequently, 10 ng/mL of phorbol myristate acetate (PMA) (eBioscience, San Diego, CA, USA) was added to the medium, and cells were incubated for 48 h at 37 °C with 5% CO2. Finally, the cultured

Table 1
Characteristics of patients recruited for the study.

Variable	Control (n = 42)	RPL-NMS patients (n = 28)	RPL-MS patients (n = 21)
Age (years)	26.31 ± 3.81	27.39 ± 4.12	27.14 ± 4.03
Recurrent Miscarriage (RM)	0	28	21
Miscarriage rate	0	4.2 ± 0.75	4.5 ± 1.2
Primary RM	0	10	8
Secondary RM	0	18	13
Blood pressure systolic mmHg	111 ± 16.35	125.9 ± 21.22	142.9 ± 24.31
Blood pressure diastolic mmHg	71.36 ± 14.87	81.89 ± 14.6	91.38 ± 17.64
BMI, kg/m^2	23.17 ± 4.33	24.61 ± 3.92	27.19 ± 6.48
Basic biochemical parameters			
Fasting Blood Sugar, mg/dl	86.24 ± 11.3	89.32 ± 11.62	125.12 ± 28.39*
Total cholesterol, mg/dl	185.16 ± 11.85	222.21 ± 32.16	230.86 ± 41.66**
HDL-C, mg/dl	54.12 ± 3.14	52.48 ± 2.96	43.14 ± 5.62*
LDL-C, mg/dl	60.21 ± 9.30	69.15 ± 8.95	115.36 ± 46.93*
Triacylglycerides, mg/dl	94.68 ± 9.14	101.49 ± 21.87	258.47 ± 55.65*

RPL-MS: Recurrent Pregnancy Loss with Metabolic Syndrome; RPL-NMS: Recurrent Pregnancy Loss without Metabolic Syndrome; BMI: Body Mass Index; HDL-C: High Density Lipoprotein C; LDL-C: Low Density Lipoprotein-C.

* ($P < 0.05$) vs RPL-NMS and Healthy Pregnant Women.

** ($P < 0.05$) vs Healthy Pregnant Women.

cells were used for RNA extraction and the supernatant was exploited for cytokine assessment using enzyme linked immunosorbent assay (ELISA). To evaluate the levels of OS and inflammatory parameters, the serum samples were implemented.

2.3. Treg and Th17 frequency evaluation

For evaluation of T-reg and Th17 cells frequency, different monoclonal antibodies against surface and intracellular antigens were used. To determine Treg cells, samples were incubated with FITC-labeled anti-human CD4, PE-labeled anti-human CD25, and PerCP-Cy5.5-conjugated anti-human CD127 (eBioscience) for 15 min at 4 °C. For intracellular IL-17A staining, cells were stimulated with PMA (25 ng/ml) and ionomycin (1 μg/ml) for 4 h in the existence of monensin (1.7 μg/ml; all from eBioscience, San Diego, CA). For Th17 study, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 at 4 °C for 15 min and then stained with phycoerythrin (PE)-conjugated anti-IL-17A (eBioscience). Cells were evaluated using FACS Calibur flow cytometer. FlowJo software (Becton Dickinson, Mountain View, CA) was used to analyze the flow cytometric data.

2.4. Gene expression analysis

Messenger RNA (mRNA) expressions of the target genes and miRNA quantification were measured by Real-time PCR using SYBR Green method. Total RNA was isolated using RNX-PLUS solution (SinaClon, Tehran, Iran), and complementary DNA (cDNA) was synthesized using a Revert Aid Reverse Transcriptase Kit (Thermo Fisher, Waltham, MA, USA). RNU6B and Beta actin was considered as an endogenous control for miRNA and mRNA respectively. The $2-\Delta\Delta CT$ method was applied to calculate gene expressions relative to the housekeeping control of RNU6B and Beta actin.

2.5. Enzyme linked immunosorbent assay (ELISA)

Quantitative levels of cytokines (IL1β, IL6, TNFα, IL17) and chemokines (CCL2, CXCL8) were measured in the supernatant culture media of PBMCs and serums using ELISA, according to the manufacturer's instructions (Mybiosource, San Diego, USA). This analysis was performed to compare the cytokine and chemokine profile of study groups. All the measurements were conducted in duplicate and the concentrations were calculated from a standard curve according to the manufacturer's protocol.

2.6. Biochemical assays of oxidative stress biomarkers

Erythrocyte catalase activity was measured by kinetic method previously described by Aebi (Aebi, 1984). Briefly, after preparation of RBC hemolysate the optical density of samples containing 2 ml of hemolysate and 1 ml of H_2O_2 was measured in 240 nm at room temperature. Superoxide dismutase activity was determined using Oyanaguis method (Ōyanagui, 1984), The absorbance was measured by Pekrin Almer analyzer at 550 nm and SOD activity was expressed in nitric unit. TAS and TOS levels in the plasma were measured by total antioxidant and oxidant activity method as previously described by Erel (Erel, 2005). The unit was reported in μmol equivalent/l (Erel, 2005). MPO activity was also measured using the protocol defined by Bradly et al. (Bradley et al., 1982). Briefly, read the formation rate of yellowish-orange product of the odianisidine oxidation with MPO in the presence of H_2O_2 was analyzed at 460 nm and the unit of this activity in plasma was reported as (U/L) (Bradley et al., 1982). AOPP was measured using spectrophotometric assay. Briefly potassium iodide (KI) was added to the plasma diluted by PBS and glacial acetic acid was added after 2 min and the absorbance of mixed reaction was immediately read at 340 nm. Chloramine-T and PBS were used as calibrator and blank, respectively (Witko-Sarsat et al., 1996). NO_2^- and NO_3^- quantitation were also estimated as NO production source. Based on Griess reaction, strong absorbance formed by NO_2^- and mixture of naphthethylenediamine and sulfanilamide reaction was detected at 540 nm (Cortas and Wakid, 1990).

2.7. Statistical analysis

Statistical analysis was done by SPSS software (version 23.0; SPSS Inc). Data analysis of three studied groups was carried out by One-Way ANOVA test. For graphs illustration, version 7.00 of GraphPad Prism software (GraphPad Software, La Jolla, CA, USA, www.graph pad.com) was used. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. The frequency of T-reg and Th17 cells

The frequency of T-reg and Th17 cells were measured with CD4 + IL17A and CD4 + CD25 + CD127- phenotypes, respectively, from peripheral blood of study groups using flow cytometry technique. Results revealed significant increase in Th17 cells frequency in both of RPL-MS and RPL-NMS patients compared to the control group. Furthermore, there was a significant increase in Th17 cells frequency in RPL-MS patients compared to RPL-NMS patients. On the other side, T-reg cells frequency were substantially decreased in both of RPL-MS and RPL-NMS compared to the control group. Additionally, T-reg cells frequency in RPL-MS patients showed a significant decrease compared to RPL-NMS (Fig. 1) (Table2).

3.2. mRNA and miRNA expression levels of relevant genes

As shown in Fig. 2, the mRNA level of IL1β, IL6, IL17, TNFα, CCL2, CXCL8, NFκB, AP1 and miRNA level of miR-21 in RPL-NMS patients increased compared to the control group. By contrast, the mRNA level of FoxP3 and miR-146a and miR-223 levels decreased in RPL-NMS patients compared to the healthy control. On the other side, a significant increase in IL1β, IL6, IL17, TNFα, CCL2, CXCL8, NFκB, and AP1 mRNA and miR-21 levels was also observed in RPL-MS patients compared to the both RPL-NMS patients and control group. Also, the expression level of FoxP3, miR-146a and miR-223 in RPL-MS patients showed a significant decrease compared to the both RPL-NMS and healthy controls (Fig. 2) (Table2).

3.3. Cytokine and chemokine secretion analysis

Evaluation of associated cytokines and chemokines in the serum of the study and control groups indicated that IL1β, IL6, TNFα, IL17 secretion levels in RPL-NMS patients were higher compared to the control group. However, no significant changes in the secretion levels of CCL2 and CXCL8 were observed between RPL-NMS and control groups. The results also indicated a significant increased secretion levels of IL1β, IL6, TNFα, IL17, CCL2 and CXCL8 in RPL-MS patients compared to both RPL-NMS and control groups (Fig. 3-A) (Table2). In addition, stimulated supernatant of isolated PBMCs from studied groups were assessed for these cytokines and chemokines. In line with serum samples results, significant increases were seen in IL1β, TNFα, IL17, CCL2 and CXCL8 levels of RPL-MS patients compared to RPL-NMS patients and healthy control groups. Subtle but not significant increase of IL6 level was observed in RPL-MS patients toward NMS-RPL patients. In addition, the levels of IL6, TNFα and IL17 revealed desirable difference between RPL-NMS patients and control group (Fig. 3-B) (Table2).

3.4. Oxidative stress biomarkers analysis

As it is noted in Table 2 and Fig. 4, no significant differences was observed in the level of TOS, TAS, AOPP and NO between RPL-NMS

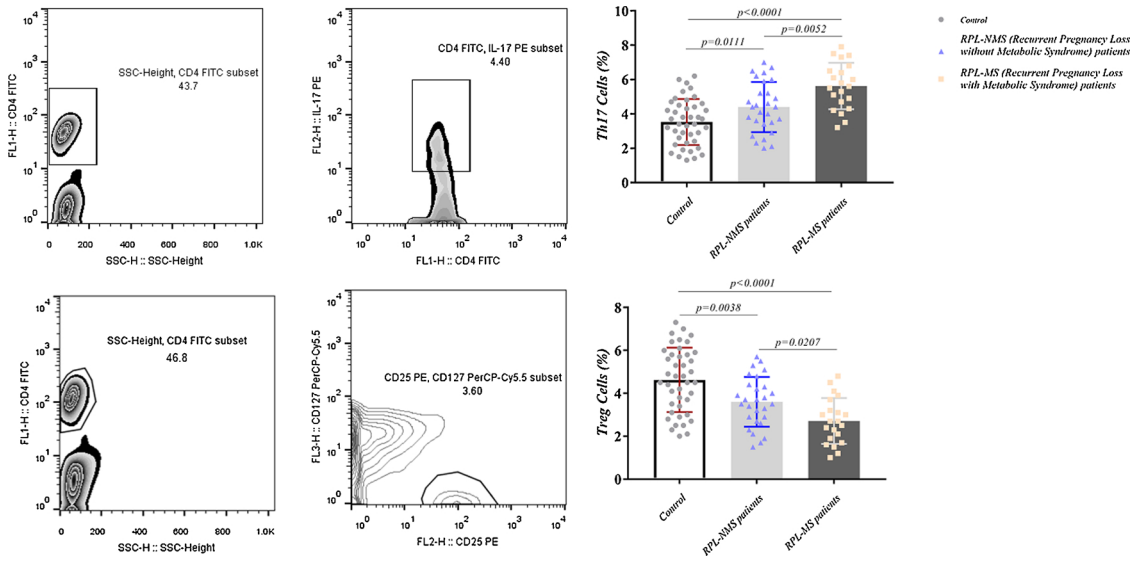

Fig. 1. Flow cytometry analysis of T-reg and Th17 cells in RPL-NMS (n = 28), RPL-MS (n = 21) and control groups(n = 42). Frequency analysis was performed using flow cytometry for evaluation of T-cell subsets in the study groups. The Th17 cells frequency in RPL-MS group was found significantly higher compared to RPL-NMS and control groups. In contrast, T-reg frequency in RPL-MS patients was lower than that of RPL-NMS and the control groups. Results are given as mean ± SD. $p < 0.05$ was considered as statistically significant. RPL: recurrent pregnancy loss; NMS: none metabolic syndrome; MS; metabolic syndrome; T-reg: regulatory T cell; Th17: T helper17; FITC: fluorescein isothiocyanate.

patients and control group. However, the level of CAT and SOD in both RPL-NMS and RPL-MS patients showed a significant decrease compared to control group. Additionally, the MPO level in RPL-NMS patients was also higher compared to the control group. The results further illustrated the significant increase in the level of TOS, MPO and NO in RPL-MS patients compared to both RPL-NMS and control groups. The findings also showed a significant increase in the level of TAS and AOPP in RPL-MS patients compared to the control group, however, the mentioned parameters showed no significant changes in RPL-NMS patients compared to control group(Fig. 4) (Table2).

4. Discussion

Recurrent pregnancy loss has a major psychological and emotional effect on couples. The immune system of pregnant women is strongly organized to protect the body against microbial infections, inflammation-like processes, fetus preservation, proper decidua differentiation, transformation and tissue growth during pregnancy. Unwanted immunological deviations may lead to reproductive disorders, such as preterm birth, preeclampsia, intrauterine fetal growth restriction, implantation failure, as well as RPL. In humans, increased and decreased concentration of the Th1 (IL-2, TNFα and IFN-γ) and Th2 (IL-10, IL4, IL5 and IL13) cytokines have been reported to be correlated with recurrent miscarriage, respectively (Lim et al., 2000). Murine studies have displayed that the predominant Th1 immunity is associated with fetal resorptions and implantation failure (Lim et al., 2000). The Th2 produced cytokines at the materno-fetal interface are supposed to be useful for pregnancy conservation because of their suppressive capabilities of cellular cytotoxicity and trophoblastic cells development stimulation (Jenkins et al., 2000; Lim et al., 2000). However, Bick et al. (Bick and Hoppensteadt, 2005) stated a higher occurrence of immunological alterations and coagulation among patients with RPL. The imbalance of serum TNF-α level is one example of a dysregulated inflammatory status in RPL patients. Serum TNF-α level have been identified to be directly related with abortion which is escalated in IL-17^+ T cells, stimulating enhanced inflammatory reactions. Calleja-Agius et al. (Calleja-Agius et al., 2011) also reported that the TNF-α serum level is elevated in euploid miscarriages compared to aneuploid counterparts. The production of IL-6, INF-γ and TNF-α were also meaningfully higher in abortions compared to enduring gestations (Wegmann et al., 1993). The proper equilibrium of cytokine and chemokine expression at the maternofetal interface can manage the function and profile of immune cells within the decidua. Evidences supporting this research demonstrated that the administration of one of the Th-1-related cytokines to normal pregnant mice causes miscarriage by enhancing embryonic resorption rates (Chaouat et al., 1990). Other investigations in mice models have revealed that enhanced levels of IL-6 at the maternal–fetal interface are associated with fetus loss (Zenclussen et al., 2003). In our study the secretion levels of cytokines (IL1β, IL6, IL17 and TNFα) were higher in RPL-NMS compared to control group and also there was significant difference in the cytokines level among RPL-NMS and RPL-MS. There are rising evidences that Th17 and T-reg cells are involved in conservation and establishment of pregnancy as effector and regulator cells, respectively (Lee et al., 2011). IL-17 is known to arouse inflammation through neutrophil infiltration and stimulation of TNF-α, IL-8, IL-1, IL-6, nitric oxide, receptor activator for nuclear factor κB ligand (RANKL), matrix metalloproteinase, and granulocyte-macrophage colony stimulating factor (GM-CSF) production (Nakashima et al., 2010). Nakashima et al. (Nakashima et al., 2010) reported the increased IL-17^+ T cells in the decidua in patients with unavoidable miscarriage. It has also been proved that peripheral blood $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells are slowly elevated during the first and second trimester of the pregnancy and in the third trimester and postpartum are decreased (Somerset et al., 2004). Several studies have illustrated that T-reg cells reduced in the deciduas and/or peripheral blood in women with RPL (Lee et al., 2012). Jasper et al. (Jasper et al., 2006) demonstrated the down-regulation of FoxP3 mRNA in endometrial tissue in women suffering from primary unexplained miscarriage. Sasaki et al. (Sasaki et al., 2004) also stated the relationship between T-reg cells and unexplained miscarriage. According to the data gotten from this study, there was significant difference in Th17 and T-reg frequency in RPL-NMS and RPL-MS patients. It has been assumed that some chemokine receptors and their ligands are involved in recruitment of regulatory T-cell into the deciduas and endometrium. Arici et al. (Arici et al., 1995) also showed that CCL2 is produced in the human endometrium. Additionally, Critchley et al. (Critchley et al., 1999) explained higher CCL2 levels in primary phase of pregnancy than that of non-pregnant women. Fest

Table 2
Comparison of cellular and molecular parameters in RPL-MS, RPL-NMS and control groups.

Measurement	Control (n = 42) A	p-value A vs. B	RPL-NMS patients (n = 28) B	p-value B vs. C	RPL-MS patients (n = 21) C	p-value A vs. C
Mean level of gene expression ± Standard Deviation in activated PBMC (fold change)						
Measurement	Control (n= 42) A	p-value A vs. B	RPL-NMS patients (n = 28) B	p-value B vs. C	RPL-MS patients (n = 21) C	p-value A vs. C
IL1β	1.01 ± 0.03758	0.0007	1.077 ± 0.07963	0.0003	1.161 ± 0.1047	< 0.0001
IL6	1.009 ± 0.06417	0.0014	1.092 ± 0.09758	0.0021	1.187 ± 0.1326	< 0.0001
IL17	1.005 ± 0.02662	0.0023	1.091 ± 0.1154	0.0004	1.207 ± 0.161	< 0.0001
TNFα	1.007 ± 0.03518	0.0027	1.098 ± 0.1164	0.0002	1.232 ± 0.1795	< 0.0001
CXCL8	1.011 ± 0.02925	0.0007	1.118 ± 0.07661	< 0.0001	1.383 ± 0.2187	< 0.0001
CCL2	1.011 ± 0.04574	< 0.0001	1.214 ± 0.1433	0.0042	1.326 ± 0.1701	< 0.0001
FoxP3	1.016 ± 0.04295	0.0149	0.9671 ± 0.05695	0.0023	0.8971 ± 0.1153	< 0.0001
NFKB	1.005 ± 0.07015	0.0075	1.111 ± 0.1424	0.0014	1.256 ± 0.2173	< 0.0001
AP1	1.015 ± 0.04608	0.0035	1.111 ± 0.139	0.0001	1.259 ± 0.1768	< 0.0001
miR-21	1.007 ± 0.04651	0.011	1.083 ± 0.1076	0.0006	1.2 ± 0.1682	< 0.0001
miR-146a	1.005 ± 0.05018	0.001	0.95 ± 0.0545	0.0026	0.89 ± 0.08343	< 0.0001
miR-223	1.009 ± 0.04242	0.0024	0.9514 ± 0.05797	0.0004	0.8729 ± 0.1106	< 0.0001
Level of cytokines and chemokines in cell culture supernatant (pg/ml)						
IL1β	299.1 ± 141.5	NS	356.3 ± 145.7	< 0.0001	653 ± 253.6	< 0.0001
IL6	359.6 ± 115.4	0.0018	476.7 ± 157.2	NS	597.3 ± 241.7	< 0.0001
IL17	197.8 ± 70.12	0.0019	288.9 ± 139.7	0.0373	400.8 ± 173.6	< 0.0001
TNFα	416.6 ± 281.6	0.0169	661.3 ± 365.4	0.0018	1027 ± 468.3	< 0.0001
CCL2	301.3 ± 169.4	NS	339.8 ± 205.8	0.0039	547.7 ± 225.6	< 0.0001
CXCL8	59.79 ± 24.22	NS	65 ± 26.75	0.02	94.57 ± 40.64	0.0002
Level of cytokines and chemokines in serum (pg/ml)						
IL1β	92.21 ± 43.45	0.0241	126.3 ± 52.45	0.0004	186.1 ± 66.37	< 0.0001
IL6	62.33 ± 19.96	0.0002	94.98 ± 30.05	0.048	116.9 ± 48.66	< 0.0001
IL17	67.58 ± 20.46	0.0021	105.3 ± 49.37	0.0005	155.7 ± 66.78	< 0.0001
TNFα	39 ± 27.11	0.0144	64.36 ± 34.56	0.0004	106.3 ± 51.75	< 0.0001
CCL2	113.9 ± 64.01	NS	154.6 ± 93.52	0.0077	226.7 ± 93.31	< 0.0001
CXCL8	19.05 ± 7.717	NS	21.46 ± 8.863	0.0069	29.96 ± 12.84	0.0001
Oxidative stress status parameter in serum/Plasma						
NO (μmol/l)	17.81 ± 8.067	NS	19.52 ± 8.791	0.0037	27.71 ± 9.177	0.0001
AOPP (μmol/l)	34 ± 6.541	NS	36 ± 7.698	NS	39 ± 7.861	0.0299
TOS (μmol equivalent/l)	14.98 ± 9.331	NS	15.99 ± 10.96	0.0202	24.38 ± 12.44	0.0038
TAS (μmol equivalent/l)	1.6 ± 0.225	NS	1.72 ± 0.2427	NS	1.783 ± 0.3038	0.0203
SOD (NU/ml)	10.92 ± 2.618	0.0015	8.604 ± 2.741	NS	8.267 ± 2.529	0.0009
CAT (U/mg Hb)	333.8 ± 103.5	0.0444	274.8 ± 101	NS	264.3 ± 109.2	0.0423
MPO (U/l)	66.86 ± 17.57	0.0003	85.86 ± 20.41	0.001	106.6 ± 21.01	< 0.0001
T Cell frequency in PBMCs (%)						
Th17	3.526 ± 1.338	0.0111	4.400 ± 1.465	0.0052	5.619 ± 1.349	< 0.0001
T reg	4.621 ± 1.499	0.0038	3.600 ± 1.155	0.0207	2.710 ± 1.066	< 0.0001

IL1β: Interleukin 1β; IL6: Interleukin 6; IL17: Interleukin 17; TNFα: Tumor Necrosis Factor α; CCL2: C-C Motif Chemokine Ligand 2; CXCL8: CXC Motif Chemokine Ligand 8; AP1: Activation Protein-1; NFκB: Nuclear Factor- κB; FoxP3: forkhead box P3; miR21: Micro RNA 21; miR146-a: Micro RNA 146-a; miR223: Micro RNA 223; NO: Nitrite Oxide; SOD: Superoxide Dismutase; CAT: Catalase; TOS: Total Oxidative Stress; MPO: Myeloperoxidase; AOPP: Advanced Oxidation Protein Products; Th17: T helper 17 cell; T reg: Regulatory T cell.

et al. (Fest et al., 2007) also reported the communication between macrophage-trophoblast in pregnancy protection, so that trophoblast cells augment the production of CCL2 which is beneficial in trophoblastic development and survival. In this study, a significant increase in CCL2 and CXCL8 levels was also observed in the RPL-MS groups. Several studies propose that the CCL2/CCR2 pathway has a significant role in insulin resistance owing to macrophage infiltration into adipose tissue, leading to systemic inflammatory and metabolic consequences (Kang et al., 2011). Although various molecular mechanisms participate in the development of obesity related complications, recent studies (Kang et al., 2011) report that inflammation, particularly that resulting from monocytes/macrophages, is an essential pivot in the pathophysiology of numerous obesity-related complaints. Macrophages are interestingly versatile cells, and their secretion, production and regulatory properties concerning inflammatory molecules are not restricted to adipose tissue; their activities also befall in other organ tissues (Kang et al., 2011).

Furthermore, elevated CCL2 levels may mediated reproductive failure by attenuation of the regulatory mechanisms between desidual NK cells and peripheral blood NK cells (Fest et al., 2007). Moreover, Li and Colleague (Li et al., 2016) demonstrated raised levels of CCL2 mRNA in both chorionic tissues and decidua, signifying an important role of CCL2 in the incidence of pregnancy loss. CCL2 has been exposed to be related to premature birth, thereby proposing its contribution in pregnancy disorders. In the first trimester of pregnancy, macrophages released high amounts of CCL2 under the induction of fetus implantation into trophocytes, thus recruiting and stimulating mononuclear-macrophages in addition to the release of cytokines for the induction of central immune suppression and fetal nutrition. The boost of CCL2 expression in decidual tissues may cause failings in macrophage recruitment and consequence in augmented secretion of inflammatory cytokines including IL-1, TNF-α and INF-γ, some of which may further enforce CCL2 expression. This would form a positive feedback loop and result in pregnancy failure. This study demonstrates a higher CCL2 concentration during the luteal phase of patients with unexplained RPL but the accurate mechanism is unidentified.

There are limited evidences on CXCL8 levels in abortion (Madhappan et al., 2003). According to Madhappan et al. (Madhappan

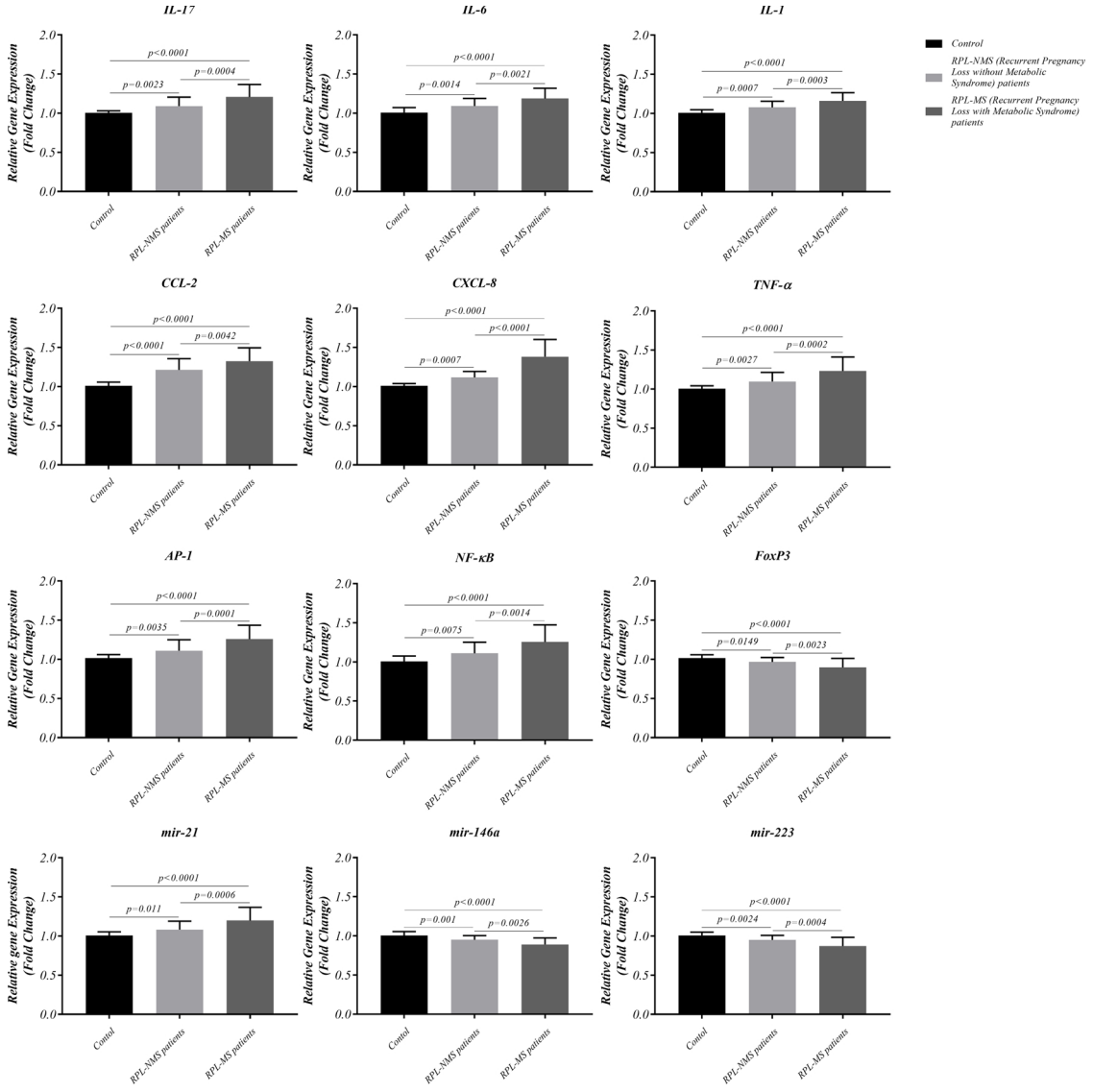

Fig. 2. The mRNA and miRNA expression level in RPL-NMS (n = 28), RPL-MS (n = 21) and control groups (n = 42). The mRNA expression level of IL1β, IL6, TNFα, IL17, CCL2, CXCL8, NFκB, AP1 and, miR-21 were found significantly higher in RPL-MS patients compared to RPL-NMS and control groups, whereas the mRNA expression level of FoxP3 as well as miR-223 and miR-146-a were revealed significantly lower in RPL-MS patients compared to other groups. The level of IL1β, IL6, TNFα, IL17, CCL2, CXCL8, NFκB, AP1 and miR-21 in RPL-NMS patients found to be slightly higher compared to the control group. The level of FoxP3, miR-223, miR-146-a in RPL-NMS group were faintly lower than that of the control group. Results are given as mean ± SD. $p < 0.05$ was considered as statistically significant. mRNA: messenger RNA; miR: microRNA; RPL: recurrent pregnancy loss; MS: metabolic syndrome; NMS: none metabolic syndrome; IL: interleukin; TNFα: tumor necrosis factor α; CCL2: chemokine (C−C motif) ligand 2; CXCL8: (C−X−C motif) ligand 8; FoxP3: forkhead box P3; NFκB: nuclear factor κB; AP1: activator protein 1.

et al., 2003) findings, higher levels in products of conception observed in women with two or more abortions compared to women with the first abortion experience or normal pregnancy. AP-1 and NF-κB are the key transcription factors that coordinate expression of numerous genes involved in lymphoid differentiation, embryonic development, inflammation, apoptosis, as well as oncogenesis (Li and Verma, 2002). According to our finding the expression level of AP-1 and NF-κB in RPL-MS patients was significant higher compared to both of RPL-NMS patients and control group. Several inflammatory stimuli such as extreme ROS/RNS are produced during oxidative metabolism and some artificial or natural chemicals have been reported to begin the inflammatory process resulting in pro-inflammatory cytokines synthesis and secretion (Roebuck, 1999). During pregnancy, development of OS could defect placental expansion and/or increase pregnancy loss. Oxidative ingredients are often free oxygen radicals and peroxides that are typically formed in small quantities. They are formed through various pathways such as oxidative phosphorylation in mitochondria, and when the tissue is exposed to ischemia/reperfusion damage, they are generated in larger amounts. Owing to their extremely reactive features, they can cause functional and structural damage to proteins, cellular DNA and cell membranes (Buonocore et al., 2010). It has been proven that, nitrative stress, as well as oxidative stress, play a significant role in pregnancy failure, following covalent DNA and proteins modification and nitration by peroxynitrite (Agarwal et al., 2012). During pregnancy, the placenta is a place of active oxygen metabolism that constantly produces OS. During the first trimester of pregnancy, oxygen pressure is about 20 mmHg in the intervillous space. Placental tissues comprise a low activity of antioxidants such as SOD, CAT, GPx, Mn and Cu/Zn, because of low concentration of syncytiotrophoblasts, sensitizing the tissue against OS (Agarwal et al., 2012). Overproduction of ROS and reactive nitrogen species (RNS) can also corrupt ordinary placental functions. Consequently, the feto-placental unit produces plentiful antioxidants to retain OS under control. Controlled oxidative species have been reported to act as crucial cellular signaling

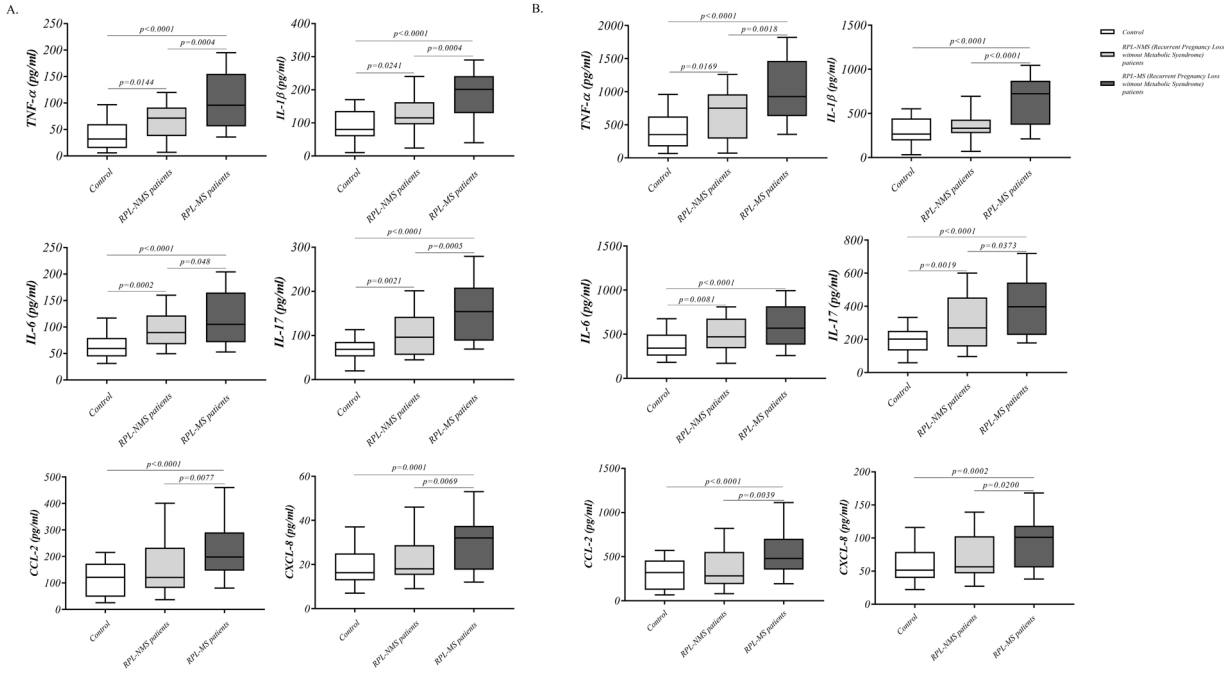

Fig. 3. The secretion level of inflammatory cytokines (IL1β, IL6, TNFα, IL17) and chemokines (CCL2, CXCL8) in RPL-NMS (n = 28), RPL-MS (n = 21) and control group (n = 42). A. The serum level of IL1β, IL6, TNFα, IL17, CCL2 and CXCL8 in RPL-MS patients was significantly increased compared to RPL-NMS and the control groups. There were no markedly increases in the level of CCL2 and CXCL8 in RPL-NMS group compared to the control group. B. The cell culture supernatant level of IL1β, TNFα, IL17, CCL2 and CXCL8 in RPL-MS patients was significantly increased compared to RPL-NMS and the control groups. No remarkable difference was seen in IL6 level between RPL-MS and RPL-NMS patients. Also, there were no markedly increases in the level of IL1β, CCL2 and CXCL8 in RPL-NMS group compared to the control group. $p < 0.05$ was considered as statistically significant. Whiskers and boxes demonstrate Min-Max and 25%–75% of data respectively in representative plots. RPL: recurrent pregnancy loss; MS: metabolic syndrome; NMS: none metabolic syndrome; IL: interleukin; TNFα: tumor necrosis factor α; CCL2: chemokine (C−C motif) ligand 2; CXCL8: (C−X−C motif) ligand 8.

messengers by adjusting the gene expression and downstream cellular activities. It has been revealed that OS may lead to complications such as preeclampsia, recurrent abortions and congenital disorders in diabetes (Agarwal et al., 2012). Anomalous placentation in the early stages of pregnancy leads to OS and subsequent endothelial dysfunction leading to advent complications of pregnancy such as miscarriage. In 2003, safronova et al. (Safronova et al., 2003) found that the production of active oxygen species in the granulocytes of RPL patients is higher compared to normal reproductive function. OS has also been implicated as a decisive cause of RPL. Lack or shortage of antioxidant defense has been demonstrated to be associated with RPL (Agarwal et al., 2012). OS is also well recognized to induce the caspase cascade leading to cell death in other systems. It has also been reported that concentration of lipid peroxides rise in the decidua of women experiencing primary pregnancy loss (Sugino et al., 2000).

Antioxidants are classified as enzymatic and nonenzymatic. Common enzymatic systems include glutathione reductase, GPX, SOD, and CAT. Nonenzymatic agents are α-tocopherol (vitamin E), ascorbic acid (vitamin C), ceruloplasmin, ferritin, and transferrin. Interestingly, decreased levels of GPX, vitamin A, vitamin E, β-carotene, SOD and CAT, and elevated levels of reduced glutathione (GSH) are generated to compensate for the enhanced ROS levels as stated in patients with RPL (Safronova et al., 2003). This may lead to unusual placentation during the early pregnancy that result in syncytiotrophoblast destruction, consequently contributing to RPL. SOD catalyzed the conversion of superoxide anions to hydrogen peroxide (H_2O_2). Then, CAT converts the produced H_2O_2 to water. Three factors have been explained for pathogenesis of missed abortion: First, the ROS can induce lipid peroxidation damage in the fetus. Second, elevated ROS can alter the oxygen partial pressure in embryonic cells. And third, enhanced production of ROS leads to a malicious circle of ischemia-reperfusion damage in the fetus and partial growth (Zhu et al., 2014). Zhu lj et al. (Zhu et al., 2014) showed that HIF-1α, ROS and SOD levels could be pivotal in missed abortion. Moreover, Ishii et al. (Ishii et al., 2016) declared that OS may cause embryopathy in early pregnancy and growth termination through extreme apoptosis leading to inflammation-mediated placental angiodysplasia and recurrent miscarriage. Results of this study shows significant increase and not remarkable change of oxidant and anti-oxidant elements respectively in RPL-MS patients toward RPL-NMS patients and control group. This condition may disrupt the balance between oxidative and anti-oxidative factors and probably lead to more oxidative and inflammatory activity in these patients that increase the risk of abortion.

A number of microRNAs have also been described as differentially regulated in systemic inflammatory disorders. Since, microRNAs act in the upstream of many immune functions, they may establish more immediate and trustworthy information on pregnancy risk compared to the generally used laboratory tests. For instance, miR-223 in addition to its outstanding function in polymorphonuclear (PMN) expansion, as well as regulating their activation, controls inflammatory responses of mononuclear phagocytes by modulating NF-κB kinase subunit α (IKKα) inhibitor expression during monocyte-to-macrophage lineage development (Hulsmans et al., 2011). Also, miR-21 is described to controls the levels of ROS by targeting SOD (Hulsmans et al., 2011), whereas, miR-146a is reported to decrease ROS and modulate macrophage polarization through CAT pathway (Hulsmans et al., 2011). The results of our study showed that the expression level of miR21 in the RPL-MS group significantly higher compared to both of RPL-NMS patients and control group while the expression level of miR146-a and miR223 in the RPL-MS patients significantly declined compared to both of RPL-NMS and control group. Enhanced body weight along with metabolic disorders including type 2 diabetes, insulin resistance and cardiovascular complications constitute metabolic syndrome (MetS) (Matsuda and Shimomura, 2014). Different factors are implicated in the MetS pathogenesis. However, a number of studies have demonstrated, chronic inflammatory status along with OS, pave the way for progress of metabolic disorders. Furukawa et al. (Fujita et al., 2006) showed an association between systemic oxidative stress and fat accumulation in humans and mice. Furukawa et al. (Redecha et al., 2009) showed that generation of free

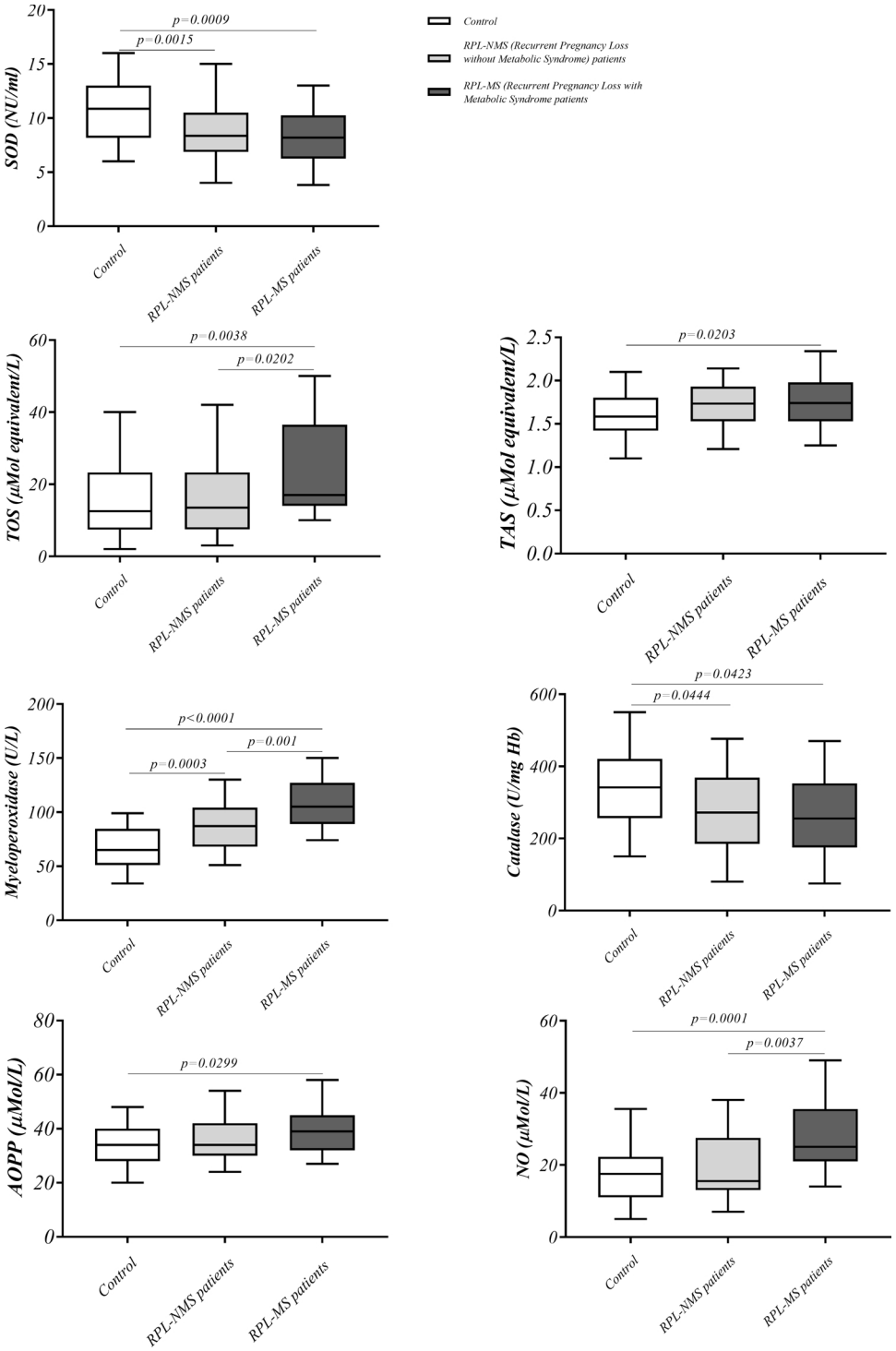

Fig. 4. The level of oxidative stress factors (NO, CAT, MPO, TAS, TOS, AOPP, SOD) in RPL-NMS (n = 28), RPL-MS (n = 21) and control groups (n = 42). The level of NO, MPO and TOS in RPL-MS patients were significantly increased compared to RPL-NMS and the control groups. The results further illustrated that, the levels of SOD and CAT in RPL-MS patients compared to the control group, have significantly decreased. There was no statistically significant differences in the level of NO, TAS, TOS and AOPP among RPL-NMS patients and control group. However, the level of MPO significantly increased in RPL-NMS patients compared to the control group. There was no substantial differences in the level of TAS among RPL-MS and RPL-NMS groups. $p < 0.05$ was considered as statistically significant. Whiskers and boxes demonstrate Min-Max and 25%–75% of data respectively in representative plots. AOPP: advanced oxidation protein products; CAT: catalase; MPO: myeloperoxidase; NO: nitric oxide; SOD: super oxide dismutase; TAS: total antioxidant status; TOS: total oxidant status; RPL: recurrent pregnancy loss; MS: metabolic syndrome; NMS: none metabolic syndrome.

radicals (RONS) enhances in adipose organ of overweight persons is concomitant by increased level of NADPH oxidase and decreased level of antioxidant enzymes. Additionally, elevated levels of RONS, small total antioxidant capacity (TAC), in obese individuals, has led to OS elevation and MetS phenotype formation. The achieved outcomes demonstrated that OS is significantly raised in obese patients, particularly in those displaying a MetS phenotype.

5. Conclusion

In our study, we found a significant increase of MPO, NO and TOS and substantial decreases of CAT and SOD in RPL women with metabolic syndrome. These parameters may be valuable in the prediction or risk assessment of RPL, however, the need for further and more extensive investigations is obvious. Oxidative stress is present in most tissues exposed to high oxygen metabolism such as the placenta. There is a developing confluence of opinion which recommends that OS is one of the major underlying mechanisms in the pathogenesis of a continuum of disorder processes such as preeclampsia, hydatidiform mole, and RPL. OS and ROS-induced injury may be the missing fragments of the puzzle of miscarriage and RPL of unexplained etiology. Establishment and conservation of pregnancy are critical challenges for the maternal immune system. Prosperous pregnancy requires a rational communication between maternal immune system and fetal cells. Each

side includes mechanisms boosting maternal tolerance of the fetus, and fetal tolerance of the mother, to inhibit inflammation and fetal loss. The maternal reproductive system displays cyclical alterations in immune features to protection the fetus as soon as possible, and once pregnancy is established, both mother and fetus cooperate via overlapping mechanisms to confirm success.

Author contribution

R. Azizi wrote the paper; M. Yousefi: supervised the work and provided the comments and additional scientific information; MS. Soltani-Zamgbar: performed the statistical analysis: G. Sheikhansari and Z. Pourmoghadam: contributed to the conception and the main idea of the work; L. Koushaein: helped with patient management and monitoring. S. Sandoghchian: Contributed to cell collection and helped with immunological tests; H. Samadikafil: Contributed to flowcytometric analysis; M. Mahdipour: Participated in the final edition of the manuscript; A. Mahdizadeh: Contributed to data analysis and study design; Sh. Danaii: Responsible for subject selection, monitoring, and inpatient and outpatient care.

Conflict of interests

Authors declare no conflict of interests.

Acknowledgements

We wish to thank all the healthy volunteers and patients who participated in this study. This study was financially supported by the grant of Stem Cell Research Center, Tabriz University of Medical Sciences, Tabriz, Iran (Grant No. 59661).

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