Chemokine (C-C motif) ligand-2 (CCL2) and oxidative stress markers in recurrent pregnancy loss and repeated implantation failure

Müberra Namlı Kalem¹ · Nilufer Akgun² · Ziya Kalem³ · Batuhan Bakirarar⁴ · Tugrul Celik⁵

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Abstract

Purpose The main purpose of this study is to evaluate the inflammatory response and oxidative stress together in the cases of recurrent pregnancy loss (RPL) and repeated implantation failure (RIF).

Methods This is a cross-sectional study conducted to compare infertile patients who have RIF and patients with RPL histories in terms of CCL2, TAS, TOS, and OSI. To this end, two study groups were formed by primary-infertile women with RIF history and women with nulliparous RPL history who consulted a university hospital between 2014 and 2016, and a control group was formed by multiparous women who had

no pregnancy loss. With 30 women in each group, 90 women in total were included in the study. CCL2, TAS, and TOS blood levels were measured and oxidative stress index was calculated in all participants.

Results The patients with RPL and RIF had higher levels of CCL2 than those in the control group. The TOS, TAS, and OSI levels did not differ in RPL and RIF groups from the control group. No statistically significant relationship was found between CCL2 and the TOS, TAS, and OSI values.

Conclusions Oxidative stress markers in the pregestational period did not have a predictive value in the RPL and RIF. CCL2 might be useful in risk prediction.

✉ Müberra Namlı Kalem
muberranamli@hotmail.com

Nilufer Akgun
niluferakgun80@hotmail.com

Ziya Kalem
ziyakalem@hotmail.com

Batuhan Bakirarar
batuhan_bakirarar@hotmail.com

Tugrul Celik
htugrulcelik@gmail.com

Keywords CCL2 · Oxidative stress index · Recurrent implantation failure · Recurrent pregnancy loss

Introduction

Inflammatory immune responses play a critical role in the female reproductive system [1–3]. Excessive or the inappropriate recruitment process of inflammatory cells in peripheral blood may cause a cytotoxic micro-environment in the uterus, impairing trophoblast proliferation and invasion, leading to abnormal vascular development and blood flow impeding the implantation and development of the embryo [4, 5].

Oxidative stress is due to the imbalance between free radical production and indigenous antioxidant defense mechanisms: an increase in reactive oxygen species (ROS) and/or decrease/deterioration in the function of the antioxidant system, causing oxidative stress. Impairment of the natural antioxidant defense system in the body may create an unsuitable hypoxic environment in the female reproductive physiology [6].

¹ Department of Obstetrics and Gynecology, Liv Hospital Ankara, Kavaklıdere, Bestekar sokak No: 8 066680, Çankaya, Ankara, Turkey

² Department of Obstetrics and Gynecology, American Hospital, Istanbul, Turkey

³ Gurgan Clinic, IVF and Women Health Center, Department of IVF, Ankara, Turkey

⁴ Department of Biostatistic, Ankara University, Ankara, Turkey

⁵ Department of Biochemistry, Cankaya Hospital, Ankara, Turkey

For a successful pregnancy to be commenced and maintained, both the inflammatory and oxidative processes need to have a certain balance and their interaction needs to be appropriate. It has been reported that defects in the interaction of these systems themselves or between each other may cause failure in fertilization or loss of pregnancy [4, 6].

Recurrent pregnancy losses (RPL) or repeated implantation failures (RIF) are problems upon which pathophysiology has yet to shed light and therefore, it is one for which a precise success cannot be promised in the management process. The immune system and oxidative processes appear to be guilty of both, although RPL and RIF pathophysiology is a quite different entity and current treatment studies are carried out through immune-modulator, anti-inflammatory, and antioxidant therapies [7, 8].

The aim of this study was to jointly evaluate inflammatory response and oxidative stress in cases of recurrent pregnancy loss and repeated implantation failure.

Chemokine (C-C motif) ligand 2 (CCL2)/monocyte chemoattractant protein-1 (MCP-1) was chosen as the indicator of inflammatory immune response (MCP-1 is the alias for the current gene symbol CCL2. In this study, the term CCL2 was preferred to MCP-1). This is because CCL2 is one of the key chemokines that is excreted in response to pro-inflammatory cytokines and that regulates monocyte/macrophage migration and leukocyte activation in the endometrium during the preparation process for implantation [9]. Including the Ca^{2+} flux and integrin expression, chemotaxis of monocytes and several cellular events related to chemotaxis are instigated by CCL2. Also, being a weak inducer of cytokine expression in monocytes, it stimulates a respiratory burst that leads to the generation of reactive oxygen species at high concentrations [10].

Instead of separate measures for oxidant molecules and antioxidants as oxidative stress markers, total antioxidant status (TAS), total oxidant status (TOS), and the oxidative stress index (OSI) were examined.

Materials and methods

This is a cross-sectional study conducted to compare infertile patients who have RIF and patients with RPL histories in terms of CCL2, TAS, TOS, and OSI. To this end, two study groups were formed with primary-infertile women with an RIF history and women with a nulliparous RPL history, who consulted a university hospital between 2014 and 2016 and a control group that was formed with multiparous women who had no pregnancy loss. As 30 women comprised each group, a total of 90 women were included in the study. The recurrent implantation failure group was formed on the basis of RIF criteria, as defined by Coughlan et al. in 2014: the “failure to achieve a clinical pregnancy after transfer of at least four good-quality embryos in a minimum of three fresh or frozen

cycles in a woman under the age of 40 years” [11]. Those who had three or more successive pregnancy losses were included in the RPL group [12]. Since different etiologies are probable in different trimester pregnancy losses, only the patients who had first-trimester losses were included in the RPL group in the study. The control group was composed of the multiparous women who had live births at least twice to term without any pregnancy loss.

The criteria that were taken as the basis for the study were that the participants were younger than 40 years of age, had a BMI lower than 29.9, would not get pregnant during the study, had no chronic or acute inflammatory disease, and had not used any steroids, anti-inflammatory, or antioxidant medication. Patients with diabetes, hypertension, chronic systemic diseases, thyroid disorders, and uterine malformation and patients who had previous uterine surgery and had diagnosed endometriosis were not included in the study. Pelvic ultrasonography (USG), hysterosalpingography (HSG), and diagnostic hysteroscopy results and uterine evaluation results of all the patients in RIF and RPL groups were normal and antiphospholipid antibody screens and karyotype analyses were concluded to be normal. As the control group comprised multiparous women who consulted for routine gynecological check, uterine work-up, antiphospholipid antibody screenings, and karyotype analyses were ignored.

Approval of the ethical committee and the written consent of all the patients were obtained for this study. The study was conducted in accordance with the principles of the Declaration of Helsinki.

All blood samples were collected at the secretory phase of the menstrual cycles for consistency. To this end, blood samples were taken 2–4 days following ovulation, from the patients whose ovulation was detected using transvaginal USG or estradiol and progesterone measurements in mid-cycle. Contraception was recommended for all the women in the cycle during which the blood samples were taken, and it was confirmed that none of them had become pregnant at the end of this cycle. Patients' peripheral venous blood samples were taken into heparin tubes from the cubital vein, following a nighttime fast of at least 8 h, and immediately centrifuged for 10 min at 3000 rpm. The plasma samples were stored at -80°C until the day of analysis. After all the blood samples had been taken, CCL2, TAS, TOS, and OSI levels were measured.

The serum CCL2 level was analyzed using the standard enzyme-linked immunosorbent assay (ELISA) method, and the ELISA kit used in the analysis was *MCP1/CCL2 pg/ml* (BOSTER Code: EK0441 Lot No: 16,011,109,510, Abcam, Cambridge, UK). The results are expressed in picograms per milliliter.

The TAS level measurement in the plasma was made using the current and automated total antioxidant activity method, defined by Erel. The results of the measurement were unitized in $\mu\text{mol Trolox equivalent/l}$ [13].

The serum TOS measurement was done using the method defined by Erel. The results of the measurement were unitized in mmol H₂O₂ Equiv./L [14].

The OSI value was accepted to be the % rate of TAS and TOS values and calculated with the formula below:

$$\text{OSI} = \frac{\text{TOS, } \mu\text{mol H}_2\text{O}_2\text{Equiv./L.}}{\text{TAS, mmol Trolox Equiv./L}} \times 100$$

The results are expressed in arbitrary units (AU).

Statistical analysis

All statistical analyses were performed using the SPSS for Windows 11.5 software program (SPSS Inc., Chicago, IL). For the quantitative variables, mean $\pm$ sd and median (min-max) were used as descriptors in the study. When identifying whether there was a statistically significant difference between the categories of a qualitative variable with three or more categories in terms of a quantitative variable, one-way ANOVA was used if the normal distribution assumptions were met; if not, Kruskal-Wallis test was used. When looking to see if there was a statistically significant relationship between two quantitative variables, Pearson's correlation coefficient was used when both of the variables met the normal distribution assumptions, whereas the Spearman's rank correlation coefficient was used when at least one of the variables did not meet the normal distribution assumptions. Significance level was set at $p = 0.05$.

Results

The participants' demographics and the laboratory results by group are given in Table 1. There is no statistically significant difference among three groups (control, RIF, and RPL) in terms of age and BMI ($p = 0.668$ and $p = 0.121$, respectively) (Table 1).

No statistically significant difference was found among the three groups in terms of TAS, TOS, and OSI variables ($p = 0.809$, $p = 0.243$, and $p = 0.175$, respectively). There is a statistically significant difference with regard to the CCL2 variable among the three groups ($p < 0.001$). The statistical differences found by the CCL2 variable were between the control group and the RIF group ($p = 0.010$), and between the control group and the RPL group ($p = 0.001$) (Table 1).

Regarding the correlations among CCL2 and TAS, TOS and OSI in all groups, no statistically significant correlations were found between the CCL2 and TAS, TOS and OSI variables in all groups, either ($p = 0.614$, $p = 0.653$, and $p = 0.748$, respectively). As for the correlations among CCL2 and TAS, TOS and OSI for each individual group, they were also found

to be statistically insignificant for the control group ($p = 0.785$, $p = 0.499$, and $p = 0.302$, respectively), RIF group ($p = 0.453$, $p = 0.824$, and $p = 0.940$, respectively) and RPL group ($p = 0.864$, $p = 0.760$, and $p = 0.734$, respectively).

Finally, we investigated if there was any correlation between age and BMI, TAS, TOS, OSI, and CCL2 values. There was no statistically significant correlation between age and TAS, TOS, OSI, and CCL2 levels ($p = 0.732$, $p = 0.375$, $p = 0.724$, and $p = 0.577$, respectively). Likewise, no statistically significant difference was found between BMI and TOS, OSI and CCL2 ($p = 0.424$, $p = 0.309$, and $p = 0.090$, respectively). The only significant correlation is between BMI and TAS ($p = 0.005$).

Discussion

It was shown in this study that the patients with RPL and RIF had higher levels of CCL2 than those in the control group. The TOS, TAS, and OSI levels did not differ in RPL and RIF groups from the control group. No statistically significant relationship was found between CCL2 and the oxidative stress markers (TOS, TAS, and OSI) in this study.

Arici et al. also showed that CCL2 is synthesized in the human endometrium. CCL2 level could not be interrelated with the cycles day, endocrine status, or gestational period in this study which included mid-trimester and term pregnant women and non-pregnant ovulatory and anovulatory women [15]. Critchley et al. showed that CCL2 level in early pregnancy are higher than non-pregnant women [16].

Caballero et al. also showed in a study on the implantation window process in human endometrium that CCL2 levels reached immunoreactive levels in luminal and glandular cells as in endothelium cells in endometrium in the receptive phase of cycle; it was also presented in the same study that human blastocyst did not excrete CCL2 on measurable levels [17]. It has been shown in subsequent studies that embryo express the CCL2 receptors [18–20]. Fest et al. revealed in their study examining the interaction between trophoblast-macrophage in pregnancy preservation that trophoblast cells increased the production of chemokine involving CCL2 and CCL2 was effective in trophoblastic growth and survival [21].

Boomsma et al. aspirated the endometrial secretion before the embryo transfer in IVF cycles and examined the cytokine profile that predicts pregnancy in IVF and the study concluded that high CCL2 levels had a negative impact on achieving pregnancy [22]. It is known that CCL2 is a potent attractant and activator for natural killer (NK) cells in uterus [23]. It has been also revealed that decidual NK cells are the key regulators in maternal-fetal interface and related to miscarriage and infertility at high levels [10]. These data are in parallel with the result that low concentrations of CCL2 are accompanied by higher implantation rates. Along with all these results, it

Table 1 Comparison of age, BMI, TAS, TOS, OSI, and in CCL2 each of three groups

	Group						
	Control		Recurrent IVF failure		Recurrent pregnancy loss		<i>p</i> value
	Mean ± sd	Median (min-max)	Mean ± sd	Median (min-max)	Mean ± sd	Median (min-max)	
Age (year)	33.1 ± 5.2	32.5 (24.0–38.3)	32.7 ± 3.4	33.0 (24.0–40.0)	31.9 ± 6.0	31.0 (21.0–39.6)	0.668
BMI (kg/m ²)	25.3 ± 3.4	25.3 (19.3–28.6)	25.2 ± 4.3	25.9 (19.5–28.8)	26.3 ± 3.5	26.5 (19.2–29.8)	0.121
TAS (μmol Trolox equivalent/l)	1.3 ± 0.1	1.3 (1.1–1.5)	1.3 ± 0.1	1.3 (0.9–1.5)	1.3 ± 0.2	1.3 (1.0–1.8)	0.809
TOS (mmol H ₂ O ₂ equivalent/l)	9.2 ± 2.2	9.1 (5.6–14.5)	9.7 ± 2.6	9.0 (6.7–14.7)	9.4 ± 6.1	8.4 (5.0–39.5)	0.243
OSI (AU)	0.2 ± 0.1	0.1 (0.1–0.2)	0.1 ± 0.1	0.1 (0.1–0.2)	0.2 ± 0.1	0.2 (0.1–0.3)	0.175
CCL2 (pg/ml)	22.7 ± 6.5	20.7 (13.4–42.0)	29.8 ± 10.3	26.8 (16.5–61.6)	38.3 ± 23.6	30.0 (16.8–108.9)	<0.001*

**p* < 0.05

would be proper to remind the following comment of Boomsma et al. in their “Non-invasive analysis of endometrial receptivity” study: “Since the expression of cytokines differs in different compartments of the body due to the strong influence of the local micro-environment, it is unlikely that this approach would generate data representative of the intrauterine milieu.” [23]. In this study, it was seen that there was increase in CCL2 levels in the RIF and RPL groups in coherence with the literature data. This result coincides with the inflammatory immune response increase in repeated reproductive failure phenomenon. Although there was no implantation failure in the RPL group, increased CCL2 levels might have caused reproductive failure by impairing the regulatory mechanisms between peripheral blood NK cells and desidual NK cells [24].

In the literature, when examining the studies on pregnancy and oxidative stress, it is seen that oxidative stress play a role in the achievement of pregnancy and its onward stages and oxidative stress increases in repeated reproductive failures [2, 25, 26].

Ishii et al. stated that oxidative stress may cause embryopathy in early pregnancy and cause discontinuation of growth through excessive apoptosis and placental oxidative stress leads to placental angiodyplasia and recurrent abortus through inflammation [27, 28]. Hempstock et al. showed that placental oxidative stress due to early start of maternal circulation causes damage in syncytiotrophoblasts, playing a role in early pregnancy losses [5].

Along with the studies showing that antioxidant levels decrease in spontaneous abortus in the literature [29, 30], it was also revealed by Yiyenoglu et al. that there is an increase in total oxidant levels and decrease in total antioxidant level and oxidative stress index in RPL [31].

Quenby et al. (2009) reported in their study which examines the RPL and RIF groups under the title of recurrent reproductive failure that increase in NK cells in endometrium is related to increased angiogenesis and pregnancy failure is developed with the early start of maternal circulation due to peri-

implantation blood flow and consequent increased oxidative stress [4].

All these studies show that regulation of uterine and endometrial blood flow is important for the achievement of pregnancy and immune and inflammatory systems as well as oxidative stress play an important role in this multifactorial process and the literature data coincide with the fact that oxidative stress increase in the cases of reproductive failure [6].

On the other hand, total antioxidant, antioxidant levels, and oxidative stress index did not differ in the RPL and RIF groups from the control group in our study. These results stand up for the fact that increase in oxidative stress markers in the blood in the RPL and RIF groups takes place not in the non-pregnancy period but after the reproduction process starts.

This is the first study in which CCL2 and oxidative stress is evaluated both in RPL and RIF at the same time. Evaluation of oxidants and antioxidants in total and inclusion of OSI in the study is thought to be increasing the value of the study.

It is thought that this study should contribute to the literature, as in this study, we desired to offer a novel approach of measuring blood markers instead of endometrial markers. This is a far more desirable testing sample-type for measuring endometrial receptivity or an indicator for pregnancy outcome. The present study does not claim that blood CCL2 levels and oxidative stress markers would sufficiently reflect uterine microenvironment. This would have been further clarified if the endometrial sampling had been performed concurrently with blood sampling to establish the correlation between tissue and serum levels, and the present study would possible have more clinical significance.

The fact that the study was conducted in the non-pregnant period distinguishes it from other studies in the literature and brings importance to the study. This subject is also an important limitation of the study. If the non-gestational results could have been evaluated together with the gestational values of the same patients, the study would have been undoubtedly more valuable. One may not really know whether those results have

any clinical meaning as the study checked those parameters but could not correlate them with pregnancy.

In conclusion, this study demonstrates a higher CCL2 concentration during the luteal phase of patients with unexplained RIF and RPL. This finding is significant and suggests an immune related mechanism for these diagnoses, which are poorly understood. Oxidative stress markers that had been looked at in the non-gestational period did not have a predictive value in the RPL and RIF phenomena. Accordingly, it is too early to decide the use of antioxidant in repeated reproductive failure. For CCL2, it was thought that it might be possible to be used in risk prediction by supporting well-designed and expanded studies in the future.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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