Int J Fertil Steril
International Journal of Fertility & Sterility
Homepage: https://www.ijfs.ir/
Original Article
Vol 17, No 4, October-December 2023, Pages: 236-241

Gene Expression Levels of *CSF-1* and *CSF-1R* Endometrial under The Influence of Prolactin Level in Unexplained Miscarriage: A Case-Control Study

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Abstract

Background: Hormones such as prolactin, by influencing expression of the endometrial genes, play a pivotal role in embryo implantation and development. The present study aimed to evaluate serum level of prolactin and its effect on altering expression level of colony-stimulating factor-1 (*CSF-1*) and colony-stimulating factor-1 receptor (*CSF-1R*) genes in endometrial tissue during *in vitro* fertilization (IVF) pregnancy in the infertile women and recurrent pregnancy loss (RPL), compared to fertile women, who lost their pregnancies at gestational age <20 weeks.

Materials and Methods: In this case-control study, 40 infertile women, 40 IVF pregnant women with RPL and 40 fertile women who lost their pregnancies at <20 weeks of gestation for unknown reasons were selected. Prolactin serum level was assessed using ELISA technique and expression of *CSF-1* and *CSF-1R* genes was determined in endometrial tissue, using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Results: Mean prolactin level of the infertile group was 24.38 ± 1.43 ng/mL and it had statistically significant relationship with the fertile group (P<0.001). Expression level of the *CSF-1* and *CSF-1R* genes were higher in the fertile than infertile groups by 2.88 times (P<0.0001) and 2.64 times (P<0.0001), while it was respectively 2.28 (P<0.0001) and 1.69 (P<0.0001) times higher compared to the RPL group. Risk factors for pregnancy loss, such as aging, increased body mass index (BMI), smoking and diabetes caused decreasing changes in gene expression (*CSF-1* and *CSF-1R*) and the differences were statistically significant, except in the infertile group.

Conclusion: The present study showed a significant relationship of *CSF-1* and *CSF-1R* expression levels with pregnancy loss. Risk factors such as aging, obesity, smoking and diabetes decreased both genes expression levels.

Keywords: Genes, Infertility, Miscarriage, Prolactin, Recurrent Pregnancy Loss

Citation: Sadeghi Jam Z, Tahmasebi Fard Z. Gene expression levels of CSF-1 and CSF-1R endometrial under the influence of prolactin level in unexplained miscarriage: a case-control study. Int J Fertil Steril. 2023; 17(4): 236-241. doi: 10.22074/IJFS.2023.555588.1316

Introduction

Recurrent miscarriage occurs in 1-2% of women with reproductive age. Different terms and guideline is proposed to describe recurrent pregnancy loss. For example, it is termed as “recurrent pregnancy loss" and “recurrent miscarriage” by respectively the European society of human reproduction and embryology (ESHRE) and royal college of obstetricians and gynaecologists (RCOG) of UK. The new definition of ESHRE and the American society for reproductive medicine (ASRM) for recurrent miscarriage is the loss of two or more consecutive pregnancies, whereas the earlier description was defined as three or more consecutive pregnancy losses (1).

Recurrent pregnancy loss (RPL) is the most common complication of pregnancy. Almost 70% of human conceptions do not survive a live birth. Approximately 50% of all pregnancies end in miscarriage before clinical diagnosis even with fetal heart activity (2). RPL is characterized by two or three consecutive miscarriages prior to the 20th week of gestation (3). Pathogenic factors which have been known in only 50% of the cases include immune, endocrine, genetic and infectious factors, as well as metabolic disorders, anatomical abnormalities and other unknown causes (2, 3).

As a hormone secreted by the anterior pituitary gland, central nervous system, immune system, uterus, the tissue involved in pregnancy, and even the mammary glands, prolactin has several chemical forms after translation. It emerges a range of chemical changes, such as phosphorylation or glycosylation (4). It relies on the estrogen, progesterone, glucocorticoids, insulin,

Received: 11/June/2022, Revised: 15/September/2022, Accepted: 11/June/2023
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thyroid hormone and parathyroid hormone. Prolactin also enhances uptake of some amino acids and glucose as well as the production of milk sugar and milk fats (5).

Prolactin is a member of the growth hormone-placental lactogen family, arisen from a common ancestral gene, about 500 million years ago. More than 300 different functions (6), including growth, development, reproduction, metabolism, water and electrolyte balance, brain and behavior, and immune system regulation, are reported for prolactin (7), most of which are related to lactation and reproduction (8). Thus, its secretion is increased during pregnancy (9). Prolactin receptors are located on endometrial cells and, by binding to the hormone, help the endometrium capability to accept egg and create a suitable environment for blastocyst implantation (10). The prolactin effects are exerted through the CSF-1R receptor. This receptor is a member of the prolactin family, belonging to the tyrosine kinase receptors which are also expressed in macrophages and dendritic cell ancestors. This receptor controls proliferation, differentiation and survival of macrophages (11). In addition, CSF-1R is more expressed in the cell columns of extravillous trophoblasts, anchoring placenta to uterus (12).

Colony-stimulating factors (i.e. M-CSF, CSF1, GM-CSF, CSF2, G-CSF and CSF3) are a family of cytokines, among which M-CS and GM-CSF are expressed during pregnancy in the oviduct and uterus (13). CSF-1 (M-CS) is a factor that promotes growth of immune cells, especially monocytes. It is mainly produced by fibroblast cells, but there are various reports of its presence in the other tissue (14). This glycosylated homodimer with a disulfide bond is also expressed in the endometrium, decidua and placenta. In the endometrial glands, high levels of CSF-1 are seen during the secretory phase compared to the proliferative phase. Decidua also shows high levels of mRNA and CSF-1 protein in the secretory phase compared to the proliferative phase endometrium. In addition, it seems that endometrial cells near the anchoring villi of trophoblast are the main sources of CSF-1 for the placental-uterine interface (12).

Recurrent miscarriage and RPL mostly occur in the first trimester of pregnancy, prior to the week 20 of gestation; in addition, abortion (28th week of gestation) or premature birth (after 28th week of gestation) events occur in 10-20% of fertile couples (15). The current study aimed to evaluate prolactin hormone change and its relationship on the expression of *CSF-1* and *CSF-1R* genes during miscarriage prior to the week 20th of gestation in three groups of women with infertility, RPL and healthy fertile.

Materials and Methods

In this case-control study, three groups of women with infertility, RPL and healthy fertile were selected from patients referred to Yazd Infertility Center (Yazd, Iran), as well as Yas and Mirza Kuchak Khan Hospitals in Tehran, Iran. Women with children who have had at least two times normal pregnancy were assigned to the fertile group. Women with unknown causes of infertility and normal menstrual cycles with passing at least five years from their marriages were enrolled in the infertile group. Additionally, women who passed at least five years from their marriages and experienced a miscarriage at least twice without any children were assigned to the RPL group. Each group included 40 subjects (the sample size was estimated based on the following assumption: type1 and 2 errors: 0.05 and 0.20, respectively; expected implantation rate in the control group: 65%; expected frequency of abortion: 35%). Women in both infertility and RPL groups attempted to conceive via IVF, but subjects in the fertile group had normal pregnancies. The inclusion criteria were as follow: having a normal ovarian function, regular menstrual cycles, normal fallopian tubes, lack of uterine abnormalities, lack of endometriosis signs in ultrasound or laparoscopic examinations, and miscarriage of unknown causes with a normal embryonic karyotype prior to the week 20th of gestation. In addition, their spouses should have a normal volume and analysis of semen, based on the World health organization (WHO) reference values.

The selected individuals were within the age range of 25 and 35 years. Serum samples were taken from all subjects before undergoing curettage and stored at -20°C. Endometrial specimens were also collected using the Novak curette/Pipelle catheter and stored at -20°C after transferring to vials containing RNA-later. Other information about their age, height, weight and blood pressure was extracted from their files.

Determining serum concentration of prolactin, using ELISA

Serum prolactin concentrations were assessed by the commercially available kits (REF: DKO011, LOT No.: 4808A, DiaMetra, Italy) based on ELISA.

RNA extraction and cDNA synthesis

Firstly, endometrial tissue samples (approximately 100-150 mg) were rinsed with saline to remove RNA-later. Then, whole RNA was extracted from the tissue using a commercially available kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. Then, the extracted total RNA was evaluated using spectrophotometry and gel electrophoresis. To synthesize cDNA, 1 mg of the total RNA of each sample was mixed with random hexamer primers, RT (Reverse Transcriptase) enzymes and enzyme buffer, according to the kit instructions (Gene All Inc., South Korea) and placed in a thermocycler.

Expression level analysis of *CSF-1* and *CSF-1R* by quantitative reverse transcription polymerase chain reaction

Beta-actin housekeeping gene was used to evaluate expression levels of *CSF-1* and *CSF-1R* genes. Sequences of the designed primers and length of the proliferated segments are shown in Table 1.

Table 1: Primer sequence and polymerase chain reaction (PCR) product of *CSF-1* and *CSF-1R* genes

Gene	Primer sequence (5'-3')	PCR product size (bp)
CSF-1	F: AAGTTTGCCTGGGTCCTCTC R: TCCACTCCCAATCATGTGGC	290
CSF-1R	F: TGAGCTCACCCTTCGATACC R: CCTCAGGGTATGGGTCATCC	188
B-actin	F: TGGGCATCCACGAAACTAC R: GATCTCCTTCTGCATCCTGT	135

SYBR Green PCR Master Mix kit was used to perform qRT-PCR. The reaction was carried out with a mixture containing 10 µl master mix SYBR Green (Qiagen, Germany), 1 µl of each primer (10 pmol/µl), 1 µl of cDNA (50 ng) and 7 µl of dH_2O based on the following program: initial denaturation at 95˚C for 10 minutes, then denaturation at 95˚C for 30 seconds, annealing at 60°C for 30 seconds and elongation at 72˚C for 20 seconds in a total of 35 cycles. The melting curve analysis was performed at the translation rate of 1°C/second from 95˚C to 60˚C for 60 seconds. To analyze the samples, the PCR cycle threshold curve was plotted based on the exponential phase, and the $2^{-\Delta\Delta Ct}$ method was used to analyze the data.

Statistical analysis

SPSS version 25 software (IBM, USA) was used for data analysis. The inter- and intra-group comparisons in terms of the mean age, BMI and prolactin levels were performed by one-way ANOVA and the post hoc Tukey test. The results were expressed as mean ± SD. Due to the non-normality of the data, nonparametric Kruskal-Wallis test was used to compare gene expression among the three groups. Pearson's correlation coefficient was used to evaluate the relationship between the prolactin level and the expression level of *CSF-1* and *CSF-1R* genes. Significant level was considered P<0.05.

Ethical considerations

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the Tehran Islamic Azad University of Medical Science (IR.IAU.TMU.REC.1397.007, Tehran, Iran). Informed consent was obtained from all participants and their information was received through a questionnaire.

Results

Demographic characteristics of the subjects in the fertile, RPL and infertile groups, including age, body mass index (BMI), smoking habit, diabetes status and prolactin levels, are shown in Table 2.

Assessment of prolactin levels in the study groups

Mean prolactin level had a significant difference between the fertile and infertile groups (41.84 ± 4.32 vs. 24.38 ± 1.43 ng/mL, P<0.001). The same result was obtained between the fertile and RPL (32.45 ± 4.16 ng/mL, P=0.121). The results are shown in Figure 1.

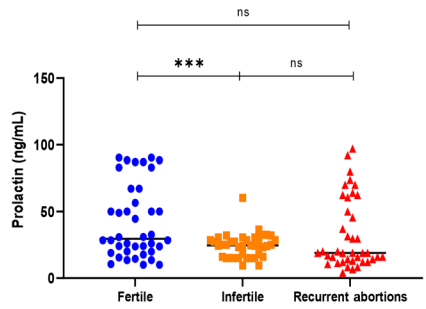

Fig.1: Comparison of the prolactin levels among fertile, infertile and miscarriage. Comparison of the three study groups showed that the highest and lowest levels of prolactin belonged to the fertile and infertile groups respectively, and a significant difference was observed among these groups. ***; P<0.0001 and ns; P>0.01.

Assessment of the expression level of *CSF-1* and *CSF-1R* genes in the study groups

Expression level of the *CSF-1* gene in the endometrial tissue was higher in the fertile than the infertile group by 2.88 times (P<0.0001). Its expression level was 2.28 times higher than the RPL ones (P<0.0001). Its expression level between the RPL was 1.26 times higher than the infertile group (P=0.011).

Expression level of the *CSF-1R* gene was 2.64 times higher in the fertile group than infertile ones (P<0.0001), and 1.69 times higher than the RPL group. The difference was statistically significant (P<0.0001). Expression level of the *CSF-1R* gene in the RPL group was 1.56 times higher than the infertile group, and the difference was statistically significant (P=0.002). Data are shown in Figure 2.

Table 2: Characteristics of the fertile, infertile and recurrent abortion women

Variable	Fertile	Infertile	Recurrent abortion	P value between the groups
Age (Y)	32.93 ± 1.25	33.20 ± 1.30	32.03 ± 1.02	0.769
BMI (kg/m²)	23.76 ± 0.56	27.43 ± 0.48	26.23 ± 0.58	<0.001
Prolactin (ng/mL)	41.84 ± 4.32	24.38 ± 1.43	32.45 ± 4.16	0.003
Smoker				0.303
Positive	8 (20)	4 (10)	9 (22.5)	
Negative	32 (80)	36 (90)	31 (77.5)	
Diabetes				0.277
Positive	3 (7.5)	6 (15)	8 (20)	
Negative	37 (92.5)	34 (85)	32 (80)	

Data are presented as mean ± SD or n (%). BMI; Body mass index.

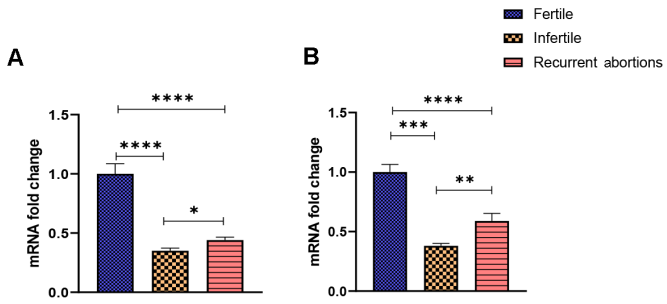

Fig.2: Comparison of *CSF-1* and *CSF-1R* gene expressions between the studied groups. Expression changes of the **A.** *CSF-1* and **B.** *CSF-1R* genes among the three study groups are shown based on the fold change. The lowest and highest expression levels of the *CSF-1* and *CSF-1R* gene were observed in the infertile and fertile groups, respectively. *; P<0.01, **; P<0.001, ***; P<0.0001, ****; P<0.00001, and ns; P>0.01.

Relationship of prolactin on the expression level of *CSF-1* and *CSF-1R* genes in the study groups

Effect of ≤40 and >40 ng/mL prolactin concentrations was evaluated on expression level of *CSF-1* and *CSF-1R* genes in the fertile, infertile and RPL groups. Expression level of *CSF-1* gene in subjects with prolactin serum concentration >40 ng/mL was increased 1.63, 1.01 and 1.36 times in the fertile, infertile and RPL groups, respectively, compared to the ones with prolactin serum levels ≤40 ng/mL. Differences for the infertile group were insignificant.

Expression level of *CSF-1R* gene in the individuals with prolactin serum concentration >40 ng/mL was respectively increased 2.86, 1.07 and 1.54 times in the fertile, infertile and RPL groups, respectively than those with prolactin serum levels ≤40 ng/mL; the differences were statistically significant unless infertile group. Data are shown in Figure 3.

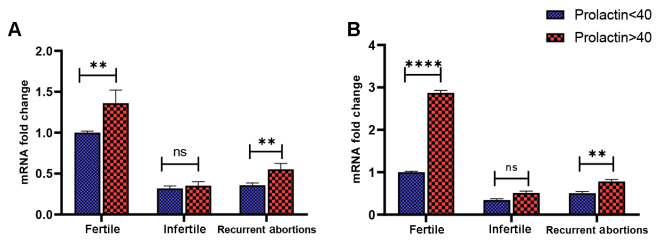

Fig.3: Changes in the expression levels of *CSF-1* and *CSF-1R* genes under the influence of prolactin hormone. Expression changes of the **A.** *CSF-1* and **B.** *CSF-1R* genes among the three study groups are shown based on the fold change. By increasing serum concentration of prolactin to >40 ng/mL, expression level of the *CSF-1R* gene was increased in the three groups compared to those with prolactin serum levels ≤40 ng/mL; although the differences were insignificant for infertile group. **; P<0.001, ****; P<0.00001, and ns; P>0.01.

Effect of different variables on *CSF-1* and *CSF-1R* expression levels in the study groups

Comparison of the subjects in the two age groups of ≤30 and >30 years showed significant differences in the *CSF-1* and *CSF-1R* gene expression levels of the fertile and RPL group. In comparison, there was no significant difference in the expression level of genes between the infertile subgroups. However in the age ≤30 of the fertile, infertile and RPL groups, expression level of the *CSF-1* gene in endometrial tissue was 1.34 (P=0.0096), 1.02 (P=0.736) and 1.23 (P=0.031) times higher than the age group >30 years. Expression level of the *CSF-1R* gene was higher 1.42 (P=0.0015), 1.33 (P=0.620) and 1.34 (P=0.031) times in the fertile, infertile and RPL groups, respectively.

Evaluation of the subjects in the BMI groups ≥25 and 25> kg/m^2 showed intragroup differences for the expression of the *CSF-1* and *CSF-1R* genes. In other words, expression level of the *CSF-1* and *CSF-1R* genes was decreased with increasing BMI. Comparison of the subjects with a BMI ≥25 kg/m^2 showed that *CSF-1* gene expression level was 1.58 (P=0.006), 1.25 (P=0.032) and 1.48 (P=0.049) times higher than the fertile, infertile and RPL groups, respectively. Additionally, comparison of the individuals with BMI ≥25 kg/m^2 showed that expression level of *CSF-1R* was 2 (P<0.0001), 1.20 (P=0.048) and 1.33 (P=0.034) times higher than the fertile, infertile and RPL groups, respectively.

Intragroup comparisons showed significant differences between diabetic and non-diabetic subjects in each group by evaluating the *CSF-1* and *CSF-1R* gene expression levels. Comparison of the diabetic subjects with non-diabetic individuals showed that *CSF-1* expression levels were decreased 2.22 (P=0.0004), 1.58 (P=0.0073) and 2.13 (P=0.0002) times in the fertile, infertile, and RPL groups respectively. Comparison for the *CSF-1R* expression levels were decreased 1.42 (P=0.0028), 1.33 (P=0.029) and 1.34 (P=0.019) times in the fertile, infertile and RPL groups, respectively.

Intragroup comparisons between smokers and non-smokers showed significant differences. Expression levels of *CSF-1* genes in smoker subjects were decreased 1.58 (P=0.017), 1.25 (P=0.045) and 1.49 (P=0.026) times in the fertile, infertile and RPL groups, respectively, compared to their counterparts with non-smokers. Expression levels of *CSF-R1* genes in the smoker subjects were 2 (P=0.039), 1.20 (P=0.025) and 1.33 (P=0.030) times lower than the non-smokers who counterparts in the fertility, infertility and RPL groups respectively.

Intragroup comparisons between gestational age ≤10 and >10 weeks showed no significant difference in the gene expression levels of the infertile and PRL groups. The fertile group with a history of miscarriage, less than ten weeks, was compared to those with a history of miscarriage more than ten weeks. CSF-1 and CSF-1R gene expression levels were increased 1.2 (P=0.002), and 1.16 (P=0.012) times, respectively. All data are shown in Figure 4.

Correlation coefficient of prolactin level with expression level of *CSF-1* and *CSF-1R* genes

Pearson correlation test for correlation between prolactin and *CSF-1* gene expression showed that the fertile group had a positive correlation (r=0.64, P=0.0017), the infertile group had no significant correlation (r=0.052, P=0.575) and the RPL group had statistically significant positive correlation (r=0.415, P=0.008). The same study, performed for the *CSF-1R* gene, showed that this correlation was only seen in the fertile group (r=0.452, P=0.015), but there was not any association between the infertile (r=0.098, P=0.285) and RPL groups (r=0.167, P=0.067).

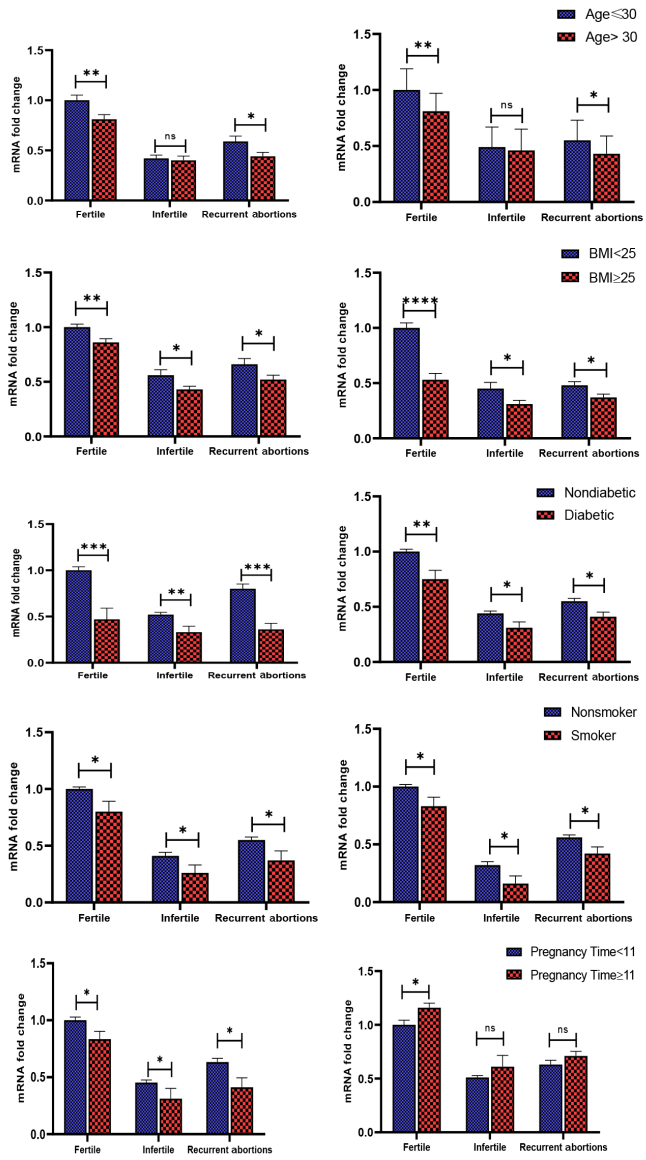

Fig.4: Changes in the expression levels of *CSF-1* and *CSF-1R* under the influence of different parameters. Effect of age, BMI, diabetes, smoking, gestational age and prolactin level on expression level of the studied genes was observed in the current study. BMI; Body mass index, *; P<0.01, **; P<0.001, ***; P<0.0001 and ns; P>0.01.

Discussion

Many factors can contribute to the success of IVF-ET in fertilization and embryo transfer. The main independent variables are age of the women, serum concentration of the anti-Mullerian hormone, number of the transferred embryos and their qualities. Some researchers showed that growth factors, hormones and cytokines, produced by macrophage cells, were involved in the implantation process (16).

During pregnancy, prolactin rises above the normal level of 10-25 ng/mL and reaches to a peak of 200-400 ng/mL within eight weeks of gestation (17). In the current study, mean prolactin serum concentration was determined in pregnancy losses at eight weeks of gestation as 41.84, 24.38 and 32.45 ng/mL for respectively the fertile, infertile and RPL groups; but in pregnancies losses <20 weeks of gestation, expression levels of *CSF-1* gene in subjects with prolactin serum concentration >40 ng/mL was increased 1.63, 1.01 and 1.36 times; additionally, expression levels of *CSF-1R* gene were 2.86, 1.07 and 1.54 times in the fertile, infertile and RPL groups respectively, compared to the individuals with prolactin serum levels ≤40 ng/mL indicating that prolactin serum concentration was increased by gestational age and it affects the embryo survival. Findings showed that increasing concentration of serum prolactin in this group was not sufficient for the survival of embryo. It also contributes to pregnancy loss. Moreover, investigating the role of prolactin biomarker confirmed its effect on fertility.

It is expected that maternal prolactin serum concentration is significantly elevated from 10 to 20 weeks of gestation (17). Although the highest and lowest average serum prolactin levels were significantly detected between the fertile and infertile groups, no significant elevation was determined between the fertile and PRL groups.

In pregnant women, elevated levels of *CSF-1* and *CSF-1R* expression were observed in the endometrial epithelium and fetal trophoblast, respectively. Studies showed that activation of trophoblast *CSF-1R* and increased level of local *CSF-1* expression were essential for the implantation of a normal fetus and placental development (18). Prolactin also affected endometrial tissue through the *CSF-1R* (10). In the studied subjects, expression level of *CSF-1* and *CSF-1R* genes in endometrial tissue, as well as the gene expression changes under the influence of prolactin concentration, were compared among the fertile, infertile and RPL groups. Expression level of the *CSF-1* and *CSF-1R* genes was higher in the endometrial tissue of the fertile group rather than the infertile ones. With increasing prolactin serum levels, expressions of *CSF-1* and *CSF-1R* were increased in the three groups. In the endometrial tissue of infertile group, expression levels of *CSF-1* and *CSF-1R* were lower than those of the fertile and RPL groups. Increased prolactin serum levels did not change expression of the *CSF-1* and *CSF-1R* genes in the infertile group. Increasing prolactin serum concentration caused increasing *CSF-1* and *CSF-1R* expression levels in the fertile women.

A recent study showed that CSF-1 expression level increased up to 1000 times during gestation in the endometrium of pregnant mice, due to its synthesis in the uterine lumen and glandular secretory epithelium controlled by the maternal endocrine hormones. Increasing local CSF-1 synthesis in the uterus was associated with proliferation and differentiation of cells. Additionally, association of CSF-1 receptor was determined between the uterus and placenta for implantation. CSF-1 also played role in regulating processes which are essential for implantation and preimplantation (19). A study, performed by Cai et al. (13), indicated that increasing M-CSF affected fetal development and increased trophectoderm (TE) cell count in mice.

Pregnancy loss risk factors are age, weight and general

health status of mother. Risk of spontaneous miscarriage was increased by increasing maternal age (20). In the present study, in addition to prolactin serum levels, other parameters, such as age, BMI, diabetes, smoking habits, and gestational age, CSF-1 and CSF-1R expression were evaluated in the all three groups. Intragroup comparisons showed significant difference between the subjects, aged > 30 and ≤30, in terms of gene expression expected the infertile group. But, expression level of the both genes was decreased by increasing age in the fertile women and those with RPL. Intragroup comparisons showed a significant difference between the subjects with BMI ≥25 and <25. Additionally, *CSF-1* and *CSF-1R* gene expressions were decreased in three groups by increasing BMI.

Intragroup comparisons showed a significant difference in the gene expression pattern between diabetic and non-diabetic subjects. However, expression level of the both genes was reduced in diabetic individuals. In addition, the difference was statistically significant. Intergroup comparisons showed significant differences between the smokers and non-smokers in gene expression levels. In terms of gestational age, expression of the both genes had a significant increase in the fertile women who lost their pregnancies at <20 weeks of gestation, but the infertile and PRL groups had no significant changes.

Conclusion

Overall, hormones, such as prolactin, are among the development factors of fetus, which can help fetal growth and successful continuation of pregnancy, through affecting the genes involved in conceptus–endometrial interactions. Decreased expression of *CSF-1* and *CSF-1R* can be considered as disruptive factors of implantation and fetal growth, leading to miscarriage in the studied groups. Factors, such as aging, increased BMI, smoking and diabetes, also caused decreasing changes in gene expression (*CSF-1* and *CSF-1R*). It seems that decreasing expression level of these genes disrupted conceptus-endometrium interaction, resulting in miscarriage. For future investigations, it is suggested to examine expression of the other genes involved in embryo implantation and their functional role in recurrent pregnancy loss. Additionally, discovery of the other molecular factors, involved in implantation, lead to increase in fertility success.

Acknowledgments

The authors would like to express their gratitude to the medical personnel at Yazd Infertility Center (Yazd, Iran), as well as Yas and Mirza Kuchak Khan Hospitals in Tehran and to all the patients who participated in this study. There is no conflict of interest in this study.

Authors' Contributions

Z.T.F.; Designed and directed the project, planned the qRT-PCR method, data and statistical analysis, in addition to interpretation of data. Z.S.J.; Contributed to sample preparation, performed the experiments, and wrote the paper. All authors read and approved the final manuscript.

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