Evaluation of leukemia inhibitory factor level in endometrial biopsy and blood samples of patient with concurrency of unexplained recurrent abortion and secondary infertility

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Objectives: Recurrent pregnancy loss (RPL) is a common disorder defined as occurrence of 3 or more unintentional abortions during the first 20 weeks of pregnancy. Uterine factors responsible for RPL have been recently brought into focus. Leukemia inhibitory factor (LIF) is one of the uterine molecular mediators associated with implantation and endometrial receptivity. In the present study the association of endometrial and serum levels of LIF with recurrent pregnancy loss and infertility was investigated.

Methods: In a prospective case control study measurements of LIF in endometrial tissue and blood samples in 4 groups of patients were performed and compared; one group consisted of 24 RPL cases, one group consisted of 24 patients with coexisting RPL and secondary infertility, one group of 20 primary infertility cases and a control group of 20 women without any fertility or pregnancy problem. LIF measurement was performed using ELISA technique.

RESULTS: There was no statistically significant variation of serum and endometrial levels of LIF among studied groups.

Conclusion and Recommendation: The present study is the first to investigate endometrial LIF levels in patients with RPL and infertility. The results failed to demonstrated significant statistical difference measurements of both serum and endometrial LIF among patients with RPL, infertility or both with controls.

Key Words: *: Recurrent abortion, Leukemia inhibitory factor, Uterus, Infertility*

ABBREVIATION: aCL: anti-cardiolipin;APS: Anti-phospholipid syndrome;CSF:Colony stimulating factor;DM:Diabetes mellitus;EGF:Epidermal growth factor;LIF:Leukemia inhibitory factor;IL: Interleukin ; LA: Lupus anticoagulant;PCOS:Poly-cystic ovary syndrome;RPL:Recurrent pregnancy loss

Recurrent abortion also known as recurrent pregnancy loss (RPL) is classically defined as three consecutive pregnancy losses prior to 20th week of gestation or a fetus weight less than 500 gr, excluding molar, ectopic and biochemical pregnancies. Failures in pregnancy after 20th week of gestation are called stillbirth or premature birth that has different pathologic causes. However The American Society for Reproductive Medicine regards recurrent pregnancy as two or more histologically and radiologically proved pregnancies and suggests a complete assessment after the third pregnancy loss (1). Primary recurrent pregnancy loss is the failure to maintain pregnancy for three or more times with no previous successful pregnancy after 20th week of gestation. Secondary RPL is defined as new occurrence of three or more pregnancy losses after at least 1 live birth. The prognosis of both types has been reported to be equal but patients with primary RPL are more susceptible to adverse obstetric and neonatal outcomes (2).

Sporadic pregnancy loss is relatively common. About 15% of women experience sporadic pregnancy loss once in their lives. Incidence of two miscarriages is 2% and only 0.4 to 1% of women develop clinically established diagnosis of RPL with three times of pregnancy loss.

Several conditions may be responsive for recurrent abortion. Major etiologies of RPL are classified as uterine factors, immunologic factors, endocrine factors, genetic factors, environmental and chemical factors, thrombophilia and fibrinolytic factors (3).

It is known that women with unexplained secondary infertility are 3 times more prone to spontaneous abortion. On the other hand, RPL may also complicate to secondary infertility in some cases (4). Etiologic factors such as thyroid autoimmune disease (5) and blood coagulation protein and platelet defects (6) have been proposed to be responsible for secondary infertility in cases of recurrent pregnancy loss. Other etiologic factors for concurrence of RPL and infertility are intrauterine adhesions (7) and celiac disease (8). Luteal phase defect has been debated as one of the responsible factors in recurrent pregnancy loss and infertility. Although some studies proposed failure of the corpus luteum to produce adequate progesterone to maintain pregnancy, to be responsible for recurrent pregnancy loss and infertility, others demonstrated no clinical evidence for efficacy of exogenous progesterone therapy in prevention of early pregnancy loss as well. Also some studies reported no significant predictive value of serum progesterone level for pregnancy outcome (9, 10).

Another entity of pathophysiologic factors proposed as the etiologic factor for recurrent pregnancy loss and infertility is defective endometrial receptivity. For successful attachment, implantation, and development of placenta, appropriate endometrial receptivity via several cellular and molecular pathways should be warranted (11). Multiple animal studies have been conducted to achieve more precise understanding of the bimolecular pathways involved in physiologic endometrial receptivity. These studies have proposed an important role for leukemia inhibitory factor (LIF) in the process of endometrial receptivity and fetal maternal immunologic tolerance (12, 13). Leukemia inhibitory factor (LIF) a member of IL-6 family is a pleiotropic cytokine playing role in multiple human systems. In hematopoietic pathways, LIF induces cell differentiation in both normal hematopoiesis and myeloid leukemia. In embryological development, LIF plays great role in neural cell differentiation and differentiation of epithelial kidney cells from mesenchymal tissue. It has been hypothesized that LIF has a basic role in immunologic mediation and tolerance during embryonic implantation and invasion to maternal endometrial tissue (14).

Also in the study by Hasegawa and colleague 2012, the authors provided evidence that there is decreased expression of leukemia inhibitory factor in abnormal uterine cavities which are responsive for poor reproductive results (15).

In the study by Aghajanova in 2004, the author provided evidence that measures of LIF expression and LIF receptor concentrations on the plasma

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membrane during the window of implantation at the mid-cycle of a healthy individual rises significantly compared to infertile women that show a declining pattern of LIF expression in the same period of menstrual cycle. The author also suggested that recombinant human LIF might be helpful in improvement of implantation in infertile individuals (16).

In the present case control study, for the first time, we evaluate the levels of leukemia inhibitory factor in serum and endometrial tissue in women with coexisting diagnosis of recurrent pregnancy loss and secondary infertility as well as patients with RPL and infertility and compare the expression of LIF between these patients and control group.

we believe that identification of specific antibody has beneficial to treatment disease with effective immuno_therapy .

PATIENTS and METHODS

The study protocol was approved and supervised by the ethics committee of the institutional review board for human medical research.

Patient Selection

In the present study, 24 patients with established diagnosis of recurrent abortion defined by 3 or more pregnancy losses and 24 patients with concurrent secondary infertility and recurrent abortions, and 20 cases of primary infertility of unknown etiology were recruited in three study groups. 20 individuals with no fertility problem and negative history of abortion where selected as the control group. Cases of primary infertility and those with recurrent abortion with or without secondary infertility were selected from patients who referred to infertility clinic between Aug 2011 and Jan 2013. The control group were selected from patients who referred to university affiliated outpatient clinic of gynecology between Aug 2011 and Jan 2013 due to gynecologic problems other than abortion and infertility. Though, the study population was arranged in 4 groups labeled as:

- Control group (n=20)
- Primary infertility with unknown cause (n=20)
- Recurrent abortion group (n=24)
- Recurrent abortion + secondary infertility (n=24)

To include patients in the control group, those with gynecologic infections, unexplained secretions or menstrual abnormalities without any problem regarding fertility who required endometrial biopsy for diagnosis, were selected. Individuals were excluded from the control group if any of the following was present:

- Positive history of fertility problem
- Endocrinologic disorders such as hypo or hyperthyroidism, adrenal or ovarian dysfunction
- Gynecologic problems which influence fertility such as polycystic ovary syndrome, luteal phase defect, hyperprolactinemia or any sign or symptom of hyperandrogenism
- History of intentional or spontaneous pregnancy loss at any stage
- History of any pregnancy related problem such as gestational diabetes mellitus, preeclampsia, etc.
- History of any previous instrumentation or manipulation of the uterus such as IUD placement or curettage
- Suspected or evident uterine anomaly
- Established or suspected immunologic disorder such as systemic lupus erythematous, anti-phospholipid syndrome or thyroid autoimmunity.

In the second group 20 patients with unexplained primary infertility were included. Any patient with identified etiology for infertility was excluded. Exclusion criteria for this group were same as those described for the control group except the first criteria regarding presence of infertility.

The third group consisted of 24 patients with isolated RPL (by definition of three or more early pregnancy losses) and 24 patients with RPL complicated by secondary infertility were allocated in group D.

Informed consent was obtained from all patients. None of the study population underwent unnecessary endometrial biopsy.

Materials

- Antibiotics (penicillin and streptomycin), GIBCO, Germany
- FBS (fetal bovine serum), GIBCO, Germany
- L-glutamine, GIBCO, Germany
- 4-RPMI, GIBCO, Germany
- Culture plate, SPL
- 6-Blue tip, SPL

Instruments

- Tip, IBL, U.S
- Eppendorf tube, SPL
- Sterile pipet 10 ml, SPL
- Sterile pipet 5 ml, SPL
- Novak currette, Cooper surgical Inc. Germany
- Human LIF ELIZA kit, Massachusetts, U.S
- CO2 incubator, Memert, Germany
- Hood, Zhal tajhiz, Iran
- Centrifuge, Hettich, Germany
- Microscope, Invert, Belgium

Study protocol

In all patients, endometrial biopsies were obtained between 19th and 25th days of menstrual cycle. Studies involving large numbers of women who had performed the endometrial biopsy during these days , showed no greater incidence of birth defects or abortion (17) .On the day of biopsy, prior to the procedure blood samples were also obtained and separated. Sera were kept at -70 degrees to be checked later for LIF levels. After preparing the patient and instruments for the procedure, in lithotomy position, endometrial biopsies were taken using Novak's curette (Cooper surgical Inc., Germany), under sterile prep and drape. Specimens were rapidly transferred to culture medium (RPMI) containing %10 fetal Bovine Serum (FBS) (Life Technologies Corporation, Carlsbad, California, U.S.) and final concentration of 100 I.U./mL penicillin and 100 µg/mL streptomycin for culturing. At Autoimmune Diseases Research Center the specimens were weighted primarily. Extracellular matrix was destructed using 10 ml of collagenase enzyme (final concentration of 2 mg/ml) and collagenase-treated cells were infiltrated through mesh filter. Filtered suspension containing scattered cells was centrifuged at 50 g for 1 minute to separate stromal cells from glandular cells. For this mission the upper phase of supernatant (12ml of 15ml solution) which contained most of the stromal cells was pulled in a separate tube. RPMI medium was added to the primary tubes to reach the primary volume of 15ml. Once again the suspension was centrifuged at 200 g for 8 minutes to completely separate glandular cells from stromal type cells. Stromal cells and glandular cells were separately cultured in 6 well tissue culture plates. For stromal cells culture 1X106 cells in the final volume of 1ml were added to each well while for glandular cells culture 4X105 cells in a final volume of 1 ml were added to each cm^2 of tissue culture wells. Tissue culture plates were incubated for 18-20 hours in 5% carbon dioxide atmosphere at 37°C. Then the round shaped glandular cells and columnar shaped stromal cells which were easily recognizable floating above the culture environment were drawn and cultured again for 48 hours in the same environment. After 48 hours, supernatant of cultured cells were collected and transferred to -80°C temperature for measurement of Leukemia Inhibitory Factor using Fitzgerald industries international ELISA kit (Massachusetts, U.S.).

From each patient 5 cc blood sample was taken in clotting tube and after excluding the serum, it was transferred to - 80 °C temperature for measurement of Leukemia Inhibitory Factor using Fitzgerald industries international ELISA kit.

Measurement of LIF in endometrial cultured specimens from the study groups performed twice to enhance the fidelity of the study. LIF level measures were equalized regarding the primary tissue weight among cases by subdividing LIF measures to specimen weight.

Statistical analysis method

Serum and endometrial biopsy levels of leukemia inhibitory factor were measured and recorded for comparative statistical analysis among study and control groups using independent T-test of IBM SPSS Statistics version 18.

RESULTS

In the present study, a total number of 88 women aging between 21 and 49 years with mean age of 33.6 years were recruited and allocated in 4 groups RPL, RPL+infertility , primary infertility ,control group.

Independent T-test analysis for evaluation of the significance of variation of LIF measures among groups was performed which revealed non-significant difference of means among case and control groups, considering the confidence interval of 95%. Further more comparison between group A and C (6.9 ± 11.6 and 10.5 ± 12.7) (Table 1) and group A and D (10.5 ± 12.17 and 5.76 ± 12.2) (Table 2) and groups A and B (mean value of 10.5 ± 12.17and 13.5 ± 20.3) (Table 3).

Serum levels of LIF were finally between group B and D (13.5 ± 20.3 and 5.76±12.2) (Table 4) which is lower in compared to control groups C and D (mean value of 6.9 ± 11.6 and 5.76 ± 12.2, respectively) (Table 5) . Measurement of LIF in cultured tissue samples similarly showed no significant variation of LIF expression.

In addition there was no correlation between the LIF levels in the culture and number of abortion for group 1 (p=0.21), group 2 (p=0.67) or total patients (groups 1 + 2; p=0.26). The same results were obtained for the correlation of LIF levels in the sera and number of abortions (Groups 1 + 2; p=0.59).

DISCUSSION

Uterine factors play important role in successful conception and their defects can be responsible for recurrent abortions. LIF, a member of interleukin-6 family, is a cell proliferation and differentiation regulatory factor which plays important role in hematopoietic differentiation and maturation, neural development and in several other human organogenesis. Serum and endometrial tissue levels of LIF has been noted to alter significantly during the window of implantation. In mammals, tightly controlled changes within the endometrial tissue vascularization, cellularity, and microenvironment are achieved after ovulation to prepare the endometrium for receiving the blastocyst. These changes are regulated by alternating balance of estrogen and progesterone. Window of implantation lasts 18-24 hours in rodents and up to several days in humans. During this window, several cytokines and mediators have been detected to undergo alterations. LIF has been reported by numerous studies as an important regulatory factor of endometrial implantation. LIF is highly expressed within the endometrial tissue and is upregulated during the window of implantation (18). The integral role of LIF during window of implantation was described by Cullinan et al. in 1996. The authors proposed that LIF plays role in regulation of trophoblastic function and placental vascular formation, although the authors failed to detect any significant variation of expression of LIF among infertile and healthy women (19). In 2002, Cheng et al. performed a study on LIF-null mice and observed that although blastocyst formation was non-problematic, implantation could not occur in these mice (20). Uterine receptivity has been reported to be tightly associated with LIF receptor signaling pathway. In 2003, Aghajanova performed a study to verify the relation of pinopode formation in endometrial cells and expression of LIF and LIF receptor. They obtained endometrial biopsy and blood samples from 26 healthy women with no gynecologic disorder and regular menstruation and measured LIF and LIF receptor protein by immune-histochemical staining as well as they observed morphological transformations of the endometrial cells by scanning electron microscopy. The results were significant for simultaneous morphological and molecular alterations within the endometrial cells during the window of implantation (21). In 2012, Xu and colleagues investigated LIF and several other biomolecules in patients with recurrent pregnancy loss. In that study, the authors measured expression of LIF, integrin-β3 and MUC1 as well as morphology of pinopodes and their coverage in 30 patients with recurrent pregnancy loss and compared those with 24 healthy individuals. The results revealed no significant variation among the two groups regarding pinopodes morphology, LIF and integrin-β3 expression (22).To compare the expression of LIF among infertile women and healthy controls, in 1998, Hambartsoumian performed enzyme-linked immunosorbent assay (ELISA) on in vitro culture endometrial biopsy samples from 32 cases of unexplained infertility and compared the findings with the same measures from 17 fertile women. The author provided evidence that endometrial LIF levels are significantly lower during both proliferative and secretory phases of the menstrual cycle in patients with unexplained infertility compared to healthy controls (23).

The present study was the first one to measure and compare both endometrial and serum levels of leukemia inhibitory factor in patients with recurrent abortion and infertility and compare the results with patients with RPL or infertility alone or with healthy controls. Considering the fact that leukemia inhibitory factor plays important role in pinopode formation during the window of implantation, we expected significant alteration of endometrial or tissue levels of LIF in women with RPL or coexisting RPL and infertility comparing to controls. As the results of this study indicate, although the mean values of serum and endometrial levels of LIF in RPL groups were as low as half of the measure for control group, statistical analysis did not show any significant difference. This finding may be resulted by the fact that in this study, standard deviations of measures of LIF within studied group is very larger than the mean value, so the effect of the confounding factors was not overridden by the population size. On the other hand one similar previous study which has compared LIF among RPL and control patients has failed to reveal any significant relation between endometrial LIF and recurrent pregnancy loss (22).

Table 1

Statistical comparison of RPL and control group

Group	Recurrent pregnancy loss	Control	P-Value
Serum LIF	6.9 ± 11.6	10.5 ± 12.17	0.3
Tissue LIF	192.3 ± 323.0	305.24 ± 468.3	0.35

Table 2

Statistical comparison of RPL + infertility and control group

Group	Recurrent pregnancy loss + infertility	Control	P-Value
Serum LIF	5.76 ± 12.2	10.5 ± 12.17	0.2
Tissue LIF	148.3 ± 254.1	305.24 ± 468.3	0.16

Table 3

Statistical comparison of primary infertility group and control

Group	Infertility	Control	P-Value
Serum LIF	13.5 ± 20.3	10.5 ± 12.17	0.53
Tissue LIF	102.83 ± 218.87	305.24 ± 468.3	0.06

Table 4

Statistical comparison of primary infertility group and RPL + infertility

Group	Infertility	RPL + infertility	P-Value
Serum LIF	13.5 ±20.3	5.76 ± 12.2	0.13
Tissue LIF	102.83 ± 218.87	148.3 ± 254.1	0.52

Table 5

Statistical comparison of RPL and RPL + infertility

Group	RPL	RPL + infertility	P-Value
Serum LIF	6.9 ± 11.6	5.76 ± 12.2	0.76
Tissue LIF	192.3 ± 323.03	148.3 ± 254.1	0.6

CONCLUSIONS

Since this investigation was the first study to compare endometrial and serum levels of LIF in patients with both RPL and infertility and controls, we recommend repetition of this study with larger study populations to determine if the results of this study reveal the fact that there is no association between LIF and recurrent pregnancy loss or the results were distorted by the study population size

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