

The assessment of LIF in uterine flushing – a possible new diagnostic tool in states of impaired fertility

Mateusz Mikołajczyk¹, Jana Skrzypczak, Krzysztof Szymanowski, Przemysław Wirstlein

Division of Reproduction, Department of Gynecology and Obstetrics, Karol Marcinkowski University of Medical Sciences, Poznań, Poland

Received: 10 September 2003; accepted: 12 November 2003

SUMMARY

The objective of this study was to assess the LIF (leukemia inhibitory factor) concentration in uterine flushing and serum (ELISA) of women with proven fertility, infertile women and women with recurrent miscarriage. In addition, progesterone level was determined in serum. A decreased production of LIF in the uterine microenvironment was found in states of impaired fertility. With a cut-off point of 8.23 pg/ml for LIF level in uterine flushings we have achieved 86.7% sensitivity and 100% specificity in detection of women with idiopathic infertility compared to fertile controls. No correlation between LIF in serum and uterine flushing was demonstrated, rendering LIF measurements in serum useless for diagnosis of impaired fertility. We conclude that LIF measurement in uterine flushing could be a useful diagnostic tool to predict unsuccessful implantation. *Reproductive Biology* 2003 3 (3): 259-270.

¹Corresponding author: Division of Reproduction, Department of Gynecology and Obstetrics, Karol Marcinkowski University of Medical Sciences, ul. Polna 33, 60-535 Poznań, Poland; email: matmikol@mail.gpsk.am.poznan.pl

Key words: infertility, miscarriage, abortion, LIF, leukemia inhibitory factor, endometriosis, idiopathic

INTRODUCTION

The desire to have a child is shared by many couples around the world, at the same time being an essential factor influencing survival of humans as species. However, it is estimated that worldwide 10-15% of couples have problems conceiving a child and 0.5-1% experience recurrent miscarriage (defined as two or more consecutive miscarriages). These problems are associated with a great deal of stress, anxiety and financial burdens for those families.

While many techniques that are used to diagnose different causes of infertility and recurrent miscarriage has been developed (ultrasound, laparoscopy, hysteroscopy) still about 50% of cases of recurrent miscarriages and 10% of infertility remain unexplained. It may be assumed that the cause of unexplained pregnancy loss might be attributed to flawed implantation. The highest rate of miscarriages in natural cycles occurs before implantation. Similarly, the implantation rate is disappointingly low (rarely exceeding 30%) in ART (artificial reproductive techniques) cycles.

Since current diagnostics methods have failed to establish the early events during implantation, the search has begun for a reliable marker of the so-called uterine receptivity. Utilizing an animal model, Psychoyos [21] has found that there is a time period during which implantation is possible. Beyond this period, endometrium is refractory to implantation. Similar characteristics of human endometrium have also been described, however, the exact time of an "implantation window" remains uncertain. Bergh and Navot [3] after experiments with blastocysts and embryos have stated that after 24th day of the cycle, implantation becomes impossible. The beginning of the window of receptivity seems to start eight days after ovulation [27].

Evaluation of the endometrium development day of the menstrual cycle, according to the criteria of Noyes et al. [19] does not always allow the chances of implantation to be predicted successfully. The exact mechanism

of an initial attachment of the blastocyst to the luminal epithelial surface is not known since most studies are performed in non-conceptual cycles.

Studies on implantation are difficult for obvious moral and ethical concerns and involve experiments with embryos. Studies of conceptual cycles require damaging the endometrium through invasive techniques such as biopsy. There is a clear need for both reliable and safe method for demonstrating the presence of molecules important for the implantation in the uterine cavity. Many molecules have been described which could serve as a marker of uterine receptivity including integrins, glycodefins, MUC1 (mucin 1) and some features of the endometrial surface like pinopodes [2, 14, 18, 22].

Leukemia inhibitory factor (LIF), a member of IL-6 family, is one of the potential candidates to be used as a predictor of implantation failure. In 1984, LIF was found to induce differentiation of the myeloid leukemic cell line M1 in macrophages [25]. Next, it was demonstrated that this pleiotrophic cytokine exerted an action on hematopoietic, embryonic, neural and bone cell lines [1, 5, 6, 23]. Bhatt et al. [4] who found its expression in mouse endometrium first noticed the role of LIF in reproduction. In 1992, Stewart et al. [24] demonstrated that mice lacking functional LIF gene are totally infertile as a result of a failed implantation. At the same time, placing recombinant LIF in mice uteri resulted in the restoration of fertility.

The expression of LIF was detected in endometrium also in humans, and the peak LIF mRNA expression was pinpointed to secretory phase of endometrium development [5]. Kojima et al. [11] have confirmed the localization of LIF in the endometrium. Delage found that endometrium from infertile women cultured *in vitro* produced less LIF than endometrium from fertile control [7]. Since many factors associated with pregnancy establishment are locally generated, it seems that the best method for studying the receptivity would be to examine the uterine cavity environment. This can be done by endometrial biopsies or “endometrial flushings”. The aim of this study was to determine whether the level of LIF in the serum and endometrial flushings differs between women with impaired fertility and those who are fertile.

MATERIALS AND METHODS

Sera and uterine flushings have been collected from the women hospitalized between January 2001 and July 2003 in the Division of Reproduction, Department of Obstetrics and Gynecology, University of Medical Sciences in Poznań. Forty-nine patients with infertility (patients with known cause of infertility, patients with unexplained infertility and patients with endometriosis), thirty patients with recurrent miscarriage (patients with idiopathic recurrent miscarriage and with known cause of miscarriages) and sixteen control women were enrolled in this study. Infertile women had performed a standard panel of tests that included hysterosalpingography, hormonal profiles and semen analysis. Women with recurrent miscarriage were additionally evaluated for the presence of anticardiolipin antibodies and karyotype anomalies. The control group comprised of fertile women (at least one live birth, no miscarriages, with regular menses) who were admitted for non-endometrial associated diseases (e.g. benign ovarian cysts). Exclusion criteria included patients submitted for hormonal replacement therapy, patients using intrauterine contraceptive devices or oral contraception within last six months and patients with serious disease (requiring use of any drugs).

The mean age of infertile women was 32 years (range: 26-43), 30 years of age for women with recurrent miscarriage (range: 23-37), and 38 years in control group (range: 30-49). Mean number of miscarriages in the recurrent miscarriage group was 2.7 (range 2-4), and the mean duration of infertility was 5.6 years (range: 1-17 years).

All women underwent serial ultrasound assessments to track follicular growth and the formation of corpus luteum. During the same cycle, 7-9 days after the ovulation (the putative implantation window) blood was drawn for evaluation of the progesterone and LIF levels. Blood was drawn into a 10 ml syringe and centrifuged at 3 000×g. Serum was stored at -20°C until progesterone measurement was completed.

At the same time a uterine flushing was performed. The procedure involved placing a sterile catheter in os of the uterine cervix, which was connected to a 20 ml syringe filled with 3.5 ml of sterile isotonic solution

of sodium chloride (0.9% NaCl). The fluid was slowly infused into uterine cavity and then gently aspirated in a repetitive fashion creating a turbulent flow (to achieve homogenous distribution of soluble factors). Next, the fluid was drawn into the syringe, which was transported to the laboratory, centrifuged and frozen in -20°C for further examination.

The progesterone level was assessed using electrochemiluminescence method (intra-assay variability 2.1%, inter-assay variability 7.4%; Elecsys 1010). LIF was evaluated by ELISA (Bender MedSystems, USA). The reference range in this assay was from 0.45 to 500 pg/ml (intra-assay variability 7%, inter-assay variability 5%). All patients signed an informed consent form approved by the local ethics committee.

Statistical analysis

Statistical analysis was performed by MedCalc for Windows v.7.2 (MedCalc Software, Belgium). Kolmogorov-Smirnov test was used to determine normality of distribution. Since values of LIF in almost all groups did not exhibit normal distribution, differences between values of parameters in different groups were calculated by Mann-Whitney test. The correlations between LIF values in blood and in serum in the same group were checked using Spearman rank correlation. The same test was used to analyze correlations between progesterone and LIF in blood or serum.

To test whether LIF values can be used for diagnostic purposes we used Receiver Operating Characteristic (ROC) curves (MedCalc for Windows v.7.2). The ROC curve displays diagnostic accuracy expressed in terms of true-positive rate against false-positive rate for different cut-off points [15]. The ROC curves were plotted for LIF concentrations in serum and uterine flushings in all examined groups.

RESULTS

All data are presented as medians and range values. The concentrations of LIF in serum and uterine flushing exhibited normal distribution in control patients as well as in serum of women with recurrent miscarriage. In other

groups, concentrations of LIF and progesterone did not exhibit normal distribution.

The level of LIF in uterine flushing and serum in women with recurrent miscarriage (n=30) ranged from 0.5 to 496.2 pg/ml (median: 35.73 pg/ml) and from 0.45 to 22.64 pg/ml (median: 4.6 pg/ml), respectively (tab. 1). In twelve women (40 %), the concentration of LIF in uterine flushing was below 10 pg/ml. In women with recurrent miscarriage, the progesterone level varied from 0.4 to 21.5 ng/ml (median: 5.3 ng/ml). No correlations were found between progesterone and LIF as well as between LIF in serum and uterine flushing.

In infertile women (n=49), serum LIF level ranged from 0.5-10.73 pg/ml (median: 4.24 pg/ml). The uterine flushing LIF level varied from 0.5 to 379.13 pg/ml (median: 11.03 pg/ml). Progesterone level in this group ranged from 0.34 to 17.86 ng/ml (median: 5.5 ng/ml). A low level of LIF (<10 pg/ml) was detected in 52% patients with infertility. Similar to group with recurrent miscarriage, there was no correlation between LIF levels in serum and flushing as well as between LIF and progesterone.

In women with idiopathic infertility (n=15), concentrations of LIF in uterine flushing and serum ranged from 0.5 to 15.91 pg/ml (median: 1.57 pg/ml) and from 0.5 to 10.15 pg/ml (median: 4.33 pg/ml), respectively. The progesterone level varied from 1.63 to 16 ng/ml (median: 8.39 ng/ml). The positive correlation between LIF levels in serum and uterine flushings ($r=0.59$; $p < 0.05$) was found in this group.

In women with identifiable cause of infertility (n=34), the LIF levels in uterine flushing and serum ranged from 0.5 to 379.13 pg/ml (median: 18.89 pg/ml) and from 0.5 to 10.73 pg/ml (median: 3.4 pg/ml), respectively. Progesterone levels varied from 0.34 to 17.86 ng/ml (median: 7.44 ng/ml).

In women with endometriosis (subgroup of women with infertility), LIF concentrations in uterine flushing and serum ranged from 0.5 to 379.14 pg/ml (median: 5.64 pg/ml) and from 0.5 to 6.29 pg/ml (median: 3.11 pg/ml), respectively. Progesterone level in this group varied from 0.34 to 15.58 ng/ml (median: 8.15 ng/ml).

Serum LIF level in all women in control group (n=16) was higher than 10 pg/ml (10.83-229.48 pg/ml). The LIF concentration in the uterine flushings ranged from 0.5 to 229.48 pg/ml (median: 26.46 pg/ml). The progesterone

Table 1. Median and range of leukemia inhibitory factor (LIF) levels in uterine flushings and serum, and progesterone level in serum of infertile and fertile women

GROUPS		LIF (pg/ml)		Progesterone (ng/ml)
		Uterine flushing	Serum	
CONTROL		26.46 (0.5-229.48)	5.28 (10.83-229.48)	2.36 (0.35-21.53)
INFERTILITY	Identified cause of infertility	11.03** (0.5-379.13)	4.24 (0.5-10.73)	7.55* (0.34-17.86)
	Idiopathic infertility	1.57* (0.5-15.91)	4.33* (0.5-10.15)	8.39 (1.63-16)
	Endometriosis	5.64 (0.5-379.14)	3.11 (0.5-6.29)	8.15 (0.34-15.58)
RECURRENT MISCARRIAGES	Identified cause of recurrent miscarriages	35.73 (0.5-496.2)	4.64 (0.45-22.64)	5.25 (0.37-21.53)
	Idiopathic recurrent miscarriages	25.36 (0.5-496.2)	4.73 (0.5-6.5)	8.85 (0.51-11.54)

* $p < 0.05$; ** $p < 0.001$

level varied from 0.35 to 21.53 ng/ml (median: 2.36 ng/ml). No correlations were found in this group between LIF levels in serum and in uterine flushing as well as between LIF level and progesterone level.

Serum progesterone was lower ($p < 0.05$) and LIF in uterine flushing ($p < 0.01$) was higher in the control group compared to all infertile patients. In addition, LIF level in uterine fluid was higher in control women in comparison to women with idiopathic infertility ($p < 0.01$).

To test the diagnostic value of LIF measurements for detection of states of impaired fertility we utilized ROC curves. With a cut-off point of 8.23 pg/ml for LIF level we have achieved 86.7% sensitivity and 100% specificity in detection of women with idiopathic infertility compared to fertile controls. All women with uterine flushing LIF level below 8.23 pg/ml will have idiopathic infertility. Moreover, this cut-off value will enable us to detect 86.7% women who are at high risk for infertility despite normal results of other tests.

DISCUSSION

Leukemia inhibitory factor has been found to affect various cell and tissue types including neurons, megakaryocytes, osteoblasts, hepatocytes and certain tumors. Discovery of LIF in the mouse endometrium have turned the attention of researchers to its role in fecundity. The fact that LIF is the key molecule in the implantation process in mice inspired similar research in humans. It was demonstrated that LIF mRNA is present in human endometrium and that the rise in expression of LIF coincides with the implantation window [11].

In the current study, LIF concentrations in uterine flushings during the implantation window were lower in women with infertility compared to healthy controls. We have found that almost half of infertile women (regardless of primary cause of infertility) secreted lower amounts of LIF into uterine cavity in comparison to control patients. Women with idiopathic infertility have shown LIF deficiency even more pronounced than other infertile patients. Delage et al. [7] found that endometrium of women with infertility produces less LIF than fertile control patients. In addition, Laird et al. [12] found lower amounts of LIF in uterine flushings around the time of implantation in women with unexplained infertility than in normal fertile women. However, their method of uterine flushing was questioned as a non-consistent and not very useful in clinical practice [10].

Utilizing the ROC curves, we aimed at approximating the threshold value of LIF in the uterine cavity necessary for successful implantation. The calculated LIF value of 8.23 pg/ml allowed us to pinpoint almost 87% of

women with idiopathic infertility with 100% specificity. Thus, we postulate that the abnormal LIF production might be a causative factor of infertility. Preliminary reports on supplementing the low LIF levels in women with infertility with recombinant human LIF are promising¹. Therefore, we suggest that LIF assessment in uterine flushing should be a standard test especially in women with idiopathic infertility.

Contrary to Laird et al. [12], we have found that women with recurrent miscarriage have had a similar percentage (40%) of low LIF values compared to infertile women. This appears to be in agreement with results of Eckert and Niemann [9] who have localized LIF receptors on blastocysts. Moreover, Ware et al. [26] found that LIF receptor deficient mice have exhibited damaged placenta, skeleton, neuronal and metabolic pathways in mammals. LIF deficiency could lead to death of an already implanted embryo and, in consequence, contribute to recurrent miscarriage. In addition, Nachtigall et al. [17] have found that LIF may be directly implicated in the modulation of implanted trophoblast differentiation. Therefore, it is possible that LIF is responsible not only for initial implantation, but also for early development of the human embryo. If this statement is true, there may be a common reason for early miscarriage and infertility at the endometrium level.

Since endometriosis is sometimes associated with infertility, we have examined LIF secretion in women with this disease. We have found that women with endometriosis have a comparable distribution of LIF levels to women with other causes of infertility.

The lack of correlation between progesterone and LIF levels is somehow surprising since progesterone level during the luteal phase is high and LIF is a progesterone dependent molecule. However, it is known that progesterone exhibits a pulsatile pattern of secretion. We have analyzed only single measurement of serum progesterone level. Therefore, the lack of correlation could be attributed to a faulty progesterone determination. It also cannot be excluded that the individual level of progesterone required for normal

¹Brinsden PR, Ndukwe G, Engrand P, Pinkstone S, Lancaster S, Macname NC, DeMoustier B **2003** Does recombinant inhibitory factor improve implantation in women with recurrent failure of assisted reproduction treatment? *Human Reproduction* **18** suppl pp XVIII 18 abstract.

LIF secretion varies among different individuals. In certain patients, even low progesterone level (below 10 ng/ml) may be sufficient to induce LIF production and normal development of the endometrium.

In the current study, we found a positive correlation between LIF levels in flushing and serum only in the idiopathic infertility group. It suggests that many cytokines potentially involved in implantation process might act in a microenvironment of uterine cavity, which justifies the need to develop relatively noninvasive tools and techniques for evaluation this environment. In women with idiopathic infertility, there might be a general LIF deficiency, either at the DNA or protein level. The used method of uterine flushing does not damage endometrium in comparison to the uterine biopsy. Moreover, Ledee-Bateille et al. [13] have demonstrated its value in assessment of soluble molecules within the uterine environment.

Studies on human embryos during IVF cycles have proven that the measurement of LIF in the uterine cavity does not adversely affect pregnancy rates and it may be used as a predictor of implantation [20]. Moreover, the addition of LIF to culture media significantly improved pregnancy rate and embryo development without exhibiting negative effects on morphological development of mouse offspring's [8, 16]. In conclusion, the assessment of LIF in uterine flushings could be another useful and safe method for predicting successful implantation as well as for diagnosing and eventually treating women with impaired fertility.

REFERENCES

1. Allan EH Hilton DJ Brown MA Evelyn RS Yumita S Metcalf D Gough NM Ng KW Nicola NA Martin TJ **1990** Osteoblasts display receptors for and responses to leukemia-inhibitory factor. *Journal of Cell Physiology* **145** 110-119.
2. Aplin JD Meseguer M Simon C Ortiz ME Croxatto H Jones CJP **2001** MUC1, glycans and cell-surface barrier to embryo implantation. *Biochemical Society Transaction* **29** 153-156.
3. Bergh P Navot D **1992** The impact of embryonic development and endometrial maturity on the timing of implantation. *Fertility and Sterility* **58** 537-542.
4. Bhatt H Brunet LJ Stewart CL **1991** Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. *Proceedings of the National Academy of Sciences of the USA* **88** 11408-11412.

5. Charnock-Jones DS Sharkey AM Fenwick P Smith SK **1994** Leukemia inhibitory factor mRNA concentration peaks in human endometrium at the time of implantation and the blastocyst contains mRNA for the receptor at this time. *Journal of Reproduction and Fertility* **101** 421-426.
6. Curtis R Scherer SS Somogyi R Adryan KM Ip NY Zhu Y Lindsay RM DiStefano PS **1994** Retrograde axonal transport of LIF is increased by peripheral nerve injury: correlation with increased LIF expression in distal nerve. *Neuron* **12** 191-204.
7. Delage G Moreau JF Taupin JL Freitas S Hambartsoumian E Olivennes F Fanchin R Letur-Konirsch H Frydman R Chaouat G **1995** In vivo endometrial secretion of human interleukin for DA cells/ leukaemia inhibitory factor by explant cultures from fertile and infertile women. *Human Reproduction* **10** 2483-2488.
8. Dungleison GF Barlow DH Sargent IL **1996** Leukaemia inhibitory factor significantly enhances the blastocyst formation rates of human embryos cultured in serum-free medium. *Human Reproduction* **11** 191-196.
9. Eckert J Niemann H **1998** mRNA expression of leukaemia inhibitory factor (LIF) and its receptor subunits glycoprotein 130 and LIF-receptor-beta in bovine embryos derived in vitro or in vivo. *Molecular Human Reproduction* **10** 957-965.
10. Hamilton JA Iles RK Gunn LK Wilson CMY Lower AM Chard T Grudzinskas JG **1998** Concentration of placental protein 14 in uterine flushings from infertile women: validation of collection technique and method of expression of results. *Human Reproduction* **13** 3357-3362.
11. Kojima K Kanzaki H Iwai M Hatayama H Fujimoto M Inoue T Horie K Nakayama H Fujita J Mori T **1994** Expression of leukemia inhibitory factor in human endometrium and placenta. *Biology of Reproduction* **50** 882-887.
12. Laird SM Tuckerman EM Dalton CF Dunphy BC Li TC Zhang X **1997** The production of leukaemia inhibitory factor by human endometrium: presence in uterine flushings and production by cells in culture. *Human Reproduction* **12** 569-574.
13. Ledee-Bateille N Lapree-Delage G Taupin JL Dubanchet S Frydman R Chaouat G **2002** Concentration of leukemia inhibitory factor (LIF) in uterine flushing fluid is highly predictive of embryo implantation. *Human Reproduction* **17** 213-218.
14. Merviel P Challier J Carbillon L Foidart J Uzan S **2001** The role of integrins in human embryo implantation. *Fetal Diagnosis and Therapy* **16** 364-371.
15. Metz CE **1978** Basic principles of ROC analysis. *Seminars in Nuclear Medicine* **8** 283-298.
16. Mitchell MH Swanson RJ Oehninger S **2002** In vivo effect of leukemia inhibitory factor (LIF) and an anti-LIF polyclonal antibody on murine embryo and fetal development following exposure at the time of transcervical blastocyst transfer. *Biology of Reproduction* **67** 460-464.
17. Nachtigall MJ Kliman HJ Freinberg RF Olive DL Engin O Arici A **1996** The effect of leukemia inhibitory factor (LIF) on trophoblast differentiation: a potential role in human implantation. *Journal of Clinical Endocrinology and Metabolism* **81** 801-806.
18. Nikas G Makrigiannakis A Hovatta O Jones H **2001** Surface morphology of the human endometrium. Basic and clinical aspects. *Annals of New York Academy of Sciences* **900** 316-324.

19. Noyes RW Hertig AT Rock J **1950** Dating the endometrial biopsy. *Fertility and Sterility* **1** 3-25.
20. Olivennes F Ledee-Bataille N Samama M Kadoch J Taupin JL Dubanchet S Chaouat G Frydman R **2003** Assessment of leukemia inhibitory factor levels by uterine flushing at the time of egg retrieval does not adversely affect pregnancy rates with in vitro fertilization. *Fertility and Sterility* **79** 900-904.
21. Psychoyos A **1986** Uterine receptivity for nidation. *Annals of New York Academy of Sciences* **476** 36-42.
22. Seppala M Koistinen H Koistinen R **2001** Glycodelins. *Trends in Endocrinology and Metabolism* **12** 111-117.
23. Sharkey AM Dellow K Blayney M Macnamee M Charnock-Jones S Smith SK **1995** Stage-specific expression of cytokine and receptor messenger ribonucleic acids in human preimplantation embryos. *Biology of Reproduction* **53** 955-962.
24. Stewart CL Kaspar P Brunet LJ Bhatt H Gadi I Kontgen F Abbondanzo ST **1992** Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* **359** 76-79.
25. Tomida M Yamamoto-Yamaguchi Y Hozumi M **1984** Purification of a factor inducing differentiation of mouse myeloid leukaemic M1 cells from conditional medium of mouse fibroblast L929 cells. *Journal of Biological Chemistry* **259** 10978-10982.
26. Ware CB Horowitz MC Renshaw BR Hunt JS Liggitt D Koblar SA Gliniak BC McKenna HJ Papaynnopoulou T Thoma B Cheng L Donovan PJ Peschon JJ Bartlett PF Willis CR Wright BD Carpenter MK Davidson BL Gearing DP **1995** Targeted disruption of the low-affinity leukemia inhibitory factor receptor gene causes placental, skeletal, neural and metabolic defects and results in perinatal death. *Development* **121** 1283-1299.
27. Wilcox AJ Baird DD Weinberg CR **1999** Time of implantation of the conceptus and loss of pregnancy. *New England Journal of Medicine* **340** 1796-1799.