Eotaxin and Interleukin-4 Levels and Their Relation to Sperm Parameters in Infertile Men

İnfertil Erkeklerde Eotaksin ve İnterlökin-4 Düzeyleri ve Sperm Parametreleri ile İlişkisi

Türkan YİĞİTBAŞI, MD,[a]
Yasemin BASKIN, MD,[b]
Gökhan AFACAN, MD,[c]
Füsun KARAARSLAN, MD,[d]
Cevat TAHERİ,[e]
Dilek ASLAN, MD[d]

[a]Clinical Biochemistry Clinic, Atatürk Training and Research Hospital,
[b]Dokuz Eylül University Institute of Oncology,
[c]Institute of Forensic Medicine, Adliye Sarayı,
[d]Irenbe IVF Center, İzmir
[e]Analiz Diagnostics Center, Kayseri

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Yazışma Adresi/*Correspondence:*
Türkan YİĞİTBAŞI, MD
Atatürk Training and Research Hospital, Clinical Biochemistry Clinic,
İzmir,
TÜRKİYE/TURKEY
turkanyigitbasi@gmail.com

ABSTRACT Objective: Male factor infertility accounts for 30% to 50% of the total infertile couples seeking for infertility treatment. In about 40-60% of these men, a specific etiology can not be found. The aim of this study was to confirm the presence of eotaxin and interleukin-4 (IL-4) in human seminal plasma, to show the differences between eotaxin and IL-4 concentrations in fertile and infertile men, and to show the potential relationship between eotaxin and IL-4 levels in semen and spermiogram parameters. In literature, this is the first study that evaluates eotaxin in the human seminal plasma. **Material and Methods:** The participant of the study was 55 infertile males with abnormal semen parameters as study group and 16 healthy volunteers with normal sperm parameters as the control group. Semen samples were classified according to criteria of the World Health Organization Laboratory Manual. The Morphology of the smears was scored using Kruger's strict criteria. Seminal eotaxin and IL-4 levels were measured by bead based immunoassay multiplex methods. **Results:** Seminal eotaxin levels were significantly higher in infertilite group compared to fertile donors. There were negative correlations between eotaxin concentrations and parameters such as motility ($r= -0.293$, $p< 0.01$) and, +4 motility ($r= -0.307$, $p< 0.01$) in the study group. IL-4 levels were similar in fertile and infertile seminal plasma. There were positive correlations between eotaxin and IL-4 levels ($r= 0.436$ $p< 0.01$). The receiver operating characteristic curve (ROC) analysis revealed a diagnostic value for eotaxin activity with respect to male factor infetility in case group, with an area under curve of 0.69 (95% confidence interval= 0.55-0.84). **Conclusion:** Increased levels of eotaxin may play a role in the pathogenesis of male infertility.

Key Words: Infertility; chemokines; interleukin-4; semen

ÖZET Amaç: Erkek faktörüne bağlı infertilite, infertilite nedeniyle tedavi almakta olan tüm çiftlerin %30-50'sinden sorumludur. Bu erkeklerin yaklaşık% 40-60'ında belirli bir etyoloji bulunamamaktadır. Bu çalışmanın amacı insan seminal plazmasında eotaksin ve interlökin-4 (IL-4) varlığını ortaya koymak, fertil ve infertil erkeklerde eotaksin ve IL-4 konsantrasyonları arasındaki farkı göstermek ve semendeki eotaksin ve interlökin-4 düzeyleri ile spermiogram parametreleri arasındaki olası ilişkiyi göstermektir. Yaptığımız literatür taramasına göre, bu çalışma insan semen plazmasında eotaksin düzeylerini değerlendiren ilk çalışmadır. **Gereç ve Yöntemler:** Çalışmaya anormal sperm parametreleri olan 55 infertil erkek çalışma grubu, normal sperm parametreleri olan 16 sağlıklı gönüllü de kontrol grubu olarak katıldı. Semen örnekleri, Dünya Sağlık Örgütü Laboratuvar Manual kriterlerine göre sınıflandırıldı. Morfoloji ve smear'ler ve Kruger'in kesin kriterleri kullanılarak skorlandı. Seminal eotaksin ve IL-4 düzeyleri multiplex immunoassay yöntemlerle ölçüldü. **Bulgular:** Seminal eotaksin seviyeleri infertilite grubunda fertil vericilere göre anlamlı oranda yüksek bulundu. Çalışma grubunda eotaksin konsantrasyonu ile motilite ($r= -0.293$, $p< 0.01$) ve +4 motilite ($r= -0.307$, $p< 0.01$) parametreleri arasında negatif korelasyonlar vardı. Çalışma grubundaki. IL-4 düzeyleri fertil ve infertil seminal plazmalarda benzerdi. Eotaksin ve IL-4 düzeyleri arasında pozitif korelasyonlar ($r= 0436$ $p< 0.01$) vardı. Alıcı işletimli karakteristik ROC eğrisi, vaka grubuna kıyasla eotaksin aktivitesi için 0.69 eğrisi altında (%95 güvenlik indeksi= 0.55-0.84) tanısal bir değer ortaya çıkardı. **Sonuç:** Yükselmiş eotaksin düzeyleri erkek infertilitesinin patogenezinde rol oynayabilir.

Anahtar Kelimeler: Kısırlık; kemokinler; interlökin-4; semen

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Male factor infertility accounts for 30% to 50% of the total infertile couples seeking for infertility treatment.[1] In about 40–60% of these men, a specific etiology can not be found.[2]

Cytokines are regulatory proteins involved in hematopoiesis, immune cell development, inflammation and immune responses. Several cytokines have direct effect on testicular cell functions, and a number of these are produced within the testis even in the absence of inflammation or immune activation events. There is compelling evidence that cytokines, in fact, play an important regulatory role in the development and normal function of the testis.[3]

Eotaxin is a member of the CC chemokine family of inflammatory and immunoregulatory cytokines.[4] Eotaxin is synthesized by a number of different cell types, and is stimulated by interleukin-4 (IL-4) which is produced by T-helper 2 (Th-2) lymphocytes.[4-6] Its role in numerous eosinophil-associated disorders such as food allergy, gastroenteritis, parasitic infections, allergic colitis and inflammatory bowel disease has been described.[4-7]

Seminal plasma is known to be responsible for orchestrating mating-induced immunomodulation. Central to this process are numerous cytokines that modulate uterine leukocyte recruitment and trafficking. Comprehensive analysis of the cytokine profile of murine seminal fluid revealed that only few cytokines included IL-4 and eotaxin levels which were significantly higher in seminal plasma when compared to those found in serum.[8] To best of our knowledge, this is the first study that evaluates eotaxin in the human seminal plasma.

IL-4 is a T-lymphocyte-derived 20-kDa glycoprotein possessing a broad spectrum of biological activity. In addition to inducing proliferation and differentiation of human B cells, it can stimulate the proliferation of a wide range of cells such as T-lymphocytes, mast cells, and hemopoietic progenitor cells.[9]

The presence of interleukins and several other cytokines in seminal plasma was reported in multiple studies, however there was no adequate information in the literature concerning IL-4.[10-15] Zhang et al has found that the content of IL-4 in the seminal plasma of the infertile group was significantly lower than that of the normal group ($p<0.01$).[16] Paradisi et al. reported the lack of IL-4 in seminal plasma.[17]

The aim of this study was to confirm the presence of eotaxin and IL-4 in human seminal plasma, to show differences between eotaxin and IL-4 concentrations in fertile and infertile men and to show the potential relationship between eotaxin and IL-4 levels and the spermiogram parameters.

MATERIAL AND METHODS

1. SUBJECTS AND SELECTION

The study population included 55 males of infertile couples who were treated in Irenbe IVF center. Sixteen healthy volunteers who had normal sperm parameters were enrolled as the control group. All the participants gave their informed consents to participate in the study, which had been approved by the local Ethics Committee (Izmir Tepecik Training and Research Hospital local Ethic Committee: 30.03.2007-67/11). None of the participants received any medication or vitamin supplementation. Instances of leukocytospermia and viscous semen samples were excluded from the study.

2. SEMEN COLLECTION AND PREPARATION

Semen specimens were collected by masturbation after 48 to 72 hours of sexual abstinence. The specimens underwent complete liquefaction at 37°C for 20 minutes. All semen samples were counted in a Mackler counting chamber (Sefi Medical Instruments, Rehovot, Israel). Samples were classified according to criteria of the World Health Organization Laboratory Manual, 4th edition.[18] The motility of sperm was classified as +1,+2,+3,+4 motile. Morphology of the smears were scored using Kruger's strict criteria.[19] All samples were centrifuged at 1000×g for 10 minutes. Clear seminal plasma was stored at –80°C until analysis.

3. MEASUREMENT OF EOTAXIN AND IL-4 IN SEMINAL PLASMA

Instrument: Luminex® 100 or 200 readers (Luminex Corp., Austin, TX) with data acquisition soft-

ware version 1.7, IS2.3, or xPONENT®, Millipore vacuum pump (Catalog# WP6111560) and Millipore MultiScreen® RESIST vacuum manifold (Catalog# MAVM0960R) HUMAN CYTOKINE LINCOplex KIT 96 Well Plate Assay (Cat. #HCYTO-60K) Assay plate: Millipore MultiScreen HTS, BV, 96-well filter plate (Catalog# MSBVN1250) was used for all assays. Antibody–Conjugated Beads: Carboxylated polystryene microspheres were purchased from Luminex Corp. (Austin, TX). Antibodies were either developed internally at Millipore or purchased commercially.

Assay Methodologies: All assays were built on sandwich format with two antibodies for each analyte. Phycoerythrin was used as the reporter on the surface of microspheres. Sample Requirement: 25 µL of samples was used per well. Data Analysis: StatLIA® software (Brendan Scientific., Inc.) was used for sample calculations with a weighted 2-parameter logistic curve-fitting method (Eotaxin sensitivity 1,23 pg/ml); (IL-4 sensitivity 0,57pg/ml).[20]

4. STATISTICAL ANALYSIS

Data differences between the study and the control group were analyzed by using Student's t-test.The Kolmogorov-Smirnov test was used to determine normality. Coefficients of correlation were calculated using Spearman's correlation analysis. All hypothesis tests were two-tailed with statistical significance assessed at the p value < 0.01 level with 95% confidence intervals. The data were expressed as the Mean ± SD. Statistical computations were calculated using SPSS 11.0 for windows software (SPSS Inc, Chicago, IL, USA). The area under the receiver operating characteristic curve (ROC) was used to assess the discriminative ability of eotaxin levels in patient with male infertility.

RESULT

Demographic characteristics, seminal eotaxin and IL-4 levels in the study and control groups were summarized in Table 1. There was not any significant difference in terms of age between the groups. Seminal eotaxin levels were significantly higher in infertile group as compared to fertile donors (Table 1).

TABLE 1: Demographics, semen characteristics, seminal eotaxin and IL-4 levels of infertile men and fertile donors.

	Fertil donors (n= 16)	Infertile men (n= 55)	P
Age (years)	33,2±6,0	33,5±6,8	0.857
Semen Parameters			
Concentrations (×10^6/mL)	49,9±28	33,2±28	0.043
Motility (% motile sperm)	55,8±7,2	34,8±12	<0.001
Morphology (Kruger's criteria % normal sperm)	15,9±5,8	6,0±2,2	<0.001
+4 Motility (%motile sperm)	25,6±5,0	8,6±4	<0.001
Eotaxin(pg/ml)	9,7 ± 8,7	20,2± 15,7	0.023
IL-4(pg/ml)	13,2±12,3	11,6±12,4	0.669

Values are Means + SD. The mean difference is significant at the .01 level.

There were negative correlations between eotaxin levels and sperm parameters such as motility and +4 motility ($p<0.01$). There were similar IL-4 levels in fertile and infertile seminal plasma. However there were positive correlations between eotaxin and IL-4 levels ($r= 0,436$; $p<0.01$).

ROC analysis revealed a high diagnostic value for eotaxin levels with respect to male factor infertility, with an area under curve (AUC) of 0.69 [95% confidence interval (CI) =0.55-0.84], sensitivity = 68% and specificity= 43% with a cut off value of 5.13 (greater than the value that was related to male factor infertility) (Figure 1).

DISCUSSION

Infertility continues to be a highly prevalent condition. The primary problem resides exclusively in the male partner in 30-50% of infertile couples. A specific cause of infertility is not determinable in 40–60% of the infertile men.[1,2]

Cytokines represent a widely defined group of bioactive polypeptides involved in the communication network of immune-competent cells.[21] In addition, cytokines have decisive activities outside of the immune system where they function as regulators of testicular steroid hormone production. Cytokines have also been implicated as novel growth and differentiation factors involved in the regulation of cells in both the endocrine and the tubular compartment of the testis.[22]

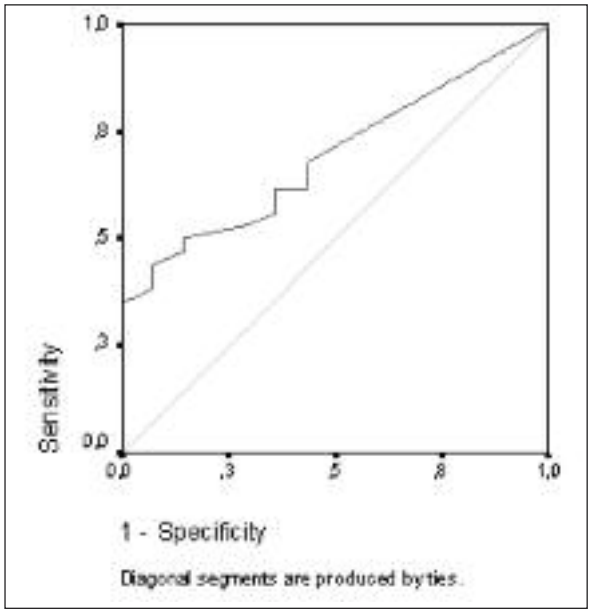

FIGURE 1: ROC of eotaxin levels in patient with male infertility.

The inflammation due to genital or systemic infections can cause alterations in the testicular function. The recognition of intratesticular antigens provokes the production of antibodies by B lymphocytes. Then, the immune system induces a cellular response, by cytokine secretion, activation of complement and T lymphocytes activation.[23] Moreover, these are produced within the testis even in the absence of inflammation or immune activation events. There is compelling evidence that cytokines, in fact, play an important regulatory role in the development and normal function of the testis.[3]

Post-mating inflammatory response has been described in mice and pigs.[24,25] The cellular changes are initiated when seminal moieties interact with cervical and uterine epithelial cells to induce a surge in synthesis of cytokines.[24] The activation of the expression of uterine cytokines and leukocyte trafficking that has been implicated in pre-implantation embryo development.[24-26]

Gopichandran et al. found that levels of IL-4, G-CSF, eotaxin, KC and RANTES in fluid drawn from the seminal vesicles of single mice were significantly higher when compared to those found in serum.[8] Based on these findings, authors proposed a model of mating-induced immunomodulation that implicated seminal eotaxin, RANTES and MIP-1 in the relocation and concentration of extravasated migrating endometrial eosinophils to the luminal epithelium.[8]

Eotaxin (condensed from eosinophil chemotaxin) microsequencing revealed a novel 73 amino acid C-C chemokine. The purified protein was a highly potent eosinophil chemoattractant *in vitro* and *in vivo,* but had no significant effect on neutrophils. The eotaxins are unusual (but not unique) in signaling via a single receptor: CCR3. Many cell types in the lung appear to be capable of synthesizing eotaxin (eg airway epithelial cells, airway smooth muscle cells, vascular endothelial cells and macrophages, as well as eosinophils themselves) [4]. Thus, cytokines that are synthesized by Th2 lymphocytes, such as IL-4, have been investigated as potential intermediaries in eotaxin production.[27]

In our study, seminal eotaxin levels were significantly higher in infertile group when compared to fertile donors (Table 1). It has been shown that motility and +4 motility decreased as eotaxin levels increased.

In our study ROC analysis revealed a high diagnostic value for eotaxin levels with respect to male factor infertility, with an area under curve (AUC) of 0.69]95% confidence interval (CI) =0.55-0.84], sensitivity = 68% and specificity= 43% with a cut off value of 5.13 (greater than the value that was related to male factor infertility) (Figure 1). A literature review revealed that no data existed regarding this matter.

IL-4 can induce eotaxin mRNA expression in human vascular endothelial cells.[5] In our study, there was a positive correlation between eotaxin and IL-4 levels. In vitro studies of Teran et al.[6] demonstrated that IL-4 synergizes with the pro-inflammatory cytokine tumor necrosis factor-α to increase eotaxin production from lung fibroblasts.

The levels of LIF, IL-4, IL-10 and M-CSF produced by decidual T cells of women suffering from

unexplained spontaneous abortion are lower than those of healthy pregnant women, and this can indicate that these cytokines may contribute to the maintenance of pregnancy. T cells from the cumulus oophorus surrounding the preimplantation embryo produce LIF and IL-4. These findings suggest that cytokines produced by maternal T cells create a suitable microenvironment for preimplantation embryo development and maintenance of pregnancy.[28,29] Seminal fluid was also characterized by higher levels of IL-4 and G-CSF compared to serum, suggesting that these too, may have a role, as yet undefined, in mating-induced immunomodulation. In man, IL-4 is thought to be necessary for the establishment and maintenance of pregnancy, by avoiding the harmful effects of cell-mediated immunity in the vicinity of putative embryo implantation sites and at the feto-maternal interface.[8]

Zhang et al. found that the content of IL-1 beta in the seminal plasma of the infertile group was obviously higher, but the content of IL-4 and IL-10 levels were significantly lower than that of the normal group.[16] Paradisi et al. reported the lack of IL-4 in seminal plasma.[17] These findings might indicate that further studies are necessary to clarify the role of IL-4 in patients with male infertility.

In our study, we found similar IL-4 levels in fertile and infertile seminal plasma.

It has been shown that the role of cytokines at post-mating inflammatory response is important. However in literature, there are no studies concerning the levels of eotaxin in human seminal plasma. Our study is the first study that evaluates eotaxin levels in the seminal plasma of infertile men using multiplex immunoassays.

In conclusion, our results demonstrated that eotaxin levels were significantly higher in patients with male infertility, and correlated with the sperm parameters such as motility and +4 motility. Increased levels of eotaxin may play a role in the pathogenesis of male infertility.

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