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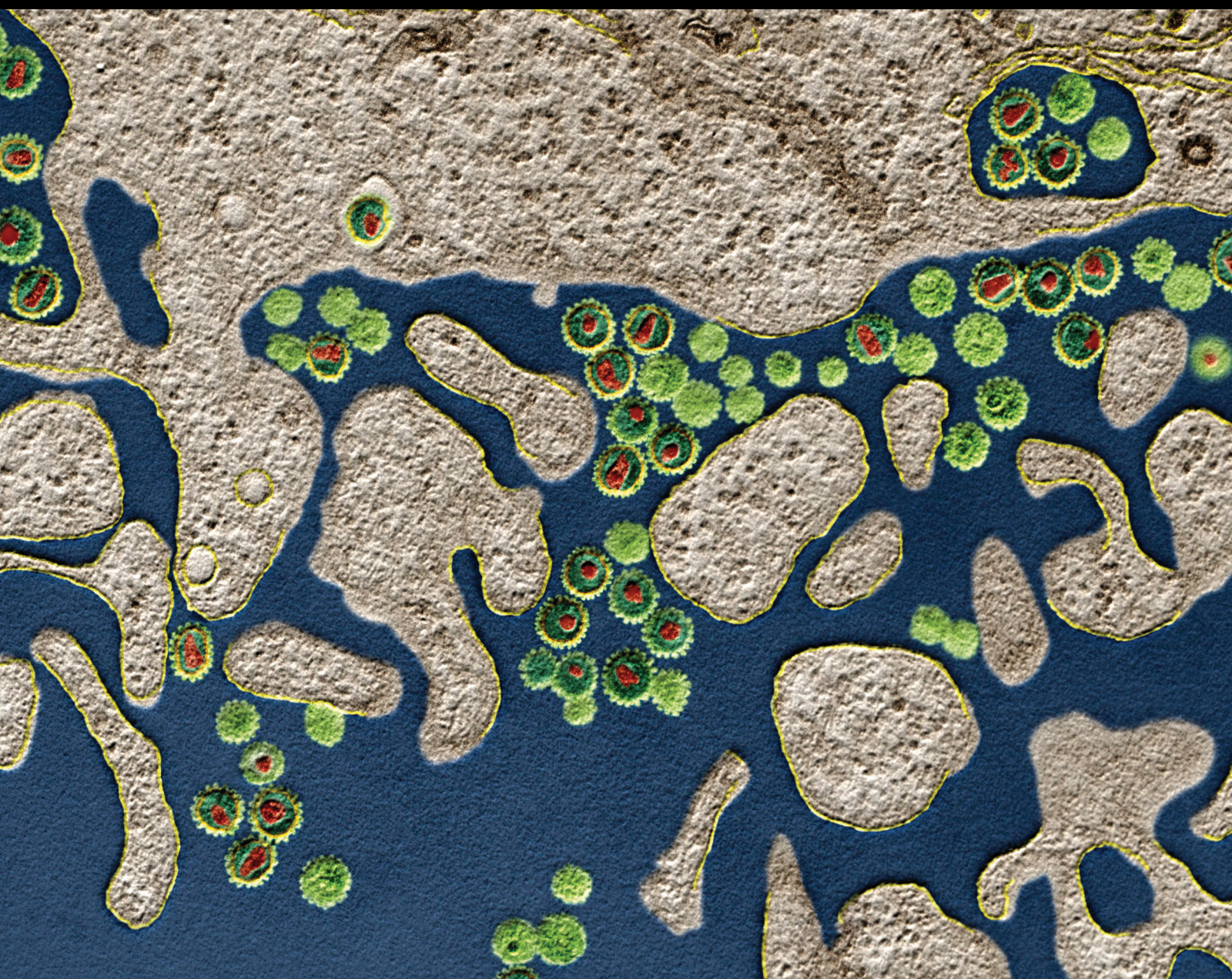
New Biomarkers of Innate and Adaptive Immunity in Infectious Diseases

Lead Guest Editor: Sergey Morzunov
Guest Editors: Levon Abrahamyan and Varough Deyde



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Editorial

New Biomarkers of Innate and Adaptive Immunity in Infectious Diseases

Sergey Morzunov,[1] Varough Deyde,[2] and Levon Abrahamyan[3]

[1]*University of Nevada, Reno, USA*
[2]*CDC, Pretoria, South Africa*
[3]*Université de Montréal, Montreal, Canada*

Correspondence should be addressed to Sergey Morzunov; smorzunov@medicine.nevada.edu

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Despite dramatic achievements in the field of infectious disease control and prevention in economically developed countries, infectious diseases still remain the leading cause of morbidity, disability, and mortality worldwide. According to the World Health Organization (WHO), 15 million fatalities were attributed to infectious diseases in 2010. It is extremely alarming that the WHO predicts 13 million infectious disease-related deaths for 2050 [1]. In particular, emerging infectious diseases are on the rise, which can be explained by many factors like microbial adaptation to new hosts, selective pressures of the treatment, migration of the natural hosts, and so on. Emerging infectious diseases often have deadly consequences which may threaten the existence of humanity. Although much has been learnt about the pathogenesis of infectious diseases, reliable early diagnosis and effective treatment for many of these diseases are still not available. These limitations could be explained by our limited knowledge of the molecular host-pathogen interactions and immune response to many particular infections, which hampers the discovery of the early diagnostic biomarkers.

Recent advances in molecular biology and immunology resulted in a rapid expansion in the field of immune biomarkers. These biomarkers can help make an early diagnosis, evaluate the efficacy of treatment, and improve or predict the disease outcome. The increase of emerging infections and spread of the antibiotic-resistant bacterial strains make the search for biomarkers especially urgent. Recently, the U.S. Food and Drug Administration addressed the need for Immune Biomarkers regarding development and approval of the new diagnostics as essential for improving the treatment of infectious diseases. The ongoing search for biomarkers is wide and includes correlation analysis between clinical presentation and genetic mutations, cytokines, receptors, growth factors, and so on found in the host or the infectious agent. Despite extensive research, the need for novel biomarkers remains critical. For example, not much is known about immune biomarkers for such emerging/reemerging infections as Zika virus-caused disease and Ebola hemorrhagic fever. There is also ongoing search for biomarkers of the immune dysfunction in HIV and severe dengue disease. Novel biomarkers for cytomegalovirus and *Mycobacterium tuberculosis* provide useful information on the human host response to the corresponding infections.

We invited prospective authors to contribute original manuscripts, case reports, clinical trials, and reviews focusing on genetic aberrations, cytokines, growth factors, and other small biologically active molecules as potential biomarkers in infectious disease. As a result, the manuscripts selected for this special issue reflect the current diversity of this research field.

The investigation of C. C. Emene et al. presents genetic markers associated with an increased chance of developing erysipelas. The authors also identified genetic markers associated with the localization of the infection on different parts of the body. In addition, elevated serum cytokines IL-1β, CCL11, IL-2Rα, CXCL9, TRAIL, PDGF-BB, and CCL4 were found in erysipelas patients. Furthermore, increased serum levels of IL-6, IL-9, IL-10, IL-13, IL-15, IL-17, G-CSF, and

VEGF were detected in patients with recurrent erysipelas. Finally, correlation analysis revealed that level variations of IL-1β, IL-7, IL-8, IL-17, CCL5, and HGF were associated with SNPs in the SOD2 gene, while variations of PDFG-BB and CCL2 were found to be associated with SNPs in the CAT gene.

N. Molaee et al. investigated the immune response in mice to the *Vibrio cholera* recombinant pili and flagella proteins. High levels of IL5 and low levels of IFNγ observed in response to injection of FlaA and TcpA suggest that these proteins stimulate immune responses toward Th2, while high levels of IL5 and high IgG1 antibody titer observed after injection of TcpB suggest that it mostly directs immune responses toward Th1. The data provided on the immune response in humans is identical; combining these recombinant antigens with potential *Vibrio cholera* vaccine can cause higher immunogenicity and better protection against cholera.

Y. A. Tyurin et al. evaluated a cytokine profile in nasal secretion and blood serum of the patients with seasonal (SAR) and perennial (PAR) allergic rhinitis. The authors showed that increased GM-CSF production is maintained in the patients with PAR sensitized to the house mite allergen components. A higher production profile of TNFα and TSLP was also detected in nasal secretion in the patients with PAR and additional high sensitization to SEs. Sensitization to mold fungi allergen components was significantly higher in patients with SAR. In particular, these patients displayed a high level of sensitization to the *Aspergillus fumigatus* component m3. A negative correlation was demonstrated between serum TGF-β level and the age of patients with SAR. TGF-β can inhibit the immune response and proliferation of immunocompetent cells. Thus, along with other clinical trials, the study performed and clarified some aspects of the molecular pathogenesis of human allergic rhinitis.

In the second investigation of Y. A. Tyurin et al., the correlation between polymorphisms of the Toll-like receptor genes TLR2 and TLR4 in relation to clinical and immunological parameters in atopic dermatitis patients was analyzed. Observed dysregulation of cytokine production (IL-4, IL-10) in the patients with heterozygous polymorphic genotypes probably reflects an imbalance of Th1/Th2/Th17 regulation of immune response in these individuals.

N. S. Zakharchenko et al. evaluated the antimicrobial efficacy of the *Kalanchoe pinnata* extract containing cecropin P1 (CecP1). *K. pinnata* extracts were tested for the treatment of wounds infected with *Candida albicans*. The therapeutic efficacy of *K. pinnata* extract was comparable with that of a commercial fungicide clotrimazole. However, *clotrimazole* neither facilitated wound healing nor caused remodeling of the scar matrix. The improved therapeutic effect of the *K. pinnata* extract was attributed to a synergism between the fungicide activity of CecP1 and wound healing (antiscar), revascularizing, and immunomodulating effect of natural biologically active components of *K. pinnata*. Taken together, data presented suggest that CecP1-enriched *K. pinnata* extracts could be a candidate drug for the treatment of dermatomycoses and fungus-contaminated wounds.

In a related investigation performed by A. A. Lebedeva et al., the therapeutic effect of *K. pinnata* water extracts containing CecP1 on healing of wounds contaminated with *S. aureus* or combined *S. aureus* with *P. aeruginosa* was evaluated. The efficacy of *K. pinnata* extract treatment of *S. aureus* turned out to be equal to that of cefazolinum; however, it performed better than cefazolinum when treating the wounds simultaneously contaminated with *S. aureus* with *P. aeruginosa*. *K. pinnata* extracts (both wild-type and transgenic) did not exhibit general toxicity while accelerating wound recovery. Immunomodulating and microbicide activity of *K. pinnata* synergize with microbicide activity of CecP1 accelerating elimination of bacteria.

In the study of E. V. Martynova et al., several important correlations were detected for the nephropathia epidemica (NE) patients. In particular, elevated levels of triglycerides and decreased HDCL were found in NE patients, while total cholesterol did not differ between NE cases and controls. Higher triglyceride levels were found in males as compared to female NE cases. Furthermore, data indicated that high triglycerides are associated with the lowest thrombocyte counts and high serum VEGF, as well as a high severity score. On the other hand, low levels of triglycerides were associated with upregulated IFN-γ and IL-12. Activation of the Th1 type immune response was suggested, since IFN-γ and IL-12 levels were increased in patients with lower severity scores. This relationship strongly indicates that Th1 lymphocytes play a protective role in NE. These data advance our understanding of NE pathogenesis and establish a clear link of the high triglyceride level with the severity of the disease.

Kh. S. Khaertynov et al. evaluated the serum levels of the proinflammatory cytokines (TNF-α, IL1-β, and IL-6) and the anti-inflammatory cytokines (IL-4 and IL-10) in neonatal sepsis cases. It was demonstrated that both the late onset of sepsis (LOS) cases and the early onset of sepsis (EOS) cases were characterized by increased serum level of TNF-α. However, increased serum levels of IL-6 and IL-10 were found in LOS cases only. Moreover, levels of the proinflammatory cytokines, such as TNF-α and IL-6, were elevated in the acute phase of sepsis, whereas the anti-inflammatory cytokines, such as IL-10, were substantially upregulated during the postacute phase of the disease. The authors concluded that the analysis of serum cytokines can provide valuable information when determining the most effective therapy for treating neonatal sepsis.

In the study by D. Akberova et al., serum cytokine levels were investigated in several autoimmune diseases including autoimmune liver diseases (AILD), autoimmune hepatitis (AIH), and overlap syndrome. AILD cases were characterized by increased levels of IL-6, IL-8, and TNF-α as compared to controls. Statistical analysis revealed a correlation between high IL-8 and diagnosis of AILD, AIH, and overlap syndrome.

Due to an excellent response, this special issue includes a number of the original research articles aimed at the detection and analysis of the various potential biomarkers (genetics, proteomics, cytokines, growth factors, hormones, and so forth) of infectious disease contributing to diagnosis, treatment, and prophylaxis. The analysis of these novel biomarkers was based on the bioinformatics approaches that were employed to characterize biomarkers associated with

the clinical presentation, severity, and treatment efficacy of infectious diseases. In addition, this issue includes data on novel therapeutic approaches for the treatment of infectious disease using both novel and presently known biomarkers. Finally, new data on the biomarkers of the immune evasion and development of resistance to viral and microbial pathogens are presented in this issue.

Sergey Morzunov
Varough Deyde
Levon Abrahamyan

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Clinical Study

Cytokine Profile of Patients with Allergic Rhinitis Caused by Pollen, Mite, and Microbial Allergen Sensitization

Yury A. Tyurin,[1,2] Svetlana A. Lissovskaya,[2] Rustem S. Fassahov,[3] Ilshat G. Mustafin,[1] Anton F. Shamsutdinov,[3] Marina A. Shilova,[4] and Albert A. Rizvanov[3]

[1]*Department of Biochemistry and Clinical Laboratory Diagnostics, Medical State University, Butlerov Street 49, Kazan, Republic of Tatarstan, Russia*
[2]*Laboratory of Immunology and the Development of Allergens, Mycology Research Laboratory, Research Institute of Epidemiology and Microbiology, Big Red Street 67, Kazan, Republic of Tatarstan 420015, Russia*
[3]*Institute of Fundamental Medicine and Biology, Kazan Federal University, Kremlevskaya Street 18, Kazan 420008, Russia*
[4]*I.M. Sechenov First Moscow State Medical University, 8-2 Trubetskaya Str., Moscow 119991, Russia*

Correspondence should be addressed to Albert A. Rizvanov; albert.rizvanov@kpfu.ru

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Allergic rhinitis (AR) is especially prevalent among the population of large cities. Immunologically, the airway epithelium is a region where the population of allergen-presenting cells concentrates. These cells actively express a group of receptors of the innate immune system. A specific cytokine profile is its representation. The study was aimed at evaluating the cytokine profile in patients with seasonal and perennial allergic rhinitis. The cytokine profile of nasal secretion and blood serum of 44 patients with AR was studied. 24 of them had seasonal allergic rhinitis (SAR), and 20 patients suffered from perennial allergic rhinitis (PAR). The patients' age ranged from 4 to 60 years. It was determined in our study that the activation of the GM-CSF production retained in patients with PAR sensitized to mite allergen components (*Dermatophagoides pteronyssinus*). There was a higher production profile of TNF-α and TSLP in nasal secretion in the patients with perennial allergic rhinitis and additional high sensitization to SEs. Sensitization to mold fungal allergen components significantly increases in patients with seasonal allergic rhinitis. They demonstrated high level of sensitization to the *Aspergillus fumigatus* component m3. Thus, along with other clinical trials, the study performed would clarify some aspects of molecular pathogenesis of human allergic rhinitis.

1. Introduction

At present, allergic rhinitis is increasingly becoming an urgent problem for primary health care as the number of visits to general practitioners for this disorder rises. There is a high prevalence of allergic rhinitis (AR) especially among the population of large cities. This allergic disorder requires that therapists and allergists should perform diagnostic tests in order to confirm its diagnosis and detect underlying bronchial asthma. The incidence of allergic rhinitis and other respiratory allergies, atopic (allergic) asthma in particular, has significantly increased over the past fifty years. This is the case in most of European countries including Russia and has been attributed to environmental factors, urbanization, and changes in a diet and a lifestyle of a modern urban dweller [1].

According to recommendations provided by the ARIA expert working group in cooperation with the World Health Organization (WHO), the World Organization of Family Doctors (WONCA), and the International Primary Care Respiratory Group (IPCRG), allergic rhinitis is a chronic airway disorder and one of the most significant risk factors for the development of bronchial asthma [2].

Pathophysiologically and immunologically, the airway epithelium is a region with a concentrated population of allergen-presenting cells (APCs) called dendritic cells

Table 1: Immunologically active aeroallergens.

Source of allergens	Identified biological activities
(1) Grass pollen	Pectate lyase, RNase, polygalacturonase, lipid transfer protein, profilin, expansin
(2) Tree pollen	Profilin, isoflavone reductase, pectin methylesterase, peptidyl-prolyl isomerase, 1,3-β-glucanase, calcium-binding protein, pectate lyase, superoxide dismutase
(3) Fungi	Protein disulfide isomerase, aldehyde dehydrogenase, RNase, vacuolar serine proteases, alkaline serine protease, enolase, aspartate proteases, dipeptidyl peptidase, subtilisin-like protease
(4) Epidermal allergens	Uteroglobin-like protein, cystatin, lipocalin, albumin
(5) House dust mites	Cysteine protease, β-glucan moiety, trypsin, amylase, chymotrypsin, chitinase, collagenase, glutathione transferase

(DCs) which express receptors of the innate immune system. In patients with allergic rhinitis, these cells are involved in binding allergens, processing them into peptides, and presenting them via the major histocompatibility complexes MHC classes I and II for T cell receptors. The intact respiratory mucosa has been established to contain no DCs at birth; however, the exposure to biologically active aeroallergens (see Table 1) activates the respiratory epithelium.

These stimuli cause the release of chemoattractants (CCL20, CCL19, and CCL27), which initiate DC migration from bone marrow to the respiratory mucosa [3, 4]. A cytokine of GM-CSF, released by the respiratory epithelium, in cooperation with IL-4 and tumor necrosis factor-α (TNF-α) causes DC maturation. Tissue basophils are supposed to be a source of IL-4. For inhaled allergens, such as those from house dust mites, it is proposed that basophils amplify the T_H2 immune response that is initiated by mucosal DCs in highly allergic individuals and influenced by innate immunity signaling through receptors such as Toll-like receptor 4 (TLR4) and C-type lectin signaling on epithelial cells and DCs [5]. Polarization toward a T_H2 subtype is also under epigenetic regulation. TLRs are key components of the innate immune system that mediate immune response to PAMPs in the form of microbial, fungal, and viral products and their ligands, including endotoxins (recognized by TLR4), microbial lipoproteins (TLR2 and TLR6), viral double- and single-stranded RNA (TLR3 and TLR7/8), and bacterial CpG-containing DNA (TLR9) [6]. Other PRRs of the immune system are activated in response to endogenously generated danger signals (DAMPs) produced during allergic tissue inflammation, such as free ATP and uric acid [7]. These immune responses are also specific for allergic inflammation of the upper respiratory airways. However, the effect of relevant microbial allergen components on an immunity-mediated inflammation response character in seasonal and perennial allergic rhinitis is not studied. Its reflection is a specific cytokine profile. The profile of those cytokines such as TSLP, TNF-α, and GM-CSF which are synthesized in the mucosal epithelial cells, when indirectly stimulated through a system of innate immunity receptors, is of utmost interest.

The study was aimed to evaluate the cytokine profile of nasal secretion and blood serum in patients with seasonal and perennial allergic rhinitis with a potential for additional sensitization with microbial allergens.

2. Materials and Methods

2.1. Patients. The diagnosis of allergic rhinitis was established on the basis of the clinical criteria and recommendations of ARIA, 2010 [8]. The inclusion criteria were as follows: a diagnosis of allergic rhinitis for at least 2 years, specific symptoms of allergic rhinitis (an allergic rhinitis questionnaire), detected sensitization, the absence of nonallergic disorders of the nasopharynx and other organs and systems, age of patients from 4 years to 60 years, and distribution by sex in the ratio 1 : 1.2. The exclusion criteria were as follows: chronic rhinosinusitis, nasal polyps, nonallergic (infectious) rhinitis, and age below 4 and over 60 years.

2.1.1. Control Group. The inclusion criteria were as follows: healthy volunteers at the age of 3–43 years without any findings of allergic disorders at examination and distribution by sex in the ratio 1 : 1.14. The exclusion criteria were as follows: chronic pathology of the nasopharynx, chronic rhinosinusitis, nasal polyposis, and age below 3 years and over 60 years.

2.2. Methods

2.2.1. Allergy Testing. Cytology of the inferior nasal concha imprint swabs was performed. Romanowsky-Giemsa staining was used. Cells were counted with direct microscopy under a Mikmed-5 microscope (Russia) with a magnification of ×80. A percentage of epithelial cells, eosinophils, neutrophils, and lymphocytes per 100 cell elements of the stained imprint swabs was determined.

The total IgE concentration was evaluated with the use of an immunoassay total serum IgE kit.

Sensitization to mite, microbial, and pollen allergens was detected with an ImmunoCAP® technology based on immunofluorescence when allergens are sorbed on a 3D cellulose sponge that increases a specific antigen-binding surface. The immunoassay was carried out with the analyzer ImmunoCAP 100, v.1 (PhadiaAB, Uppsala, Sweden). Reagents for enzyme immunofluorescence assay of specific immunoglobulins (Ig) to house dust mites *Dermatophagoides pteronyssinus* (d1) and *Dermatophagoides farina* (d2) and their allergen component *Der p 1* being mite serine proteinase; to fungi *Penicillium notatum* (m1), *Cladosporium herbarum* (m2), *Aspergillus fumigatus* (m3), *Alternaria alternata* (m6), *Aspergillus terreus* (m36), *Rhizopus nigricans* (m11), and *Fusarium proliferatum* (*F. moniliforme*) (m9); to birch (*Betula verrucosa*) pollen allergens rBet v 1 and rBet v 2

(profilin, t216); to common wormwood allergens (w6); and to microbial allergens staphylococcal enterotoxin A (m80) and staphylococcal enterotoxin B (m81) were used.

2.3. Immunoassay. Immunoassay was performed in the clinical and diagnostic laboratory of the Kazan Research Institute of Epidemiology and Microbiology. In order to evaluate the innate and adaptive immunity, the cytokine profile of blood serum (IL-4, IL-10, and TGF-β) and nasal secretion (TSLP, IL-1β, TNF-α, and GM-CSF) was determined.

2.4. Nasal Secretion. A nasal secretion was sorbed on a cotton swab in the middle nasal concha for 30 sec, transferred into 0.25 ml of physiological saline, and centrifuged at 1500 rpm for 10 min to precipitate cellular elements, and the supernatant was collected and frozen at $T = -20^{\circ}$C.

2.5. Cytokine Concentration Assessment. Cytokine concentrations in secretion and serum samples were determined with the use of enzyme immunoassay kits "interleukin-1 beta-EIA-BEST" (AO Vector-Best, Novosibirsk, Russia), "interleukin-4-EIA-BEST," and "TNF-α-EIA-BEST" (AO Vector-Best, Novosibirsk, Russia) in accordance with the manufacturer's instructions. To determine TGF-β, IL-10, and GM-CSF concentrations, enzyme-linked immunosorbent assay kits were used (eBioscience, Bender MedSystems). The Human TSLP Quantikine ELISA Kit (R&D Systems, MN, USA) designed to measure human thymic stromal lymphopoietin in cell culture supernates, serum, and plasma with ELISA was used for the quantitative assessment of TSLP.

2.6. Statistical Analysis. The median of the parameter, standard deviation (SD), and the arithmetic mean were calculated. The nonparametric one-way ANOVA "Tukey's multiple comparison test" was applied to compare measurable characteristics between groups. The differences were considered significant at $p < 0.05$. To calculate statistical functions, the GraphPad Prism v.5 analysis program was used.

3. Results

3.1. General Characteristics of Patients with AR. Symptoms of mild and moderate rhinitis were the specific characteristic of patients with allergic rhinitis. Runny nose, nasal itching, and nasal congestion of various degrees prevailed. The characteristics of patients with AR enrolled into the study are given in Table 2.

In the study group of perennial allergic rhinitis (PAR), patients suffered from the main symptoms (sneezing, nasal congestion and impaired nasal breathing, nasal itching, and rhinorrhea) regardless of the season. The symptoms exacerbated from time to time depending upon the change of a place of habitation and exposure to initiating agents (cigarette smoke, cold air, and occupational factors). 10 patients with PAR had intermittent and mild symptoms in duration, with 10 others having persistent and moderate ones. In 5 patients, PAR was combined with other allergic pathologies (atopic asthma, atopic dermatitis).

Table 2: Characteristics of patients with allergic rhinitis.

Parameter	Form of AR	
	PAR	SAR
Number of patients, N	20	24
Age, years*	27.8 ± 4.0 17.9 (4–60)	18.2 ± 2.86 14.01 (3–43)
Gender (M/F)	8/12	12/12
Serum total IgE level, IU/ml*	100.65 ± 20.48 91.63 (14–412)	92.3 ± 6.91 33.86 (45–212)
Blood eosinophilia, %	4.91 ± 0.99 3.44 (2–12)	6.33 ± 1.08 3.7 (1–12)
Eosinophil portion in nasal mucosa imprint swab, %*	27.65 ± 4.54 20.34 (3–80)	19.5 ± 3.32** 16.3 (2–57)

*M ± m, SD: standard dev., Xmin–Xmax; **$p < 0.05$.

In the study group of patients with seasonal allergic rhinitis (SAR), the main symptoms (sneezing, nasal congestion and impaired nasal breathing, nasal itching, and rhinorrhea) debuted in early summer and persisted throughout the summer to early autumn. 11 patients with CAR had intermittent and mild symptoms in duration, while 13 others had persistent and moderate ones. Seven patients with SAR had other allergic pathologies (atopic asthma, atopic dermatitis, and allergic conjunctivitis).

4. Sensitization Profile in SAR

12 patients with SAR were found out to have sensitization to a wormwood allergen component (w6) (Table 3).

There was sensitization to the main birch allergen component rBet v 1 in the majority of SAR patients examined ($N = 24$). One patient had an allergen-specific IgE level more than 50 kUA/l. It was a combination of SAR with persistent moderate atopic asthma. It was detected that 5 patients with SAR exhibited a high level of sensitization to allergen components of both wormwood (w6) and birch (rBet v 1).

The level of allergen-specific IgE to w6 was high ranging from 23.5 to 45.7 kUA/l, while that to rBet v 1 ranged from 5.8 to 31.8 kUA/l. It was determined that 14 patients with SAR and a high level of allergen-specific IgE to the main component, rBet v 1, had sensitization to the minor birch allergen component, rBet v 2 (profilin). An average level of allergen-specific IgE to the minor birch allergen component profilin (rBet v 2) was 2.01 kUA/l, while that of IgE to the main allergen component rBet v 1 was 23.16 kUA/l.

5. Sensitization Profile in PAR

The distribution of patients along the profile of sensitization to allergic components of house dust mites is presented in Table 4. Sensitization to an allergen component (d1) of the house dust mite *Dermatophagoides pteronyssinus* was detected in 15 patients with PAR. Sensitization to an allergen

Table 3: Distribution of patients with seasonal allergic rhinitis (SAR) according to the level of sensitization to pollen allergen components of *Artemisia vulgaris* and birch ($N = 24$).

Level of IgE (sensitization), kUA/l	Allergen components			
	w6	rBet v 1	w6 + rBet v 1	rBet v 2
0.7–3.5 (very low)	1	2	—	14
3.6–17.5 (sensitization revealed)	4	9	—	—
17.6–50.0 (high level of sensitization)	7	11	5	—
50–100.0 (very high)	0	1	—	—
Total	12 (50.0%)	23 (96.0%)	5 (21.0%)	14 (58.0%)

Table 4: The distribution of patients with PAR in terms of the level of sensitization to allergen components of house dust mites and bacterial microflora ($N = 20$).

Level of IgE (sensitization), kUA/l	Allergen components			
	House dust mite			Bacteria
	d1	d2	d1 + d2	m81
0.3–0.69 (very low)	—	—	—	1
0.7–3.5 (low)	2	1	—	2
3.6–17.5 (sensitization revealed)	3	4	10	
17.6–50.0 (high level of sensitization)	6	5	—	5
50–100.0 (very high)	4	4	4	—
Total	15 (75.0%)	14 (70.0%)	14 (70.0%)	8 (40.0%)

component (d2) of the house dust mite *Dermatophagoides farina* was detected in 14 patients with PAR. Eleven patients with PAR were detected to have a high sensitization level to two house dust mite allergen components (d1 and d2) simultaneously.

The majority of the patients ($N = 14$) with PAR examined had sensitization to the two allergen components (d1 and d2) of the house dust mites *Dermatophagoides pteronyssinus* and *Dermatophagoides farina*. At the same time, the concentration of allergen-specific IgE to the allergen component d2 was more than 89.0 kUA/l in 4 patients, 2 of them had PAR combined with atopic dermatitis and recurrent bronchitis. The concentration of allergen-specific IgE to the allergen component d1 was more than 70.0 kUA/l in 4 patients, two of whom had combined severe persistent atopic asthma and allergic conjunctivitis.

In patients with PAR, an average concentration of allergen-specific IgE was 43.37 kUA/l to the allergen component d2 of the mite *Dermatophagoides farina* and 36.9 kUA/l to d1. At the same time, the concentration of allergen-specific IgE to the minor allergen (d202) Der p 1, being cysteine protease, was 16.9 kUA/l in these patients.

6. Additional Sensitization to Microbial Allergens in PAR

The distribution of patients with PAR at the sensitivity profile to allergens of *S. aureus* is presented in Table 4. From the point of view of pathophysiology, we have studied sensitization to allergens (m80, m81) of *Staphylococcus aureus* in these patients. A potential of additional sensitization to microbial allergens cannot be ruled out in patients with PAR. These allergens are staphylococcal superantigens SEB and SEA.

7. Additional Sensitization to Fungal and *Staphylococcus aureus* Allergens in Patients with SAR

Sensitization to mold fungal allergen components was evaluated in the study group of patients with seasonal allergic rhinitis (Table 5). We have determined that the patients with SAR studied had additional sensitization to allergen components of mold fungi such as *Penicillium notatum* (m1), *Cladosporium herbarum* (m2), and *Aspergillus fumigatus* (m3).

There was sensitization to the *Staphylococcus aureus* allergen component m81 in 12 patients, while allergen-specific IgE concentrations ranged from 1.0 to 17.0 kUA/l; that is, it was moderate and high. Seven patients demonstrated sensitization to the *Staphylococcus aureus* allergen component m80, having allergen-specific IgE concentrations at the range of 2.1 to 10.2 kUA/l; that is, it was moderate and high.

8. TSLP Concentrations in Nasal Secretion of Patients with PAR

TSLP is a cytokine which is of great interest in allergic disorders. It has been established at present that in allergen-sensitized persons, the epithelial cells of the upper airways are capable of synthesizing TSLP where this cytokine affects DCs causing their maturation and activation. We have noticed a significant correlation ($r = 0.46$, $p = 0.014$)

Table 5: Distribution of patients with seasonal allergic rhinitis according to the level of sensitization to allergen components of mold fungi ($N = 24$).

Level of IgE (sensitization), kUA/l	Allergen components		
	m1	m2	m3
0.3–0.69 (very low)	—	—	—
0.7–3.5 (low)	3	—	1
3.6–17.5 (sensitization revealed)	—	4	—
17.6–50.0 (high level of sensitization)	—	—	3
50–100.0 (very high)	—	—	—
Total	3 (12.5%)	4 (16.7%)	4 (16.7%)

between the TSLP concentration in nasal secretion and that in allergen-specific antibodies (IgE) to a *Staphylococcus aureus* enterotoxin (the allergen component m80) in patients with PAR. There was a significant correlation ($r = 0.56$, $p = 0.008$) between TSLP and GM-CSF cytokine concentrations in nasal secretion of these patients.

9. TSLP Concentrations in Nasal Secretion of Patients with SAR

There was a significant correlation dependence between TSLP cytokine concentrations in nasal secretion and those in allergen-specific antibodies (IgE) to the allergen component m3 of the mold fungus *Aspergillus fumigatus* in the patients with SAR ($r = 0.43$, $p = 0.023$).

10. TNF-α, IL-1β, and GM-CSF Cytokine Concentrations in Nasal Secretion in AR

We have studied the relationship between the level of specific sensitization to allergens of mite, fungi, and bacteria in contact with the mucosa of AR patients and concentrations of cytokines, particularly TNF-α, IL-1β, and GM-CSF, in secretion. Human granulocyte macrophage colony-stimulating factor (GM-CSF) is a small glycoprotein that primarily stimulates the production and functioning of eosinophils, monocytes, and neutrophils. The GM-CSF cytokine is produced by the airway epithelial cells in an allergic inflammation. There was a significant correlation ($r = 0.58$, $p = 0.007$) between GM-CSF concentrations in nasal secretion and those in allergen-specific IgE antibodies to the allergen component d1 of the house dust mite *Dermatophagoides pteronyssinus* in patients with PAR. At the same time, a significant correlation between GM-CSF concentrations in nasal secretion and a level of allergen-specific antibodies (IgE) to the minor house dust mite allergen d202 was determined in these patients. The allergen component d202 is the cysteine protease of house dust mites.

There was a similar significant correlation dependence ($r = 0.53$, $p = 0.014$) between GM-CSF concentrations in nasal secretion and those in allergen-specific IgE to enterotoxin (m81) of *Staphylococcus aureus*.

There was also a dependence between secretion TNF-α concentrations and sensitization (antibody levels) to a *Staphylococcus aureus* enterotoxin (the allergen component m81) ($r = 0.43$, $p = 0.049$).

In patients with SAR, there was a significant dependence between nasal secretion concentrations of TSLP and GM-CSF ($r = 0.54$, $p = 0.006$). In patients with SAR, there was a significant dependence between GM-CSF concentrations in nasal secretion and a level of allergen-specific antibodies (IgE) to mold fungal allergen components m3 and m2 ($r = 0.66$, $p = 0.0003$ and $r = 0.58$, $p = 0.002$, resp.).

Table 6: Serum cytokines in allergic rhinitis.

Form of AR	Cytokine concentration, pg/ml[1]		
	IL-4[2]	IL-10	TGF-β[3]
SAR ($n = 24$)	0.54 ± 0.03 0.17 (0.21–0.9)	0.36 ± 0.04 0.22 (0.11–0.87)	$35{,}900.0 \pm 471.0^{**}$ 23,090 (600.0–89,000.0)
PAR ($n = 20$)	0.4 ± 0.05 0.24 (0.11–0.87)	$0.42 \pm 0.04^{*}$ 0.17 (0.11–0.78)	$17{,}000.0 \pm 306.0$ 13,700 (1700–45,008)
Control ($n = 15$)	0.19 ± 0.05 0.17 (0.1–0.34)	0.41 ± 0.04 0.22 (0–0.5)	$40{,}300 \pm 17{,}700$ 17,000 (11,000–51,000)

[1]Data are presented as M ± m, SD, and Xmin–Xmax. [2]Differences between patients with allergic rhinitis and control group are significant (SAR versus control, PAR versus control), $^{*}p < 0.05$. [3]Differences between groups of patients (SAR versus PAR, PAR versus control), $^{**}p < 0.01$.

11. Serum Cytokine Profile in AR Patients

The exacerbation-specific serum cytokine profile of patients with AR is shown in Table 6.

It was determined that concentrations of TGF-β, lymphocyte Treg and T cell proinflammatory cytokine T_H2/T_H9, and IL-4 were almost twice as high in patients with SAR than in those with PAR. Significantly high IL-10 concentrations were observed in patients with PAR when compared to those with SAR. It should be noted that the serum level of IL-4 in patients with allergic rhinitis is 2.1–2.8 times higher than that in the control group.

In the patients with PAR, there was a negative significant dependence between serum concentrations of IL-4 and TGF-β ($r = -0.49$, $p = 0.026$). A negative correlation between blood serum total IgE levels and those of IL-10 ($r = -0.63$, $p = 0.002$) and TGFβ ($r = -0.49$, $p = 0.01$) was also demonstrated in these patients.

The SAR serum cytokine profile (in a period of seasonal exacerbation) was markedly different from that of patients with PAR. There was a negative correlation dependence between the total IgE level and a patient's age ($r = -0.51$, $p = 0.025$) in SAR. There was a similar dependence between TGF-β levels and age ($r = -0.45$, $p = 0.025$) in this group of patients.

12. Discussion

Molecules of allergen components of anemophilous plants, fungal spores, house dust mites, and microbial antigens start actually contacting with the airway from the nasal mucosa,

and it is in fact an active process. By interacting with IgE, sorbed via FcεRI receptors to mast cells, basophil and eosinophil allergens retain a high affinity to IgE [9]. At birth, the airways contain no DCs [3]. It is the activation of the respiratory epithelium by microbial components and irritating agents that initiates ingression of immature DCs from the bone marrow to mucosal membranes [4]. These stimuli cause the release of a number of chemoattractants, such as CCL20, CCL19, and CCL27, which direct dendritic cell migration toward the epithelium and underlying mucosa [10]. The epithelial cells of the mucosal membrane are able to modulate functions of immunocompetent cells [11]. The expression of PRRs for soluble regulatory molecules is specific for these cells, and these receptor-mediated systems can activate and produce cytokines which directly regulate a functional state of granulocytes, lymphocytes, and mononuclear phagocytes [12]. It has been established that activation of TLSP production by the epithelium during the action of aeroallergens can underlie the formation of allergic bronchial asthma in the future [13].

A significant relationship between the level of sensitization to staphylococcal superantigens (SEA) and TSLP concentrations in nasal secretion has been determined to occur in PAR. This might be due to pronounced changes in the bacteriocenosis (normal flora) of the nasal mucosa in this form of allergic rhinitis that releases gram-positive microflora superantigenic toxins. A potential for additional sensitization to microbial allergens cannot be ruled out in these patients. That was demonstrated in our study where 8 out of 20 patients had high concentrations of allergen-specific IgE antibodies to an enterotoxin. Staphylococcal superantigens might be one of the stimuli of local TSLP hyperproduction by the epithelium. There was a significant correlation between GM-CSF concentrations in nasal secretion and the intensity of sensitization to a staphylococcal enterotoxin (SEB) in the patients with perennial allergic rhinitis. Staphylococcal superantigens, SEB in particular, belong to a group of polyclonal activators of T cells that might account for increased concentrations of cytokines such as GM-CSF locally within the system of mucosal immunity. The patients with perennial allergic rhinitis and additional high sensitization to SEs demonstrated a higher TNF-α production profile due to macrophage and T cell activation by these toxins. With seasonal allergic rhinitis, an inflammatory response is limited in time, as a rule, by a contact season with pollen allergens and is short term. The bacteriocenosis of the nasal mucosa is not so markedly impaired in seasonal allergic rhinitis; however, sensitization of these patients to mold fungal allergen components significantly increases. There was particularly high sensitization to the *Aspergillus fumigatus* component m3. At the same time, mold fungal allergens can also stimulate a TSLP production by the epithelium. In this form of rhinitis, aeroallergen components of mold fungi can in addition lead to macrophage and T cell activation that can be observed as an increased level of factors such as TSLP and GM-CSF in the local cytokine profile of these patients. In turn, these factors can cause the maturation of dendritic cells into "inflammatory" DCs actively involved in the development and maintenance of an inflammatory response. House dust mite allergens play a significant role in sensitizing patients with perennial allergic rhinitis. A group of Acarina mites, the Pyroglifidae and Tyroglyfidae families, mainly prevails. Six main house dust mite allergen components are known; most of them were small proteins, for example, the allergen component Der p1. It was determined in our study that patients with PAR sensitized to mite allergen components (*Dermatophagoides pteronyssinus*) had the same activation of the GM-CSF production possibly by macrophages and epithelial cells.

Thus, a selective activation of TLRs on epithelial cells by bacterial, fungal, and mite allergens can enhance DC mobility within the respiratory mucosa and their invasion into the lower respiratory airways and thereby expand antigen processing. This process is mediated by the production of T_H2 chemokines (CCL17 and CCL22) and cytokines (GM-CSF, TSLP) [14, 15]. Describing the serum cytokine profile especially in seasonal allergic rhinitis, one can note that immune-mediated inflammatory response of the upper respiratory airway mucosa is characterized by a more significant production of IL-4. In most cases, it occurs due to the selective diversity of Th2 cells, which secrete a cluster of cytokines encoded on the chromosome 5q31-33, including interleukins IL-3, IL-4, IL-5, IL-9, and IL-13 and granulocyte macrophage colony-stimulating factor (GM-CSF) [16].

In seasonal allergic rhinitis, it might be due to more pronounced allergic properties of anemophilous plant pollen. Pollen of anemophilous grass, an allergen component of common wormwood (w6), and a birch pollen allergen component are capable of causing high sensitization in patients with seasonal AR. There was a high homology (more than 80%) between the birch allergen component Bet v 1 and other allergens including allergen components of timothy and wormwood. A high TGF-β concentration in seasonal AR is another relevant finding of our study. TGF-β in cooperation with IL-10 has been established to facilitate/promote the development of regulatory T cells (Treg) [17].

We have determined that there was a negative correlation between TGF-β concentrations and a patient's age in seasonal rhinitis. The elder the patients, the less evident this cytokine concentration in blood serum in the development of allergic inflammation in seasonal AR. It should be noted that TGF-β is one of the cytokines which provide the inhibition of immune responses and the proliferation of immunocompetent cells. Thus, along with other clinical trials, the study performed would clarify some aspects of molecular pathogenesis of human allergic rhinitis.

Ethical Approval

The Institutional Review Board of the Kazan Research Institute of Epidemiology and Microbiology approved this study (Protocol no. 2, the Local Ethics Committee; date 26 May 2015).

Consent

Informed consent was obtained from each study subject according to the guidelines approved under this protocol

(article 20, Federal Law "Protection of Health Right of Citizens of Russian Federation" N323-FZ, 21 November 2011).

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

Immunomodulating and Revascularizing Activity of *Kalanchoe pinnata* Synergize with Fungicide Activity of Biogenic Peptide Cecropin P1

N. S. Zakharchenko,[1] A. S. Belous,[2,3] Y. K. Biryukova,[4,5] O. A. Medvedeva,[2] A. V. Belyakova,[5] G. A. Masgutova,[6] E. V. Trubnikova,[2,3] Y. I. Buryanov,[1] and A. A. Lebedeva[1]

[1]*Branch of Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Science Avenue 6, Pushchino, Moscow Region 142290, Russia*
[2]*Kursk State Medical University, K. Marksa St 3, Kursk 305041, Russia*
[3]*Kursk State University, Radisheva St 33, Kursk 305000, Russia*
[4]*Emanuel Institute of Biochemical Physics, Kosygina St 4, Moscow 119334, Russia*
[5]*Chumakov Institute of Poliomyelitis and Viral Encephalitides, 27 km Kievskogo shosse, Moscow 142782, Russia*
[6]*Kazan Federal University, Kremlevskaya St 18, Kazan 420008, Russia*

Correspondence should be addressed to Y. K. Biryukova; kudykina_yuliya@mail.ru

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Previously transgenic *Kalanchoe pinnata* plants producing an antimicrobial peptide cecropin P1 (CecP1) have been reported. Now we report biological testing *K. pinnata* extracts containing CecP1 as a candidate drug for treatment of wounds infected with *Candida albicans*. The drug constitutes the whole juice from *K. pinnata* leaves (not ethanol extract) sterilized with nanofiltration. A microbicide activity of CecP1 against an animal fungal pathogen in vivo was demonstrated for the first time. However, a favorable therapeutic effect of the transgenic *K. pinnata* extract was attributed to a synergism between the fungicide activity of CecP1 and wound healing (antiscar), revascularizing, and immunomodulating effect of natural biologically active components of *K. pinnata*. A commercial fungicide preparation clotrimazole eliminated *C. albicans* cells within infected wounds in rats with efficiency comparable to CecP1-enriched *K. pinnata* extract. But in contrast to *K. pinnata* extract, clotrimazole did not exhibit neither wound healing activity nor remodeling of the scar matrix. Taken together, our results allow assumption that CecP1-enriched *K. pinnata* extracts should be considered as a candidate drug for treatment of dermatomycoses, wounds infected with fungi, and bedsores.

1. Introduction

Kalanchoe pinnata (*Bryophyllum pinnatum*) (Lamarck) Persoon (Crassulaceae) is a succulent perennial plant native to Madagascar. It is used for healing wounds in a traditional medicine to treat psychiatric disorders and as a tocolytic agent to prevent premature labour [1]. Extracts of *K. pinnata* and some other Kalanchoe species (*K. crenata*, *K. brasiliensis*, and *K. daigremontiana*) are reported to exhibit antimicrobial [2, 3] and virucide [4–6] activity. Further, they were found to kill leishmanias [7] and malaria plasmodium [8]. It induces the relaxation of smooth muscles [9]; exhibits antimutagenesis, antihistamine [10], and hepatoprotective activity [11]; causes immunomodulatory and anti-inflammatory effects [12, 13]; and inhibits thyroid peroxidase [14]. Baginskaya and Leskova [15] reported a positive effect of *K. pinnata* extract towards experimental gastric ulcer in mice. Eventually, it demonstrates an oncolytic activity on certain models [16]. To our best knowledge, primary experimental data about testing antifungal activity of Kalanchoe extracts have not been published, but occurrence of this effect is mentioned in a review by Kutsik and Zuzuk [17].

Like other species of the genus *Kalanchoe*, *K. pinnata* contains a number of biologically active compounds which may contribute to pharmacological properties of its extracts:

(1) Flavonoid glycosides: two phenolic glucosides, syringic acid β-D-glucopyranosyl ester and 4′-O-β-D-glucopyranosyl-cis-p-coumaric acid; nine flavonoids including kaempferol, quercetin, myricetin, acacetin, and diosmetin glycosides; and flavonol glycosides: quercetin (3-O-α-L-arabinopyranosyl-(1 → 2)-α-L-rhamnopyranoside 7-O-β-D-glucopyranoside) and myricetin (3-O-α-L-arabinopyranosyl-(1 → 2)-α-L-rhamnopyranoside) [18].

(2) Four bufadienolides: bersaldegenin-1-acetate, bryophyllin A, bersaldegenin-3-acetate, and bersaldegenin-1,3,5-orthoacetate [18].

(3) Blood-agglutinating lectins with Mr 44–47 kDa containing ~1.5% carbohydrate [19, 20].

Over the last decade, ethanol extracts became the most popular form of Kalanchoe medicinal application [21–23]. It means that lipophilic constituents of the fresh juice (bufadienolides, polyphenols, and flavonoids) are suggested to confer essential part of their total activity. However, an evident biological effect of Kalanchoe lectins has been formerly documented. Lapchik et al. demonstrated a high mitogenic and blast-transforming activity of lectins from different Kalanchoe species, particularly from *K. blossfeldiana* [24]. A study of lectins from 52 Kalanchoe species cultivated in Kiev State University's botanical garden in 1982 demonstrated a drastic difference in their virucide activity. For instance, *K. daigremontianum* broadly used in pharmacy did not exhibit virucide activity. In contrast, *K. velutina*, *K. blossfeldiana*, *K. pinnata*, and *K. crenata* demonstrated a high neutralizing activity in vitro towards nonenveloped RNA-containing viruses (vaccine poliovirus Sabin type II, Coxsackie B1, and Coxsackie B6). 50% reduction of the virus titter was achieved after incubation of these viruses with fresh juice of *K. velutina* diluted with cultural medium in ratio 1 : 32768, and *K. pinnata*, *K. blossfeldiana*, and *K. crenata* diluted 1 : 16384–1 : 8192 times. An enveloped influenza virus A (Hong Kong) 1/68/H3N2 could be 50% inactivated by a juice of *K. velutina* in dilution 1 : 400, by juice of *K. crenata*—in dilution 1 : 200, and *K. pinnata*, *K. blossfeldiana*, and *K. beharensis*—in dilution 1 : 100 [20]. The rate of the virus neutralization did not depend on the temperature. Electronic microscopy demonstrated the contact of the viral particles with Kalanchoe juice induced their deformation and aggregation. Taken together, these observations may be explained by agglutination of the viral particles by Kalanchoe lectins or blocking their cell-specific receptors.

Hence, lectins may contribute to both antipathogenic and immunomodulatory activities of Kalanchoe extracts by binding carbohydrate moieties of extracellular receptors. Immunomodulatory functions may be mediated by nonproteinaceous components of these extracts as well. Costa et al. [25] reported inhibition of human lymphocyte proliferation with patuletin acetylrhamnosides from *K. brasiliensis*. Da-Silva et al. [26] communicated about the induction of Th1-mediators (IL-2 and IFN-γ) and suppression of Th2-mediator IL-4 in mice after the administration of *K. brasiliensis* ethanol extract.

Recently, transgenic plants of *K. pinnata* producing antimicrobial peptide (AMP) cecropin P1 (CecP1) from *Ascaris suis* was described [27]. An antimicrobial, antifungal, and antiviral activity was attributed to this AMP [28]. However, data about biological trials of CecP1 towards human or animal microbial pathogens are scarce due to poor availability of the synthetic peptide and difficulty of its biogenic synthesis in bacteria or yeast which suffer from its toxicity [29].

This work pursues testing antifungal and wound healing activity of *K. pinnata* plants producing CecP1 towards highly prevalent human fungal pathogen *Candida albicans* on a model of infected wounds in rats. Taking into account the probability of existence of an intrinsic antifungal activity in *K. pinnata* juice (although not formerly reported), a juice of nontransgenic *K. pinnata* was tested in parallel as a negative control. Since CecP1 is not soluble in ethanol, a water extract of the plants was tested. In this respect, occurrence of lectins exhibiting lymphoproliferative and immunomodulatory properties along with bufadienolides and flavonoids in this type of preparations may be noteworthy. A commonly used commercial medical fungicide clotrimazole was used as a positive control (standard).

2. Methods

2.1. Producing K. pinnata Extracts. *K. pinnata* extracts were prepared form leaves of *K. pinnata* transgenic plants bearing a binary vector pBM-cecP1 for *Agrobacterium tumefaciens* vector with T-element randomly integrated to a plant genome. The vector was free from drug-resistance markers. The transgenic plants were selected after Agrobacterium-mediated transformation by a direct immunological testing exhibited a steady yield of CecP1 for at least two years as checked by three independent methods (immunoblotting, plate test for microbicide activity, and HPLC combined with mass-spectroscopy detection). 5.07 L of extract was produced from 3 kg of the recombinant plant leaves using deionized water as a solvent. After heating the fresh extract at +80°C and its sterilizing filtration through a nylon membrane with 0.22 μm pores, the extracts were adjusted to a total protein concentration ~1 mg/ml. Usually, they contained 0.69–0.82 μg/ml CecP1. The extracts of the wild-type (nonrecombinent) *K. pinnata* was produced by the same method and adjusted to the same concentration of the total protein.

2.2. Animals. In vivo trial was designed according to the European Convention about defense of the vertebrates used for experiments or for another scientific aims (Strasbourg, Mar. 18, 1986) of ETS N123. In total, 120 adult male Wistar rats weighing 180 ± 20.0 g 3-4 months old were allocated for the experiment. After quarantine, they were kept in individual cages. All animals were contained in equal terms on a standard diet and photoperiod (twelve hours of darkness and twelve hours of light). They had a ready access to water and food.

The animals were randomly divided into experimental, positive control, reference, and negative control groups (30 animals per each). All 120 animals after wounding were infected with *C. albicans*. The experimental group was treated with CecP1-enriched recombinant *K. pinnata* extract (*K. pinnata* + CecP1). The positive control group was treated with clotrimazole. The negative control group was subjected to mock treatment with a saline, and the reference group was treated with wild-type *K. pinnata* extract. Each group was divided into three echelons withdrawn from the experiment at 3th, 10th, and 14th day after the beginning of the curing.

2.3. Fungal Pathogen Strain. The human fungal pathogen *C. albicans* (NCTC 2625) formerly isolated from a human clinical specimens was purchased from a type strain collection of Tarasevich Research Institute for Standardization and Control of Medical Biological Preparations. *C. albicans* culture was obtained by cultivation on a slant meat-peptone nutrient agar supplemented with 1% glucose for at 37°C for 18–20 h. The cells were washed from the slant agar with a sterile saline, thoroughly suspended, adjusted to a concentration ~10^9 CFU per ml by using an optical turbidity standard CCA 42-28-29-85 and used for infecting the wounds in rats.

2.4. Surgical Manipulations, Treatment, and Planimetry Assay of the Wounds. A purulent infection of wounds was modeled in rats using a method described previously [30]. The animals were anesthetized with diethyl ether. A square shape 20 × 20 mm skin area on the back of each animal was thoroughly shaved, treated by a disinfectant (70% ethanol), and then derma and epidermis were surgically removed. 1 ml of the yeast suspension containing 10^9 CFU/ml *C. albicans* NCTC 2625 was distributed over the surface of the wound. For standardizing the wound healing conditions, the wound cavity was closed with a gauze bandage coupled to the skin.

In 36 h after wounding and infection, all animals exhibited clear symptoms of suppuration and inflammation. In this moment, the stitches and the bandage were removed, and the wound cavity was thoroughly washed from the pus. The initial wound area was determined by its lineation at a sterile transparent polyethylene film. Then the wounds were treated with 3% hydrogen peroxide as described by [31] and subjected to a specific treatment. The described medical procedure was repeated daily for 14 days after the beginning of the curing.

The negative control group was treated with 3% hydrogen peroxide and the mock medicine (a sterile saline). Other groups were treated in the same way using 10% clotrimazole or undiluted *K. pinnata* extract containing 1 mg/ml total protein (experimental preparation contained 0.7 μg/ml CecP1) instead of the saline.

The animals were examined daily, and stages of wound healing (inflammation, granulation, and maturation (marginal epithelization)) were fixed.

The planimetric analysis of the wound recovery percentage was carried out at 3th, 10th, and 14th day after the beginning of curing. After this, one echelon (10 animals from each group) was withdrawn from the experiment. The animals were sacrificed with overdose of the ether anesthesia.

Residual wound square was measured individually in each animal as described formerly [32], using an imprint on a transparent polyethylene sheet which was scanned at resolution 200 pcs/inch using a common office scanner. The images were acquired in a format Adobe Photoshop CS5 Extended. The object was selected using the abovementioned standard software, and its square was automatically calculated by selecting a menu command "Analysis." An average mean and a standard deviation (M ± Std. Dev.) were calculated for each group/echelon (10 animals). The recovery percentage was evaluated with following formula:

Recovery percentage = (initial wound square − wound square on the day X) × 100/(initial wound surface), where X = the day of wound square measurement (0 = beginning of the curing).

2.5. Preparing Histological Slides, Their Staining, and Analysis. After the completion of treatment, animals were sacrificed, and tissue samples were prepared as described previously [32]. The resulting biological material was fixed in 10% neutral formalin. After fixation, the tissues dissected for 1 × 1 cm fragments, washed, dehydrated, and impregnated with paraffin by standard methods. 5–7 μm thick microtome sections were stained with hematoxylin and eosin to assess the density of collagen fibers. A light microscope Leica CME with magnifications ×40, ×100, ×200, and ×400 and camera DCM-510 were used. Ten sections from each wound were investigated and evaluated. Image tools 3 software (UTHSCSA ImageTool) was used for accumulating and analyzing the images.

Microscopy and photographing the slides were performed using an optical system by Leica comprising an eyepiece camera and software for documenting images FUTURE WINJOE (supplied by the manufacturer of the hardware). Cell content nearby the wound edge and underlying tissue were examined at each micrograph. The cells were categorized into neutrophils, macrophages, mast cells, fibroblasts, and capillary endotheliocytes according to their morphological features (shape of the cell, shape of the nucleus, and cytoplasm/nucleus ratio) as described previously [33]. Regularity of the collagen fiber alignment was evaluated. The percentage of those representatives of a cell population was calculated after counting 100 cells in several nonoverlapping visual fields (at least 10).

2.6. Microbiological Analysis of the Wound Bed Microbial Contamination. App. 0,5 g tissue (fibrous mass, infiltrate, and underlying derma) was sampled from each sacrificed animal under aseptic conditions, weighed at analytical grade balances (accuracy 0.1 mg), placed into a sterile porcelain mortar, mixed with a sterile saline in a weight ratio 1 : 10, and homogenized with a sterile pestle for 3 min at room temperature. The homogenate was diluted 1000 times with a sterile saline (with three consequent steps 1 : 10 using 1 mL samples) and 100 μl aliquot of each dilution was inoculated to Petri dishes with meat-peptone nutrient agar supplemented with 0.1% glucose. The inoculated dishes were incubated at 37 ± 1°C for 72 h and then 1 day more at a room

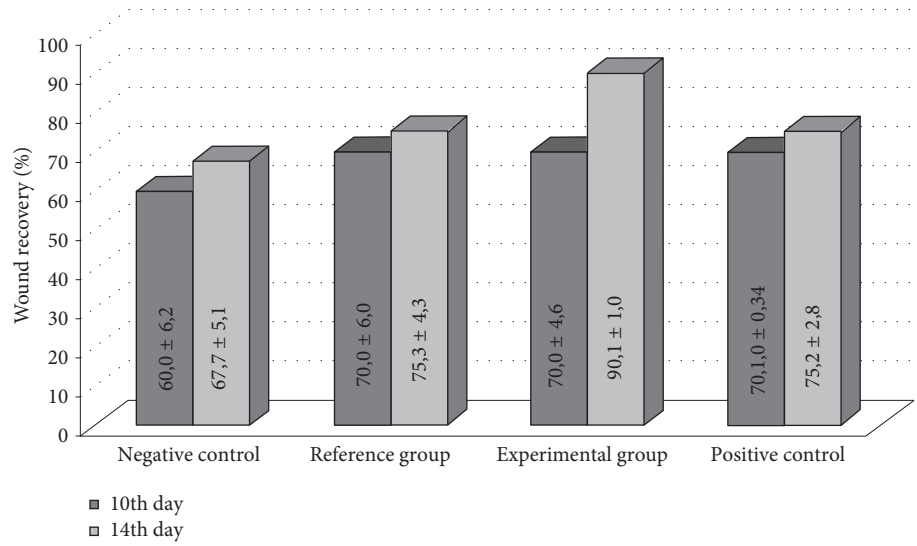

Figure 1: Planimetry examination of healing of the wounds in rats infected with *C. albicans*. Wound recovery percentage at 10th and 14th days after beginning of curing is shown (M ± Std. Dev.).

temperature. The colonies were counted and number of CFU recalculated per 1 g tissue. The count was suggested to be valid if number of colonies was between 30 and 300 per dish.

2.7. Statistical Analysis. Statistical analysis was provided by Microsoft Excel 2007 and program "Statistics" 8.0 StatSoft. The average values and standard deviations of quantitative indexes obtained by planimetry and microbiological methods were calculated for each group/echelon (10 animals). The statistical significance of differences between the groups/echelons was estimated by the Mann–Whitney U test ($p < 0.05$).

3. Results

3.1. Study of the Wound Healing Activity by Planimetry. The wound healing process was divided into three partially overlapping stages: inflammation, granulation, and maturation. The stage of inflammation was considered to be finished once perifocal edema around the wound disappeared. Start of the granulation stage was registered once the wound bed began filling with a newly tissue of a pale pink color. Start of the maturation stage was registered once a marginal epithelialization became visible. Each animal was analyzed daily in order to reveal perifocal edema, granulation, and marginal epithelialization (Figure 1). Residual square of the wound was measured at 10th and 14th days after the beginning of curing (Figure 2). Significance of the difference between the groups is shown on Figure 3.

3.2. Histological Analysis of the Wound Healing Activity. Three days after the beginning of curing, the wound beds in all four groups were filled with purulent-necrotized cell mass (Figure 3). Leucocyte infiltration was visible deeply in the derma. An edema and plethora of dilated capillaries were found in the underlying tissues. The collagen fibers were disorganized and pushed away from each other due to interstitial edema.

Ten days after the beginning of curing, the negative control group exhibited rising of the inflammation that was manifested as an appearance of a polynuclear inflammatory infiltrate, composed of neutrophils, macrophages, and must cells (Figure 4).

This period was characterized with melting of the necrotic tissue, their removal, and partial emptying of the wound cavity. The interstitial edema was conserved beneath the wound cavity indicating an increased permeability of the capillaries. Single microabscesses were found in deep layers of the derma. In the same period, noninfected animals did not exhibit traits of the inflammation. The surface of the wound was covered by separate islands of new-formed thin epithelium growing from the margins of the wound. Layers of derma beneath the wound were impregnated with fibrin and contained single fibroblasts. A wound bed in the positive control group (clotrimazole) contained granulation focuses constituted from inflammatory and proliferative cells and enriched with newly collagen fibers. A thin epithelial layer without abnormalities appeared along the margins of the wound. The reference group (extract of the wild-type *K. pinnata*) contained much more collagen fibers and fibroblasts beneath the edge of the wound then the negative and positive control groups. Marginal epithelialization was more pronounced in the reference group. However, the inflammatory cell component was more abundant than in the positive control group. The animals of the experimental group (CecP1) were characterized with the fastest progression in the collagen matrix formation, its optimal structuration, the fastest marginal epithelialization, and the minimal number of the polynuclear inflammatory infiltrate in the wound bed.

Fourteen days after the beginning of curing, the wound cavity of the negative control group was still filled with polynuclear inflammatory infiltrate (Figure 5).

Only single spindle-shaped fibroblasts were found. The new-formed granulation tissue was located in the bottom of the wound bed. The inflammatory infiltration and the interstitial edema were conserved in the depth of the derma.

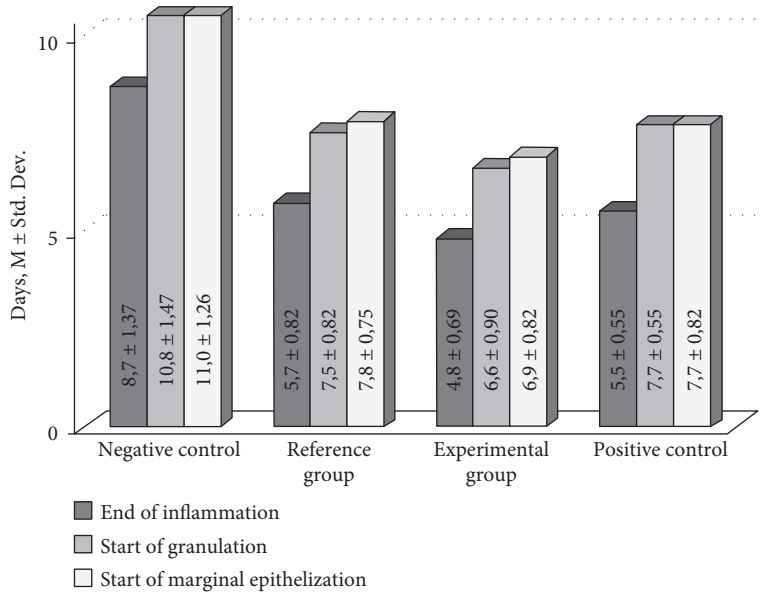

Figure 2: Duration of the wound recovery phases in rats (infected with *C. albicans*) after beginning of curing (M ± Std. Dev.).

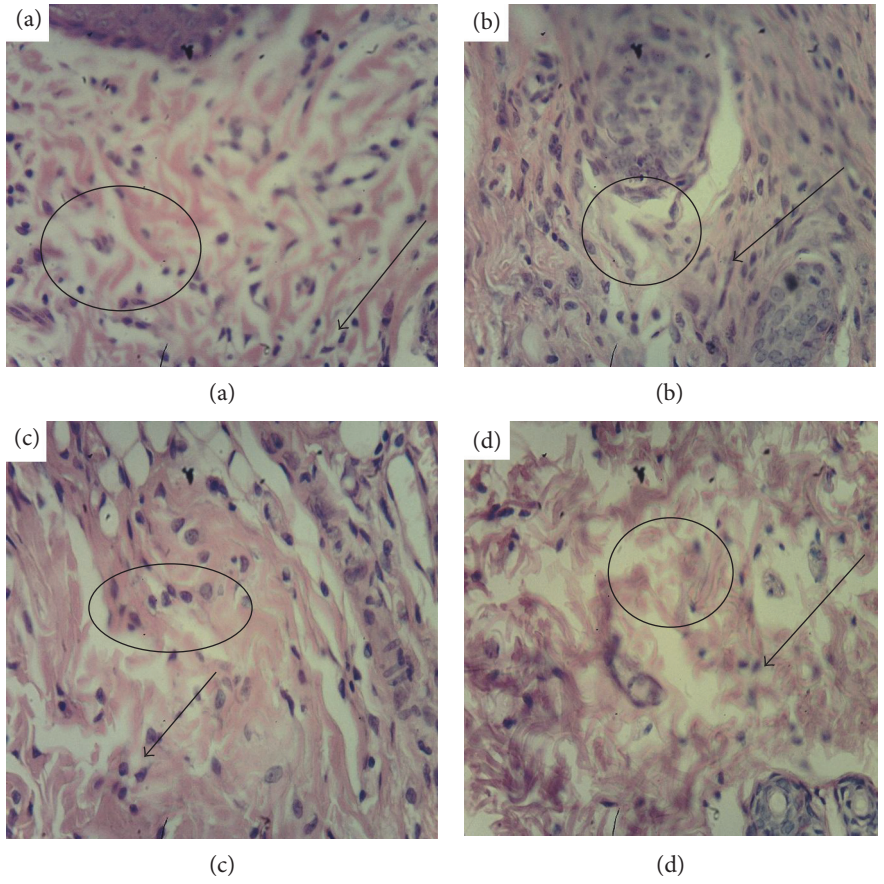

Figure 3: Histological analysis of derma in the wound bed, day 3 after beginning of curing (magnif. ×280). (a) Positive control (clotrimazole); (b) negative control (mock); (c) reference group (wild-type *K. pinnata*); (d) experimental group (CecP1). Selected area: circle/oval—edema in derma, arrow—leucocyte infiltration in the derma.

The singular thin collagen fibers were located irregularly. Most fibroblasts were clustered around them. A scar formation appeared as a solid layer of collagen fibers without vessels and skin appendages. The epidermis was much thicker than normal. The positive control group exhibited a similar picture although the collagen fibers were more

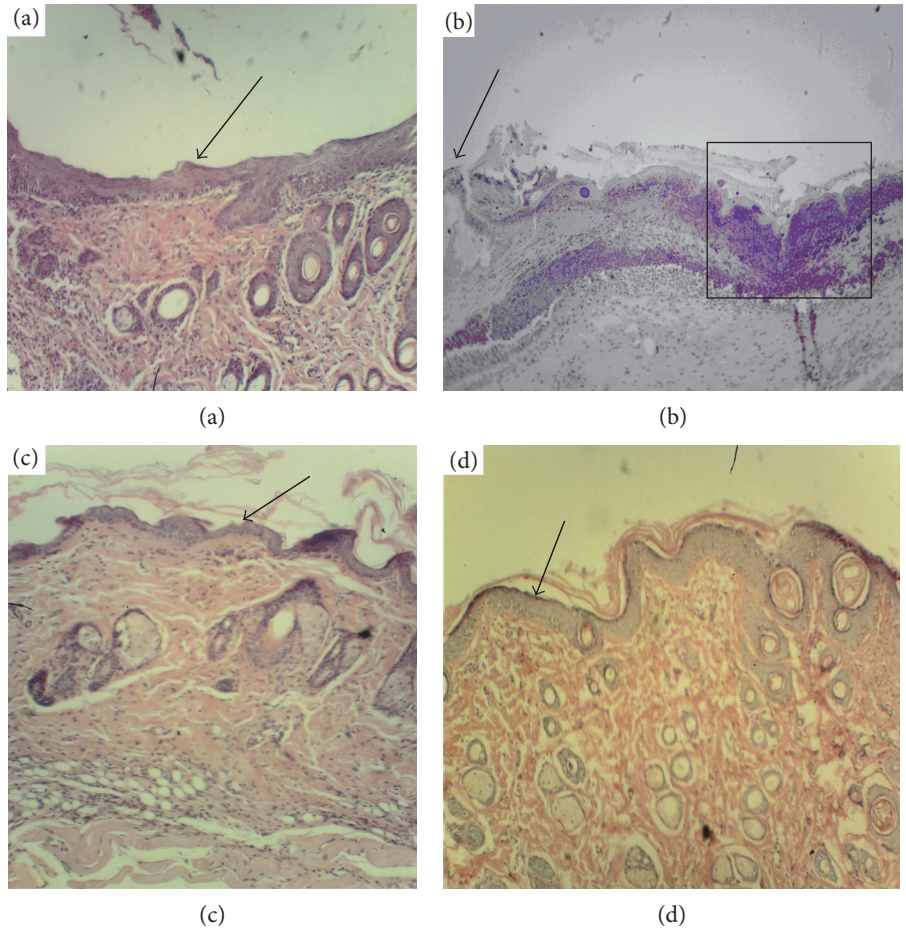

Figure 4: Histological analysis of derma within the wound bed, day 10 after the beginning of curing (magnif. ×70). (a) Positive control (clotrimazole); (b) negative control (no specific treatment); (c) reference group (wild-type *K. pinnata* extract); (d) experimental group (CecP1). Selected area: arrow—new-formed thin epithelium, square—area of melting of the necrotic tissue.

abundant and less regular than in the negative control. The reference group (wild-type *K. pinnata* extract) did not contain solid collagen layer neither focuses; isolated inflammatory infiltrate focuses were conserved. However, overall structure of the collagen was more regular that in positive and negative control group. Revascularization of the scar was beginning. The experimental group combined positive traits of the positive control and the reference group. In this period, solid collagen layer completely covered the former wound cavity in this group. This layer was of the most regular, partially revascularized, and was completely free from the residual inflammation focuses.

3.3. Analysis of C. albicans Cell Survival In Vivo. Fungicide activity of the tested medicines was evaluated as a decrease of alive *C. albicans* cell number per g of the granulate tissue determined by microbiological method as described in Method (Figure 6). An evident fungicidal activity of *K. pinnata* extract in vivo was found, and it was substantially increased by CecP1. The load of yeast cells in the granulate tissue at the 3rd day upon a treatment with the experimental preparation was even less than in the positive control (clotrimazole). In contrast, at the 10th day, the fungicide effect of clotrimazole was stronger than in the experimental *K. pinnata* extract with CecP1. In this period, distribution of the pathogen in the granulate tissue of the experimental group was much less homogeneous than the positive control group. At 14th day after the beginning of curing, *C. albicans* was eliminated over the most surface of the wound bed in the positive control and reference and experimental groups; however, single focuses of infection were conserved. In contrast, the infection evolved in the negative control group.

4. Conclusion

An evident fungicidal activity of *K. pinnata* against the most common human fungal pathogen *C. albicans* has been demonstrated for the first time. Moreover, the data of histological analysis make an evidence that *K. pinnata* exhibits a positive effect on remodeling of the collagen matrix and reepithelizaton of the wound cavity along with its repairing. As specified in Introduction, *K. pinnata* is known with the production of bufadienolides and polyphenols which exhibited a toxic activity of animal cells in vitro. However, our results give show that these compounds do not compromise wound healing activity when administrated within the water extracts of *K. pinnata* leaves normalized by the total protein load 1 mg/ml. CecP1 within the recombinant *K. pinnata* extract

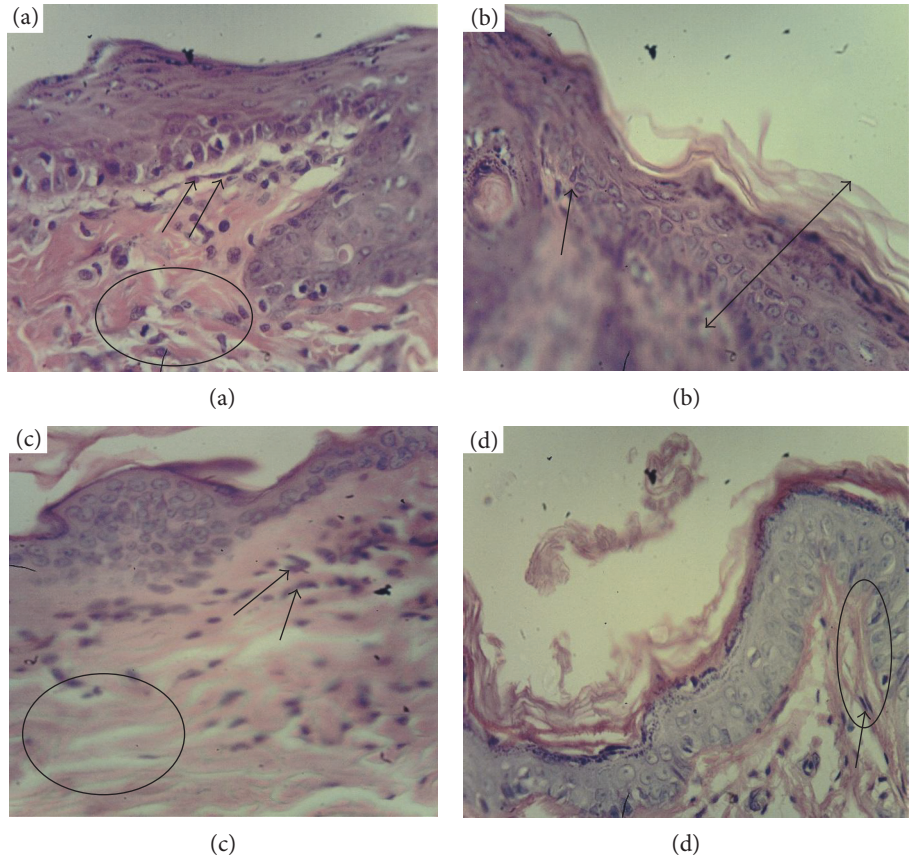

Figure 5: Histological analysis of derma in the wound bed, day 14 after the beginning of curing (magnif. ×280). (a) Positive control (clotrimazole); (b) negative control (no specific treatment); (c) reference group (wild-type *K. pinnata* extract); (d) experimental group (CecP1). Selected area: arrow—fibroblasts, circle—collagen fibers, and double arrow—thickened epidermis.

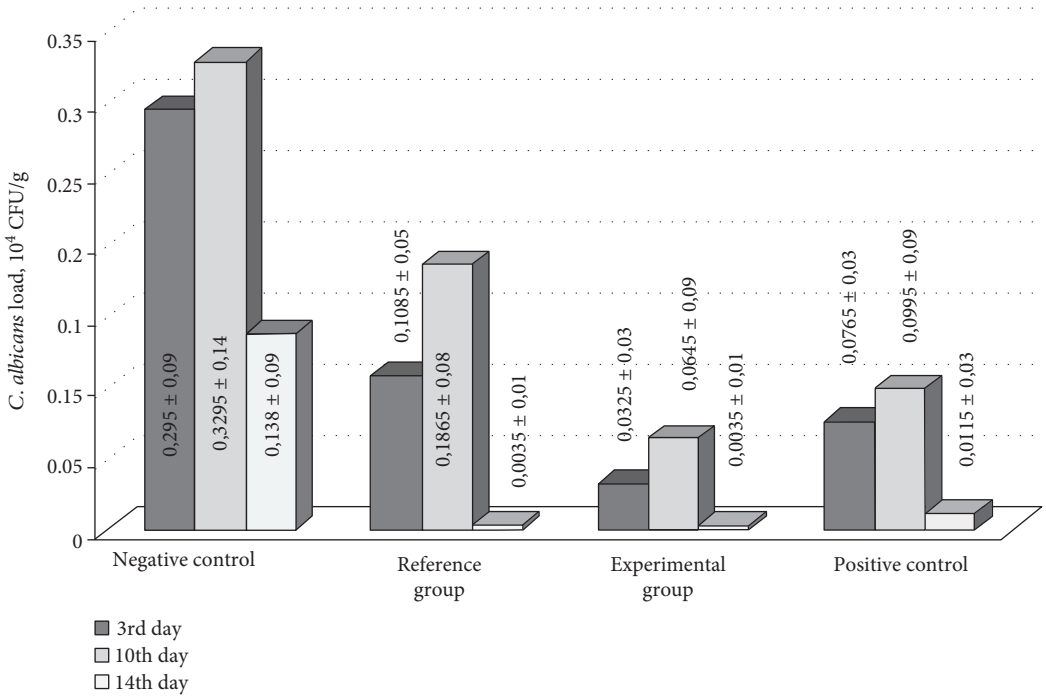

Figure 6: Dynamics of the wound bed contamination with *C. albicans*.

substantially ameliorates its natural antifungal properties making it comparable to the commonly used commercial antifungal preparation. In a certain extent, this result is unexpected, since many formerly studied AMP with α-helical secondary structure, on the one hand, exhibited unacceptable side-toxicity and, on the other hand, were unstable in vivo [34]. Contribution of immunomodulating activity of *K. pinnata* natural compounds to *C. albicans* elimination in vivo is confirmed by the fact that although CecP1 exhibits 80–300-fold lower molar activity against *C. albicans* in vitro than clotrimazole, CecP1-enriched extract of *K. pinnata* provides even more fast and complete elimination of the fungal pathogen from the infected wound than the commercial fungicide. Taken together, our data demonstrate a high perspectivity of pharmaceutical application of *K. pinnata* extracts containing CecP1 for curing *C. albicans* infection and possibly other fungal infections. This approach is technologically and economically affordable. However, an extensive toxicological trials and testing of rising resistance mechanisms in the fungal pathogens against the novel potential drug are required before it can be recommended for a practical implementation.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Clinical Study

Association of Toll-Like Cell Receptors TLR2 (p.Arg753GLN) and TLR4 (p.Asp299GLY) Polymorphisms with Indicators of General and Local Immunity in Patients with Atopic Dermatitis

Yury A. Tyurin,[1,2] Anton F. Shamsutdinov,[3] Nikolay N. Kalinin,[4] Alsou A. Sharifullina,[2] and Irina D. Reshetnikova[2]

[1]*Department of Biochemistry and Clinical Laboratory Diagnostics, Kazan State Medical University, 49 Butlerov Street, Kazan 420012, Russia*
[2]*Laboratory of Immunology and the Development of Allergens, Kazan Research Institute of Epidemiology and Microbiology, 67 Big Red Street, Kazan 420015, Russia*
[3]*Institute of Fundamental Medicine and Biology, Kazan Federal University, 18 Kremlevskaya Street, Kazan 420008, Russia*
[4]*The I.M. Sechenov First Moscow State Medical University, 8-2 Trubetskaya Street, Moscow 119991, Russia*

Correspondence should be addressed to Yury A. Tyurin; tyurin.yurii@yandex.ru

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A whole group of polymorphisms of genes involved in the formation of the epidermal barrier, immune responses, and their regulation is important in the formation of atopic phenotype. The purpose of the study is to determine the relationship of polymorphisms of genes of Toll-like receptors TLR2 and TLR4 with clinical and immunological parameters in atopic dermatitis patients in a "case-control" study. Polymorphisms of genes TLR2 (p.Arg753Gln) and TLR4 (Asp299Gly) were detected by PCR. Parameters of the state of innate and adaptive immunity were assessed by the level of local production of sIgA, cytokine profile of blood serum for IL-4, IL-10, and IFN-γ. Biological samples from 50 people with allergic pathology, aged 4.5 to 35 years, and 100 healthy individuals (controls) were analyzed. Observed dysregulation of cytokine production (IL-4, IL-10) in patients with heterozygous polymorphic genotypes probably reflects an imbalance of Th1/Th2/Th17 regulation of immune system response in these individuals.

1. Introduction

Human allergic diseases are considered today a medical issue of global significance which leads to a considerable reduction in the quality of life and public health. Extensive studies of the last two decades on the pathogenesis of allergic diseases suggest that they are associated with a number of genetic and environmental factors and also with the interaction of these factors, all of which lead to a quite complex pathogenesis and considerable difficulties for a rational therapy. Molecular and genetic studies of the last 20 years have shown that there are several hundreds of gene mutations involved in the development of an atopic phenotype [1]. In the case of atopic dermatitis, for example, there were identified significant associations with gene polymorphism regarding those genes involved in the development of the epidermal barrier, the appearance of immune responses, and their regulation [1]. Also important are the studies on associations between different polymorphic forms of genes controlling innate and adaptive immune responses in the pathogenesis of allergic diseases. Dysfunctions of the skin barrier are significant factors in the pathogenesis of atopic dermatitis, being this a matter recognized by the majority of researchers [1, 2]. Toll-like receptors (TLR) constitute one of the groups of receptors in the immune system involved in inflammatory responses of various cell types to microbial antigens [3]. It has been shown that TLR2 and TLR4 cell receptors participate in the development of innate and adaptive immune responses to lipoteichoic acids forming the cell wall of Gram-positive bacteria and also to viral proteins and LPS of

Gram-negative bacteria [4]. A number of studies have shown that the clinical significance of the Toll-like receptor 2 rs5743708 (c.2258G>A) mutation (polymorphism) is characterized by the substitution of a guanine nitrogenous base with adenine in the TLR2 gene at nucleotide 2258 from the start codon (missense mutation), which leads to a change in the primary amino acid sequence at position 753 with a replacement of arginine (Arg) with glutamine (Gln). The rs4986790 (c.896A>G) mutation is another clinically significant Toll-like receptor 4 missense mutation, consisting of a nonsynonymous substitution of a nitrogenous base of adenine (A) with guanine (G). As a result, Asp is substituted with Gly at position 299 (Asp299Gly) of the primary amino acid sequence of the TLR4 protein receptor [5]. The goal of the present research (actually, a random case-control study) was to elucidate how the polymorphisms of genes TLR2 and TLR4, which encode the TLR2 and TLR4 receptors of the immune response system, are associated with clinical and immunological indicators of atopic dermatitis.

2. Material and Methods

2.1. Patients and Samples. The study considered biological samples obtained from two groups of individuals. The first group was composed of 50 individuals, comprising 25 people with atopic dermatitis and 25 with atopic dermatitis combined with either allergic rhinitis or allergic bronchial asthma. The age of patients in this group ranged from 4,5 to 35 years. In accordance with the modern classification of atopic dermatitis, the distribution of the study patients by diagnosis was as follows:

(1) Atopic dermatitis, pediatric, common, moderate (SCORAD 50–89), with a high serum total IgE level (>350 IU/mL), and continuously recurrent ($n = 15$)

(2) Atopic dermatitis, adolescent, common, moderate, (SCORAD 45–75), recurrent, with a moderately high total IgE level (200–350 IU/mL) ($n = 10$)

(3) Atopic dermatitis, localized, moderate (SCORAD 45–70), recurrent, combined with allergic persistent rhinitis, household sensitization, and a total IgE level of >250 IU/mL ($n = 15$)

(4) Atopic dermatitis, localized, moderate (SCORAD 50–65), combined with atopic bronchial asthma, persistent, mild, without signs of respiratory failure, household sensitization, and a total IgE level of >280 IU/mL ($n = 10$).

The gender ratio is 1 : 1.5 (20 male and 30 female). All patients of the study belong to the European group by race, and by ethnicity, they are Tatar (55%), Bashkir (5%), and Russian (40%) (based on the questionnaire findings).

The second group (control group) consisted of 100 persons (medical personnel) without symptoms of atopic dermatitis and aged from 21 to 39 years. The gender ratio is 1 : 1.5 including 40 men and 60 women. By race, the controls (100 persons) belong to the European group, the ethnic composition is Tatars (60%) and Russians (40%). The control and study groups selected were comparable by gender and ethnic composition.

2.2. Genotyping. The TLR2 (p.Arg753Gln) and TLR4 (Asp299Gly) receptor polymorphisms were determined by polymerase chain reaction (PCR) using the "SNP-express" kits with electrophoretic detection (NPO "Lytech," Moscow, Russia) following the manufacturer's instructions. The amplification of DNA fragments was accomplished with a "Tertsik" amplifier (DNK-Tekhnologia, Russia). The amplification products were analyzed by horizontal electrophoresis in an agar gel medium, which was visualised and imaged using a transilluminator together with the image processing system "Biotest-1" (Russia). Genomic DNA was isolated from whole blood leukocytes, and buccal epithelial cells were collected with informed consent from patients during clinical and allergic investigations at Kazan Scientific Research Institute of Epidemiology and Microbiology (KSRIEM). Genomic DNA isolation and purification were accomplished by use of the "DNA-express" and "DNK-ekspresskrov-plus" kits (NPO "Lytech," Russia).

2.3. Immunological Studies. The immunological studies were performed in the clinical diagnostic laboratory at KSRIEM. In order to determine the innate and adaptive immunity conditions, we studied the local production of sIgA and found the serum cytokine profile for such cytokines as IL-4, IL-10, and IFN-γ. Nasal secretion was collected with a cotton swab introduced in the middle nasal concha for 30 seconds, and then it was added with 0,25 ml of physiological solution, centrifuged for 10 minutes at 1200 rpm to precipitate cell components, collecting subsequently the supernatant and freezing it at −20°C. The concentrations of sIgA and cytokines in the secretion and serum samples were determined by immunoassay using the "IgA sekretornyi-IFA-BEST," "Interleukin-4-IFA-BEST," "Gamma-Interferon-IFA-BEST," and "Interleukin-10-IFA-BEST" kits (ZAO "Vektor-Best," Novosibirsk, Russia) according to the manufacturer's recommendations.

2.4. Statistical Analysis. The statistical analysis of the data included a test to estimate the genotype distribution deviations from the Hardy–Weinberg–Castle law using the χ^2 test with Yate's correction. Subsequently, we constructed a 95% confidence interval (CI) for the mean values (M) and calculated the standard deviation of the mean values in the sample (SD). The significance of different quantitative indicators was assessed by a t-test for unequal variances [6].

3. Results

The distribution of alleles and genotypes of the *TLR2* and *TLR4* receptor polymorphisms in the tested groups was consistent with the Hardy–Weinberg–Castle law and did not deviate from the equilibrium. The distribution of genotypes in the general sample for the *TLR2* and *TLR4* receptors polymorphisms was as follows: homozygous—91,63%, heterozygous—8,37%, and homozygous for a mutant allele—0%.

The frequency distribution of alleles and genotypes of the polymorphic variants of TLR2 (*rs5743708*) and TLR4 (*rs5743708*) receptor genes in the study groups is comparable to the results of previous studies performed in the Russian Federation on the Russian population (the Chelyabinsk region) [7]. It is also consistent with the frequency distribution of alleles and genotypes of these polymorphisms in other geographic populations [8].

3.1. Distribution of the Frequency of Appearance of the TLR2 Receptor Gene Polymorphism rs5743708 in the Tested Groups. The frequency of the *G*/*G* homozygous genotype in patients with atopic dermatitis was 80,0% (40 patients), while in the control group of healthy individuals it was 94,0% (94 individuals). For the *G*/*A** heterozygous genotype, the frequency in patients with atopic dermatitis was 20,0% (10 patients), while in the control group it was 6,0% (6 individuals); therefore, the differences were significant ($\chi^2 = 7{,}4$, p value $\chi^2 <$ 0,01). Thus, we found that the *G*/*G* homozygous genotype was 1,17 times less frequent in the group of patients with atopic dermatitis than it was in the group of healthy individuals, whereas the *G*/*A** (heterozygous) genotype was found to be 3,3 times more frequent in the first group than in the second.

When comparing the serum levels of cytokine *INF-γ* for patients with atopic dermatitis having different genotypes, we detected a significant reduction of cytokine *INF-γ* (more specifically, it was 1,5 times lower in the group of patients having the polymorphic (heterozygous) genotype) and also an increase in the levels of *IL-4* and *IL-10* (correspondingly, 1,4 and 1,8 times higher; see Table 1). In the group of patients having the polymorphic genotype, we found a significant reduction in the level of *sIgA* in nasal secretion, being 1,4 times lower than it was in the group with the homozygous genotype (*GG*-131 genotype: 1 μg/mL and *GA* genotype: 93,5 μg/mL; $p < 0{,}05$); see Figure 1.

The *A**/*A** polymorphic homozygous genotype was not found in the sample of sick and healthy individuals. In general, the *TLR2* gene *G* allele frequencies were 90,0% in the group of patients with atopic dermatitis and 97,0% in the control group. Furthermore, the *A** (rs5743708) polymorphic allele was found in 10 patients (10,0%) with atopic dermatitis and in 3 healthy individuals (3,0%) (p value $\chi^2 <$ 0,05).

3.2. Distribution of Allele and Genotype Frequencies for the TLR4 Receptor Gene Polymorphism rs5743708 in the Tested Groups. In the group of patients with atopic dermatitis, the *TLR4* *A*/*A* genotype frequency was equal to 87,0% (43 persons), while in the control group it was 91,0% (91 persons). The *A*/*G** polymorphic heterozygous genotype was found in 7 patients with atopic dermatitis (12,0%) and also in 9 individuals from the control group (9,0%) (p value $\chi^2 p > 0,05$). We did not detect the *G**/*G** polymorphic homozygous genotype in either group. The *TLR4* gene *A* rs4986790 allele had a frequency of 92,4% in the group of sick individuals and 94,0% in the control group; the *G** mutant allele had frequency 3,5% in the first group and 4,5% in the second, hence revealing no significant difference (p value $\chi^2 >$ 0,05).

Table 1: Serum cytokine profile of patients with atopic dermatitis with different genotypes for the TLR2 receptor polymorphism rs5743708.

Cytokine profile	Genotype	
	GG ($n = 40$) "protective"	GA ($n = 10$) "polymorphic"
INF-γ (pg/mL)	11,69 (95% CI 11,0–11,7)	7,82 (95% CI 7,0–8,7)*
IL-4 (pg/mL)	14,63 (95% CI 13,2–16,11)	20,4 (95% CI 10,8–30,3)**
IL-10 (pg/mL)	13,2 (95% CI 11,7–14,7)	23,9 (95% CI 15,6–32,2)**

t-test, differences are significant: *$p < 0{,}05$, **$p < 0{,}001$, and 95% CI.

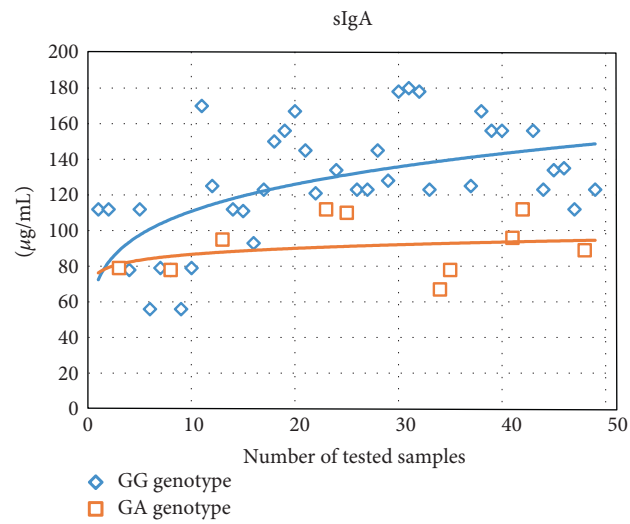

Figure 1: Distribution of sIgA concentration in nasal secretion in patients with atopic dermatitis with different genotypes for the TLR2 receptor polymorphism *rs5743708*.

By comparing the serum levels of interferon INF-γ in patients with atopic dermatitis, we detected a significant reduction of this cytokine level in the group of patients with polymorphic genotype (i.e., heterozygous), being 1,6 times lower than its level in the homozygous group (see Table 2). Also, we noticed a significant increase in the concentrations of serum interleukins IL-4 and IL-10 for the heterozygous group, specifically 1,3 and 1,6 times higher than the corresponding concentrations in the homozygous group (Table 2). In the group of patients having the polymorphic genotype (heterozygous group), we observed a reduction of the *sIgA* level in nasal secretion, being 1,4 times lower than that in the group of homozygous patients (AA genotype: 129,1 μg/mL and AG genotype: 90,0 μg/mL; $p < 0{,}05$); see Figure 2.

Subsequently, we compared the serum levels of cytokines and secretory immunoglobulin A in nasal secretions for the groups of patients with atopic dermatitis having different genotypes for the TLR2 and TLR4 gene polymorphisms

Table 2: Serum cytokine profile of patients with atopic dermatitis with different genotypes for the TLR4 receptor polymorphism rs4986790.

Cytokine profile	Genotype	
	A/A ($n = 43$) "protective"	A/G* ($n = 7$) "polymorphic"
INF-γ (pg/mL)	12,2 (95% CI 11,7–17,9)	7,9 (95% CI 7,2–8,4)*
IL-4 (pg/mL)	15,7 (95% CI 14,1–17,6)	20,4 (95% CI 10,8–30,0)**
IL-10 (pg/mL)	13,8 (95% CI 12,1–15,3)	21,8 (95% CI 16,2–27,4)

t-test, differences are significant: $*$ for $p \leq 0{,}05$ and $**$ for $p \leq 0{,}01$.

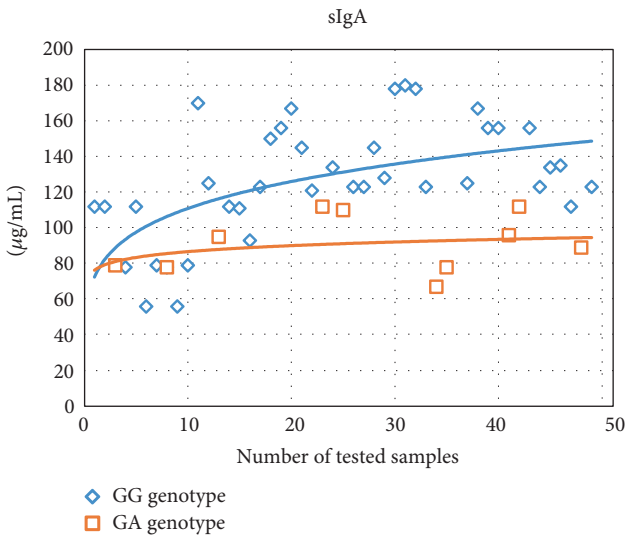

Figure 2: Distribution of sIgA concentration in nasal secretion in patients with atopic dermatitis with different genotypes for the TLR4 receptor polymorphism rs4986790.

and also for the control group (group of healthy individuals without symptoms of atopy).

We found that serum levels of interferon (INF-γ) in healthy individuals were higher than those in the case of patients with atopic dermatitis (see Table 3). Moreover, there were significant differences in the level of serum interferon INF-γ in healthy individuals compared to the corresponding level in patients with atopic dermatitis having either homozygous or heterozygous genotypes (Table 3). Additionally, we proved that serum level of IL-4 and IL-10 was 1,6 times higher but only in the groups of sick individuals with heterozygous genotypes for the gene polymorphisms considered here (compared to the corresponding levels in healthy individuals). No significant differences were detected in healthy individuals for the serum levels of cytokines depending on genotype.

When comparing serum cytokine INF-γ concentrations, there was a significantly decreased level of this cytokine in the atopic group. It was almost 1.4 times as high as compared to that in the control group. This pattern is also observed in the group of healthy individuals with polymorphic genotypes of the TLT-2 receptor (G/A*) and TLR4 (A/G*). Serum concentrations of this cytokine were as a rule higher in this group than those in patients. When studying the INF-γ concentrations, it was also found out that concentrations of this cytokine were even lower in the group of patients with atopic dermatitis, having polymorphic genotypes of Toll-receptors, than in the patients without a polymorphic allele in the genotype.

A comparison of the recurrence rate of atopic dermatitis over the last 3 years in subgroups of patients with a polymorphic heterozygous genotype (G/A*) and a homozygous "protective" (GG) TLR2 receptor demonstrated that clinical episodes of dermatitis recurrence were significantly more often recorded (as twice as often, $p = 0.03$) in a subgroup of patients with atopic dermatitis and a mutant allele of the TLR2 gene.

4. Discussion and Conclusion

Toll-like receptor activation leads to the development of transcription factors in the cell cytoplasm; subsequently, these factors enter the nucleus, where they bind to promoter elements of genes involved in the expression of inflammatory mediators and cytokines, major histocompatibility complex II types, adhesins, and costimulating cells [9]. Genetic mutations in the genes encoding this type of receptors are associated with an increase in the risk of developing immune-mediated diseases such as atopic dermatitis, bronchial asthma, and allergic rhinitis, as well as autoimmune and oncological processes [10].

The *TLR2* receptor gene is located on chromosome 4. In this study, we detected the *TLR 2* gene *rs5743708* polymorphism, which is characterized by a substitution of guanine (*G*) with adenine (*A*) at nucleotide position 2257 from the start codon, this leads to a replacement of *Arg* with *Gln* at position 753 in the amino acid sequence of the receptor. The presence of this polymorphism causes a dysfunction of cell activation processes through the TLR2 signaling pathway [11].

The frequency of the *G/A** polymorphic (heterozygous) genotype and, correspondingly, that of the *TLR2* gene A* (*R753Q*, rs5743708) polymorphic allele was higher in the studied group of patients with moderate atopic dermatitis than the corresponding frequencies in the group of healthy individuals. Possibly, the presence of the specified polymorphic allele is, as reported by Ahmad-Nejad, P., 2004, a predictor of a more severe course of atopic dermatitis [4]. When we determined the serum levels of interleukins in patients with the heterozygous genotype containing the *TLR2* gene *A** (*R753Q*, *rs5743708*) polymorphic allele, we also detected quite high levels of *IL-4* and *IL-10* compared to those found in healthy individuals and patients with homozygous genotype, without the mutant allele.

The *TLR4* receptor gene is located on chromosome 9 (9q33.1). As a result of the *rs4986790* (c.896A>G) missense mutation in the *TLR4* receptor gene, a replacement of *Asp* with *Gly* at position 299 (*p.Asp299Gly*) occurs in the primary sequence of the receptor protein, leading to a disruption of both the ligand-binding and the coreceptor functions of the

Table 3: Comparative characteristics of serum cytokine profile in healthy individuals and patients with atopic dermatitis with different polymorphic genotypes.

Cytokine profile	Genotype							
	TLR2 (rs5743708)				TLR4 (rs4986790)			
	GG		GA		AA		AG	
	Atopic dermatitis, $n = 40$	Healthy, $n = 94$	Atopic dermatitis, $n = 10$	Healthy, $n = 6$	Atopic dermatitis, $n = 43$	Healthy, $n = 91$	Atopic dermatitis, $n = 7$	Healthy, $n = 9$
INF-γ (pg/mL)	11,67 ± 3,7	17,1 ± 3,1*	7,82 ± 3,7	17,4 ± 2,9*	12,2 ± 3,8	17,8 ± 2,8*	7,9 ± 4,2	15,0 ± 1,5*
IL-4 (pg/mL)	14,63 ± 2,2	14,4 ± 3,2	20,4 ± 3,8	13,7 ± 4,3**	15,7 ± 4,1	14,4 ± 3,2	20,4 ± 3,1	15,1 ± 4,5**
IL-10 (pg/mL)	13,2 ± 3,6	14,2 ± 5,4	23,9 ± 3,5	14,5 ± 3,3**	13,8 ± 3,3	13,8 ± 3,7	21,8 ± 4,2	14,3 ± 3,5**

M ± SD, t-test, differences are significant: $*$ for $p \leq 0{,}05$ and $**$ for $p \leq 0{,}01$.

TLR4 receptor, and as a consequence inducing a weak cell response to microbial antigens [12, 13]. We did not find the G^*/G^* polymorphic homozygous genotype in neither the tested groups of patients with atopic dermatitis nor the group of healthy individuals. In the case of the *TLR4* polymorphic allele *A rs4986790*, we did not detect significant differences between the group of sick individuals and that of healthy persons.

Atopic patients with genetic polymorphisms (p.Arg753Gln, rs5743708) of TLR2 and (Asp299Gly, TLR4, and rs4986790) TLR4, as our study has demonstrated, have higher levels of Th2 cell activation cytokines (IL-4 and IL-10). Cytokine IL-10 is produced by Th2-cells and at present can be considered as an antagonist to several cytokines. In particular, it suppresses IFN-γ production. Moreover, it inhibits the proliferative response of T cells to allergens and mitogens and also suppresses the secretion of monocyte-activated IL-1β, IL-6, and TNF. At the same time, IL-10 stimulates IgE secretion and synthesis.

The study of serum levels of cytokines in our investigation showed that patients with heterozygous genotypes containing polymorphic alleles present a lower concentration of interferon (INF-γ) than patients without the polymorphic allele in the genotype. It is possible that the lower concentration of interferon INF-γ in these patients reduces the stimulatory effect of cytokine on the neutrophil (neutrocytes) system, which is likely to increase the susceptibility to viral and bacterial infections in these patients. A reduction of the activating effect of interferon *INF-γ* combined with a lower *sIgA* level in the upper respiratory tract may account for a low colonization resistance of mucous membranes against conditionally pathogenic microbiota and a low capacity of secretions to neutralize antigens and allergens of microorganisms.

The reduction of both the skin barrier function and the activity of its immune antimicrobial factors observed in patients with atopic dermatitis is caused by numerous genetic mutations affecting genes controlling the synthesis of cytokines and Toll-like receptors [2].

The decline in the stability of the skin to microorganisms, which is observed in patients with atopic dermatitis and is caused by dysfunctions of the immune regulation generated by mutations of the TLR receptors, is characterized by the development of severe and difficult-to-treat forms of dermatitis; at the same time, it leads to an increase in the flow of allergens contained in the epidermis components, ticks (mites), and microorganisms, adding complications of this disease linked to local infections [2, 3].

Cytokine INF-γ is actively produced by NK cells; it increases the expression of the major histocompatibility complex (MHC) class II molecules on a cell surface and enhances phagocytosis and mechanisms of innate immunity. The decreased baseline concentrations of this cytokine detected in atopic patients might be to some extent due to an imbalance in the Th1/Th2-cell system, with a prevalence of Th2-activated lymphocytes in atopy. Taking into account that this cytokine is produced by Th1 cells, their suppression in an atopic phenotype might result in a decreased baseline INF-γ level. Genetic polymorphisms (p.Arg753Gln, rs5743708) of TLR2 and (Asp299Gly, TLR4, and rs4986790) TLR4 genes are probably to contribute to a decrease of cell activation that further modulates a suppressor effect on Th1 cell activation and enables decreased levels of proinflammatory Th1 cytokines. Cells of the Th1 differentiation serve as suppressors of IgE response and IL-4 secretion. This activity of Th1 is mainly associated with IFN-γ. Therefore, any factor contributing to the Th1 differentiation by itself inhibits the development of Th2 and allergic processes. These factors also include IL-12 and IFN-γ.

Finally, the high degree of dysregulation detected by our study in the production of cytokines (IL-4, IL-10) in patients with polymorphic heterozygous genotypes may reflect a Th1/Th2/Th17 imbalance in the regulation of the immune system response in these individuals.

Thus, we have established that the TLR2 gene polymorphism (*p.Arg753Gln*, *rs5743708*) and the TLR4 gene polymorphism (p.Asp299Gly, rs4986790) are significant for the pathogenesis of dysregulations in the expression of cytokines by cells (immune system, skin, dendritic cells, and keratinocytes) expressing the TLR2 and TLR4 receptors, which control, differentiate, and regulate the balance of Th1/Th2/Th17 subpopulations of lymphocytes involved in immune responses in cases of atopy. Given that the TLR2 and TLR4 receptors are in a certain way cellular "sensors" able to act when exposed to exogenous stress signals and microbial antigens (pathogen-associated molecular patterns (PAMPs)), including significant allergens, we claim that the genetic variations of Toll-like receptors can notably influence the susceptibility, severity, and outcome of allergic diseases in humans.

Ethical Approval

This research was conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the Local Ethics Committee at Kazan Scientific Research Institute of Epidemiology and Microbiology (2015), and all data were managed anonymously.

Consent

Informed consent was obtained from all participants.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Research Article

Analysis of Serum Cytokines and Single-Nucleotide Polymorphisms of SOD1, SOD2, and CAT in Erysipelas Patients

Charles C. Emene,[1] Irina E. Kravchenko,[2] Gulnaz I. Aibatova,[2] and Albert A. Rizvanov[1]

[1]*Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Russia*
[2]*Department of Infectious Diseases, Kazan State Medical University, Kazan, Russia*

Correspondence should be addressed to Charles C. Emene; emene.charles@gmail.com

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Increased free radical production had been documented in group A (β-hemolytic) streptococcus infection cases. Comparing 71 erysipelas patients to 55 age-matched healthy individuals, we sought for CAT, SOD1, and SOD2 single polymorphism mutation (SNPs) interactions with erysipelas' predisposition and serum cytokine levels in the acute and recovery phases of erysipelas infection. Whereas female patients had a higher predisposition to erysipelas, male patients were prone to having a facial localization of the infection. The presence of SOD1 G7958, SOD2 T2734, and CAT C262 alleles was linked to erysipelas' predisposition. T and C alleles of SOD2 T2734C individually were linked to patients with bullous and erythematous erysipelas, respectively. G and A alleles of SOD1 G7958A individually were associated with lower limbs and higher body part localizations of the infection, respectively. Serum levels of IL-1β, CCL11, IL-2Rα, CXCL9, TRAIL, PDGF-BB, and CCL4 were associated with symptoms accompanying the infection, while IL-6, IL-9, IL-10, IL-13, IL-15, IL-17, G-CSF, and VEGF were associated with predisposition and recurrence of erysipelas. While variations of IL-1β, IL-7, IL-8, IL-17, CCL5, and HGF were associated with the SOD2 T2734C SNP, variations of PDFG-BB and CCL2 were associated with the CAT C262T SNP.

1. Introduction

Erysipelas is an acute bacterial infection of the upper part of the dermis and superficial lymphatic vessels. In addition to nonspecific symptoms of fever, chills, nausea, and vomiting, erysipelas has unique clinical features of infection including skin lesions, intense erythema, tenderness, and swelling of the lymph nodes. The legs and faces are the most common areas of localization for erysipelas [1, 2]. Group A (β-hemolytic) streptococcus (GAS) bacteria are the main causative agent for erysipelas that often began with breakages on the skin barriers allowing the microorganism to penetrate into the skin [3]. Diagnosis of erysipelas is largely based on clinical findings, but laboratory diagnosis of group A streptococcal infections is still largely used to identify the causative organism. GAS-related erysipelas has often been diagnosed in old and immunocompromised individuals as well as in neonates and young children [3]. The commonly accepted antibiotic for the treatment of erysipelas is penicillin, but it is important to note that since the 1980s, cases of severe invasive penicillin-resistant GAS diseases have been on the rise [4] and that to date, no licensed vaccines against GAS have been designed. A major clinical problem that has been encountered with erysipelas is its recurrence [3]. There are 18 million cases of severe GAS-related infections annually reported, with approximately 2.3% fatality [5]. It is believed that the severity of the disease and its fatality rate are associated with penicillin-resistant GAS [6]. Despite intense research, our knowledge of the GAS pathogenesis is incomplete. It has been shown that GAS-induced production of free radicals plays several roles in the pathogenesis of infections [1]. An increased production of free radicals can affect cell vitality by disrupting the integrity and function of cytoplasmic membranes, damaging membrane lipids, proteins, and nucleic acids. Additionally, free radicals can inhibit the immune system's ability to identify and eliminate GAS.

These negative effects are referred to as oxidative stress and can be reduced with the aid of exogenous or endogenous antioxidants [2].

There are three main endogenous antioxidant systems: superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), and catalase (CAT). SOD1 is found in the cytosol and nucleus, as well as in the intermembrane space of the mitochondria. SOD1 is a major cytoplasmic antioxidant enzyme that metabolizes superoxide radicals to molecular oxygen and hydrogen peroxide (H_2O_2), thus protecting the cells against oxygen-related toxicity. SOD2 is the only antioxidant located exclusively in the mitochondria [7], where it scavenges the superoxide radicals and converts them to molecular oxygen and peroxide. Catalase (CAT) is generally found in the liver, kidney, and erythrocytes [5]. CAT catalyzes the hydrogen peroxide decomposition to harmless compounds such as water and oxygen. Therefore, antioxidants are important in preventing harmful effects of free radicals. Disturbed function of superoxide dismutase antioxidants can reduce cell protection against free radicals released during the course of infection [8].

Although cytokines play an important role in host defenses against infection, inflammatory cytokines may favor production of free radicals and facilitate oxidative stress. For example, overexpression of inflammatory cytokines TNFα, IL-6, IL-8, and IL-1β has been found in oxidative stress-induced melanomas [9]. Also, TNF-α-induced oxidative stress and subsequent redox system dysfunction have been described in patients with cardiac dysfunction [10]. In another study, the overexpression of IL-6 and TNF-α was shown to be associated with an increased severity of GAS infection, higher rate of toxic shock, and tissue necrosis [11, 12].

Currently, our knowledge on the association between SOD1, SOD2, and CAT gene expression and severity of erysipelas is limited. Relationships between serum cytokine levels and erysipelas infection are also scarcely known. Also, correlation between expression of SOD1, SOD2, and catalase genes and activation of inflammatory cytokine in erysipelas still remain unknown. Therefore, we sought to determine whether single-nucleotide polymorphisms (SNPs) in the genes encoding SOD1, SOD2, CAT, and cytokine levels and their interaction can serve as susceptibility markers for erysipelas. For that, we analyzed clinical characteristics of the disease in conjunction with SNPs in the selected genes and cytokine levels among residents of the Republic of Tatarstan, Russia. Additionally, relationships between the studied SNPs and serum cytokine profile were studied in erysipelas patients.

2. Materials and Methods

2.1. Subjects and Sample Collection. Seventy-one erysipelas subjects (average age 63.89 ± 10.33) admitted into the "Republican Clinical Infectious Diseases Hospital named after Professor A. F. Agafonova" of The Ministry of Health of the Republic of Tatarstan were enrolled into this study. Diagnosis was established based on clinical symptoms and laboratory bacterial culture test results. Subjects with concomitant severe and chronic inflammatory diseases in the acute phase were excluded. One blood sample was collected from each of the 71 erysipelas cases. Additionally, serum samples were collected from 50 patients, each of whom, one sample was taken from at the acute and another at the convalescent phase of the disease. The clinical characteristics of the subjects are summarized in Table 1. Fifty-five age-matched controls were recruited (average age 58.34 ± 6.93) as well. Blood samples were collected from 55 controls, while serum was obtained from 26 controls.

The Ethics Committee of Kazan State Medical University approved this study (N6, 06.25.2012), and informed consent was obtained from each study subject, in accordance with the Declaration of Helsinki and the Article 20, Federal Law "Protection of Health Right of Citizens of Russian Federation" (N323-Ф3, 11.21.2011).

2.2. Genetic Analysis. Genomic DNA was extracted from the blood sample using phenol-chloroform protocol [13]. DNA was stored at −40°C until further use. DNA was analyzed for the presence of the SNPs in the SOD1 (G7958A; rs4998557), SOD2 (C60T, rs4880), SOD2 (T2734C; rs11575993), and CAT (C262T; rs101179) using SNP diagnostic kits 01327-100, 01333-100, 01281-100, and 01342-100, respectively (JSC "Lytech," Russia). Amplification was performed according to the manufacturer's instructions.

2.3. Cytokine Analysis. A total of 44 cytokines were analyzed in the serum samples using Bio-Plex Pro™ Human Cytokine 27-plex and Bio-Plex Pro Human Cytokine 21-plex Assay multiplex kits according to the manufacturer's instructions. These cytokines include IL-1ra, IL-1β, IL-2, IL-2Rα, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-16, IL-17, IL-18, CCL2, CCL3, CCL4, CCL5, CCL7, CCL11, CCL27, CXCL1, CXCL9, CXCL10, CXCL12, FGF, G-CSF, GM-CSF, HGF, IFN-α2, IFN-γ, LIF, β-NGF, PDGF-BB, SCF, SCGF-β, TNF-α, TRAIL, and VEGF. A fifty-microliter serum sample was used to determine cytokine concentration according to the manufacturer's instructions. Data collected was analyzed using MasterPlex CT control software and MasterPlex QT analysis software (MiraiBio, Division of Hitachi Software, San Francisco, CA, USA).

2.4. Statistical Analysis. Statistical analysis of genotypes of the studied SNPs in our case-control study was carried out comparing the additive, dominant, and recessive models as earlier defined [14]. The Pearson x^2 test, analysis of variance, and logistic regression were used to analyze the clinical differences between the subject groups and the control. Allele and genotype frequencies in the subject and control groups were compared with those predicted by the Hardy-Weinberg equations using x^2 test. The Hardy-Weinberg equilibrium was satisfied in most of the distributions. The odds ratio (OR) and 95% confidence intervals (95% CI) were calculated to assess the association between the alleles and the risk of the studied infection. The association between cytokine levels, genotype, and clinical presentation of the study group was calculated using logistic

Table 1: Distribution of clinical characteristics of erysipelas in male and female patients.

		Male $n = 23$ (%)	Female $n = 48$ (%)	Total $n = 71$ (100.0%)
Multiplicity of infection	Primary	14 (60.9%)	24 (50.0%)	38 (53.5%)
	Recurrent	9 (39.1%)	24 (50.0%)	33 (46.5%)
Severity	Moderate	22 (95.7%)	40 (83.3%)	62 (87.3%)
	Severe	1 (4.3%)	8 (16.7%)	9 (12.7%)
Form	Erythematous	10 (43.5%)	23 (47.9%)	33 (46.5%)
	Erythematous-bullous	13 (56.5%)	25 (52.1%)	38 (53.5%)
Site of infection	Lower limbs	18 (78.3%)	41 (85.4%)	59 (83.1%)
	Face	5 (21.7%)	4 (8.3%)	9 (12.7%)
	Higher limbs	0 (0.0%)	3 (6.3%)	3 (4.2%)

Clinical characteristics of the patients were analyzed based on sex. Females have a higher chance of acquiring the infection and also severe forms than males, while a higher number of males have the infection on their faces than that of females.

regression and linear regressions using an earlier described web application SNPStats (http://bioinfo.iconcologia.net/SNPstats) [15]. Statistical difference (p) and Pearson's correlation coefficient (K) were used to analyze the cytokine profile of the patients. A value of $p < 0.05$ signifies a statistical significant difference in cytokine levels between groups and subgroups. The value of $K > 0.4$ was used to indicate a high degree of correlation in changes of a cytokine in infected individuals when the level of the cytokines during the acute phase is compared to the level of the same cytokine during the convalescent phase. Statistical analysis was performed using SPSS software 22 (IBM).

3. Results

3.1. Clinical Presentation. All erysipelas patients enrolled in this study were grouped based in the frequency of the infection: those without previous diagnosis of erysipelas (primary) within the last 365 days (38 patients) and others with more than one episode of erysipelas (recurrent) within the last 365 days (33 patients) (Table 1). Forty-eight patients were females, with an equal number of females having the primary form erysipelas (24 cases) and the recurrent form erysipelas (24 cases). Twenty-three were males, where 14 cases had primary and 9 had recurrent form of the infection. Erysipelas cases were analyzed based on the severity (moderate and severe) of the infection. A total of nine patients were diagnosed with the severe form of erysipelas, and 62 had a moderate form of the infection. The number of male and female patients with the moderate form of erysipelas was fairly similar (22 males; 95.7% of all males, versus 40 females; 83.3% of all females). However, a higher proportion of females had the severe form of the infection (8 females; 16.7%) than that of males (1 male; 4.3%). Based on the clinical manifestation of erysipelas, the subjects were also grouped based on the form of the infection into having erythematous form—33 patients (46.5%), and bullous form—38 patients (53.5%), of the disease. Erysipelas patients were also divided based on the localization of infection. The majority of patients were having infection on the lower limbs (83.1%), followed by the face (12.7%), and, least of all, on the upper limbs (4.2%). Additionally, a higher proportion of male patients had facial localization of the infection when compared to that of females (5 patients; 21.7% versus 4 patients; 8.3%). There were no significant differences between men and women having infection on the limbs.

From the observed results, we suggest that erysipelas has a higher chance to develop the severe form of erysipelas in females than in males. Also, more significantly higher number of female adults are prone to have the recurrent form of the infection than that of male adults but a higher proportion of males are prone to having erysipelas on their faces than that of females.

3.2. SNP's Predisposition to Erysipelas

3.2.1. SOD1 G7958A. Association analysis revealed significantly higher predisposition to erysipelas in patients with G/G genotype (OR = 10.63, 95% CI = 4.45–25.40, $p < 0.0001$) as compared to patients with A/A or A/G genotype. When the G/G genotype was compared to combined G/A and A/A genotypes, the association with erysipelas was still significant (OR = 8.79, 95% CI = 3.76–20.56, $p < 0.0001$) in patients with G/G genotype (Table 2). These results suggest that individuals with the G/G genotype are predisposed to erysipelas as compared to other genotypes.

3.2.2. SOD2 C2734T. An association was found between SOD2 C2734T SNP and the risk of erysipelas (Table 3). The T/T and C/T genotypes had a significant association with erysipelas. The T/T genotype had the highest predisposition to erysipelas (OR = 8.33, 95% CI = 2.44–28.41, $p < 0.0007$), followed by the C/T genotype (OR = 5.55, 95% CI = 1.98–15.55, $p < 0.0011$), and then the C/C genotype (OR = 1). It appears that the presence of a T allele (in T/T and C/T) increases the predisposition to erysipelas (OR = 6.19, 95% CI = 2.28–16.84, $p < 0.0004$) than that of C/C genotype. There was a lack of predisposition to erysipelas in SOD2 T/T genotype (OR = 2.3, 95% CI = 0.93–5.72, $p < 0.0724$) when compared to the C/T and C/C genotypes combined (Table 3). Our data suggest that individuals with the T allele (homozygous T/T or heterozygous C/T genotype) have a higher predisposition to erysipelas as compared to those with C/C genotype.

Table 2: Distribution of SOD1 G7958A in control and erysipelas groups.

	SOD 1 G7958A			
Genotype	Control	Erysipelas	OR (95% CI)	p
G/G	10 (18.9%)	47 (67.1%)	*10.63 (4.45–25.40)*	
G/A	43 (81.1%)	19 (27.1%)	1	<0.0001
A/A	0 (0%)	4 (5.7%)	NA	
G/G	10 (18.9%)	47 (67.1%)	*8.79 (3.76–20.56)*	<0.0001
G/A + A/A	43 (81.1%)	23 (32.9%)	1	

Patients with the G/G genotype had a higher frequency of the infection.

Table 3: Distribution of SOD2 SNP in the control and erysipelas groups.

	SOD2 C2734T			
Genotype	Control	Erysipelas	OR (95% CI)	p
T/T	8 (14.6%)	20 (28.2%)	8.33 (2.44–28.41)	0.0007
C/T	27 (49.1%)	45 (63.4%)	5.55 (1.98–15.55)	0.0011
C/C	20 (36.4%)	6 (8.4%)	1	—
T/T + C/T	35 (63.6%)	65 (91.5%)	6.19 (2.28–16.84)	0.0004
C/C	20 (36.4%)	6 (8.4%)	1	
T/T	8 (14.6%)	20 (28.2%)	2.3 (0.93–5.72)	0.0724
C/T + C/C	47 (85.5%)	51 (71.8%)	1	

People with the T allele have a higher chance of developing the infection than people with the C allele.

3.2.3. SOD2 C60T. The frequency of the SOD2 C60T SNP allele and genotype distribution in the erysipelas patients and controls were not significantly different (Table 4). Therefore, we conclude that the SNP SOD2 C60T has a limited effect on the predisposition to erysipelas.

3.2.4. CAT C262T. When analyzed individually, no association between genotypes C/C, C/T, and T/T and predisposition to erysipelas was found. However, when the predisposition was compared between patients with C/C genotype against those with T/T and C/T genotypes together, a significantly higher frequency of erysipelas was found in patients with C/C genotype (OR = 2.58, 95% CI = 1.24–5.38, $p < 0.01$) than in those with T/T and C/T genotypes combined (Table 5). Also, analysis revealed a lack of predisposition to erysipelas when patients with T/T genotype were compared to those with C/C and C/T genotypes. We conclude that the C/C genotype of CAT C262T may be linked to a higher predisposition to erysipelas than the C/T and T/T genotypes.

3.3. SNPs' Association with Clinical Characteristics of Erysipelas. The subjects were grouped based on gender, multiplicity of infection, lesion location, severity of the disease, and form of infection (Table 6). There were lack of differences in the distribution of SOD1 (G7958A), SOD2 (T2734C), SOD2 (C60T), and CAT (C262T) SNPs in subjects with erysipelas based on gender, multiplicity, and severity of the disease ($p > 0.05$). However, when SNPs were analyzed based on the form of erysipelas, a significant difference ($p < 0.001$) in frequency of SOD2 (T2734C) was found between the bullous and erythematous forms of erysipelas. There was a higher frequency of C allele in patients with erythematous erysipelas (48 patients—33.8%) as compared to that in patients with bullous form (37 patients—26.1%). In contrast, the frequency of patients with the erythematous form having the T allele was lower (18 patients—12.7%) than that in the patients with bullous form (39 patients—27.5%). Therefore, we concluded that the presence of C allele could define development of erythematous form of the disease, while T allele delineates bullous erysipelas. There were no differences in the distribution of the SOD1 (G7958A), SOD2 (C60T), and CAT (C262T) SNPs between patients diagnosed with erythematous and bullous forms.

Next, SNPs were analyzed based on the localization of erysipelas: the lower limbs, face, or the upper limbs (Table 6). We observed an association between SOD1 (G7958A) and localization of infection, where higher proportion of G/A and A/A genotypes was found in patients who had erysipelas on the upper limb, ($p = 0.041$). Conversely, a higher number of patients with the G/G genotype had the infection predominantly on the lower limbs. There was a lack of the association between the erysipelas location and SOD2 (T2734C), SOD2 (C60T), or CAT (C-262T) genotypes (Table 6).

Table 4: Distribution of SOD2 C60T SNP in the erysipelas and controls.

	SOD2 C60T			
Genotype	Control	Erysipelas	OR (95% CI)	p
C/C	37 (67.3%)	57 (81.4%)	1.03 (0.16–6.44)	0.97
C/T	16 (29.1%)	10 (14.3%)	0.42 (0.06–2.94)	0.38
T/T	2 (3.6%)	3 (4.3%)	1	—
C/C + C/T	53 (96.4%)	67 (95.7%)	0.84 (0.13–5.23)	0.85
T/T	2 (3.6%)	3 (4.3%)	*1*	
C/T + T/T	18 (32.7%)	13 (18.6%)	*0.47 (0.21–1.07)*	0.07
C/C	37 (67.3%)	57 (81.4%)	*1*	

No significant association was observed between the SOD2 C60T and the infection.

Table 5: Distribution of CAT C262T SNP in the control and study groups.

	CAT C262T			
Genotype	Control	Erysipelas	OR (95% CI)	p
C/C	18 (33.3%)	40 (56.3%)	6.67 (0.65–68.56)	0.11
C/T	33 (61.1%)	30 (42.2%)	*2.73 (0.27–27.66)*	0.40
T/T	3 (5.6%)	1 (1.4%)	1	—
T/T + C/T	36 (66.7%)	31 (43.7%)	1	0.01
C/C	18 (33.3%)	40 (56.3%)	*2.58 (1.24–5.38)*	
C/C + C/T	51 (94.4%)	70 (98.6%)	4.12 (0.42–40.73)	0.23
T/T	3 (5.6%)	1 (1.4%)	*1*	

The C/C genotype increases the predisposition to getting erysipelas infection.

Table 6: Association between SNPs and characteristics of patients and erysipelas symptoms.

		Gender (*n* (% genotype))		Multiplicity of infection (*n* (% genotype))		Lesion location (*n* (% genotype))			Severity (*n* (% genotype))		Form (*n* (% genotype))	
		Male	Female	Recurrent	Primary	Lower limbs	Face	Upper limbs	Low to mid	Severe	Erymanthus	Bullous
Genotype distribution												
SOD1 (G7958A)	G/G	13 (56.5)	34 (72.3)	23 (71.9)	24 (63.2)	42 (72.4)	5 (55.6)	0 (0.0)	41 (67.2)	6 (66.7)	18 (56.3)	29 (76.3)
	G/A	8 (34.8)	11 (23.4)	6 (18.8)	13 (34.2)	13 (22.4)	4 (44.4)	2 (66.7)	16 (26.2)	3 (33.3)	11 (34.4)	8 (21.1)
	A/A	2 (8.7)	2 (4.3)	3 (9.4)	1 (2.6)	3 (5.2)	0 (0.0)	1 (33.3)	4 (6.6)	0 (0.0)	3 (9.4)	1 (2.6)
p		0.406		0.203		0.041*			0.538		0.165	
Allelic distribution												
	G	34	76	52	61	97	14	2	98	15	47	66
	A	12	15	12	15	19	4	3	24	3	17	10
Genotype distribution												
SOD2 (T2734C)	C/C	6 (26.1)	14 (29.2)	8 (24.2)	12 (31.6)	15 (25.4)	2 (22.2)	3 (100.0)	16 (25.8)	4 (44.4)	16 (48.5)	4 (10.5)
	C/T	14 (60.9)	31 (64.6)	22 (66.7)	23 (60.5)	39 (66.1)	6 (66.7)	0 (0.0)	40 (64.5)	5 (55.6)	16 (48.5)	29 (76.3)
	T/T	3 (13.0)	3 (6.3)	3 (9.1)	3 (7.9)	5 (8.5)	1 (11.1)	0 (0.0)	6 (9.7)	0 (0.0)	1 (3.0)	5 (13.2)
p		0.644		0.789		0.090			0.276		0.001**	
Allelic distribution												
	C	26	59	38	47	69	10	6	72	13	48	37
	T	20	37	28	29	49	8	0	52	5	18	39

Only SOD1 (G7958A) and SOD2 (T2734C) with significant difference showed in some properties of the patients and their symptoms. Observed significance difference ($p < 0.05$) between the location of infection and SOD 1 (G7958A)*. Significant difference was also found between the form of the infection and SOD2 (T2734C)**.

Table 7: Cytokine profile of different phases of erysipelas infection.

	Binary logistic regression of cytokines comparing values in control to values in both phases of infection			95% confidence interval of cytokine values		Pearson's coeff (K) between both phases
	Phase	p	Average cytokine value	Lower	Higher	
Group 1						
IL-1β	Acute	0.046	1.516	1.008	2.280	*0.436
	Convalescent	0.158	1.419	.873	2.308	
CCL11	Acute	0.002	.963	.941	.987	*0.432
	Convalescent	0.962	.000	.000	7.555E + 216	
IL-2Rα	Acute	0.009	1.072	1.017	1.130	*0.495
	Convalescent	0.055	1.055	.999	1.115	
HGF	Acute	0.024	1.011	1.001	1.021	0.296
	Convalescent	0.099	1.010	.998	1.023	
CXCL9	Acute	0.016	1.015	1.003	1.028	*0.436
	Convalescent	0.050	1.014	1.000	1.028	
TRAIL	Acute	0.049	1.053	.999	1.109	*0.677
	Convalescent	0.091	1.043	.993	1.095	
Group 2						
IL-7	Acute	0.107	2.744	.805	9.357	0.340
	Convalescent	0.045	3.741	1.031	13.567	
IL-8	Acute	0.107	1.010	.998	1.023	0.340
	Convalescent	0.045	1.013	1.000	1.026	
PDGF-BB	Acute	0.072	1.010	.999	1.022	*0.504
	Convalescent	0.044	1.016	1.000	1.031	
CCL4	Acute	0.052	1.579	.996	2.505	*0.584
	Convalescent	0.044	1.016	1.000	1.031	
CXCL12	Acute	0.964	1.000	.988	1.013	−0.186
	Convalescent	0.031	.980	.961	.998	
Group 3						
IL-5	Acute	0.007	178.828	4.035	7925.340	0.001
	Convalescent	0.037	61.093	1.289	2896.100	
IL-6	Acute	0.007	1.053	1.014	1.094	*0.500
	Convalescent	0.037	1.042	1.003	1.083	
IL-9	Acute	0.017	2.053	1.136	3.711	*0.470
	Convalescent	0.033	2.641	1.082	6.449	
IL-10	Acute	0.017	1.075	1.013	1.140	*0.470
	Convalescent	0.033	1.102	1.008	1.205	
IL-13	Acute	0.013	1.092	1.019	1.171	*0.574
	Convalescent	0.012	1.091	1.019	1.169	
IL-15	Acute	0.006	1.119	1.034	1.211	*0.420
	Convalescent	0.001	1.157	1.058	1.265	
IL-17	Acute	0.018	1.094	1.015	1.178	*0.442
	Convalescent	0.020	1.106	1.016	1.204	
G-CSF	Acute	0.019	1.153	1.024	1.297	*0.607
	Convalescent	0.015	1.134	1.025	1.255	
GM-CSF	Acute	0.014	1.014	1.003	1.025	−0.167
	Convalescent	0.031	1.011	1.001	1.021	
CXCL10	Acute	0.011	1.005	1.001	1.009	0.296
	Convalescent	0.040	1.004	1.000	1.008	

Table 7: Continued.

	Binary logistic regression of cytokines comparing values in control to values in both phases of infection			95% confidence interval of cytokine values		Pearson's coeff (K) between both phases
	Phase	p	Average cytokine value	Lower	Higher	
CCL2	Acute	0.035	1.225	1.014	1.481	0.171
	Convalescent	0.010	1.337	1.071	1.671	
CCL3	Acute	0.035	675657538.718	4.009	113865012433100720.000	
	Convalescent	0.010	4221003831075.196	914.082	194915392050391530000000.000	0.171
VEGFr	Acute	0.019	1.036	1.006	1.067	0.767
	Convalescent	0.040	1.036	1.002	1.071	
MIF	Acute	0.027	1.017	1.002	1.032	0.360
	Convalescent	0.019	1.021	1.003	1.039	
SCF	Acute	0.020	1.058	1.009	1.110	0.386
	Convalescent	0.006	1.124	1.034	1.222	
SCGF-β	Acute	0.039	1.003	1.000	1.006	0.293
	Convalescent	0.021	1.003	1.000	1.006	
Group 4						
IL-1ra	Acute	0.323	1.022	.979	1.067	0.363
	Convalescent	0.549	1.015	.966	1.068	
IL-2	Acute	0.085	1.679	.930	3.030	0.108
	Convalescent	0.068	1.677	.963	2.922	
IL-4	Acute	0.093	2.670	.850	8.392	0.131
	Convalescent	0.380	1.618	.552	4.743	
IL12 (p70)	Acute	0.093	1.035	.994	1.077	0.237
	Convalescent	0.634	.989	.945	1.035	
FGF	Acute	0.116	1.010	.998	1.022	*0.792
	Convalescent	0.421	1.005	.993	1.017	
IFN-γ	Acute	0.093	1.003	.999	1.007	−0.010
	Convalescent	0.764	1.001	.997	1.005	
CCL5	Acute	0.106	1.004	.999	1.009	−0.235
	Convalescent	0.059	1.007	1.000	1.015	
TNF-α	Acute	0.325	1.030	.971	1.092	*0.825
	Convalescent	0.092	1.123	.981	1.284	
IL-3	Acute	0.086	1.020	.997	1.043	0.179
	Convalescent	0.647	1.005	.984	1.027	
IL12 (p40)	Acute	0.650	1.002	.993	1.012	0.244
	Convalescent	0.606	.998	.988	1.007	
IL-16	Acute	0.323	1.010	.990	1.031	−0.205
	Convalescent	0.688	.996	.975	1.017	
IL-18	Acute	0.697	1.013	.950	1.080	0.163
	Convalescent	0.556	1.020	.956	1.088	
CCL27	Acute	0.299	1.009	.992	1.027	*0.428
	Convalescent	0.209	1.015	.992	1.038	
CXCL1	Acute	0.108	1.162	.968	1.397	0.005
	Convalescent	0.252	1.167	.896	1.521	
IFN-α2	Acute	0.157	1.092	.967	1.233	0.264
	Convalescent	0.502	1.042	.924	1.174	
CCL7	Acute	0.269	.983	.953	1.014	0.230
	Convalescent	0.120	.967	.927	1.009	

Table 7: Continued.

	Binary logistic regression of cytokines comparing values in control to values in both phases of infection			95% confidence interval of cytokine values		Pearson's coeff (K) between both phases
	Phase	p	Average cytokine value	Lower	Higher	
β-NGF	Acute	0.056	7.255	.949	55.459	0.071
	Convalescent	0.489	2.543	.181	35.767	

Column p indicates the statistical difference of cytokine values between the respective phases and the cytokine values found in the controls. (Group 1) cytokine values in studied patients in the acute phase of the infection significantly differed from values observed in the controls ($p < 0.05$). During the convalescent stage, the values of the cytokines recovered to levels observed in the healthy patients ($p > 0.05$). *Coefficient of changes in cytokine values, except in HGF, of the patients between the two phases was high ($K > 0.04$). (Group 2) cytokine values in the studied patients did not differ from values observed in the healthy participants ($p > 0.05$) but differed from the values in the controls during the convalescent stage ($p < 0.05$). *Coefficient of changes in cytokine values of PDGF-BB and CCL4 of the patients between the two phases was high ($K > 0.04$). (Group 3) cytokine levels in the acute and convalescent phases significantly differed from the values of these cytokines in healthy participants ($p < 0.05$). Similar changes between both phases in the patients were observed in IL-6, IL-9, IL-10, IL-13, IL-15, IL17, and G-CSF ($K > 0.04$). (Group 4) these include cytokines which had no statistically significant difference in values between both stages in the values of the studied patients and the healthy participants ($p > 0.05$). However, FGF, TNFα, and CCL27 had a similar change in level in the patients.

3.4. Cytokine Status in Patients with Erysipelas. All cytokines analyzed in the erysipelas patients and control group were divided into four based on their expression levels during the acute phase and convalescent phase of the infection compared to their expression levels in the control group (Table 7).

Group 1 consists of IL-1β, CCL11, IL-2Rα, HGF, CXCL9, and TRAIL. Serum level of these cytokines was significantly higher during the acute phase of the infection in the controls ($p < 0.05$). There was a lack of differences in these cytokine levels during the convalescent phase as compared to the controls ($p > 0.05$).

Group 2 included IL-7, IL-8, PDGF-BB, CCL4, and CXCL12 cytokines. Serum level of these cytokines did not differ in the acute phase of the disease when compared to the controls ($p > 0.05$); however, these cytokine levels differed significantly in the convalescent phase of erysipelas as compared to the controls ($p < 0.05$). The values of IL-7, IL-8, and PDGF-BB in patients were higher in the convalescent phase of the disease than those in controls. Interestingly, the serum level of CCL4 and CXCL12 was higher in the acute phase of the disease as compared to that in the convalescent phase.

Group 3 contained IL-5, IL-6, IL-9, IL-10, IL-13, IL-15, IL-17, G-CSF, GM-CSF, CCL2, CCL3, CXCL10, VEGF, MIF, SCF, and SCGF-β cytokines, which differed significantly in the acute phase and as well in the convalescent phase of the disease when compared to values in the control group ($p < 0.05$).

Finally, group 4 included IL-1ra, IL-2, IL-3, IL-4, IL12 (p40), IL-12 (p70), IL-16, IL-18, CCL5, CCL7, CCL27, CXCL1, FGF, IFN-α2, IFN-γ, TNF-α, TRAIL, and β-NGF. Serum level of these cytokines remained unchanged during the course of the disease and was similar to that in the controls ($p > 0.05$).

Since serum level of cytokines in groups 1 and 2 differed depending on the phase of the disease, we concluded that changes in these cytokines could be related to the disease pathology. Therefore, we focused our analysis on these cytokines. From cytokines in group 1, we found that the amount of reduction in values of IL1-β, CCL11, IL-2Rα, CXCL9, and TRAIL from the acute phase to the convalescent phase was significantly similar in all the patients ($K > 0.4$). In group 2 cytokines, similar changes of serum levels of cytokines PDGF-BB and CCL4 from the acute phase to the convalescent phase were observed in all the patients ($K > 0.4$). Although the change was a similar reduction from the acute phase in CCL4, serum levels of PDGF-BB were on an average higher during the convalescent phase than the acute phase. Other cytokines of groups 1 and 2 did not express a similar change in values among the patients ($K < 0.4$). Therefore, we suggest that serum level of IL1-β, CCL11, IL-2Rα, CXCL9, TRAIL, PDGF-BB, and CCL4 could serve as markers of clinical symptoms associated to erysipelas infection.

Since serum levels of group 3 cytokines in the patients differed from the values observed in the controls during the acute phase and also in the convalescent phase, we could suggest that these cytokines are associated to the predisposition of erysipelas and possible recurrence of the infection. Serum levels of IL-6, IL-9, IL-10, IL-13, IL-15, IL-17, G-CSF, and VEGFr expressed a similar change in values among the patients ($K > 0.4$) with the values of IL-6, IL-13, and G-CSF having a similar decrease in the patients from the acute phase to the convalescent phase; VEGFr is maintaining a similar level in the acute and convalescent phases, and IL-9, IL-10, IL-15, and IL-17 expressing a similar value increase from the acute phase to the convalescent phase. Other cytokines of group 3 did not express a similar change in value between the acute phase and the convalescent phase.

Cytokines in group 4 did not differ significantly in values between the patients and the controls, but a similar reduction in value from the acute phase to the convalescent phase in the patients was observed in FGF ($K = 0.79$), while a similar increase in value from the acute phase to the convalescent phase was observed in TNF-α ($K = 0.83$) and CCL27 ($K = 0.43$).

3.5. Correlation Analysis between Studied SNPs and Serum Cytokine Levels. Serum cytokines were analyzed in 50

Table 8: Cytokine concentration respective on genotypes of CAT C262T.

Cytokines	Genotype	Cytokine mean value μg/ml of serum (standard error) Control	Acute phase	Recovery phase
PDGF-BB	C/C	43.22 (15.26)	122.84 (62.44)	79.32 (34.93)
	C/T	43.35 (12.68)	252.26 (61.5)	253.61 (53.45)
CCL2	C/C	42.81 (25.15)	5.71 (3.21)	1.34 (0.1)
	C/T	13.08 (8.1)	16.34 (6.24)	10.44 (4.26)

The table displays cytokines with significant differences in mean values between the control values and/or the acute phase and convalescent phase. The p values were not included in the table.

Table 9: Cytokine concentration respective on genotypes of SOD2 (T2734C).

Cytokines	Genotype	Cytokine (μg/ml) mean value (standard error) Control	Acute phase	Recovery phase
IL-1β	C/C	4.3 (2.4)	6.43 (1.7)	3.82 (1.28)
	C/T	3.14 (0.35)	4.96 (0.7)	4 (0.43)
	T/T	3.03 (0.29)	12.2 (2.1)	11 (1.94)
IL-7	C/C	0.7 (0.29)	1.25 (0.5)	1.78 (0.63)
	C/T	0.82 (0.18)	1.81 (0.46)	1.29 (0.26)
	T/T	0.46 (0.09)	8.71 (0.31)	1.55 (0.27)
IL-8	C/C	69.5 (29.5)	124.67 (49.59)	178 (62.77)
	C/T	82.5 (18.27)	180.76 (45.65)	129.07 (25.9)
	T/T	46.33 (9.13)	871 (36.96)	155.05 (27.42)
IL-17	C/C	19.8 (11.3)	28.57 (8.58)	21.93 (3.64)
	C/T	16.32 (3.79)	30.72 (3.89)	26.59 (2.57)
	T/T	17.03 (2.51)	103.2 (23.37)	50.3 (3.62)
CCL5	C/C	291.9 (11.8)	669.7 (253.4)	656.97 (146.29)
	C/T	303.23 (44.96)	627.41 (184.25)	438.27 (66.07)
	T/T	232.13 (19.79)	15620.2 (7505.73)	198.1 (52.73)
HGF	C/C	69.95 (54.15)	709.16 (333.39)	86.23 (19.14)
	C/T	68.56 (18.55)	188.71 (40.41)	136.95 (30.75)
	T/T	45.48 (38.55)	28.36 (7.32)	20.7 (6.26)

The table displays cytokines with significant differences in mean values between the control values and/or the acute phase and convalescent phase.

erysipelas patients and 26 relatively healthy (control) individuals. Two serum samples were collected from each erysipelas case at the time of the admission (acute phase) and the time of discharge (convalescent phase). Erysipelas patients were grouped based on the identified SNPs in SOD T2734C and CAT C262T. Correlation between serum cytokines and SOD C60T was not analyzed since there was a lack of predisposition between this SNP and erysipelas. Also, the SOD 1 G7958A SNP correlation with serum cytokines was not analyzed due to a lack of control individuals with the A/A genotype.

3.5.1. CAT C262T SNP and Serum Cytokines. The frequency of C/C and C/T genotypes within the 50 erysipelas patients was 25 (50%) and 25 (50%), respectively, while in controls, genotype distribution was 6 (25%) and 18 (75%), respectively.

Significant differences in the cytokine values when compared to the genotypes of CAT C262T were observed only in PDGF-BB and CCL2. Cytokine analysis revealed increased PDGF-BB level in serums of patients with C/T genotype (in the acute and convalescent phases) compared to that with controls ($p = 0.045$) (Table 8). Serum levels of CCL2 were significantly lower in the acute ($p = 0.02$) and the convalescent phases ($p = 0.018$) in patients with the CC genotype than in controls (Table 8).

3.5.2. SOD2 T2734C SNP and Serum Cytokines. Distribution of C/C, C/T, and T/T alleles within the 50 erysipelas patients was 9 (18%), 38 (76%), and 3 (6%), respectively. The values observed in the 26 controls were 4 (15.4%), 17 (65.4%), and 5 (19.2%), respectively.

Although IL-1β level was elevated in the serum of erysipelas with T/T genotype as compared to the controls, differences in IL-1β levels were not significant between the acute and convalescent phases in the patients. There was a T allele dependency on IL-7, IL-8, IL-17, and CCL5 serum level in the patients during the acute phase of the infection. Interestingly, only HFG serum level was associated with the presence of C/C genotype in the erysipelas patients (Table 9).

4. Discussion

4.1. Demographic Distribution of Erysipelas. Erysipelas is a superficial skin infection with its etiology often linked to group A streptococci (GAS). The skin of the infected portion is often red, swollen, soft to the touch, and with clearly visible boundaries. Associated clinical symptoms may vary depending on the form of the infection, but typical symptoms could include fever, chills, malaise, and sometimes accompanied by nausea and vomiting [16]. A major problem of erysipelas to clinicians is its recurrence, which occurs in 8 to 23 percent of cases [17]. Erysipelas is mostly affected but restricted to the elderly; it has also been observed in younger people [18]. Our observed age distribution of the study group could be on one hand related to the fact that more elderly than younger people with erysipelas are prone to seeking medical care or that the elderly are at a greater risk [19]. Other research had also observed a similar distribution in the age of their studied group [18, 20]. Our study, which was restricted to people who could be categorized as elderly, showed that there was a sex dependence on the recurrence and severity of the infection with more elderly females having more of the recurrent and severe forms of the infection. The elderly female predisposition to recurrence could be associated to treatment or the presence of other ailments such as post breast cancer treatment [21], osteoporosis [22], and vaginal colonization of related microbes [23]. Gender-associated differences in the susceptibility to erysipelas could also be attributed to hormonal variations between male and female as has been previously shown by Bellanti et al. [24]. Our observation also revealed that a higher proportion of male patients had facial localization of the infection when compared to that of females.

4.2. SNPs' Association with Predisposition to Erysipelas. To date, our knowledge on SOD1 G7958A significance is limited to Yi et al. data presenting the association between the mutant allele (A) and increased expression of SOD1 mRNA, while the presence of the wild type (G) was related to reduced gene transcription and consequent increase and accumulation of free radicals in the cells [25]. Our data corroborates this observation, as a significant association was demonstrated between G allele of SOD1 G7958A and predisposition to erysipelas. We propose that the presence of G allele reduces transcription of SOD1 gene leading to decreased protein synthesis of the SOD1 enzyme. As a result, oxidative stress could develop leading to reduced antibacterial defense. Another finding showed that a dysfunctional or reduction in SOD1 expression favored the pathogenesis of H5N1 influenza virus [26] and amyotrophic lateral sclerosis [27, 28]. In another research, an in vitro model of amyotrophic lateral sclerosis showed that cells transfected with the mutant SOD1 were more vulnerable to infectious stimuli of the bacteria than cells overexpressing normal SOD1 [29]. We observed an association between SOD1 (G7958A) and localization of infection, where higher proportion of G/A and A/A genotypes were found in patients who had erysipelas on the upper limb. Conversely, a higher number of patients with the G/G genotype had the infection predominantly on the lower limbs. This differential distribution of the infection relative to SNP cannot be explained at the moment but needs to be studied.

The SOD2 C2734T polymorphism was shown to cause an amino acid substitution from leucine to phenylalanine at codon 84 of the SOD2 gene [30]. Our data provides evidence suggesting this SOD2 substitution of leucine to phenylalanine increases susceptibility to erysipelas and was linked to the more severe bullous form of the infection. It was proposed that due to this substitution, the produced protein subunits fail to properly interact, causing reduction of SOD2 activity and accumulation of free radicals in vitro [31]. Not much data is available about the role of the SOD2 C2734T, but an earlier research had showed that the SNP influenced susceptibility to vitiligo, a skin phenomenon characterized by long-term patches on the skin and loss of skin colour [32]. Other studies had shown that a dysfunctional SOD2 is positively associated with cardiovascular disease risk [33], neurodegeneration [34], diabetes [35], and different forms of cancers [36–38].

The C to T substitution in the 60th amino acid position of the SOD2 leads to an alanine to valine substitution. Our data demonstrated lack of association between the SOD2 C60T SNP and predisposition to erysipelas. Therefore, we suggest that SOD2 C60T SNP has limited role in pathogenesis of erysipelas.

The CAT C262T SNP is located in the promoter region regulating gene transcriptional activity. It has been shown that the C allele of CAT causes reduction of the transcriptional activity as compared to the T allele [9]. Additionally, C262C genotype of CAT was shown to produce more single-strand DNA breaks than C/T and T/T genotypes [9]. Our data demonstrate that erysipelas patients with the C allele have a higher risk of developing erysipelas. We believe that patients with C262C genotype have lower CAT activity, which can cause accumulation of free radicals and oxidative stress. The association between the CAT C262T SNP was found to have an effect on the predisposition to male infertility [39], *Shigella flexneri*-related infection [40], asbestosis [41], vitiligo [42], and cancer although it remains highly contradictory and inconclusive [43].

4.3. Cytokine Role in Erysipelas Pathogenesis. Our data suggest that IL1-β, IL-2Rα, CCL4, CCL11, CXCL9, TRAIL, IL-1 β, and PDGF-BB cytokines could be linked to the clinical symptoms displayed during erysipelas infection. We have found that serum cytokine levels of these cytokines change during the course of the disease. The values of these cytokines during the acute phase in the patients significantly differed from that in the controls. However, during the convalescent phase, these cytokine levels returned to levels similar to that found in the controls. We also suggest that IL-6, IL-9, IL-10, IL-13, IL-15, IL-17, G-CSF, and VEGF could play a role in the clinical patterns and susceptibility of erysipelas infection. This suggestion was based on our finding that serum levels of cytokines in the acute and convalescent phases of the infection significantly differed from values observed in the controls. More research is however suggested to be able

to draw out a more direct link between these cytokines and erysipelas infection.

4.4. Cytokine—SNPs' Interaction in Erysipelas. Activation of proinflammatory cytokines such as IL-1β is a hallmark of bacterial infections that is crucial for host-defense responses to infection and injury [10, 44]. The T allele of SOD2 C2734T was shown to be associated with decreased antioxidant capacity in the mitochondria and, subsequently, an increased oxidative stress. Our results show that increased serum IL1-β level in erysipelas patients is the T allele of SOD2 SNP C2734T dependent. Based on these observations, we propose that the T allele of SOD2 C2734T could trigger the upregulation of anti-inflammatory cytokines such as IL-1β, which would in turn attempt to stimulate the production of a functional SOD2 production which is downregulated in individuals with the T allele. This assumption is supported by our observation that serum IL-1β levels were higher in erysipelas patients homozygous for T allele than in patients with the heterozygous form. Therefore, we believe that serum levels of IL-1β could serve as a surrogate marker for predisposition to erysipelas and impaired antioxydase system. More study into the role of antioxydase system in erysipelas will help to understand the mechanisms of cytokine activation and their role in disease pathogenesis.

IL-7 regulates the development of T cells, enhances cytolytic T lymphocyte activity, and induces lymphokine-activated killer cells [45, 46]. Bacterial invasion induces IL-7 expression in T cells [47]. The role of IL-7 in oxidative stress remains largely unknown. However, studies suggest that IL-7 could be upregulated in patients with increased mitochondrial activity and oxidative stress [48, 49]. Our observation supports the notion that decreased mitochondrial activity is associated with upregulation IL-7. We have found upregulated serum IL-7 in patients with SNP in SOD2 suggesting decreased mitochondrial activity and increased ROS formation.

IL-8 is a chemoattractant for inflammatory leukocytes [50]. We found increased IL-8 in patients with the T allele of SOD2 C2734T that causes a decreased antioxidant activity. This observation supports previous observations which showed that IL-8 gene expression can be upregulated by free radicals, while antioxidants reduce the cytokine transcription [51]. We propose that infection could trigger oxidative stress and subsequently IL-8 production. These would exacerbate the generation of the reactive oxygen species and, when combined with decreased SOD2 activity, lead to perpetuate upregulation of IL-8.

The cytokine IL-17 is known to recruit leukocytes such as neutrophils to the site of infection thus aiding in the host's defense mechanism against extracellular bacteria. IL-17 also induces neutrophilic release of antimicrobial substances and ROS [52]. Our observation supports this observation as high IL-17 values were associated to the TT genotype of SOD2 C2734T and thus elevated oxidative stress.

CCL5 plays an active role in recruiting leukocytes and natural killer cells into inflammatory sites. A strong positive association between CCL5, ROS generation, and systemic lupus erythematosus had earlier been observed [53]. Our observation linked the high levels of expressed CCL5 in erysipelas patients who were linked to the homozygous T genotype in SOD2 C2734T SNP.

Interestingly, we have found increased serum level of HGF in erysipelas with C/C genotype of SOD2 C2734T. The C/C genotype is associated with the production of the functional SOD2 protein. Therefore, we suggest that expression of functional SOD2 protein, which is associated with properly functioning antioxidant, is linked to normal cell growth and differentiation. Our conclusion corroborates Borawski et al. data showing increased HGF in individuals with properly functioning SOD [54].

PDGF-BB is known for its participation in cell growth and differentiation. The mitogenic stimulus function of PDGF was shown to be mediated via ROS to activate cell proliferation [55]. Our research showed that while elevated values of PDGF-BB was observed in the erysipelas patients with the C/T genotype of CAT C262T during the acute phase and convalescent phase, the elevated value of PDGF-BB was observed in the patients with the C/C genotype only during the acute phase of the infection. As mentioned earlier, the C allele of CAT C262T was considered poorly functional and contributed to the predisposition of erysipelas. We propose that the functional T allele of CAT C262T contributed to maintaining high PDGF-BB values during the convalescent stage of the infection. Thus, the PDGF-BB-mediated T allele of CAT C262T aided in cell growth and differentiation to replace the erysipelas damaged cells.

CCL2 otherwise known as monocyte chemoattractant protein-1 (MCP-1) has been demonstrated to mediate the migration of monocytes from the blood stream across the vascular endothelium for routine immunological surveillance of tissues, as well as in response to inflammation [56]. The cytokine values of CCL2 were observed to be significantly lower in erysipelas patients with the C/C genotype of CAT C262T than in the individual in the control group with the C/C genotype. Values of the cytokine in patients with the C/T genotype were not significantly different from the values in the individuals of the control group with the C/T genotype. These observations lead us to propose that the C allele of CAT C262T which has been earlier shown to code for a poorly functioning catalase and higher predisposition to erysipelas is linked to low functioning of CCL2, thus hampering immunological surveillance of tissues in erysipelas patients.

5. Conclusion

Our research has been able to demonstrate that erysipelas infection predisposition and its clinical characteristics are affected by age, sex, and SNPs found in SOD1, SOD2, and catalase genes. These SNPs have an influence on an array of cytokines whose values were found to be different from values found in individuals not suffering from erysipelas.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

Comparative Assessment of Cytokine Pattern in Early and Late Onset of Neonatal Sepsis

Kh. S. Khaertynov,[1] S. V. Boichuk,[1] S. F. Khaiboullina,[2,3] V. A. Anokhin,[1] A. A. Andreeva,[4] V. C. Lombardi,[2,3] M. A. Satrutdinov,[4] E. A. Agafonova,[4] and A. A. Rizvanov[2]

[1]*Kazan State Medical University, Kazan, Russia*
[2]*Kazan Federal University, Kazan, Russia*
[3]*Nevada Center for Biomedical Research, Reno, NV, USA*
[4]*Republic Children's Clinical Hospital, Kazan, Russia*

Correspondence should be addressed to S. V. Boichuk; boichuksergei@mail.ru and A. A. Rizvanov; albert.rizvanov@kpfu.ru

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Neonatal sepsis is a significant health issue associated with high mortality. Immune responses associated with neonatal sepsis, such as proinflammatory cytokine production, are believed to play a central role in the pathogenesis of this disease. In the present study, serum levels of the proinflammatory cytokines TNF-α, IL1-β, and IL-6 and the anti-inflammatory cytokines IL-4 and IL-10 were evaluated for 25 subjects with neonatal sepsis. We observed that subjects with late onset of sepsis (LOS), as well as those with early onset of sepsis (EOS), had a substantial increase in serum TNF-α. In contrast to EOS, subjects with LOS demonstrated a significant increase in serum levels IL-6 and IL-10. Additionally, we observed a significant difference in cytokine profiles between acute and postacute cases of neonatal sepsis. For instance, the level of proinflammatory cytokines, such as TNF-α and IL-6, was elevated in the acute phase, whereas the production of anti-inflammatory cytokines, such as IL-10, became substantially upregulated during the postacute phase. Additionally, no correlation was observed between cytokine levels and CRP levels or lymphocyte counts. Thus, in contrast to CRP levels and lymphocyte counts, examination of the cytokine profile can provide valuable information when determining the most effective therapy for treating neonatal sepsis. This information may be useful to physicians when determining if anti-inflammatory or immune stimulatory therapy is warranted.

1. Introduction

Neonatal sepsis presents a significant health issue and is often associated with a high mortality rate [1]. Very low birth weight infants are especially vulnerable and often tend to develop severe complications, leading to a fatal outcome [2]. Therefore, early diagnosis and implementation of appropriate antibiotic therapy play a crucial role in improving the survival rate of infants with sepsis [3]. The "gold standard" for a diagnosis of the systemic bacterial or fungal infection is the isolation of pathogens from peripheral blood. Unfortunately, the sensitivity of this method is low and thus, a diagnosis of sepsis cannot be excluded even when these results are negative [4, 5].

It is believed that the types of immune responses that occur during a bacterial infection play a central role in the pathogenesis of neonatal sepsis. There are two principal types of such responses: systemic inflammatory response (SIRS) and compensatory anti-inflammatory response (CARS) [6, 7]. Acute-phase proteins such as C-reactive protein (CRP) and procalcitonin (PCT) are known as common biomarkers for SIRS. Accordingly, serum levels of these proteins are significantly upregulated during EOS [8, 9]. Proinflammatory cytokines are also considered sensitive biomarkers of neonatal sepsis. For instance, tumor necrosis factor alpha (TNF-α), interleukin-1-beta (IL-1β), interleukin-6 (IL-6), and CXCL8 (interleukin-8) levels become rapidly and substantially increased during neonatal sepsis [4, 6, 7, 10, 11]. It

is believed that a moderate increase of these cytokines in circulation provides a protective role and promotes an antimicrobial immune response, whereas excessive upregulation of proinflammatory cytokines (often referred to as a "cytokine storm") is commonly associated with a severe and often fatal outcome due to multiple organ failure [7]. For these reasons, it has been proposed that a serum cytokine profile could be used as a prognostic biomarker to predict the severity of the disease [12].

In the present study, we investigated serum cytokine expression, with respect to disease severity, in subjects with neonatal sepsis. The information afforded by this study may provide useful knowledge for physicians when determining if an anti-inflammatory or immune stimulatory therapy is warranted.

2. Methods

Subjects. This study was conducted over a period of 10 months between February 2013 and November 2013. In this retrospective study, serum specimens from 25 cases with a diagnosis of neonatal sepsis and eight healthy controls (five full-term newborns and three premature newborns from a period of 32 to 36 weeks' gestation) were provided by the Children's Republican Clinical Hospital of the Ministry of Health, of the Republic of Tatarstan (RHC).

In accordance with the Report of the Expert Meeting on Neonatal and Pediatric Sepsis (8 June 2010, EMA, London) [13], sepsis was defined as the presence of at least two clinical and two laboratory criteria or as a result of suspected or proven infection (positive blood culture). The clinical criteria are (1) body temperature instability; (2) cardiovascular instability; (3) presence of the skin and subcutaneous lesions such as petechial rash or sclerema; (4) apnea or increased oxygen requirement, requirement for ventilation support; (5) feeding intolerance or abdominal distension; and (6) irritability, lethargy, or hypotonia. The laboratory criteria were (1) a white blood cell (WBC) count of <4 or $>20 \times 10^9$ cells/L; (2) an immature to total neutrophil ratio (I/T) of >0.2; (3) a platelet count of $<100 \times 10^9$/L; (4) C-reactive protein (CRP) levels of >15 mg/L; (5) blood glucose values of >180 mg/dL or hypoglycemia (<40 mg/dL) confirmed at least 2 times; and (6) metabolic acidosis as characterized by a base excess (BE) of ≤10 mmol/L.

The Institutional Review Board of the RHC approved this study and informed consent was obtained from each subject's respective guardian, according to the guidelines approved under this protocol (Federal Law "Protection of Health Right of Citizens of Russian Federation" N323- FL, 11.21.2011).

2.1. Serum. Peripheral blood was collected into serum-separator tubes and separated immediately and aliquots (100 μL) were made and stored at −80°C until being used. Specimens were collected during the first 2 days of the onset of clinical symptoms and laboratory signs of sepsis and seven days later. Control serum samples collected from eight healthy neonates were collected at a single-time point.

2.2. Cytokine and CRP Analysis. Serum cytokine levels were analyzed on a Luminex 200 analyzer (Austin, TX) with Millipore Human Milliplex® MAP Single-Plex cytokine kits (Millipore, Billerica, MA, USA). Single-plex kits specific for TNF-α, IL1-β, IL-4, IL-6, and IL-10 were used in combination according to the manufacturer's instructions. Serum CRP levels were determined using the Randox Full Range CRP immunoturbidimetry assay (Randox Laboratories, Crumlin, Northern Ireland, UK), also according to the manufactures instructions.

2.3. Statistical Analysis. Statistical analysis was made using the Kruskall-Wallis and Wilcoxon nonparametric methods with Statistica 6.1 for Windows (Statsoft, Tulsa, OK, USA). Significance was established at a value of $p < 0.05$. Correlation analysis was performed using Spearman method.

3. Results

3.1. Study Subject Characteristics. It is well documented that newborns developing EOS become infected during the intrapartum period. For newborns with EOS, 85% of cases present within the first 24 hours, whereas 5% of cases present at 24 to 48 hours, and the balance mostly presents within 48 to 72 hours [14]. Therefore, in our study, we classified the onset of sepsis within the first three days of life as EOS [15]. In contrast, LOS has been defined as infection between 4 and 28 days of life and is due to the horizontal transmission of pathogens during the postnatal period [15].

In the present study, neonates were grouped into two categories: 10 cases presented with EOS and 15 presented with LOS. In both groups, the majority of the neonates were male: 70% with EOS and 60% with LOS. The EOS cohort had five premature neonates (50%), whereas LOS cohort had 10 preterm neonates (66%). Preterm is defined for infants that were born before 37 weeks of gestational and having a birth weight of less than 2.5 kilograms. In the group of newborns with EOS, all preterm infants were born at less than 32 weeks of gestation. In the LOS cohort, 70% of preterm infants were born at less than 32 weeks of gestation, and another three (30%) in the period from 32 to 36 weeks of gestation. Disease patterns of EOS and LOS neonates were as follows: EOS clinically manifested with pneumonia (eight cases), microcirculatory dysfunction (two cases), and urinary tract infection (one case). LOS was associated with pneumonia (seven cases), enterocolitis (six cases), microcirculatory dysfunction (four cases), cholestatic hepatitis (four cases), urinary tract infection (three cases), and pyoderma (two cases) (Table 1). Bacteremia was detected in 12 cases (48%), 10 of which belonged to the LOS cohort. Low percentage of septicemia in the EOS cohort might be due to the early administration of antibacterial therapy. *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* were isolated in EOS cases with septicemia only. In contrast, a plethora of infectious agents were found in LOS cohort and included *Candida krusei* (three cases), *Candida albicans* (two cases), *Staphylococcus epidermidis* (two cases), and one case for each *Klebsiella pneumoniae, Enterococcus faecalis,* and *Staphylococcus haemolyticus* (Table 2). Changes in white

Table 1: Clinical manifestations associated with different types (EOS and LOS) of neonatal sepsis.

Clinical manifestation	Neonatal sepsis Number of cases (%)	EOS Number of cases (%)	LOS Number of cases (%)
Pneumonia	15 (60)	8 (80)	7 (46)
Pyoderma	4 (16)	2 (20)	2 (13)
Enterocolitis	6 (24)	0 (0)	6 (40)
Cholestatic hepatitis	4 (16)	0 (0)	4 (26)
Urinary tract infection	4 (16)	1 (10)	3 (20)
Microcirculatory dysfunctions	8 (32)	4 (40)	4 (26)

Table 2: Pathogenic spectrum of blood-culture proven sepsis episodes.

Pathogen	EOS ($n = 10$) Number of cases (%)	LOS ($n = 15$) Number of cases (%)
Staphylococcus epidermidis	1 (10)	2 (13.3)
Staphylococcus haemolyticus	1 (10)	1 (6.7)
Klebsiella pneumoniae	—	1 (6.7)
Enterococcus faecalis	—	1 (6.7)
Candida albicans	—	2 (13.3)
Candida krusei	—	3 (20.0)

blood cell counts (WBCs) included leukocytosis (20% of cases) and leukopenia (40%); 10% of cases have no changes in WBCs. Serum CRP levels were used to examine the inflammatory status. In subjects with sepsis, CRP was higher than the laboratory ranges for healthy donors (1.5 μg/dL). Seven cases (70%) with EOS and 12 cases (80%) with LOS were passively ventilated. Two cases of neonatal sepsis were fatal; each neonate in this category was born with extremely lower body weight. A total of 23 neonates with sepsis successfully recovered.

3.2. Cytokine Analysis. Cytokine levels in the control group were not dependent on the gestational age ($p < 0.05$). In contrast to healthy controls, a significant increase in TNF-α and IL-6 proinflammatory serum cytokines was observed in LOS and EOS cohorts (Figure 1) ($p < 0.05$). Interestingly, serum level of IL-1β did not differ between control and LOS and EOS groups of neonates. In contrast to TNF-α and IL-6, level of the anti-inflammatory cytokine IL-10 was significantly different in these two cohorts ($p = 0.003$), as well as between the LOS cohort and healthy controls ($p = 0.002$). Indeed, we observed a significant increase of IL-10 in the LOS cohort only, whereas the levels of IL-10 between controls and EOS cases did not differ from each other. The level of another anti-inflammatory cytokine IL-4 was increased only in LOS cohort ($p = 0.02$). It was found that the fungal sepsis is associated with substantial increase in all cytokines levels (TNF-α, IL-1β, IL-4, IL-6, and IL-10), when compared with bacterial sepsis (Table 3).

To examine the cytokine dynamics of neonatal sepsis, we next stratified cases by those who presented as acute cases and those who presented as postacute cases (Figure 2). For acute cases, the inflammatory cytokines TNF-α and IL-6 were significantly upregulated when compared to the postacute cases ($p = 0.043$). Conversely, the anti-inflammatory cytokines IL-10 and IL-4 were upregulated in the postacute phase when compared to the acute cases but it has not been statistically significant ($p > 0.05$). CRP is a pentraxin family protein that is synthesized by the liver in response to factors released by macrophages and adipocytes [14]. Serum levels of CRP rise in response to acute inflammation; consequently, CRP is the most commonly used clinical marker of acute inflammation [16]. To evaluate the association of CRP and inflammatory cytokines in the context of neonatal sepsis, we conducted correlation analysis between CRP and the cytokines TNF-α, IL-6, and IL-10 (Table 4). Unexpectedly, we observed no correlation between the CRP and the inflammatory cytokines. We then stratified subjects by the presence or absence of lymphopenia. Cases having a lymphocyte count of $< 2.0 \times 10^9$/L were considered lymphopenic and those with a lymphocyte count of $> 2.0 \times 10^9$/L were considered normal (no cases presented with lymphocytosis). Upon stratification, we observed that levels of TNF-α and IL-6 were, on average, increased in the group with lymphopenia and levels of IL-10 were slightly decreased; however, upon correlation analysis we observed no statistically significant correlation between lymphopenia and cytokine levels (Table 5).

4. Discussion

In contrast to previous studies, which reported *Streptococcus group B* as a predominant etiological factor in early neonatal sepsis, we were unable to identify this group of pathogens in our study. The principal reason for this issue might be due to the early initiation of antibacterial therapy (e.g., Ampicillin) in newborns. In our LOS cohort, a low incidence of Gram-negative bacteria in the peripheral blood, as well as *Candida* (33%), might also be related to the early initiation of antibacterial therapy. Moreover, other types of the pathogens present in hospitals and, in particular, intensive care units also might affect the etiology of neonatal sepsis.

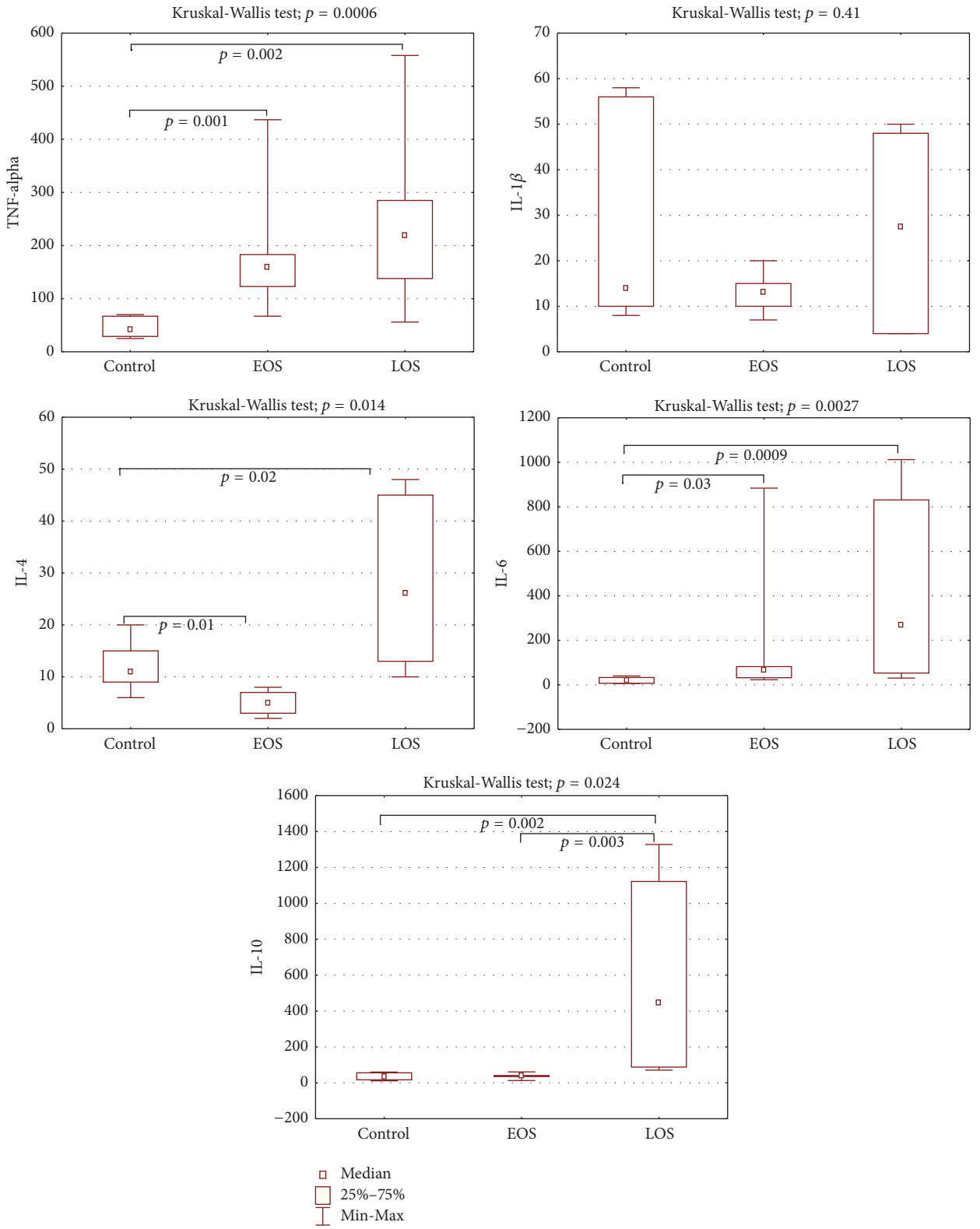

Figure 1: Serum cytokine level (pg/mL) in EOS and LOS types of neonatal sepsis (mean; quartile range). Kruskal-Wallis test was performed for comparison of control group and neonates with EOS and LOS.

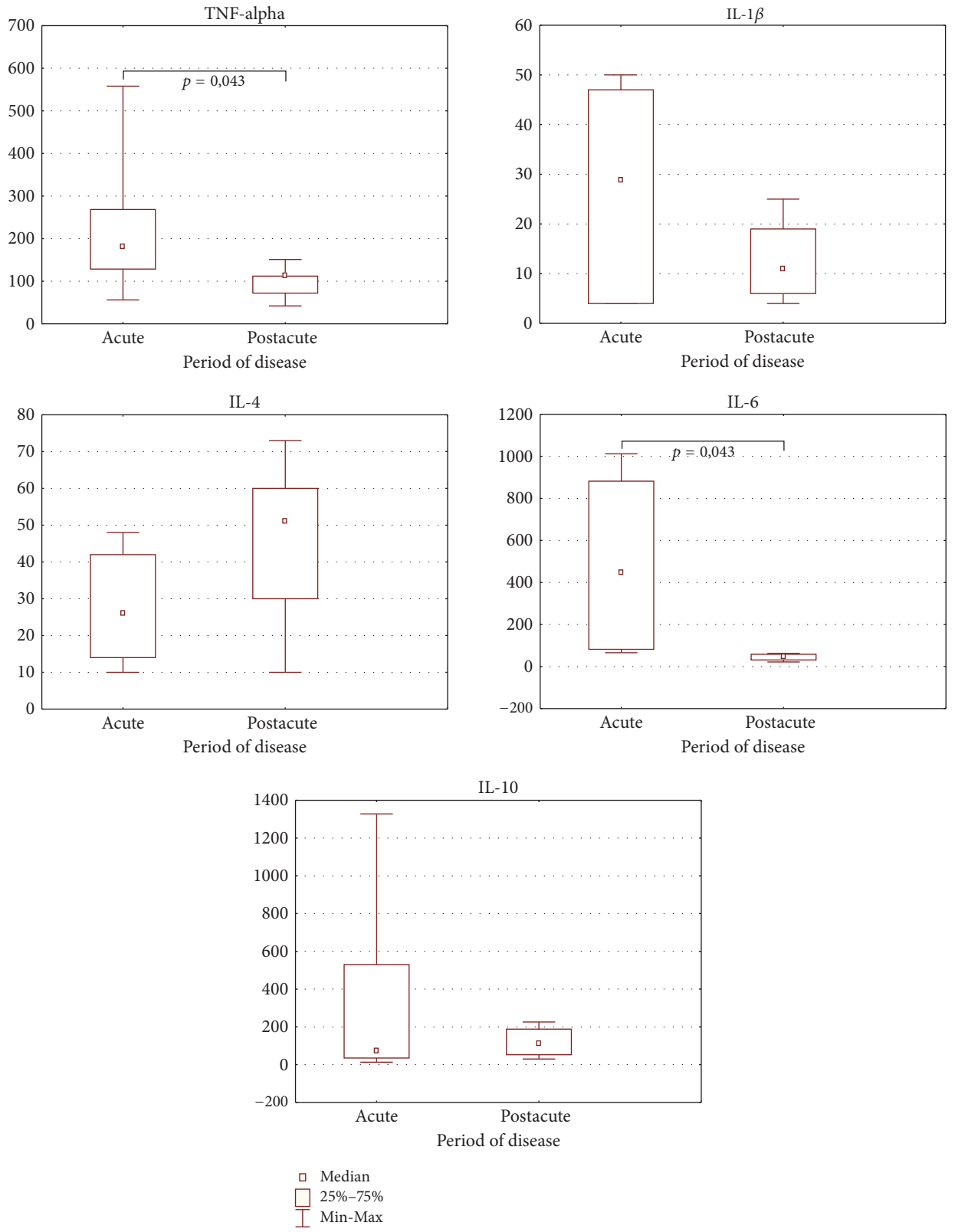

Figure 2: Dynamics of serum cytokine levels (pg/mL) during neonatal sepsis (mean; quartile range). p value was calculated by using Wilcoxon method.

Table 3: Serum cytokine level (pg/mL) in confirmed bacterial and fungal neonatal sepsis (mean; quartile range).

Cytokines	Bacterial sepsis Mean (quartile range)	Fungal sepsis Mean (quartile range)	p-value
TNF-α	112.0 (56–177.8)	281.5 (177.5–450)	0.007
IL-1β	15.0 (13–27.5)	25.0 (12–39)	0.007
IL-4	43.5 (24.5–46.5)	13.0 (10–26)	0.01
IL-6	26.7 (22.5–41.2)	445.0 (53–491)	0.01
IL-10	88.0 (17–317)	849.0 (323–1225)	0.01

p value was calculated by using Wilcoxon method.

Table 4: Correlation between serum cytokine (pg/mL) and C-reactive protein (mg/dL) levels.

CRP	TNF-α Mean (quartile range)	IL-6 Mean (quartile range)	IL-10 Mean (quartile range)
<3 mg/dL	136.8 (105–342)	219.5 (52–445)	194.0 (35–576)
>3 mg/dL	223.8 (164–285)	477.5 (69–947)	61.0 (41–88)
R; p value	$R = 0.15$; $p = 0.57$	$R = 0.38$; $p = 0.26$	$R = 0.18$; $p = 0.6$

Correlation analysis was performed using *Spearman* method.

Table 5: Relationship between cytokine levels and lymphocyte count.

Lymphocyte count	TNF-α (pg/mL) (Median; quartile range)	IL-6 (pg/mL) (Median; quartile range)	IL-10 (pg/mL) (Median; quartile range)
$>2.0 \times 10^9$/L	168; 117–231	77; 30–270	102; 35–317
$<2.0 \times 10^9$/L	253; 149–386	445; 53–882	88; 71–1122
R; p value	$R = 0.29$; $p = 0.16$	$R = 0.29$; $p = 0.25$	$R = 0.28$; $p = 0.38$

Correlation analysis was performed using *Spearman* method.

Based on the currently accepted viewpoints, the pathogenesis of neonatal sepsis is characterized by a bimodal (i.e., two-phase) immune response [6, 7]. The first phase is predominantly related to SIRS and associated with an excessive release of the proinflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) into the bloodstream. The highest levels of proinflammatory cytokines are referred to as a "cytokine storm" and often associated with a single or multiple organ dysfunctions [7]. The second phase of the immune response is characterized by CARS and is mediated by the secretion of anti-inflammatory cytokines (IL-4, IL-10) [7]. The profound immune suppression in neonatal sepsis is considered to be one of the most important factors of morbidity and mortality of newborns during this period of the disease [17]. TNF-α, Il-1β, and IL-6 are not considered to be "gold standard" biomarkers of sepsis due to their short half-life [18]. Nonetheless, these cytokines are typically increased very rapidly during neonatal sepsis, even more so the well-known proinflammatory marker C-reactive protein [18, 19]. Previous studies demonstrated procalcitonin, TNF-α [10, 20], and IL-6 [21] to be the most sensitive and specific diagnostic markers of neonatal sepsis. We found that the levels of proinflammatory cytokines (IL-6, TNF-α) were increased at both EOS and LOS. Importantly, an increase of cytokines levels in our LOS cohort was much more substantial when compared with EOS. This difference might be due to the degree of activation of the immunocompetent cells, known to be frequently involved in pathological processes in the bowel during LOS. The various rates of cytokine increases might be also due to the type of the pathogen, as an etiological factor of sepsis. For instance, it has been shown that fungal sepsis in neonates is associated with substantial increase in IL-6 and TNF-α levels, when compared with bacterial sepsis [8, 22]. In our study, fungal sepsis was diagnosed in 33% of LOS cases. Surprisingly, no differences in IL-1β levels were found between healthy controls and both groups of infants with NS (EOS and LOS). This fact might be due to the extensive recruitment of the IL-1β-producers into affected tissues such as the lungs, bowel, and kidneys. Also, it could be the consequence of the profound dysfunction or suppression of the immune system. Notwithstanding, it is also possible that the mild increase in the proinflammatory cytokines during EOS is due to the immature state of the immune system during the neonatal period [23, 24]. Indeed, the defense mechanisms in neonates are predominantly related to the innate immune reactions, whereas the adaptive immune

mechanisms are not yet well established [25]. This fact might be also responsible for the high incidence of NS in premature infants [24]. Intensity of the immune response in neonatal sepsis may be due to decreased expression of innate immunity factors. Reduced expression of the innate immune factors and proinflammatory cytokine synthesis could be due to gene polymorphism that causes a genetic predisposition to various infections, including sepsis [26]. The meta-analysis showed that genetic polymorphisms of IL-1β is associated with sepsis susceptibility [27].

It is believed that the expression of anti-inflammatory cytokines usually takes place during the second phase of neonatal sepsis and thus reflects the upregulation of immunosuppressive mechanisms. With this in mind, it is noteworthy that we observed the most significant increase of IL-10 production in the group of LOS neonates. Moreover, we also detected an increase in IL-10 for 20% of cases in the EOS cohort. In an earlier study, IL-10 was reported to be highly sensitive and specific in the diagnosis of neonatal sepsis [28]. These findings indicate that immune reactions associated with EOS and LOS in neonatal sepsis are more complex and do not display a "so-called" bimodal distribution and therefore might develop simultaneously.

Interestingly, the most significant IL-10 increase in peripheral blood was observed for the group of neonatal sepsis subjects with fungal infection, thereby confirming previous findings that fungal infection is commonly associated with immune suppression [29]. Indeed, the frequency of fungal infections in our study was up to 20%. Similarly, an increase of IL-10 in peripheral blood was detected in 28% of cases with neonatal sepsis associated with fungal infections, thus reflecting an immunosuppressive state. The elevated levels of anti-inflammatory cytokines in patients with neonatal sepsis are usually interpreted as a compensatory mechanism, reflecting the activation of systemic inflammation as a response to generalized infection. Accordingly, attempts to suppress innate immune reactions in neonatal sepsis have led to complications such as multiorganic dysfunctions and secondary infections [16]. On the other hand, anti-inflammatory cytokines are known as potent proapoptotic factors [30, 31]. Given that anti-inflammatory cytokines are overproduced in neonatal sepsis, cytokine-induced apoptosis of immune cells might play an important role in immune suppression in neonates with neonatal sepsis [32]. Taken together, these data illustrate the complexity of neonatal sepsis pathogenesis and therefore highlight an importance of the appropriate use of anti-inflammatory and/or immunosuppressive therapy for neonatal sepsis. This is especially true for corticosteroids that are commonly used for neonatal sepsis therapy. On the other hand, the immunosuppression observed during sepsis might be an indication for immune simulative therapy. Therefore, the measurement serum pro- and anti-inflammatory cytokines might be useful in determining a strategy for pathogenic therapy for neonatal sepsis.

5. Conclusions

Sepsis is characterized as a complex and dynamic disease that involves an excessive and suppressed inflammatory and immune response. The immune response in neonatal sepsis associated with proinflammatory and anti-inflammatory cytokines production plays an important role in pathogenesis of this disease. Our data indicates that cytokine profiles provide valuable information for neonatal sepsis therapy and are even more informative when compared with routine CRP and lymphocyte numbers assessment. This information may be useful for physicians when determining if anti-inflammatory or immune stimulatory therapy is indicated.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Bactericide, Immunomodulating, and Wound Healing Properties of Transgenic *Kalanchoe pinnata* Synergize with Antimicrobial Peptide Cecropin P1 In Vivo

A. A. Lebedeva,[1] N. S. Zakharchenko,[1] E. V. Trubnikova,[2,3] O. A. Medvedeva,[3] T. V. Kuznetsova,[4] G. A. Masgutova,[5] M. V. Zylkova,[1,6] Y. I. Buryanov,[1] and A. S. Belous[3]

[1]*Russian Academy of Sciences, Branch of Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Pushchino, Moscow Region, Russia*
[2]*Kursk State University, Kursk, Russia*
[3]*Kursk State Medical University, Kursk, Russia*
[4]*National Institute for Health Development, Tallinn, Estonia*
[5]*Kazan Federal University, Kazan, Russia*
[6]*Emanuel Institute of Biochemical Physics, Moscow, Russia*

Correspondence should be addressed to T. V. Kuznetsova; tatiana.kuznetsova@tai.ee

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Procedure of manufacturing *K. pinnata* water extracts containing cecropin P1 (CecP1) from the formerly described transgenic plants is established. It included incubation of leaves at +4°C for 7 days, mechanical homogenization of leaves using water as extraction solvent, and heating at +70°C for inactivating plant enzymes. Yield of CecP1 (after heating and sterilizing filtration) was 0.3% of total protein in the extract. The water extract of *K. pinnata* + CecP1 exhibits favorable effect on healing of wounds infected with *S. aureus* (equal to Cefazolin) and with a combination of *S. aureus* with *P. aeruginosa* (better than Cefazolin). Wild-type *K. pinnata* extract exhibited evident microbicide activity against *S. aureus* with *P. aeruginosa* but it was substantially strengthened in *K. pinnata* + CecP1 extract. *K. pinnata* extracts (both wild-type and transgenic) did not exhibit general toxicity and accelerated wound recovery. Due to immunomodulating activity, wild-type *K. pinnata* extract accelerated granulation of the wound bed and marginal epithelialization even better than *K. pinnata* + CecP1 extract. Immunomodulating and microbicide activity of *K. pinnata* synergizes with microbicide activity of CecP1 accelerating elimination of bacteria.

1. Introduction

Due to a broad use of antibiotics, drug resistance of microbial pathogens became one of the greatest problems of the modern medicine [1]. It is of a particular importance in complicated chronic conditions, for example, in trophic ulcers in patients with diabetes mellitus where long-term antimicrobial therapy is mandatory [2]. Due to a peculiar mechanism of action, antimicrobial peptides (AMP) are considered to be a promising alternative to the traditional antibiotics [3]. Data about velocity of rising resistance to them in microbial pathogens in comparison to the antibiotics is contradictory since practical application of AMP is much narrower than one of the antibiotics [4]. A high side toxicity and low proteolytic stability often figure as a general reason of a poor AMP (e.g., magainins/bombinins and defensins) applicability in pharmaceutics [5].

AMP cecropin P1 (CecP1) was found for the first time in a swine intestine [6]. Then it was reattributed to a swine helminth *Ascaris suis* abundant in the gut of pigs [7]. In contrast to most other known AMP, CecP1 is naturally adapted to act in a medium with a high level proteolytic activity exhibited by duodenal enzymes. Ubiquitous abundance of *A. suis* in pigs provides an evidence that CecP1 does not impair viability of the mammalian host even when accumulated in a high dosage [8]. Therefore application of CecP1 in pharmacy looks

promising. However, for a long time, CecP1 was not available for clinical trials due to absence of an appropriate way for its producing. Solid-phase chemical synthesis is too expensive, liquid-phase synthesis has not been established, and biosynthesis is precluded by a toxicity of CecP1 to potential producers (bacteria, yeast, and micellar fungi). Sophisticated methods of its producing by recombinant strains within fused proteins with decreased toxicity are expensive and difficult for technological implementation [9]. However, there is a broad range of data about microbicide activity of CecP1 towards bacterial and fungal phytopathogens [10] and human/animal pathogens [11]. Virucide and antitumor activity was also attributed to CecP1 [12, 13].

Recently engineering transgenic plants of *Kalanchoe pinnata* expressing CecP1 synthetic gene and accumulating the recombinant AMP in cytoplasm was reported [10]. Bactericide efficiency of pure CecP1 and the wild-type *K. pinnata* extract against model bacterial strains is described [14]. However, data about antimicrobial efficiency of the recombinant CecP1 in vivo and its possible side toxicity are not available. Here we report results of testing medicinal activity of *K. pinnata* extracts containing CecP1 (compared with an extract of a wild-type *K. pinnata*) in a rat model of wounds infected with *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains recently isolated from patients with a purulent infection. Antisuppurative, wound healing, and antimicrobial effect were assessed and used as criteria for comparison of antimicrobial and toxic effects of CecP1 within *K. pinnata* extract in vivo.

K. pinnata seems to be a promising bioreactor for CecP1 production since its extract may be used for epicutaneous application without special purification. *K. pinnata* (Lam.) Pers. (syn. *Bryophyllum pinnatum*; family Crassulaceae) is a popular plant used in traditional medicine in many temperate regions of the world and particularly in South America [15]. There are a number of reports about activity of *K. pinnata* extracts against bacteria [16, 17], *Leishmania* [18], insects [19], and even viruses [20, 21].

Wild-type *K. pinnata* extracts harbor a number of bioactive compounds, for example, bufadienolides [22], flavonols (polyphenols and glycosides of syringe acid) [23], and hemagglutinating lectins [24, 25]. They confer complex immunomodulatory [26, 27], lymphoproliferative [18], antioxidant [28, 29], microbicide [30], and cytotoxic [22, 31, 32] properties on the extracts. Some of these effects look directed oppositely. For instance, Da Silva et al. [18] reported activation of Th1-type response (IL-2 and IFN-γ overproduction) and suppression of Th2-type response (downregulation of IL-4) by a juice of *Kalanchoe brasiliensis* upon a systemic administration to mice, whereas Umbuzeiro-Valent et al. [33] and El Abdellaoui et al. [22] found an anti-inflammatory (antihistamine) effect in the same preparation. Shirobokov et al. [20] described induction of a blast-transformation in lymphocytes of peripheral blood by a lectin from *Kalanchoe blossfeldiana* likely due to concanavalin A whereas Costa et al. [34] described an antiproliferative effect of patuletin acetylrhamnosides from *Kalanchoe brasiliensis* on human lymphocytes. Taken together, these data do not allow a comprehensive prediction of a synergistic or antagonistic effect of antimicrobial effects of CecP1 and *K. pinnata* juice natural ingredients towards bacterial pathogens in vivo (with involvement of innate immune mechanisms of the animal).

We addressed a problem of producing a highly stable *K. pinnata* extract bearing CecP1 and testing its therapeutic activity towards *Staphylococcus aureus* purulent mono infection and its combination with *Pseudomonas aeruginosa* (as an example of typical nosocomial infection). The experiment was carried out in a model of infected planar wounds in rats. Experimental samples of *K. pinnata* extracts bearing CecP1 were compared with a wild-type *K. pinnata* extract and with a commonly used antibiotic Cefazolin (first-generation semisynthetic cephalosporin with a predominant activity towards Gram-positive bacteria) [35]. A subgroup of nontreated rats infected with the same bacterial pathogens was used as a negative control. The therapeutic effect was estimated by wound healing activity (planimetric method) and by microbicide activity (microbiological study of tissue samples in the wound cavity).

2. Methods

2.1. Producing K. pinnata Extracts. *K. pinnata* extracts were prepared from leaves of *K. pinnata* transgenic plants bearing a binary vector for *Agrobacterium tumefaciens* vector with T-element randomly integrated to a plant genome. The vector did not contain a drug resistance marker. The plants selected by a direct immunological testing exhibited a highly stable yield of CecP1 for at least two years as proved by immunoblotting, antimicrobial plate test, and HPLC combined with mass-spectroscopy detection. 5.07 L of extract was produced from 3 kg of recombinant plant leaves using deionized water as an extracting solvent. After sterilizing filtration through nylon membrane with 0.22 μm pores the extract contained 1 mg/mL total protein and 0.7 μg/mL CecP1. The extract of wild-type *K. pinnata* was produced by the same method and adjusted to the same concentration of the total protein. Synthetic CecP1 peptide described previously [14] was used as a standard for quantification of CecP1 in the recombinant *K. pinnata* extracts.

2.2. Animals. This experimental study in vivo was organized according to European Convention about defense of the vertebrates used for experiments or for another scientific aims (Strasbourg, Mar 18, 1986) of ETS N123. 240 adult male Wistar rats weighing 180 ± 20.0 g and aged 3-4 months were totally allocated for the experiment. After quarantine they were placed into individual cages. All animals were contained in equal terms on a standard diet: twelve hours of darkness and 12 hours of light were available. They had a ready access to water and food.

The animals were randomly divided into groups (60 animal each) treated with wild-type *K. pinnata* extract, recombinant *K. pinnata* extract containing CecP1 (*K. pinnata* + CecP1), Cefazolin (positive control), respectively, and a negative control group (mock treatment with a saline). Each group was randomly divided into two subgroups (30 animals each) infected with (1) *S. aureus* and (2) *S. aureus* + *P. aeruginosa*. Each subgroup was divided into three echelons

withdrawn from the experiment at 3rd, 10th, and, 14th day after beginning of curing.

2.3. Bacterial Strains. *Erwinia carotovora* subsp. *carotovora* ATCC 15713 (type strain) was cultivated at plates with LB medium (peptone bacto (Difco) 10 g/L, yeast extract (Difco) 5 g/L, NaCl 10 g/L, and agar 15 g/L) at 25°C.

The bacterial pathogens *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853) isolated from clinical specimens were purchased from type strain collection of Tarasevich Research Institute for Standardization and Control of Medical Biological Preparations. The strains were grown for 18–20 h at slant IPA (meat-peptone nutrient agar) supplemented with 0.1% glucose. The fresh cultures were rinsed out with a sterile saline, thoroughly suspended, adjusted to a concentration ~10^9 CFU per mL by using an optical turbidity standard CCA 42-28-29-85, and used for inoculation of wounds.

2.4. Surgical Manipulations, Treatment, and Planimetry Assay of the Wounds. A purulent infection was modeled in rat using a method described previously [36]. The animals were anesthetized with ether. A square shape 20 × 20 cm skin area at the animal back was thoroughly shaved and treated by a disinfectant (70% ethanol); derma and epidermis were surgically removed. 1 mL of bacterial suspension containing 10^9 CFU/mL *S. aureus* ATCC 25923 or 10^9 CFU/mL *S. aureus* ATCC 25923 and 10^9 CFU/mL *P. aeruginosa* ATCC 27853 was distributed at the surface of the wound. For standardizing the wound healing conditions the wound cavity was closed with a gauze bandage coupled to the skin.

36 h after wounding and infection all animals exhibited clear symptoms of suppuration and inflammation. In this moment the stitches and the bandage were removed, and the wound cavity was thoroughly washed from the pus. The wound area was determined by its lineation at a sterile transparent film. Then the wounds were treated with 3% hydrogen peroxide and subjected to a specific treatment. The described wound treatment was repeated daily for 14 days after beginning of the curing.

The wounds in the control subgroup were treated with 3% hydrogen peroxide and with a sterile saline. Other subgroups instead of the saline were treated with 10% Cefazolin or with undiluted *K. pinnata* extract containing 1 mg/mL total protein (experimental preparation contained 0.7 μg/mL CecP1).

The animals were examined daily and stages of wound healing (inflammation, granulation, and maturation (marginal epithelialization)) were fixed.

The planimetric analysis of the wound recovery percentage was carried out at 3rd, 10th, and 14th day after beginning of curing. After this one echelon (10 animals from each subgroup) was withdrawn from the experiment. The animals were sacrificed with overdose of the ether anesthesia.

Wounds on different days were measured as described formerly [37] by transparent sheet which was scanned at resolution 200 pcs/inch. The image was acquired in a format Adobe Photoshop CS5 Extended. The object was selected and its square was automatically calculated by selecting a menu command "Analysis." An average mean and a standard error (M ± Std. Err.) were calculated. The recovery percentage was evaluated with following formula:

recovery percentage = (wound surface on the day zero of curing − wound surface on day X)/wound surface on the day zero of curing × 100, where X = day of wound surface measurement.

2.5. Bacteriological Analysis of Microbial Load in Wound Cavity Tissue. 0,1–0,5 g tissue (fibrous mass, infiltrate, and underlying derma) was sampled from a sacrificed animal under aseptic conditions, weighed at analytical grade balances (accuracy 0.1 mg), placed in a sterile porcelain mortar, mixed with a sterile saline in a ratio 1 : 10, and homogenized with a sterile pestle for 3 min. The homogenate was diluted 1000 times with a sterile saline (with three consequent steps 1 : 10 using 1 mL samples) and 100 μL aliquot of each dilution was inoculated to Petri dishes with IPA (meat-peptone nutrient agar) supplemented with 0.1% glucose. The inoculated dishes were incubated at 37 ± 1°C for 20 h and then 1 day more at a room temperature. The colonies were counted and number of CFU recalculated per 1 g tissue. The count was suggested to be valid if number of colonies was between 30 and 300.

2.6. Statistical Analysis. Statistical analysis of research results was performed by Microsoft Excel 2007 and program "Statistics" 8.0 StatSoft. The averages of quantitative indexes and standard errors of mean were calculated. Authenticity of distinctions of averages between the series of comparison and other series was estimated by the Mann–Whitney U test ($p < 0.05$).

3. Results and Discussion

3.1. Producing K. pinnata Extracts. Three independent *K. pinnata* transgenic lines (~1-year-old plants height 2–2,5 m) and a wild-type parental *K. pinnata* of the same age cultivated in a green-house under a constant light/darkness and temperature regime were used for producing the extracts. Middle tier leaves were used for preliminary testing of CecP1 yield. 200–300 mg specimens were sampled, placed in Eppendorf tubes, and weighed and mixed with NEB buffer (10% glycerol, 4 mM EDTA, 150 mM NaCl, 100 mM NH_4Cl, 10 mM Tris-HCl, pH 7.5, 0.2 mg/mL leupeptin, and 0.2 mg/mL trypsin inhibitor) in ratio 20 μL per 100 mg leaf tissue. The leaf specimens were homogenized with a glass stick in the Eppendorf tubes and total protein was measured by Bradford method (Sigma-Aldrich B6916 ready-to-use kit was used following instructions of manufacturer). The extracts were adjusted to the total protein concentration 1 mg/mL with NEB buffer and used for determination of antimicrobial activity by diffusion in agar against *E. carotovora* subsp. *carotovora*. 10^8 CFU/plate were distributed at 90 mm Petri dishes with LB medium using top agarose method. Wells with diameter 5 mm were pierced in the agar and 100 μL aliquots of the plant extracts were dripped to each well. A synthetic CecP1 (1 μg/mL) in amount 100 μL per well was used as a standard. Results of the testing are shown at Figure 1.

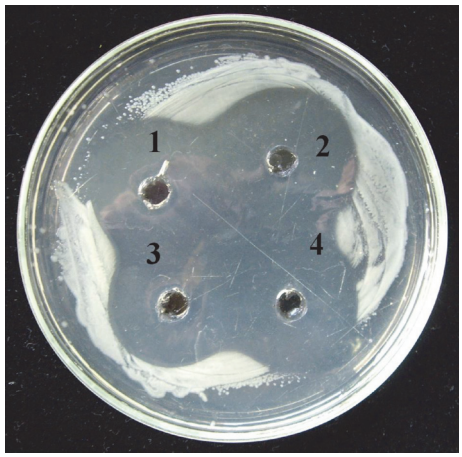

Figure 1: Agar diffusion test for determination of antibacterial activity towards *E. carotovora* in extracts of *K. pinnata* transgenic lines. (1) *K. pinnata*, line #1, halo 29 mm; (2) synthetic CecP1 (1 μg/mL), halo 34 mm; (3) *K. pinnata*, line #2, halo 26 mm; (4) *K. pinnata* line #3, halo 28 mm.

Figure 2: Immunoblotting analysis of *K. pinnata* extracts. (K+) synthetic CecP1, Mr = 3.4 kDa (30 ng); (1) *K. pinnata* transgenic line 1 after the cold stress; (2) *K. pinnata* transgenic line 2 after the cold stress; (3) *K. pinnata* transgenic line 2 before the cold stress; (4) *K. pinnata* transgenic line 2 before the cold stress.

Data of Figure 1 allow estimating yield of CecP1 contents in the leaves of *K. pinnata* transgenic lines ~0.3–0.5 of the total soluble protein. Transgenic line (1) was chosen for preparative manufacturing of the extract. An extract of the wild-type parental *K. pinnata* was prepared in parallel. 3.0 kg cut leaves were incubated at +4°C for 7 days in darkness. The leaves were mixed with the ice-cold water (1.0 L water per 1 kg leaves) and homogenized with a hand electric blender. The homogenate was dark-green. It was clarified by centrifugation at 6000g for 30 min. The green pallet was discarded. The straw-yellow extract was placed in 0.5 L glass flasks and incubated at +70°C in a water bath for inactivating enzymes. The extract was clarified by centrifugation at 6000g for 30 min and sterilized by filtration through a 0.22 μm filtering cartridge (Hydrophilic PNN Membrane Filter, Hangzhou Kosma Membrane Technology Co., PRC) under aseptic conditions. The extracts were stored at +4°C in culture flasks of nominal volume 0.5 L hermetically closed with rubber plugs and aluminum caps until used for biological testing.

Presence of CecP1 in the extracts was tested using immunoblotting as described formerly [10] (Figure 2). Briefly, 10.0 μL of extract and 30.0 μL of the control synthetic CecP1 (1 μg/mL) were pelleted with 10% trichloroacetic acid, denatured and separated in 15% polyacrylamide gel, blotted to nitrocellulose membrane, and stained with rabbit polyclonal antibodies.

Table 1: Reproducibility of manufacturing *K. pinnata* extracts containing CecP1.

Period of operation	*K. pinnata* leaves, kg	Total yield of CecP1, mg
Mar 15, 2016–Mar 29, 2016	3.04	3.63
Mar 30, 2016–Apr 5, 2016	3.02	3.45
Apr 6, 2016–Apr 20, 2016	3.04	3.34

The immunoblotting demonstrated upregulation of CecP1 expression in *K. pinnata* leaves under cold stress conditions. Further antimicrobial activity of the extracts was confirmed in the agar diffusion test at *E. carotovora* model as described above. Overall report about manufacturing *K. pinnata* extracts containing CecP1 is shown in Table 1.

3.2. Testing Healing of the Wounds Infected with S. aureus. *S. aureus* is the most common causative agent of the nosocomial purulent infections. It is prone to raising multiple drug resistance. As found in 3-month epidemiological study to determine the prevalence and antibiotic resistance of *S. aureus* nosocomial infections in 52 centers throughout Italy in 2012, the prevalence of *S. aureus* among all nosocomial pathogens isolated in that period was 11.6% (n = 2541), whilst the prevalence of methicillin-resistant *S. aureus* (MRSA) among the *S. aureus* was 35.8% (n = 910) [38]. However, the collection *S. aureus* strain ATCC 25923 exhibited a high susceptibility to Cefazolin in vitro and was chosen as a model object for in vivo studies of efficiency of *K. pinnata* extracts which was compared with this antibiotic. Data at Figure 3 demonstrate that the wound recovery percentage is significantly higher in *K. pinnata* + CecP1 subgroup than in the control subgroup (mock treatment): the difference is characterized with the third threshold of validity ($p < 0.001$) at 10th and 14th days. The final wound healing activity of *K. pinnata* + CecP1 did not differ from one of Cefazolin groups: $p > 0.05$ at days 10 and 14 when two subgroups were compared. Wild-type *K. pinnata* extract exhibited a certain efficiency (difference with the negative control was characterized with the first threshold of significance at 10th and 14th days after beginning of curing). However, the wound healing activity of *K. pinnata* + CecP1 extract was significantly higher than in the wild-type *K. pinnata* extract ($p < 0.01$ at 10th day and $p < 0.05$ at 14th day). This gives an evidence of a clear CecP1 contribution to the own *K. pinnata* extract therapeutic efficiency.

Positive results were obtained when timing of wound recovery phases was compared between the animal subgroups. Data of Figure 4 demonstrate that the inflammation phase ends about 2 times faster in all three experimental subgroups than in the control subgroup without specific treatment. However, start of granulation phase in *K. pinnata* subgroup (but not in *K. pinnata* + CecP1) was found about 1 day earlier than in Cefazolin subgroup (difference between the subgroups first threshold is characterized with the first threshold of significance, $p < 0.05$). Marginal epithelialization (maturation phase) was found almost 2 days faster in *K. pinnata* and *K. pinnata* + CecP1 subgroups than in Cefazolin

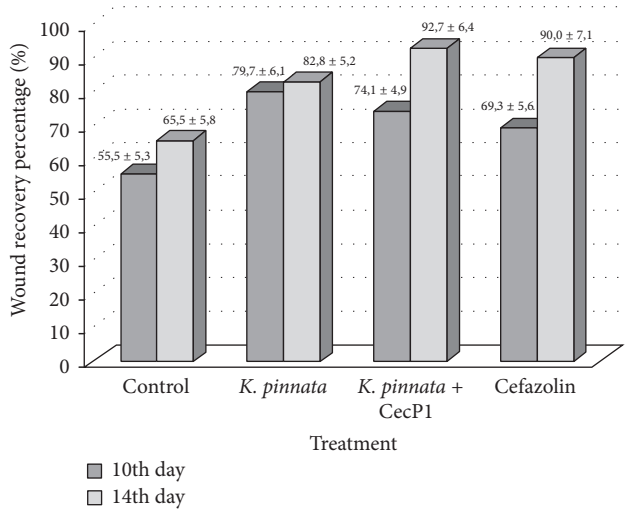

Figure 3: Testing healing of the wounds infected with *S. aureus*. Wound recovery percentage at 10th and 14th days after beginning of curing (M ± Std. Err.) is shown.

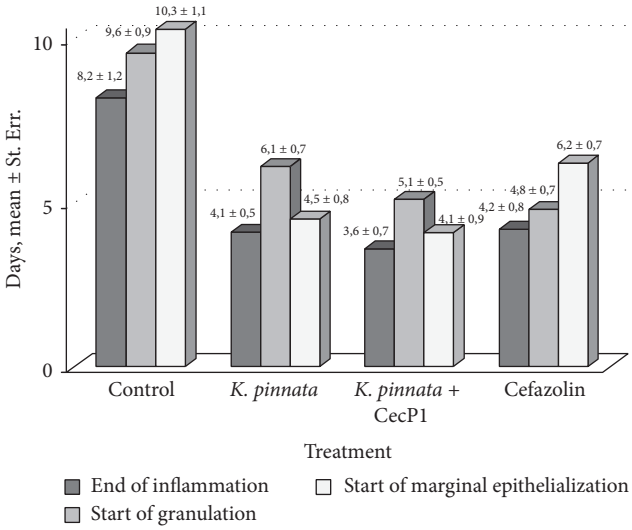

Figure 4: Testing healing of the wounds infected with *S. aureus*. Timing of wound recovery phases after beginning of curing (M ± Std. Err.) is shown.

subgroup ($p < 0.01$). Taken together these data prove a favorable effect of *K. pinnata* extract components for healing of the infected wounds which cannot be substituted with antibiotic and strengthening the antimicrobial activity.

3.3. Testing Healing of Wound Infected with a Combination of S. aureus and P. aeruginosa. Mixed purulent infections usually are heavier and difficult for curing than mono infections. Men'shikov et al. [39] reported that complex infection of *S. aureus* and *P. aeruginosa* is associated with the most unfavorable prognosis in patients with burns. In contrast to *S. aureus, P. aeruginosa* is usually resistant to Cefazolin [35]; therefore outcome of treatment of a mixed infection of *S. aureus* and *P. aeruginosa* is poorly predictable.

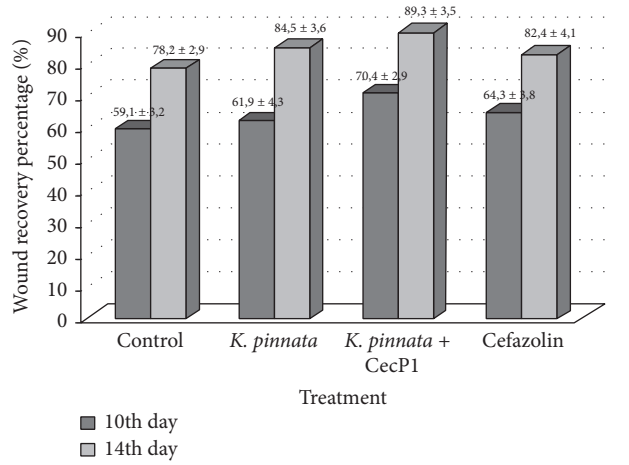

Figure 5: Testing healing of the wounds infected with combination of *S. aureus* and *P. aeruginosa*. Wound recovery percentage at 10th and 14th days after beginning of curing (M ± Std. Err.) is shown.

Experimental study of wounds infected with equal doses of *S. aureus* and *P. aeruginosa* demonstrated that their recovery in the control subgroups (without treatment) does not differ significantly from *S. aureus* mono infection and even goes somewhat faster (Figure 5). However, treatment of *S. aureus* + *P. aeruginosa* with Cefazolin is significantly less efficient than *S. aureus* mono infection ($p < 0.01$). Comparison of the control and Cefazolin subgroups within the mixed infection group demonstrated no significant difference ($p > 0.05$). Therefore, Cefazolin can be acknowledged to be inefficient for treatment of the mixed purulent infection.

Using the wild-type *K. pinnata* extract gives somewhat better results than Cefazolin for curing the mixed infection. Difference between *K. pinnata* and the control subgroups is characterized with $p < 0.05$ for 14th day ($p > 0.05$ for 10th day). At this background, efficiency of *K. pinnata* + CecP1 extract with the control looks highly promising: $p < 0.01$ for 10th day and $p < 0.001$ for 14th day after beginning of curing.

When timing of the wound healing was compared in the mixed infection group of animals, Cefazolin was found to be much more efficient than for the wound recovery percentage (Figure 6). Difference between Cefazolin and the control subgroups was characterized with $p < 0.001$ for all three parameters (end of inflammation, start of granulation, and start of marginal epithelialization).

On the other hand, comparison of *K. pinnata* and *P. pinnata* + CecP1 subgroups demonstrated no difference in timing of end of inflammation ($p > 0.05$), whereas granulation and marginal epithelialization were found to start earlier in *K. pinnata* subgroup. This ambiguous result cannot be explained by effect of CecP1 only. Apparently, immunomodulating effect of own *K. pinnata* biologically active compounds (flavonoids or lectins) for granulation and epithelialization was more pronounced than antimicrobial; effect of CecP1 and production of these compounds was higher in the control *K. pinnata* plants than on the transgenic plants expressing CecP1. In turn, this hypothesis is not in a good agreement with an evident impact of Cefazolin on the

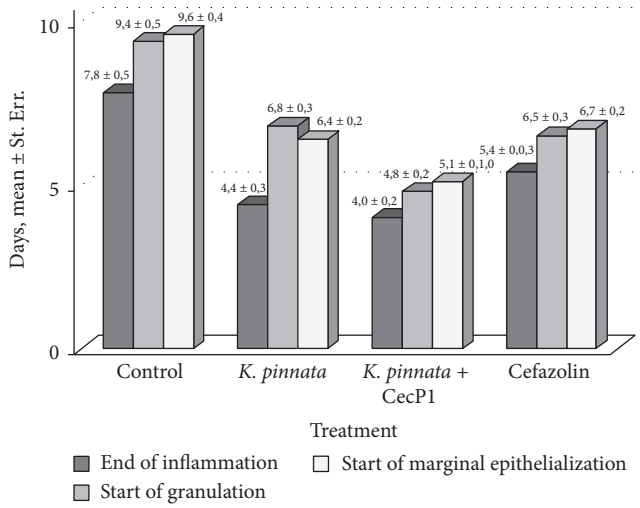

Figure 6: Testing healing of the wounds infected with combination of *S. aureus* and *P. aeruginosa*. Timing of wound recovery phases after beginning of curing (M ± Std. Err.) is shown.

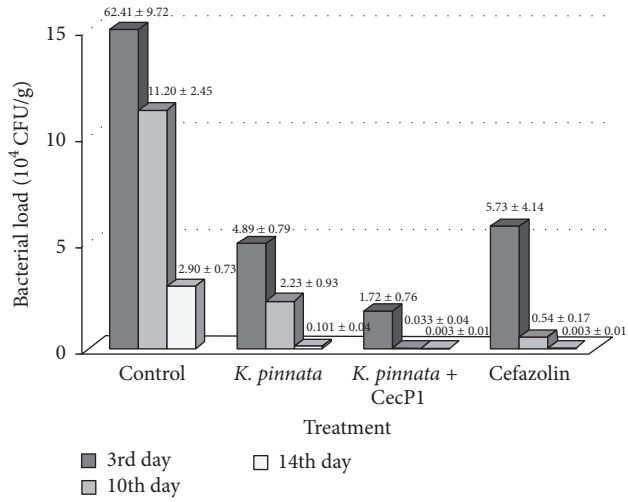

Figure 7: Testing microbicide activity of *K. pinnata* extracts against *S. aureus* mono infection in vivo.

timing of the wound healing phases but not on the wound recovery rate.

3.4. Testing Microbicide Activity of K. pinnata Extracts In Vivo. Planimetrical studies of wound healing provide key information about therapeutically efficiency of the antimicrobial means. However, they must be supplemented with direct data about survival of the microbial agents in the wound bed. These data are important for optimization of the treatment scheme.

Data at Figure 7 demonstrate collapsing bacterial load of *S. aureus* along with the wound healing. This phenomenon is evident even in the control subgroup (without treatment); however it is much greater in the experimental subgroups. On the other hand, residual bacteria are found in all subgroups even at 14th day of the experiment. All four subgroups exhibit statistically significant differences between each other in the bacterial load at 3rd and 10th day of the experiment ($p < 0.01$) following in the order: control > Cefazolin > *K. pinnata* > *K. pinnata* + CecP1. In contrast, no valid differences between the subgroups in the bacterial load are found at 14th days after beginning of curing. This observation proves existence of refugees in vivo where the pathogen can take a cover even against drugs with high microbicide potency. This effect may contribute to rising drug resistance in bacteria by providing time necessary to the pathogen for adaptation to the therapy.

Picture of the microbicide activity of *K. pinnata* extracts against combination of *S. aureus* and *P. aeruginosa* roughly repeat the picture of the microbicide activity against *S. aureus* mono infection (Figure 8). There is a statistical significance of the bacterial load between all groups ($p < 0.01$) at 10th (not 3rd) day of experiment. Microbicide effect of Cefazolin against the mixed infection was weak but not zero. Extract of the wild-type *K. pinnata* was less efficient than *K. pinnata* + CecP1 ($p < 0.05$) at 10th and 14th days of experiment. However, residual bacterial load at 14th day of the experiment was much higher in the mixed infection than in *S. aureus* mono infection.

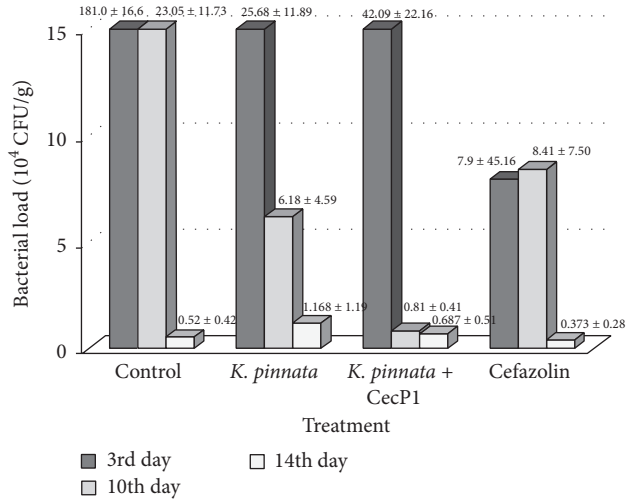

Figure 8: Testing microbicide activity of *K. pinnata* extracts against combination of *S. aureus* and *P. aeruginosa* in vivo.

4. Conclusions

Transgenic line of *K. pinnata* with the highest CecP1 was selected 1 year after its obtaining. A cold stress induction of CecP1 synthesis in cut *K. pinnata* leaves incubated at +4°C for 7 days allowed increased yield of CecP1 1.5–2 times. A simple and efficient procedure of water extraction CecP1 from *K. pinnata* leaves was established. In contrast to the commonly used ethanol extraction [21, 22], it allowed preservation of hemagglutinating lectins described in *Kalanchoe* [25] and exhibiting lymphoproliferative activity [20]. However, these proteins may be lost due to denaturation in the course of the extract heating at +70°C. Heating the extract was mandatory

for inactivation of proteases which otherwise degraded CecP1 under the storage for 7 days at +4°C.

Following our data, the extracts of *K. pinnata* (wild and transgenic) did not exhibit any cytotoxic activity towards the wound bed or general toxicity at animals in the applied concentration (1 mL of the extract per dose, total protein concentration 1 mg/mL) although such toxicity was previously ascribed to both *K. pinnata* extracts [19] and synthetic CecP1 [3]. Water extract of wild-type *K. pinnata* exhibited both microbicide and wound healing activity, although its own microbicide activity was lower than in Cefazolin. In contrast, *K. pinnata* + CecP1 extract exhibited the same microbicide activity against *S. aureus* mono infection as Cefazolin. Activity of *K. pinnata* + CecP1 extract against combination of *S. aureus* and *P. aeruginosa* was much better than in Cefazolin.

Noteworthily, none of the examined preparations did provide complete elimination of bacteria in the wound bed even on the 14th day of the experiment. Residual bacterial load at this period was close for all three microbicides. This effect, despite a low number of the residual alive bacteria, may contribute to rising drug resistance in pathogens and should be controlled.

Taken together, the extract of *K. pinnata* transgenic plants containing 0.7 μg/mL CecP1 obtained following the described method may be suggested to be promising for external use. First of all, it should be tested as a candidate drug for treatment of trophic ulceration in patients with diabetes mellitus.

Competing Interests

The authors declare no conflict of interests.

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Research Article

Serum Cytokine Levels and Their Relation to Clinical Features in Patients with Autoimmune Liver Diseases

Dilyara Akberova,[1] Andrei P. Kiassov,[2] and Diana Abdulganieva[1]

[1]*Kazan State Medical University, Kazan, Russia*
[2]*Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Russia*

Correspondence should be addressed to Diana Abdulganieva; diana_s@mail.ru

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Serum cytokine levels were explored in a combined group of patients with autoimmune liver diseases (AILDs) and separately in patients with autoimmune hepatitis (AIH) and overlap syndrome. Overall, 60 patients with AILD, among them 32 patients with AIH and 28 patients with overlap syndrome, were included in the cross-sectional study. Serum cytokine levels were measured at baseline and compared to those of 21 healthy controls. Patients with AILD had significantly higher levels of IL-6 (0.70 (range 0.17–99.86) in patients with AILD compared to 0.40 (range 0.14–2.65) in controls, $p < 0.01$), IL-8 (1.66 (0.45–34.58) versus 0.53 (0.35–2.38), resp., $p < 0.01$), and TNF-α (2.61 (0.23–120.88) versus 1.65 (0.21–7.54), resp., $p < 0.01$). Adjusted logistic regression analysis revealed a pronounced relation of IL-8 and AILD, 48.36 (3.63–643.60), as well as AIH, 18.54 (1.08–318.54), and overlap syndrome, 23.85 (2.37–240.23), while the associations between the level of other cytokines and AILD were assessed as nonsignificant. In the language of absolute numbers, the increase of IL-8 serum level by 1 pg/mL had increased the chance for a patient to find himself in a group of AILD by 48.36 times. Also, high IL-8 serum levels were strongly related to clinical parameters.

1. Introduction

Autoimmune liver diseases (AILDs) include a broad range of autoimmune disorders affecting the liver and biliary system, with primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), and autoimmune hepatitis (AIH) being the classic types of AILD. While AIH targets the hepatocytes and is mainly associated with hepatocellular injury, the targets of the autoimmune attack in PBC and PSC are the biliary epithelial cells, which leads to a predominance of cholestatic features [1, 2]. AIH and PBC are the most frequent AILDs with an incidence around 1-2 per 100000 population per year for each disease [3] and a prevalence of 2–40 cases per 100000 population in different parts of the world, with the highest incidence and prevalence found in the United States and northern Europe [4]. Conditions exhibiting features of 2 different AILDs are commonly designated as overlap syndromes. They are not uncommon, with a combination of PBC and AIH occurring in 2–19% of all patients with AIH, and usually show a progressive course towards cirrhosis and liver failure without adequate treatment [2].

Although the exact mechanisms of the AILDs are unclear [5], broadly similar pathogenic pathways of injury have been postulated for AIH, PBC, and PSC, and these comprise a combined influence of environmental triggers, genetic predisposition, and failure of immune tolerance mechanisms, which, in turn, collaborate to induce an antibody- and *T* cell-mediated immune attack against liver-specific targets, leading to a progressive necroinflammatory and fibrotic process in the liver [2]. Persistent liver injury, associated with chronic AILD, leads to persistent inflammation, cell proliferation, and the deposition of extracellular matrix proteins by hepatic stellate cells and portal myofibroblasts. Liver cirrhosis, and the resultant loss of normal liver function, inevitably ensues [1].

Although it is widely recognized that AILDs are mainly *T* cell mediated diseases [6], it is also shown that cytokines, such as interleukins (IL), play one of the key roles in the pathogenesis of the disease and in liver innate lymphoid cells and natural killer *T* cells activation [7]. The exact role of the certain cytokines and their utility as biomarkers for

predicting disease outcomes, or as a diagnostic tool, as well as their potential use as a treatment target are yet to be explored, although monoclonal antibodies, for example, antibodies directed against IL-12/IL-23, are even now considered to be one of the potential treatment options for AILD [8].

The aim of the present study was to reveal the differences in the serum cytokine levels between patients with AIH and overlap syndrome, and between the combined group of patients with AILD and healthy controls, and to explore the associations between the cytokine levels and the clinical features of the disease in patients with AILD.

2. Patients and Methods

2.1. Patients. Overall, 60 patients with AILD (females, 54 (90%), mean age 48.2 ± 15.1 years), among them 32 patients with AIH (females, 28 (87.5%), mean age 43.2 ± 15.5 years) and 28 patients with overlap syndrome (females, 26 (92.8%), mean age 53.9 ± 12.6 years), were consecutively included in the cross-sectional noninterventional cohort study. Inclusion was performed in a single center (Department of Gastroenterology, Republican Clinical Hospital, Kazan, Russia) and took place between April 2015 and May 2016. The inclusion criteria were age of 18 years and above, diagnosis of AILD established according to the actual recommendations: PBC-EASL (2009) [9], AIH-AASLD (2010) [10], AIH-PBC overlap syndrome, IAIHG position statement (2011) [11], and patients' ability and willingness to provide the informed consent for the study. Patients were not included if they had any of the following: viral hepatitis B or C (serum markers or history), Wilson's disease, nonalcoholic steatohepatitis, alcoholic liver disease, drug-induced hepatitis, pregnancy, and any severe conditions or other conditions that would in the opinion of the study physician preclude participation in the study. Every patient, who was observed at the department with AILD and was satisfying the requirements of inclusion criteria, was invited to participate in the study.

As a control group, 21 healthy age- and sex-matched volunteers (females, 18 (85.7%), mean age 47.8 ± 10.6 years) were examined. All of them had an inconspicuous medical history without diagnosed active gastrointestinal diseases, infections, and liver diseases, no history of major intestinal surgery during the past 12 months or alcohol abuse, and no history of antibiotics or anti-inflammatory drugs intake within the last month. The aforementioned exclusion criteria were also applied to the controls. Most of the controls in this study were healthcare workers, who volunteered to be included in the control group.

2.2. Study Design. All patients underwent complete clinical and laboratory examination. Demographic data (such as disease duration, age, and gender), clinical manifestations, (such as presence and severity of ascites, portal hypotension, hepatic encephalopathy, and extrahepatic symptoms), and vital parameters of the patients were assessed on the day of inclusion. Ongoing and previously taken treatments and concurrent medications were recorded. Laboratory examinations included common blood count and biochemical blood tests, as well as immunological tests (antinuclear, antimitochondrial, antismooth muscle titers, levels of circulating immune complexes and gamma-globulins), according to standard clinical protocols.

2.3. Cytokine Analysis. Serum samples were collected at baseline from each enrolled subject. Serum cytokine levels were analyzed using ⟨⟨Bio-Plex 200⟩⟩ (Bio-Rad, Hercules, CA, USA) Multiplex Immunoassays following the manufacturer's instructions. Serum levels of seven cytokines were measured: IL-2, IL-4, IL-6, IL-8, IL-10, interferon-gamma (IFN-γ), and tumour necrosis factor-alpha (TNF-α). Median fluorescence intensities were measured using Luminex 200 analyzers (Luminex, Austin, TX, USA). Collected data were analyzed using with MasterPlex CT control software and MasterPlex QT analysis software (MiraBio, San Bruno, CA, USA). Standard curves for each analyte were generated using standards provided by manufacturer.

2.4. Statistical Analysis. Statistical processing and analysis of obtained data were performed by IBM SPSS Statistics version 23 (Chicago, IL, USA), Statistica version 12.5 (Statsoft), and Microsoft Excel 2013. The descriptive data are presented as median and range (for cytokine levels), or M ± SD, where M is the mean and SD is the standard deviation (for demographic and clinical data). The groups were compared using Mann–Whitney *U* test and Kruskal-Wallis test. Dichotomous parameters were compared between groups using the chi-square test. Obtained differences were considered statistically significant if $p < 0.05$. The relation between the chance for disease and serum level of cytokines was assessed using logistic regression analysis. Variables that were significant on univariate analysis were further analyzed using multivariate analysis to adjust for confounders, such as the level of other cytokines, age, and gender.

2.5. Ethics Committee Approval. The study protocol was approved by the local ethics committee of the Kazan State Medical University (protocol number 10, 23 December 2014). All patients signed the informed consent form before being included in the study.

3. Results

3.1. Clinical Characteristics of Patients. The clinical and laboratory data for the patients at baseline are shown in Table 1. There were some differences observed between the groups. Overall, patients with overlap syndrome, as compared to patients with AIH, had shorter duration of symptoms (4.7 ± 4.5 versus 6.9 ± 6.3 years, resp.) and higher levels of alanine aminotransferase (ALT) (147.8 ± 183.6 versus 68.8 ± 72.8 U/L) and alkaline phosphatase (337.8 ± 176.9 versus 107.7 ± 71.4 U/L), as well as higher erythrocyte sedimentation rates (ESR) (33.12 ± 13.3 versus 17.3 ± 12.1 mm/h), and took glucocorticoids less frequently (15 (53.6%) versus 25 (78.1%), resp.), although mean doses of corticosteroids were overall comparable (17.8 ± 15.5 versus 26.1 ± 6.2 mg/day, resp.).

Table 1: Patients' characteristics.

	AILD ($n = 60$)	AIH ($n = 32$)	Overlap syndrome ($n = 28$)	p
Age, years (M ± SD)	48.2 ± 15.1	43.2 ± 15.5	53.9 ± 12.6	$p > 0.05$
Gender, females (%)	54 (90%)	28 (87.5%)	26 (92.8%)	$p > 0.05$
Symptom duration, years (M ± SD)	5.9 ± 5.6	6.9 ± 6.3	4.7 ± 4.5	**p = 0.02**
Jaundice (%)	36 (60%)	17 (53.1%)	19 (67.9%)	$p > 0.05$
Abdominal discomfort (%)	34 (56.7%)	16 (50%)	18 (64.3%)	$p > 0.05$
Extrahepatic signs (%)	41 (68.3%)	23 (71.9%)	17 (60.7%)	$p > 0.05$
Cirrhosis (%)	43 (71.7%)	23 (71.9%)	20 (71.4%)	$p > 0.05$
Hemoglobin, g/L (M ± SD)	120.9 ± 15.1	118.1 ± 20.2	124.2 ± 18.3	$p > 0.05$
ESR, mm/h (M ± SD)	24.7 ± 14.9	17.3 ± 12.1	33.12 ± 13.3	**p = 0.000**
ALT, U/L (M ± SD)	107.6 ± 143.1	68.8 ± 72.8	147.8 ± 183.6	**p = 0.047**
AST, U/L (M ± SD)	84.4 ± 68.0	72.9 ± 68.4	96.7 ± 66.6	$p > 0.05$
Alkaline phosphatase, U/L (M ± SD)	220.4 ± 68.0	107.7 ± 71.4	337.8 ± 176.9	**p = 0.000**
GGT, U/L (M ± SD)	258.9 ± 296.1	134.3 ± 135.8	389.1 ± 359.9	$p > 0.05$
Total bilirubin, μmol/L (M ± SD)	58.9 ± 100.5	59.6 ± 91.47	58.2 ± 111.0	$p > 0.05$
Total IgG, mg/mL (M ± SD)	18.2 ± 5.3	18.6 ± 5.6	17.8 ± 4.8	$p > 0.05$
Circulating immune complexes, U/L (M ± SD)	241.1 ± 165.3	205.2 ± 133.6	283.5 ± 190.8	$p > 0.05$
Albumin, g/L (M ± SD)	48.3 ± 5.3	47.9 ± 5.9	48.9 ± 4.3	$p > 0.05$
Gamma-globulin, g/L (M ± SD)	24.9 ± 5.7	26.1 ± 6.2	23.2 ± 4.5	$p > 0.05$
UDCA intake (%)	52 (86.7%)	25 (78.1%)	27 (96.4%)	**p = 0.037**
GC intake (%)	20 (33.3%)	25 (78.1%)	15 (53.6%)	**p = 0.044**
GC dose, mg/day (M ± SD)	17.0 ± 11.8	26.1 ± 6.2	17.8 ± 15.5	$p > 0.05$
AZA intake (%)	6 (10%)	5 (15.6%)	1 (3.6%)	$p > 0.05$

AILD: autoimmune liver disease; AIH: autoimmune hepatitis; ESR: erythrocyte sedimentation rate; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamyl transpeptidase; UDCA: ursodeoxycholic acid; GC: glucocorticoids; AZA: azathioprine.

3.2. Comparison of Serum Levels of Cytokines between the Patients with AILD and Controls. Circulating serum cytokine levels were examined in all AILD patients and were compared to the following levels of healthy controls. Overall, patients with both AIH and overlap syndrome, as well as the whole combined group of AILD, had significantly higher levels (as compared to controls) of IL-6 (0.70 (range 0.17–99.86) in patients with AILD compared to 0.40 (range 0.14–2.65) in controls, $p < 0.01$), IL-8 (1.66 (0.45–34.58) versus 0.53 (0.35–2.38), resp., $p < 0.01$), and TNF-α (2.61 (0.23–120.88) versus 1.65 (0.21–7.54), resp., $p < 0.01$). Distributions of IL-6, IL-8, and TNF-α levels are shown in Figure 1. In the group of patients with overlap syndrome, as well as in a general group of AILD, but not in patients with AIH, levels of IL-2 were significantly lower compared to controls (2.07 (range 0.11–111.87) in patients with AILD compared to 2.76 (range 0.45–4.10); $p < 0.05$). There were no differences detected in serum levels of other cytokines compared to control and in levels of all examined cytokines between the groups of AIH and overlap syndrome. Full data are shown in Table 2.

Revealed statistically significant associations were further analyzed by a regression analysis. Unadjusted logistic regression confirmed that the serum levels of IL-6, IL-8, and TNF-α were significantly higher in AILD patients compared to controls. This association was especially pronounced for IL-8 (odds ratio (OR) with 95% confidence interval (CI) – 57.54 (6.15–538.08)). Associations between IL-2 level and the disease were no longer significant. In subgroup analysis, OR for IL-8 were still high and statistically significant: 30.39 (3.15–293.12) for AIH and 22.12 (3.07–159.55) for overlap syndrome versus healthy controls. The combined adjusted regression model, which included all 4 cytokines and was adjusted for age and gender, also showed a pronounced marked relation of IL-8 and AILD, 48.36 (3.63–643.60), while the associations between the level of other cytokines and AILD were assessed as nonsignificant. In subgroup analysis, OR for IL-8 were still high and statistically significant after adjustment: 18.54 (1.08–318.54) for AIH and 23.85 (2.37–240.23) for overlap syndrome versus healthy controls. Full data are shown in Table 3.

3.3. Cytokine Levels and Their Association with Clinical and Laboratory Parameters. Parameters of liver damage, such as levels of liver enzymes, albumin, and bilirubin levels, and parameters of active ongoing inflammation, such as ESR and level of gamma-globulins, were explored for their association

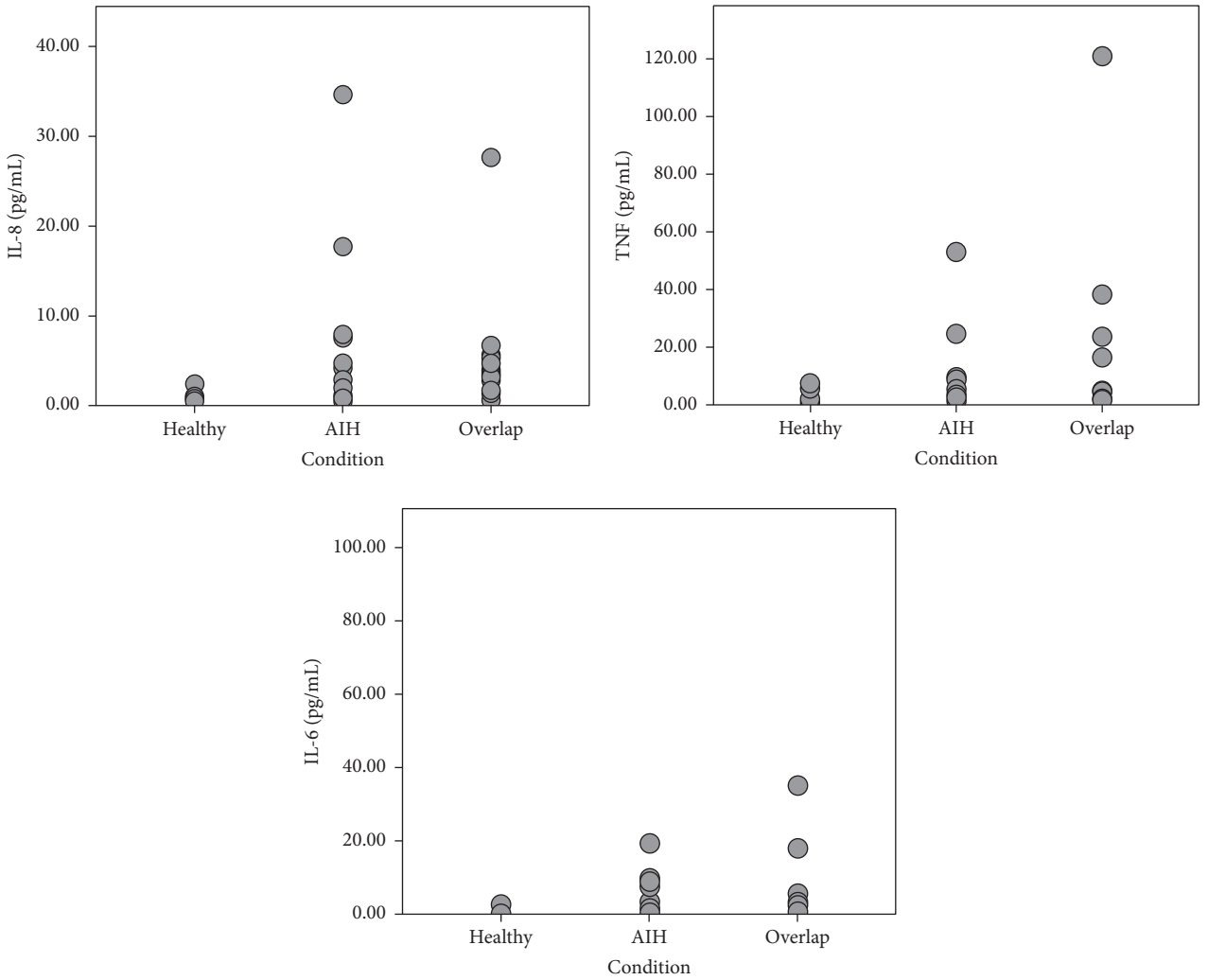

Figure 1: Circulating serum interleukin-8, tumour necrosis factor-α (TNF-α), and interleukin-6 concentrations in patients with autoimmune hepatitis (AIH) and overlap syndrome and in healthy controls. Differences between the AIH and control groups and between overlap and control groups are significant for all cases, $p < 0.01$.

Table 2: Serum circulating levels of cytokines in patients with AILD and in healthy controls. Numbers are presented as median (range).

	IL-2, pg/mL	IL-4, pg/mL	IL-6, pg/mL	IL-8, pg/mL	IL-10, pg/mL	IFN-γ, IU/mL	TNF-α, pg/mL
AILD ($n = 60$)	2.07 (0.11–111.87)[a]	0.18 (0.11–1.25)	0.70 (0.17–99.86)[b]	1.66 (0.45–34.58)[b]	0.57 (0.11–951.96)	4.77 (0.59–493.96)	2.61 (0.23–120.88)[b]
AIH ($n = 32$)	2.33 (0.19–41.33)	0.18 (0.11–0.34)	1.14 (0.20–19.4)[b]	1.50 (0.45–34.58)[a]	0.62 (0.22–29.94)	4.60 (0.59–133.32)	2.38 (0.56–52.96)[a]
Overlap syndrome ($n = 28$)	1.97 (0.11–111.87)[a]	0.18 (0.12–1.25)	0.60 (0.17–99.86)[a]	2.15 (0.60–27.60)[b]	0.52 (0.11–951.96)	5.12 (1.54–493.96)	2.66 (0.23–120.88)[b]
Control ($n = 21$)	2.76 (0.45–4.10)	0.17 (0.11–0.53)	0.40 (0.14–2.65)	0.53 (0.35–2.38)	0.42 (0.13–51.67)	5.65 (0.59–26.22)	1.65 (0.21–7.54)

AILD: autoimmune liver disease; AIH: autoimmune hepatitis; IL: interleukin; IFN-γ: interferon-gamma; TNF-α: tumour necrosis factor-alpha.
[a] $p < 0.05$ compared with healthy controls group (Mann–Whitney U test);
[b] $p < 0.01$ compared with healthy controls group (Mann–Whitney U test).

Table 3: Association (represented as odds ratios and 95% confidence intervals from the logistic regression analysis) between serum levels of cytokines (IL-2, IL-6, IL-8, and IL-10) and autoimmune liver diseases.

Parameter	Unadjusted model (all parameters analyzed separately)	Adjusted* combined model
	AILD	
IL-2	1.01 (0.95–1.08)	0.58 (0.32–1.03)
IL-6	3.86 (1.04–14.41)	1.56 (0.75–3.25)
IL-8	57.54 (6.15–538.08)	*48.36 (3.63–643.60)*
TNF-α	1.55 (1.06–2.36)	1.37 (0.93–2.00)
Age	—	0.99 (0.94–1.05)
Gender (male)	—	3.13 (0.28–35.58)
	AIH	
IL-2	1.03 (0.91–1.17)	0.65 (0.31–1.37)
IL-6	5.04 (1.25–20.32)	1.90 (0.70–5.13)
IL-8	30.39 (3.15–293.12)	*18.54 (1.08–318.54)*
TNF-α	1.41 (0.99–2.01)	1.35 (0.91–2.02)
Age	—	0.99 (0.92–1.05)
Gender (male)	—	1.59 (0.10–24.98)
	Overlap syndrome	
IL-2	1.02 (0.96–1.08)	0.53 (0.26–1.08)
IL-6	2.17 (0.81–5.59)	1.68 (0.53–5.30)
IL-8	22.12 (3.07–159.55)	*23.85 (2.37–240.23)*
TNF-α	1.35 (0.88–2.08)	1.17 (0.58–2.38)
Age	—	1.02 (0.93–1.12)
Gender (male)	—	7.47 (0.29–192.75)

*The model adjusted for the levels of IL-2, IL-6, IL-8, and TNF-α, age, and male gender. AILD: autoimmune liver disease; AIH: autoimmune hepatitis; IL: interleukin; IFN-γ: interferon-gamma; TNF-α: tumour necrosis factor-alpha.

with circulating serum cytokine levels in whole group of AILD patients and in subgroups (AIH and overlap syndrome). The majority of correlations were observed for the level of IL-8, which has positively correlated with the level of ESR ($r = 0.41$; $p = 0.002$), AST ($r = 0.32$; $p = 0.018$), alkaline phosphatase ($r = 0.45$; $p = 0.001$), and total bilirubin ($r = 0.42$; $p = 0.001$) and negatively correlated with serum albumin level ($r = -0.33$; $p = 0.026$). Table 4 gives all revealed correlations between serum cytokine levels and disease activity. No correlations were revealed for other laboratory or clinical parameters or serum cytokine levels.

4. Discussion

To our knowledge, this is the first study that compared the levels of a wide range of pro- and anti-inflammatory cytokines in patients with AILD, especially in patients with overlap syndrome, to healthy controls. In our study, serum levels of 4 cytokines, IL-2, IL-6, IL-8, and TNF-α, were significantly different in patients with AILD compared to healthy controls. The adjusted regression model has shown the central importance of IL-8, as this was the only cytokine, for which the difference between the patients with AILD and controls was still significant after adjustment. In the language of absolute numbers, the increase of IL-8 serum level by 1 pg/mL has increased the chance for a patient to find himself in a group of AILD by amazing 48.36 times, and in a group of overlap syndrome, by 23.85 times. Together with that, high IL-8 serum levels were strongly related to clinical parameters. Patients with AILD and higher serum IL-8 levels tended to have higher levels of total bilirubin, AST, alkaline phosphatase, and ESR and lower level of serum albumin, indicating lower liver synthetic function (albumin), higher grades of cytolysis (AST), and cholestasis (alkaline phosphatase), as well as higher grade of systemic inflammation (ESR). This association was almost lost in the subgroup analysis, which we hypothesize to be mainly due to a small sample size. Considering this, it seems that IL-8 levels might be of use for both establishing the diagnosis of AILD (i.e., as a diagnostic marker) and measuring the diseases activity and severity (i.e., as a clinical marker), which needs to be confirmed and explored in detail by further studies and supported by calculation of diagnostic utility parameters in future research.

Some other reports also showed significantly higher levels of all proinflammatory cytokines when compared to controls in patients with similar conditions. For example, a study in patients with primary biliary cirrhosis revealed the increased levels of IL-1β, IL-6, and TNF-α in such patients compared to healthy controls [12]. Another study revealed the possible association between the altered function of T cells and increased tissue level of TNF-α in patients with primary sclerosing cholangitis [13]. Considering the central pathogenetic role of Th17 cells in AIH, it is also important that Th17 cell-related gene expressions of such cytokines as IL-17, IL-23, IL-21, IL-1β, and IL-6 are reported to be significantly

Table 4: Spearman rank correlations between laboratory variables and serum cytokine levels measured. Numbers given are $r(p)$ values. Statistically significant correlations are marked with italic font.

	Total bilirubin	ESR	AST	AlkP	Albumin	γ-Globulin
			AILD			
IL-2	*−0.28 (0.034)*	0.03 (0.84)	−0.17 (0.21)	−0.16 (0.27)	0.07 (0.67)	0.09 (0.57)
IL-4	−0.04 (0.75)	0.16 (0.25)	−0.04 (0.80)	0.07 (0.65)	0.14 (0.37)	−0.04 (0.80)
IL-6	0.23 (0.08)	0.10 (0.46)	0.02 (0.88)	−0.12 (0.43)	*−0.30 (0.044)*	0.25 (0.10)
IL-8	*0.42 (0.001)*	*0.41 (0.002)*	*0.32 (0.018)*	*0.45 (0.001)*	*−0.33 (0.026)*	0.18 (0.23)
TNF-α	0.18 (0.17)	−0.07 (0.59)	0.11 (0.44)	0.12 (0.40)	−0.13 (0.41)	0.08 (0.61)
			AIH			
IL-2	−0.25 (0.18)	−0.13 (0.50)	−0.37 (0.052)	−0.18 (0.39)	0.13 (0.53)	−0.06 (0.79)
IL-4	−0.21 (0.27)	−0.14 (0.47)	−0.14 (0.48)	*−0.45 (0.023)*	0.26 (0.20)	−0.07 (0.72)
IL-6	0.24 (0.20)	0.02 (0.90)	0.20 (0.30)	0.22 (0.29)	−0.29 (0.15)	0.02 (0.92)
IL-8	*0.43 (0.017)*	0.32 (0.09)	*0.40 (0.032)*	0.37 (0.07)	−0.32 (0.11)	0.20 (0.32)
TNF-α	−0.09 (0.63)	−0.36 (0.05)	0.05 (0.79)	0.03 (0.90)	−0.14 (0.50)	0.15 (0.47)
			Overlap syndrome			
IL-2	−0.33 (0.08)	0.38 (0.07)	−0.03 (0.87)	−0.19 (0.38)	0.05 (0.86)	0.11 (0.65)
IL-4	0.21 (0.28)	0.31 (0.14)	−0.07 (0.74)	−0.01 (0.97)	−0.12 (0.62)	0.20 (0.41)
IL-6	0.27 (0.16)	*0.49 (0.014)*	−0.09 (0.67)	−0.20 (0.36)	−0.39 (0.10)	*0.46 (0.046)*
IL-8	0.34 (0.08)	0.27 (0.19)	0.09 (0.66)	0.19 (0.37)	−0.41 (0.08)	0.25 (0.31)
TNF-α	*0.56 (0.002)*	0.28 (0.17)	0.22 (0.27)	0.21 (0.32)	−0.12 (0.62)	0.09 (0.90)

AILD: autoimmune liver disease; AIH: autoimmune hepatitis; IL: interleukin; TNF-α: tumour necrosis factor-alpha; ESR: erythrocyte sedimentation rate; AST: aspartate aminotransferase; AlkP: alkaline phosphatase.

increased in the liver of AIH patients compared to healthy controls [14]. A study of cytokine levels in children with autoimmune hepatitis discovered a correlation between the levels of TNF-α, IL-6, and IL-8 and disease activity in patients with type 1 AIH. The levels of abovementioned cytokines were increased in children with active disease, as compared to controls, and decreased in patients with remission [15]. Our report, thus, confirms the hypothesis of a changed cytokine profile in patients with AILD, where the levels of proinflammatory cytokines, such as IL-6, IL-8, and TNF-α, would be increased compared to a healthy person, although the association between IL-8 serum level and AILDs, to the best of our knowledge, was never extensively studied, and such pronounced association was never reported before.

An elevation of IL-8 serum levels was shown before in patients with other liver diseases. Recently, a study of immune function in patients with cirrhosis has reported increased serum levels of proinflammatory cytokines, including IL-8. In this study, IL-8, together with IL-6, was higher in both patients with stable and those with decompensated cirrhosis and stayed high after development of acute-on-chronic liver failure [16]. It has been also shown that IL-8 levels are increased intrahepatically and in the serum of patients with alcoholic liver disease, probably contributing to hepatic neutrophil accumulation and also exerting systemic actions [17]. In patients with nonalcoholic fatty liver disease, interleukin-8 expression was also increased [18].

Of interest, patients with overlap syndrome in our study cohort had more severe and more rapidly progressing disease according to the clinical and laboratory parameters, with shorter disease duration, but the same frequency of severe fibrosis and higher levels of ALT, ESR, and alkaline phosphatase, compared to patients with isolated AIH. This was not an expected finding. It has been showed previously that AIH patients are significantly more likely to present with jaundice and have higher AST levels compared to patients with overlap syndromes [19]. This discrepancy might be partially explained by the age difference of nearly 10 years between the groups (nevertheless not statistically significant) and by lower percentage of patients with overlap syndrome receiving systemic treatment, such as glucocorticoids and cytostatics, at the time of inclusion.

Despite the difference in clinical parameters, the difference in IL-6 levels with the controls was somehow more marked in patients with AIH and the whole group of AILD, but not in patients with overlap syndrome. Nevertheless, in a subgroup of patients with overlap syndrome, but not in other patients, levels of IL-6 correlated with immunologic parameters, such as ESR and the serum level of γ-globulins. This comes in line with the differences of these two groups of patients. It is known that an autoimmune response is more pronounced in patients with overlap syndrome, leading to a higher production of autoimmune antibodies and to significant reduction of expected life duration [19]. IL-6, as the key cytokine mediating systemic immune response [20], may play a role in that.

5. Conclusions

In patients with AILD, cytokine profile significantly differs from that in healthy subjects, with increased serum levels of

proinflammatory cytokines, such as IL-6, IL-8, and TNF-α. IL-8 seems to have the strongest relation to the disease and also correlates with the severity of laboratory abnormalities in patients with AILD. Possible use of IL-8 as a potential diagnostic or clinical marker, or a treatment target, is still to be explored.

Competing Interests

The authors declare that they do not have anything to disclose regarding conflict of interests with respect to this study.

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Research Article

Evolution of the Immune Response against Recombinant Proteins (TcpA, TcpB, and FlaA) as a Candidate Subunit Cholera Vaccine

Neda Molaee,[1] Ghasem Mosayebi,[1] Alireza Amozande-Nobaveh,[1] Mohammad Reza Soleyman,[1] and Hamid Abtahi[2]

[1]*Department of Microbiology and Immunology, School of Medicine, Arak University of Medical Sciences, Arak, Iran*
[2]*Molecular and Medicine Research Center, Department of Microbiology and Immunology, School of Medicine, Arak University of Medical Sciences, Arak, Iran*

Correspondence should be addressed to Hamid Abtahi; abtahi@arakmu.ac.ir

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Vibrio cholerae is the causative agent of cholera and annually leads to death of thousands of people around the globe. Two factors in the pathogenesis of this bacterium are its pili and flagella. The main subunits of pili TcpA, TcpB, and FlaA are the constituent subunit of flagella. In this study, we studied the ability of pili and flagella subunits to stimulate immune responses in mice. After amplification of TcpA, TcpB, and FlaA genes using PCR, they were cloned in expression plasmids. After production of the above-mentioned proteins by using IPTG, the proteins were purified and then approved using immunoblot method. After injection of the purified proteins to a mice model, immune response stimulation was evaluated by measuring the levels of IgG1 and IgG2a antibody titers, IL5 and IFN-γ. Immune response stimulation against pili and flagella antigens was adequate. Given the high levels of IL5 titer and IgG1 antibody, the stimulated immune response was toward Th1. Humoral immune response stimulation is of key importance in prevention of cholera. Our immunological analysis shows the appropriate immune response in mice model after vaccination with recombinant proteins. The high level of IL5 and low level of IFN-γ show the activation of Th2 cell response.

1. Introduction

Vibrio cholerae is a noninvasive, Gram-negative bacterium that causes acute gastroenteritis in humans. Millions of people around the globe are suffering from cholera, and annually more than 100,000 cases of mortality are reported from this disease [1, 2].

The main structures of the bacteria which play an important role in the disease process include bacterial toxin, cell wall, flagella, and pili [3, 4]. Pilus plays a major role in the development and progression of infection. Several proteins are involved in formation of pilus, but only a limited number of them are on the bacterial cell surface. The most important of these proteins are toxin coregulated pilus subunit A (TcpA) and TcpB [5]. Several studies have demonstrated that antibodies against TcpA and TcpB can affect immunogenicity by reducing *Vibrio cholerae* colonization. A study performed by Rollenhagen et al. (2006) showed that immunization of mice with pilin proteins can induce protective immunity [6, 7].

TcpA is able to stimulate Th1-type immune responses, so that Tcp antigens have the ability to stimulate IL-4 cytokines and produce IgG1 antibodies. There is limited information on TcpB; this subunit is assumed to be effective in immune responses to cholera. Also, flagella-A (Fla-A) of *Vibrio cholerae* has a main role in pathogenicity of disease [8, 9].

Although bacterial pili and flagellar antigens are of major significance in the development and progression of cholera, there is a scarcity of studies on the type of immune responses induced by these antigens. In addition, when simultaneously inoculated, the effect of each of these antigens on the other has not been investigated yet. Therefore, the aim of this study is evaluation of immune responses against recombinant

proteins TcpA, TcpB, and FlaA or combination of them in animal model.

2. Materials and Methods

2.1. Bacteria and Sera. *V. cholerae serotype Inaba* (a gift from the Pasteur Institute of Iran) was grown on TCBS (thiosulfate-citrate-bile salts-sucrose agar, Merck, Germany) for 24 hours. For recombinant protein production, prokaryotic expression vector pET32a (Novagene) and pGEX4T1 were used. *E. coli* strain DH5α (Stratagene) was used for initial cloning and *E. coli* BL21 (DE3) pLysS and *E. coli* BL21 were used as host strains for protein production.

The required antibiotics (ampicillin and chloramphenicol) were added to LB media according to the reference recommendation [10]. We received standard rabbit anti-*V. cholerae* sera from Tarbiat Modares University (Department of Microbiology, Tehran, Iran). All chemicals were obtained from Merck Co. (Germany).

2.2. Gene Amplification, Expression, and Purification of Recombinant Proteins. *V. cholerae* chromosomal DNA was prepared according to standard CTAB/NACL method [11].

Primers were designed according to published sequence for TcpB (accession number: FJ209011), TcpA (accession number: U09807), and FlaA (accession number: Af019213) of *V. cholerae* as follows: TcpB: forward: 5′TCGGATCCATGAGAAAATACCAA3′ and reverse: 5′ACTCGAGATTTTCACACCATTGA3′; TcpA: forward: 5′-AGGGATCCATGACATTACTCGAAG-3′ and reverse: 5′-AACTCGAGGCTGTTACCAAATGC-3′; FlaA: forward: 5′CTGGATCCATGACCATTAACGTAAA3′ and reverse: 5′CCTCGAGCTGCAATAACGHAGATT3′. All primers contained BamHI (forwards) and XhoI (reverses) sites, respectively. The restriction enzyme sites (underlined) were added to the primers for subsequent cloning procedure. PCR amplification was performed (separately) and was analyzed by horizontal agarose gel electrophoresis in 1x TBE buffer and visualized by ethidium bromide staining on UV transilluminator.

The PCR products were digested with BamHI and XhoI and ligated to pGEX4T1 (TcpA-pGEX4T1) and pET32a (TcpB-pET32a and FlaA-pET32a), which were digested by the same restriction enzymes, using T4 DNA ligase at 16°C overnight. *E. coli* BL21 and *E. coli* BL21 (DE3) pLysS competent cells were prepared by calcium chloride method and were used for transformation of TcpA-pGEX4T1, TcpB-pET32a, and FlaA-pET32a plasmids, respectively [12].

E. coli BL21 (DE3) pLysS (transformed by TcpB-pET32a and FlaA-pET32a plasmids) and *E. coli* BL21 (transformed by TcpA-pGEX4T1) were grown in 2 mL nutrient broth medium being supplemented with ampicillin (100 mg/mL) and chloramphenicol (35 mg/mL for *E. coli* BL21 (DE3) pLysS) on shaking incubator overnight at 37°C. In the next day, 500 μL of culture was inoculated in 50 mL of nutrient broth medium 0.5 g yeast extract, 1 g bactopepton, 0.1 g glucose, 0.5 g NaCl, 0.05 g KCl, 0.025 g $MgCl_2{\cdot}6H_2O$, 0.025 g $CaCl_2$, 0.25 g nutrient broth, ampicillin (100 mg/mL), and chloramphenicol (35 mg/mL for *E. coli* BL21 (DE3) pLysS) at 37°C with vigorous agitation at 220 rpm. The cells grew until the OD (optical density) at 600 nm reached 0.6. Expression of the recombinant proteins was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, Fermentas) to a final concentration of 1 mM and incubated for four hours [13].

TcpB and FlaA were purified using Ni-NTA column (Qiagen) and TcpA was purified by GST column (Bio-Sciences) according to manufacturer's instructions. The purified protein was dialyzed twice against PBS (pH 7.5) at 4°C overnight. The quality and quantity of purified recombinant proteins were analyzed by SDS-PAGE (15%) and Bradford methods, respectively.

2.3. Immunoblot Analysis. The integrity of the purified recombinant proteins was confirmed by Western blot analysis. Western blotting was performed according to the standard protocol [14] using sera from rabbit immunized against *Vibrio cholerae* and negative sera (as the negative controls) as the primary antibody at 1 : 100 dilution and HRP-conjugated (horseradish peroxidase) goat anti-rabbit IgG (Abcam, United Kingdom) at 1 : 2500 dilution in 1x TBST buffer (10x: 15 mMNaCl, 10 mMTris-HCl (pH = 7.4), 0.1% Tween 20) as secondary antibody. The reactions were developed by diaminobenzidine (DAB) solution (Roche, Germany).

2.4. Evaluation of the Immune Responses against Recombinant Proteins. To investigate immune responses against the recombinant proteins, six groups of BALB/c male mice were studied ($n = 10$) with a mean age of six weeks and weight of about 60 g. The groups were injected with poly(butylene succinate) (PBS, negative control), TcpA, TcpB, TcpA+TcpB, FlaA, and TcpA+TcpB+FlaA. Injections were performed intradermally in three doses with an interval of two weeks. The first dose of protein injection was administered with complete Freund's adjuvant, and, in the next sessions, the proteins were injected with incomplete Freund's adjuvant. Each mouse was injected with 75 μg of the proteins per injection.

2.5. Separation of Mononuclear Cells from Spleen. At day 28 after immunization, mice were sacrificed by CO_2 inhalation, and the spleens were removed. Spleens were washed in RPMI (modification with 5 mM HEPES, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (all from Gibco, Life Technologies, Inc., Gaithersburg, MD). The spleens were punctured repeatedly with a pair of forceps to release the spleen cells. Low-density mononuclear cells were collected after standard separation on Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) and washed in RPMI with 10% heat-inactivated fetal bovine serum (FBS). The number of viable cells was assessed by trypan blue exclusion. The cells were then resuspended in RPMI supplemented with 10% FBS and used for proliferation assay and cytokines detection.

2.6. Cytokines Assay. The levels of cytokines (IL5 and IFN-γ) were measured in serum and supernatant of MNCs (mononuclear cells) cultured. MNCs isolated from spleen of mice at day 28 after immunization were incubated in 1 mL

cultures at a density of ~2 × 106 cells/mL in the presence or absence of specific recombinant protein (20 μg/mL) and placed in a 5% CO_2, 37°C incubator for 72 h. For quantitative IFN-gamma and IL5 productions, supernatants were collected and the amounts of IFN-gamma and IL5 in the supernatants were quantified by ELISA kit (R&D Systems) according to the manufacturer's protocol. 96-well plates were coated with anti-mouse IFN-gamma and IL5 antibody in coating buffer and incubated overnight. After blocking, samples and standards at 1 : 2 serial dilutions of IFN-gamma and IL10 (standard curve) were added to the plates and incubated for 60 min at room temperature. After that, biotinylated anti-mouse IFN-gamma and IL5 mAbs were added and incubated for another 60 min. HRP-conjugated streptavidin was then added for 30 min. After further washings, TMB substrate was added to incubate for 30 min, followed by addition of 0.18 M H_2SO_4 solution to stop the reaction and reading at 450 nm was obtained.

2.7. Measurement of IgG1 and IgG2a Antibodies. ELISA method was employed to measure IgG1 and IgG2a antibodies in the vaccinated animals. In this method, 10 μg of recombinant proteins TcpA, TcpB, and FlaA was first bound in 96-well plate for 4 h. After washing, the proteins were kept at 4°C. After incubation with 2% BSA solution for an hour, blocking stage was performed. Thereafter, 100 μL of serum from the vaccinated mice was added to each 96-well microplate (NUNC). The plates were kept at 37°C for 2 h. After washing, 50 μL of 1/10000 diluted antibodies against IgG1 and IgG2a conjugated classes was added to HRP (Abcam, UK) and they were kept at 37°C for 2 h. Finally, light absorption at wavelength of 490 nm was determined by adding 100 μL of o-phenylenediamine (OPD) substrate solution to each well and incubation for 10 min.

2.8. Proliferative Response Check by MTT. Proliferation was checked by MTT method [15]. A total of 1-2 × 10^3 cells/well in 100 μL RPMI 1640 supplemented with 10% FBS were stimulated with 20 μg/mL specific antigen (TcpA, TcpB, and FlaA) or 1 μg/mL PHA (phytohemagglutinin) concentration (as a mitogen) used for T-cell activation and proliferation. The plates were then placed in a 5% CO_2, 37 °C incubator for 72 h. Ten microliters of 5 mg/mL MTT (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, Germany) was added to the cells, followed by incubation for 4 h. After centrifugation, the medium was removed, and 200 μL of DMSO was added to each well. Then, the optical density at 570 nm was measured by microtiter plate reader (Stat Fax 2100, USA). The experiments were performed in triplicate sets. Blastogenic responses for the MTT assay were expressed as a mean stimulation index (SI) by dividing OD values of stimulated cells (C) minus relative cell numbers of unstimulated cells (C) by relative OD values of unstimulated cells. SI = (C − C)/C_{MTT}.

2.9. Statistical Analysis. Statistical analysis included independent sample *t*-test to evaluate differences between variables in the groups. Comparisons between groups were assessed using Mann–Whitney *U* test. Data were analyzed by SPSS software version 16.0 (SPSS, Inc., Chicago, Illinois, USA) and were represented as mean ± SD. *P* values < 0.05 were considered statistically significant.

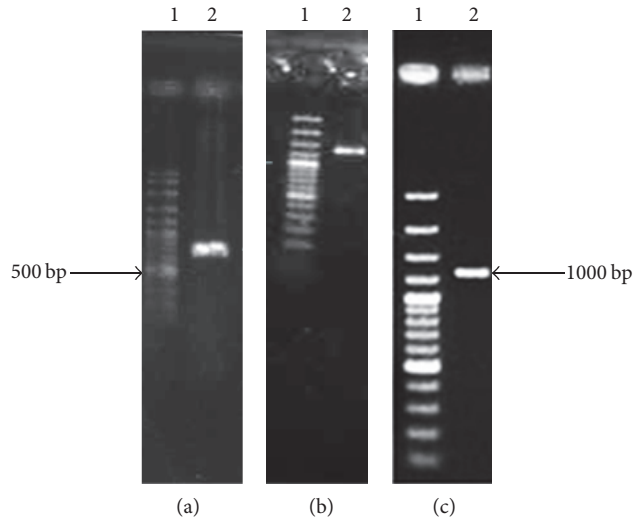

Figure 1: Results of amplification of TcpA, TcpB, and FlaA genes. (a): lane 1: DNA marker 100 bp (Fermentas); lane 2: TcpA gene PCR result (598 bp). (b): lane 1: DNA marker 100 bp; lane 2: TcpB gene PCR result (1295 bp). (c): lane 1: DNA marker 100 bp; lane 2: FlaA gene PCR result (1139 bp).

3. Results

3.1. Quality of Expressed Protein. The results of polymerase chain reaction (PCR) consisted of gene fragments in the desired size (Figures 1(a), 1(b), and 1(c)). Sequencing and analysis using BLAST indicated the accuracy of the obtained sequence. The amplified DNA fragments (TcpA, TcpB, and FlaA) were cloned in pET32a and pGEX4T1 vectors.

TcpA, TcpB, and FlaA proteins were produced in *Escherichia coli*. The produced FlaA and TcpA proteins were purified using Ni-NTA kit and TcpB protein was purified using GST-Sepharose kit; the produced proteins were in the expected size. The amounts of proteins produced after purification and dialysis (against PBS buffer, in pH 7.5) were measured to be 2.1, 2, and 1.5 mg/mL. Western blotting of the produced proteins is exhibited.

According to the result of the Western blotting, the purified proteins reacted against the serum of rabbit infected with *Vibrio cholerae*. Nevertheless, no reactions were observed in the serum of the healthy controls and the normal rabbit serum in the Western blotting (Figures 2(a), 2(b), and 2(c).

3.2. Cytokine Response (Serum and Supernatant). The type of cytokines produced in the lymphocyte culture (serum and supernatant) of the mice immunized by recombinant proteins was determined three weeks after the last inoculation. Figures 3(a) and 3(b) show the levels of IFN-gamma and IL5 produced in the cultured lymphocytes (supernatant) of the immunized animals. As noted in Figure 3(a), IFN-gamma level increased in all of the studied groups except the FlaA. In

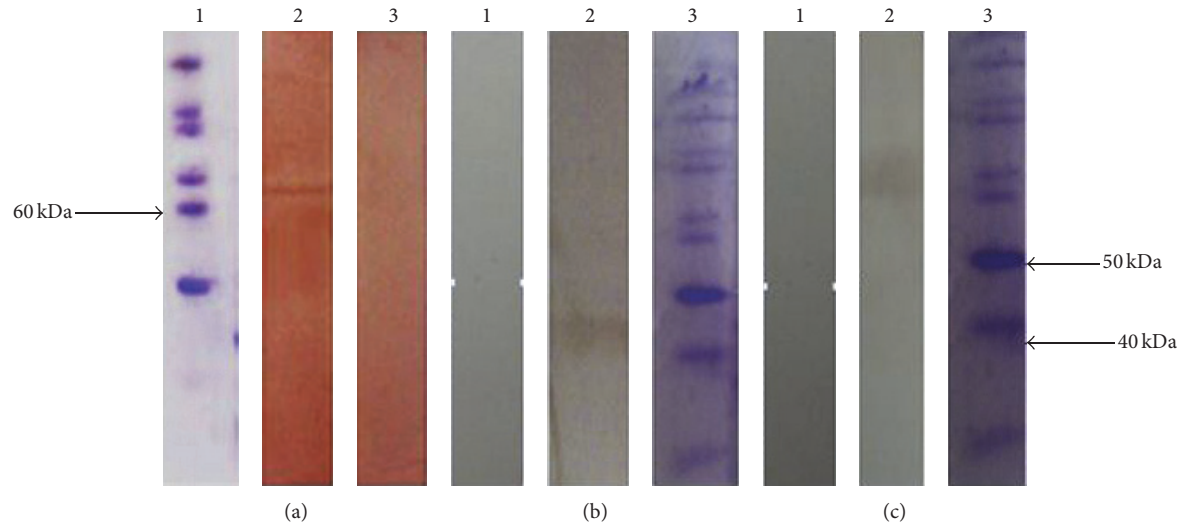

Figure 2: Western blot analysis of the recombinant proteins (a, b, c). (a) lane 1: protein marker (Fermentas); lane 2: interaction between serum of immunized rabbit and purified recombinant FlaA protein. (b) lane 1: protein marker; lane 2: interaction between serum of immunized rabbit and purified recombinant TcpA protein. (c) lane 1: protein marker; lane 2: interaction between serum of immunized rabbit and purified recombinant TcpB protein.

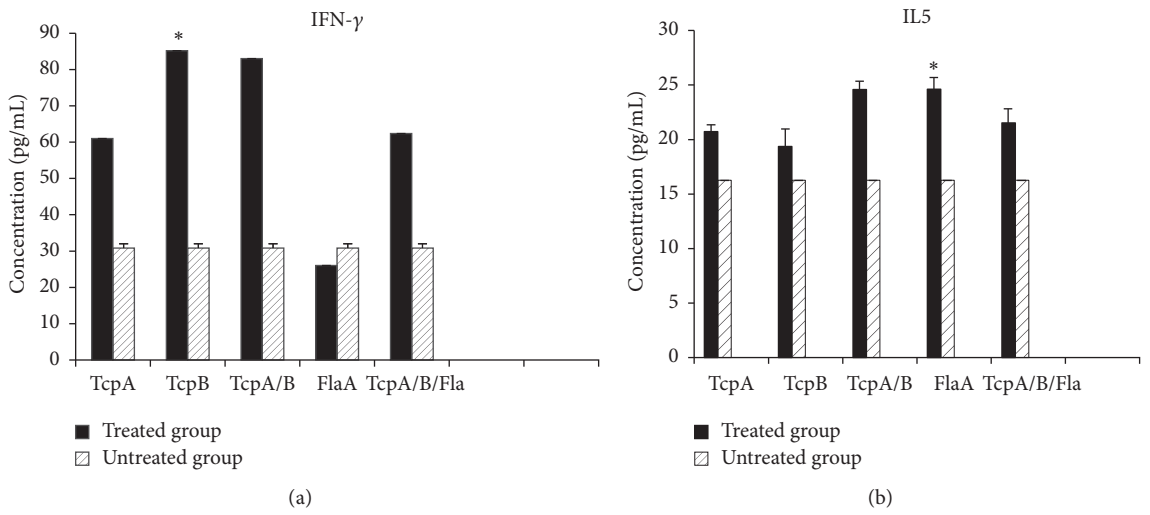

Figure 3: Quantitative ELISA analysis of IFN-γ (a) and IL5 (b) measured in the supernatant of the MNCs isolated from the spleen of mice immunized with specific recombinant proteins. Significant differences were designated as $^{*}P < 0.05$.

Figure 3(b) curve, IL5 level is higher in all the experimental groups compared to the untreated group. This increase, particularly in FlaA and TcpA/B groups, is higher than the other groups. In serum (Figures 4(a) and 4(b)), IFN-gamma level in the serum was not significant. In Figure 4(b) curve, IL5 level is higher in FlaA compared to the untreated group.

3.3. Antibody Response. Serum collected after the last vaccination was evaluated against the purified recombinant proteins TcpB, TcpA, and FlaA with regard to IgG2a and IgG1 antibody titers using ELISA method. As seen in Figures 5(a) and 5(b), the level of IgG1 antibody titer in all the vaccinated groups, compared to the untreated group, is higher than IgG2a antibody titer. In TcpB IgG2a antibody titer level is higher than the untreated group. However, IgG2a antibody titer level in groups receiving TcpA/B+FlaA is lower than the untreated group. IgG1 to IgG2a ratio is shown in Figure 5(c).

3.4. Lymphocyte Proliferation. According to the outcomes, the highest cell response in the experimental groups is observed in the TcpA+TcpB group. The lowest rate of cell proliferation is noted in the FlaA group. Results of MTT can be observed in Figure 6.

4. Discussion

The results show that pili and flagella antigens of *Vibrio cholerae* can cause strong humoral immune responses

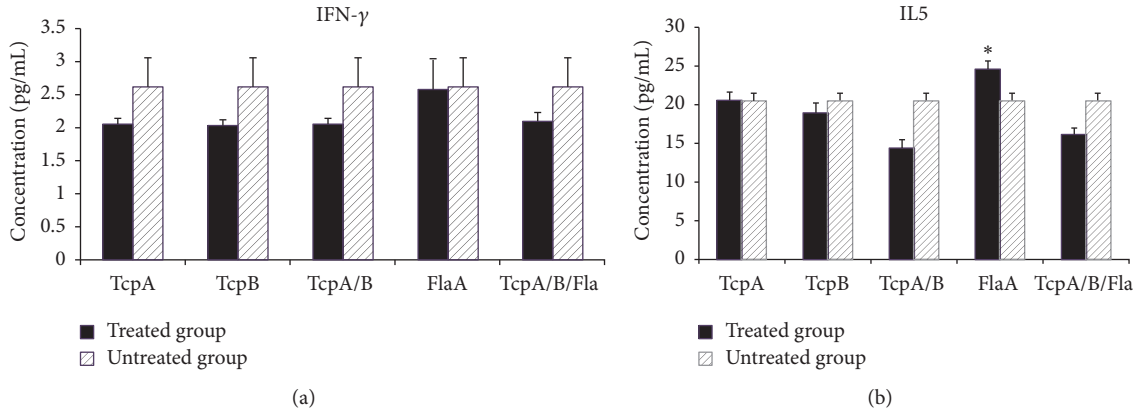

Figure 4: Quantitative ELISA analysis of IFN-γ (a) and IL5 (b) measured in the serum of mice immunized with specific recombinant proteins. Significant differences were designated as $^{*}P < 0.05$.

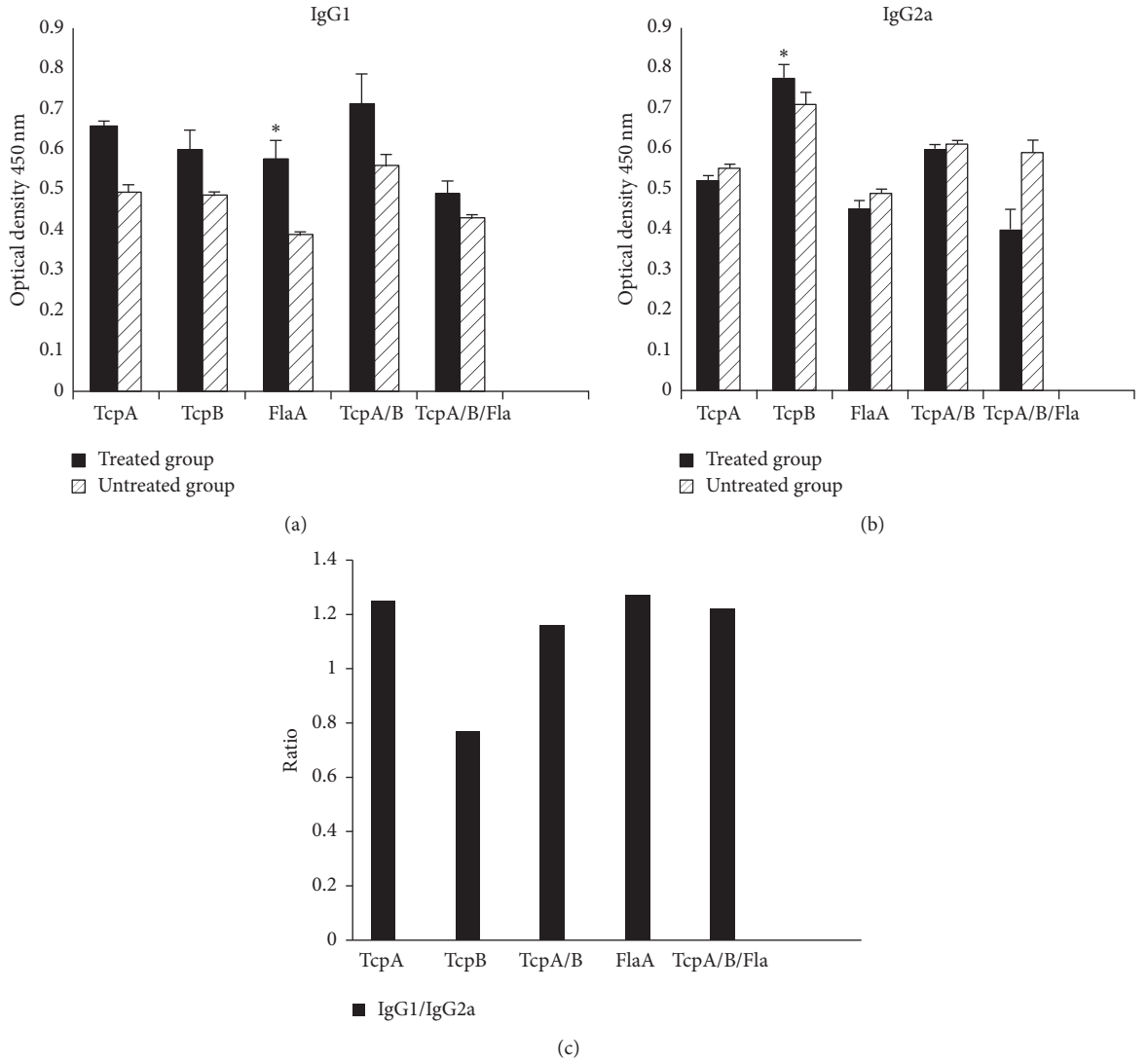

Figure 5: Measuring IgG1 (a) and IgG2a (b) and IgG1/IgG2a (c) antibodies in the vaccinated mice using ELISA method three weeks after immunization. Significant differences were designated as $^{*}P < 0.05$.

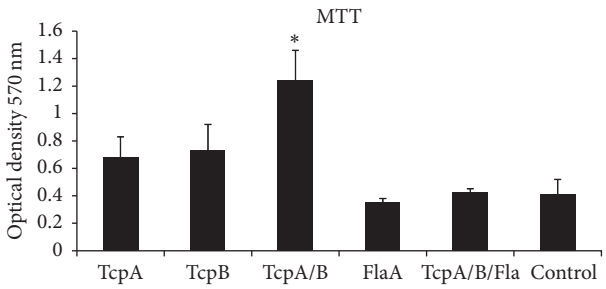

Figure 6: The MTT assay of the MNCs of BALB/c male immunized with specific recombinant proteins. Significant differences were designated as $^{*}P < 0.05$.

after immunization. However, TcpB antigen drives immune response away from the humoral immune response.

Cholera is an acute diarrheal infection caused by *Vibrio cholerae*. Following ingestion of contaminated water or food and after colonization of the bacteria in the small intestine, toxins are secreted by the bacteria, and, therefore, the patient loses great amounts of water and electrolytes. Given the high rates of mortality of the disease, prevention is crucial, especially in high-risk areas [16].

A large number of live attenuated or inactivated vaccines have already been studied, but none of these types of vaccines have been able to successfully prevent this disease, especially in endemic areas. Accordingly, more attention is drawn to subunit vaccines of *Vibrio cholerae* [17]. Adhesion and motility play a very important role in the process of *Vibrio cholerae* infectivity [18].

Pili and flagella are two important structures of *Vibrio cholerae* which help with adhesion and motility of the bacterium. TcpA and TcpB are the main subunits of *pilus* in *Vibrio cholerae* which can be seen on its outer surface [19].

TcpA is the main subunit of *pilus* in *Vibrio cholerae*. This antigen plays the main role in attachment of the bacterium to intestinal cells. In studies on synthetic version of the protein, it has been shown that peptides made from TcpA induce protective antibodies in mice and that these antibodies have a high protective effect. However, the level of antibody titer against this protein is not high in those vaccinated with live attenuated or inactivated vaccines, which is due to the sharp decline of this antigen in *Vibrio cholerae* culture [20].

TcpB is another component of *pilus* which helps with attachment of *Vibrio cholerae* to intestinal cells. Despite studies on the immunogenicity of TcpA, there are only few studies on the immunogenicity of the subsidiary subunit of TcpB. Only the study by Kiaie et al. showed that TcpB antibody exists in cholera patients as well as in animals vaccinated with killed bacteria [21].

In addition to adhesion, motility of *Vibrio cholerae* is considered as one of the most important indicators of bacterial pathogenicity. Flagella play a key role in the bacterium's access to the bacterial colonization location. As it was proven, mutant is not capable of causing infection without flagella. According to former studies, flagella are able to enhance expression of some pathogenicity genes such as the gene encoding the cholera toxin (CT), genes encoding pilus (TCP), and other genes involved in this disease. Therefore, flagella in *Vibrio cholerae* are considered as a suitable subject for immunological studies [22–24].

FlaA is the most important constituent of the outer surface of flagella. This protein is recognized by the normal immune response system, such that the body's first line of defense would respond against the bacterium. In a study conducted in 2008, it was revealed that recombinant flagella of *Vibrio cholerae* are able to stimulate IL8 production through the activation of Toll-like receptor 5 (TLR5) and nuclear factor kappa B. Thus, flagella of *Vibrio cholerae* are considered one of the main targets of the immune system and act as a ligand for Toll-like receptors, that is, TLR5, of the host immune cells. TLR5 stimulation by various pathogens leads to the activation of the innate immune response and, in turn, adaptive immunity [25].

In immunogenicity studies on pili and flagella antigens, the effects of immunogenicity of antigens have been studied separately. In the present study, the immunogenicity of these antigens was studied. The vaccinated groups (six groups) included mice vaccinated with TcpA, TcpB, FlaA, TcpA+TcpB, and TcpA+TcpB+FlaA. After the full course of immunization, humoral immunity and cellular immunity were evaluated in the groups.

Evaluation of cytokine responses after stimulation of spleen cells with recombinant proteins TcpA, TcpB, and FlaA exhibited the ability of these proteins to produce IFN-gamma and IL5. Stimulation level of the production of IFN-gamma after stimulation of lymphocytes of mice immunized by the proteins TcpA and TcpB can be observed. Furthermore, the lowest level of IFN-gamma production is observed in mice immunized with FlaA. However, in mice immunized with all the three proteins (TcpA+TcpB+FlaA), the level of IFN-gamma slightly increased compared to the FlaA group.

Measurement of serum IFN-gamma in the immunized mice showed that there was no significant different between the groups.

IL5 production rate in the vaccinated groups showed that the highest rate of cytokine production was observed in mice immunized with FlaA protein. The level of IL5 in serum and cell culture in the vaccinated animals increased. Statistical results also demonstrated that *P* value obtained from the FlaA group was less than 0.05. IL5 in cell culture and serum of mice given FlaA was more than the level of this cytokine in the untreated group. Nevertheless, the level of IL5 in the cell culture of the TcpB group was lower than the other groups. In addition, the level of IL5 in the serum of TcpB and TcpA+TcpB and TcpA+TcpB+FlaA groups was less than the untreated group.

Thus, according to the obtained results regarding cytokine (in the lymphocyte culture), it is indicated that the immune system in TcpA and FlaA groups tends toward humoral immune responses, while TcpB drives the immune responses away from humoral immunity.

The levels of IgG1 and IgG2a antibody titers in the studied groups partly confirm the cytokine results. IgG1 antibody titer increased in all the studied groups. Moreover, IgG1 titer was high in the groups receiving FlaA and TcpA proteins, such

that the IgG1 to IgG2a ratio was more than one in these groups. Increased level of IgG1 titer was also noted in the TcpB group. The promoted level of antibody titer can be observed even in the groups that received several antigens with TcpB.

High levels of IgG2a titer can be clearly observed in the TcpB group. Additionally, this increase can be observed in the groups where the antigen was injected with other antigens, especially in the TcpA+TcpB group. In the FlaA+TcpA/B group, the level of this antibody was less than the untreated groups.

IgG1 to IgG2a ratio in the studied groups demonstrates that, in the groups inoculated with the FlaA and TcpA antigens, this ratio was higher than one. However, this ratio was less than one in the TcpB group. Combination of TcpB with other antigens lowered this ratio, compared to the other groups.

High levels of IgG2a titer and IFN-gamma are observed in the groups vaccinated with TcpB. Therefore, it is inferred that TcpB mostly plays a role in stimulating cellular immune responses.

According to the results, it can be concluded that FlaA and TcpA stimulate immune responses toward Th2; however, high levels of TcpB mostly direct these responses toward Th1. Since using vaccines consisting of pathogen subunits is more efficient when two or more subunits are used, the use of FlaA and TcpA antigens in *Vibrio cholerae* vaccination can cause higher immunogenicity.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

High Triglycerides Are Associated with Low Thrombocyte Counts and High VEGF in Nephropathia Epidemica

Ekaterina V. Martynova,[1] Aygul H. Valiullina,[1] Oleg A. Gusev,[1,2] Yuriy N. Davidyuk,[1] Ekaterina E. Garanina,[1] Venera G. Shakirova,[3] Ilsiyar Khaertynova,[3] Vladimir A. Anokhin,[3] Albert A. Rizvanov,[1] and Svetlana F. Khaiboullina[1,4]

[1] *Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Tatarstan, Russia*
[2] *RIKEN, Yokohama, Japan*
[3] *Kazan State Medical Academy, Kazan, Tatarstan, Russia*
[4] *University of Nevada, Reno, NV, USA*

Correspondence should be addressed to Albert A. Rizvanov; albert.rizvanov@kpfu.ru and Svetlana F. Khaiboullina; skhaiboullina@medicine.nevada.edu

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Nephropathia epidemica (NE) is a mild form of hemorrhagic fever with renal syndrome. Several reports have demonstrated a severe alteration in lipoprotein metabolism. However, little is known about changes in circulating lipids in NE. The objectives of this study were to evaluate changes in serum total cholesterol, high density cholesterol (HDCL), and triglycerides. In addition to evaluation of serum cytokine activation associations, changes in lipid profile and cytokine activation were determined for gender, thrombocyte counts, and VEGF. Elevated levels of triglycerides and decreased HDCL were observed in NE, while total cholesterol did not differ from controls. High triglycerides were associated with both the lowest thrombocyte counts and high serum VEGF, as well as a high severity score. Additionally, there were higher levels of triglycerides in male than female NE patients. Low triglycerides were associated with upregulation of IFN-γ and IL-12, suggesting activation of Th1 helper cells. Furthermore, levels of IFN-γ and IL-12 were increased in patients with lower severity scores, suggesting that a Th1 type immune response is playing protective role in NE. These combined data advance the understanding of NE pathogenesis and indicate a role for high triglycerides in disease severity.

1. Introduction

Nephropathia epidemica (NE) is a mild form of hemorrhagic fever with renal syndrome (HFRS), characterized by kidney insufficiency and hemorrhagic disorders. The causative agent is *Puumala virus* (PUUV), member of *Hantavirus* genus, family Bunyaviridae. PUUV targets endothelial cells as viral antigens have been frequently found in endothelial cells, in postmortem tissue [1, 2]. PUUV infection is not cytopathic as cell death attributed to virus replication has not been reported either in vivo or in vitro [1–4]. Therefore the *Hantavirus* pathogenesis remains largely unknown.

NE is endemic in the Republic of Tatarstan where the highest annual prevalence of 64.4 cases per 100,000 was registered in 1997 [5]. Clinically, NE is characterized by an acute onset of high fever, headache, and abdominal pain. Soon, back pain and decrease urine output are followed indicating the impaired renal function. This disease is characterized by 4 periods, febrile, oliguric, diuresis, and convalescence. The oliguric period is the most critical due to the high likelihood of developing life threatening complications. Recovery begins with the onset of diuresis [1, 2]. At this stage, complications can include disseminated encephalomyelitis and hypopituitarism. NE is characterized by a low fatality rate (0.4%). Postconvalescent sequelae are rare and may involve increased glomerular permeability and moderate hypotension [6, 7].

There are 3 clinical forms of NE: mild, moderate, and severe. The severe form of NE is characterized by prominent hemorrhagic symptoms including petechial and nasal and internal bleeding. In some cases, disturbed blood coagulation

presents as disseminated intravascular coagulation (DIC) [8]. Laboratory findings for the severe form include high blood urea and creatinine levels, blood urea nitrogen (BUN) >20 mmol/L, and creatinine up to 600 μmol/L. The moderate form of NE has similar but subtler symptoms, with BUN and creatinine levels over 19 mmol/L and 200–300 mmol/L, respectively. The mild form often remains undiagnosed and characterized by mild headache and fever, with hemorrhagic symptoms restricted to small petechia on mucosa and skin.

Increased vascular permeability is a hallmark of NE pathogenesis. Clinically, this increased vascular leakage manifests as petechia, subconjunctival and gastrointestinal hemorrhaging, and, in severe cases, DIC [9, 10]. Additionally, signs of disturbed hemostasis are evident in laboratory tests including prolonged bleeding, increased prothrombin time, and activated partial thromboplastin times [8]. Furthermore, decreased thrombocyte counts are commonly found in NE patients [11, 12]. Extreme thrombocytopenia has been suggested to be predictive of disease severity. Thrombocytopenia ($<60 \times 10^9$ platelets/L) was found in patients with serum creatinine >620 μmol/L and was an early prognostic marker for acute renal failure [11]. Thrombocytopenia in NE is associated with platelet consumption, due to endothelial cell activation and repair [11, 13]. Recently, a correlation has been shown between thrombocyte counts and serum VEGF levels. Xu et al. demonstrated that thrombocytopenic disorders, characterized by increased platelet destruction, can exhibit increased levels of plasma VEGF [14]. Although serum VEGF has been shown to be upregulated in HFRS, the severe form of *Hantavirus* infection [15, 16], little is known about the association between VEGF and thrombocyte counts in NE cases.

Elevated serum lipase has been documented in some NE patients [17], as well as increased serum cholesterol, total phospholipids, and triglycerides [18], together with upregulated lipid peroxidation [19]. Another study demonstrated low HDCL and total cholesterol, as well as high levels of triglycerides in NE patients [20]. However, little is known about gender differences in lipidemia or any association between serum lipids and cytokine activation in NE.

The objectives of this study were to evaluate changes in serum total cholesterol, HDCL, triglycerides, and cytokine activation in NE patients based on gender, thrombocyte counts, and VEGF and further analyze the association between the severity of the disease and serum lipid, cytokine, VEGF levels, and gender of NE cases. Increased triglycerides were found in NE cases, while total cholesterol levels did not differ significantly between patients and controls. These data indicated that high triglycerides were associated with the lowest thrombocyte counts and high serum VEGF. Furthermore, we found higher triglycerides in male as compared to female NE. Additionally, low triglycerides were associated with upregulation of IFN-γ and IL-12, suggesting activation of Th1 helper cells. Patients with lower severity scores had increased IFN-γ and IL-12 suggesting that a Th1 type immune response plays protective role in NE.

2. Materials and Methods

2.1. Patients. Two hundred and twenty-eight NE patients (190 male, 38 female; 38.6 ± 3.1 years) admitted to Republican Clinical Hospital for Infectious Disease named after Agafonov, Republic of Tatarstan, were recruited. Serum from 64 NE patients was collected twice (early (2.1 ± 1.5) and late (7.5 ± 2.3)), while a single serum sample was obtained from 164 patients. Diagnosis of NE was established based on clinical presentation and was serologically confirmed by detection of anti-*Hantavirus* antibodies. In some cases, diagnosis was confirmed using PCR. Serum samples from 56 controls matched for gender, age (32.7 ± 2.3 years), and region were collected. The Institutional Review Board of the Kazan Federal University approved this study and informed consent was obtained from each study subject according to the guidelines approved under this protocol (article 20, Federal Law "Protection of Health Rights of Citizens of Russian Federation" N323- FZ, 11.21.2011).

2.2. Multiplex Analysis. Serum cytokine levels were analyzed using Bio-Plex (Bio-Rad, Hercules, CA, USA) multiplex magnetic bead-based antibody detection kits following manufacturer's instructions. Multiplex kits, Bio-Plex Pro Human Cytokine 27-Plex Panel (IL-1β, IL-1Ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17A, CCL2, CCL3, CCL4, CCL5, CCL11, CXCL10, TNF-α, G-CSF, GM-CSF, IFN-γ, PDGF-BB, and VEGF) and Bio-Plex Human Cytokine 21-Plex Panel (IL-1α, IL-2Ra, IL-3, IL-12(p40), IL-16, IL-18, CCL7, CCL27, CXCL1, CXCL9, CXCL12, HGF, IFN- α2, LIF, M-CSF, MIF, β-NGF, SCF, SCGF-β, TNF-β, and TRAIL), were used for detection of a total of 48 analytes. Serum aliquots analyzed were 50 μL. A minimum of 50 beads per analyte was acquired. Median fluorescence intensities were collected using Luminex 100 or 200 analyzer (Luminex, Austin, TX, USA). Data collected was analyzed using MasterPlex CT control software and MasterPlex QT analysis software (MiraiBio, San Bruno, CA, USA). Standard curves for each analyte were generated using standards provided by manufacturer.

2.3. Serum Lipid Profile. Fasting serum samples were collected early in the morning. Total cholesterol levels were determined using Novochol-200 kit (Vector-Best, Russia) according the manufacturer's instructions. The optical density (OD 520 nm) of the test serum and calibration sample (provided by the manufacturer) was determined using an Infinite M200 PRO analyzer (Tecan, Port Melbourne, VIC, Australia). Cholesterol levels were calculated using the formula: $C = (E/E_k) * 4.65$ (mmol/L), where C is concentration of cholesterol; E is optical density of tested serum; E_k is optical density of calibration sample; and 4.65 mmol/L is concentration of cholesterol in calibration sample.

Serum triglycerides were determined using the Triglyceride-Novo kit (Vector-Best, Russia) according to manufacturer recommendations. The optic density (520 nm) of serum sample and calibrator (provided by the manufacturer) was

determined using Tecan Infinite M200 PRO analyzer (Australia). Triglyceride concentrations were calculated using the formula: $C = (E/E_k) * 2.29$ (mmole/L), where C is concentration of triglycerides; E is optical density of serum sample; E_k is optical density of calibration sample; and 2.29 mmol/L is concentration of triglycerides in calibration sample.

Serum levels of HDCL were determined using HDCL-Cholesterol-Novo-A kit (Vector-Best, Russia). Briefly, serum sample (3 μL) or calibrator was mixed with reagent 1 (300 μL), incubated for 5 min at 37°C, and used to determine the optic densities E_{s1} and E_{cal1} for the sample and calibrator (provided by the manufacturer), respectively. Then 100 μL of reagent 2 was added and optical densities E_{s2} and E_{cal2} were measured for the sample and calibrator, respectively. The optic density (OD 650 nm) was determined using Tecan Infinite M200 PRO analyzer (Australia). The HDCL level was calculated using formula: $C = (\Delta E_s/\Delta E_{\text{cal}}) * C_{\text{cal}}$, where C is concentration of HDCL; $\Delta E_s = E_{s2} - E_{s1}$; $\Delta E_{\text{cal}} = E_{\text{cal2}} - E_{\text{cal1}}$; C_{cal} is 1.08 mM/L which is concentration of HDCL in calibration sample.

The HDCL concentrations were calculated using the formula: $C = (\Delta E_s/\Delta E_{\text{cal}}) * C_{\text{cal}}$, where C is concentration of HDCL; $\Delta E_s = E_{s2} - E_{s1}$; $\Delta E_{\text{cal}} = E_{\text{cal2}} - E_{\text{cal1}}$; C_{cal} is 1.08 mmol/L which is concentration of HDCL in calibration sample.

2.4. RT-PCR Detection of Puumala virus (PUUV) Transcripts. Total RNA from 100 μL of serum was extracted using TRIzol® reagent (Life Technologies, Carlsbad, CA, USA). cDNA was transcribed using Super Script kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Two rounds of PCR were conducted. PCR products were sequenced to confirm *Hantavirus* strain. Primers used were 5′-GTGAGAA ACACACCACAATACTATG-3′ forward and 5′-CTCTGCGT CGTTGGAGTCGTTC-3′ reverse, which amplifies the S segment RNA giving a first-round product of 328 bp, and 5′-CGGACACACAAAGGACAGGG-3′ forward and 5′-GACGCAGAGAAACACAAGTATAATA-3′ reverse, which amplified a second-round product of 302 bp.

2.5. Statistical Analysis. Statistical analysis was conducted using Minitab software (Minitab, State College, PA, USA); differences between the medians of compared groups were calculated using the Mann-Whitney test for nonparametric data and were considered significant at $P \leq 0.05$.

The differential abundance of metabolite concentration was calculated using the Subio Platform (Subio Inc., Kagoshima, Japan) with a differentiation expression threshold of 1.5-fold (t-test, $P < 0.05$).

3. Results

3.1. Clinical Presentation. Two hundred and thirty-six NE cases (190 male, 38 female) were recruited for this study (Table 1). The average period of hospitalization was 13.5 ± 2.7 days. NE diagnosis was based on clinical presentation, epidemiological data, and serological confirmation. Additionally, all 228 NE serum samples were analyzed for presence of PUUV RNA. PUUV RNA was detected in the initial serum samples in 49 cases (21.5%). At this time there were decreased platelet counts (159.4 ± 32.1), which, by the end of hospitalization, was restored to control levels (449 ± 40). Bleeding and varying degrees of blood coagulation disturbances were detected in 46 patients (20.1%). Six cases developed DIC (2.5%).

Table 1: Clinical characteristics of NE cases.

Variables	Value
Antibody titer (1st)	885 ± 115
Antibody titer (2nd)	2163.5 ± 263
Hospitalization (day)	13.5 ± 2.7
Serum urea (mmol/L)	11.6 ± 0.8
Serum creatinine (μmol/L)	148.8 ± 5.4
Thrombocytes (1st) (×1000 cells/μL)	105.1 ± 4.2
Thrombocytes (2nd) (×1000 cells/μL)	466.9 ± 29.9
Bleeding (number of patients)	46 out of 236
Sex (M/F)	198/38

Patients were grouped based on the presence of signs of disturbed hemostasis. Symptoms were evaluated to assign severity points: 0, absence of bleeding (100 cases; 42.4%); 1, few skin petechia (102 cases; 43.2%); 2, multiple skin petechia, scleral hemorrhages, and gastrointestinal bleeding (28 cases; 11.9%); and 3, DIC (6 cases; 2.5%).

Patients were also grouped based on disease severity based on clinical presentation, mild, moderate, or severe forms. Each presentation was assigned severity points: 0, mild (100 cases; 42.4%); 2, moderate (102 cases; 43.2%); and 3, severe (31 cases; 14.4%).

3.2. Serum Lipid Profile Analysis. Significant upregulation of triglycerides and HDCL was found in patients at the early stage of the disease (Table 2) compared to controls. In contrast, levels of triglycerides remained significantly upregulated in the late stage of the disease, whereas HDCL were similar to controls. Serum total cholesterol did not change and remained similar to controls.

It has been previously demonstrated that severe *Hantavirus* cases are characterized by a "lipid paradox," with serum cholesterol lower but triglycerides higher in HFRS cases than controls [21]. Therefore, we sought to determine whether similar serum lipid profiles are found in severe NE. A total of 6 NE patients (5 male, 1 female; age 30.5 ± 0.7 years) were identified as having severe NE based on the diagnosis of DIC (Table 3). Serum total cholesterol and HDCL levels did not differ from controls; however, triglycerides were significantly higher in severe NE cases than controls. These data support Clement et al. observation of the high triglycerides in severe NE cases [21].

3.3. Gender Differences in Serum Lipid and Cytokine Activation Profile in NE Patients. When serum lipid profiles

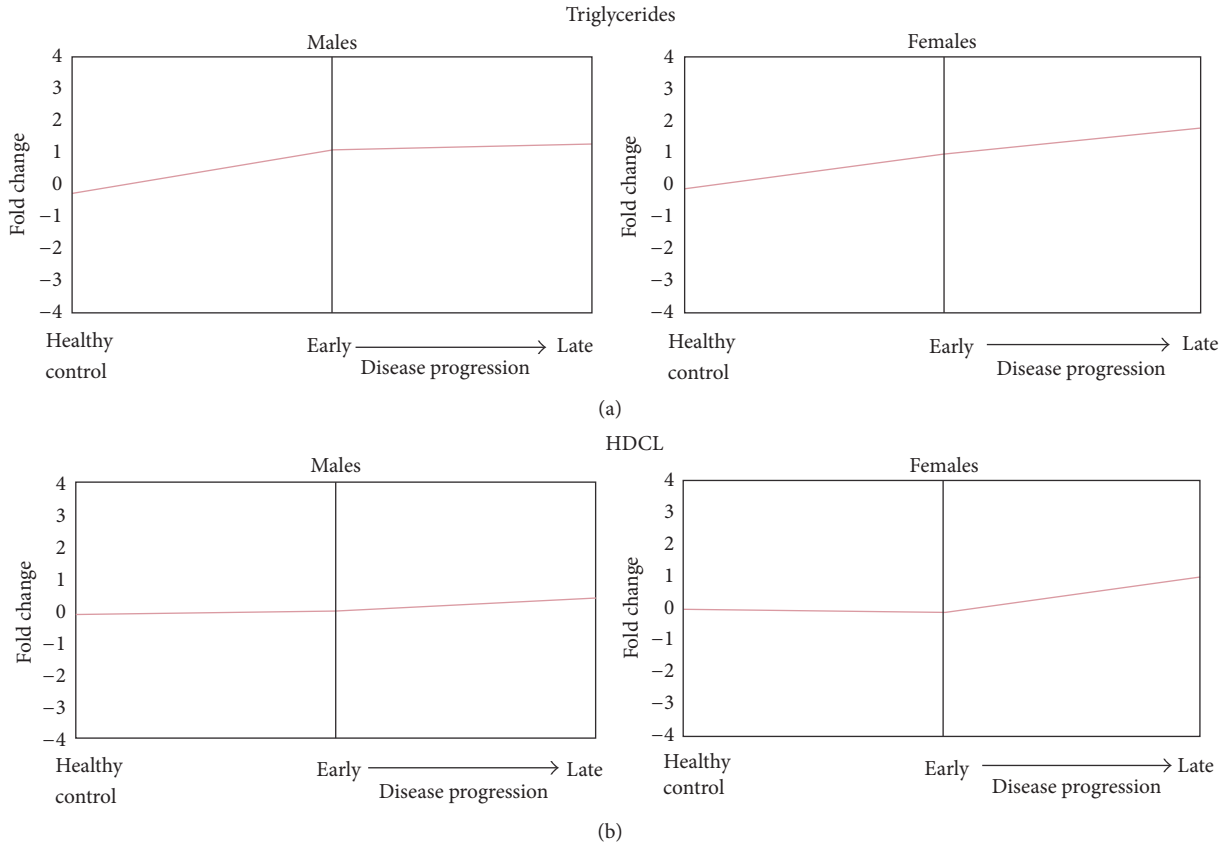

Figure 1: Subio analysis of triglycerides and HDCL in male and female NE cases. Differential abundance of the metabolites concentration was calculated using the Subio Platform (Subio Inc., Kagoshima, Japan) with a differentiation expression threshold of 1.5-fold (t-test, $P < 0.05$).

Table 2: Lipid profile in NE patients.

Analyte	Control	NE early stage	NE late stage	P
HDCL (mmol/L)	1.6 ± 0.1	1.2 ± 0.1	1.8 ± 0.9	*0.05
Total cholesterol (mmol/L)	3.6 ± 0.2	4.1 ± 0.5	4.2 ± 0.6	
Triglycerides (mmol/L)	1.7 ± 0.2	3.5 ± 0.1	3.5 ± 0.3	*0.0001; **0.0001

*P differences are between NE 1st and control.
**P differences between NE 2nd and control.

Table 3: Lipids analysis in NE cases with high bleeding score.

Analyte	Control	NE	P
HDCL (mmol/L)	1.6 ± 0.1	1.8 ± 0.6	
Total cholesterol (mmol/L)	3.6 ± 0.2	3.1 ± 0.3	
Triglycerides (mmol/L)	1.7 ± 0.2	2.8 ± 0.4	0.05

were analyzed based on gender, only male cases had significantly higher triglyceride levels than male controls (Table 4). Although upregulated in female NE serum, triglycerides did not differ significantly when compared to female controls. There were no differences between serum level of triglycerides and total cholesterol between male and female NE patients. HDCL levels were significantly lower in female NE compared to female controls, while HDCL was not significantly different in all male patients. Total cholesterol levels did not differ between male and female NE patients as well as between corresponding gender controls.

Serum triglyceride levels were significantly increased in NE patients as compared to controls (Figure 1). Also, triglyceride levels differed in NE males and females when compared to controls. It should be noted that once they increased in early stage of the disease, triglycerides remained steady through the convalescent stage in male patients, while female triglycerides continued increasing through early and late stages of the disease (Figure 1(a)). When gender differences in HDCL levels were analyzed, only a slight increase in HDCL level was detected in male NE patients compared to controls, while a steep increase of the HDCL was found in female patients (Figure 1(b)).

Further analysis revealed that more cytokines were significantly upregulated in females than male NE cases when compared to their corresponding controls (33 versus 9)

Table 4: Gender based lipid (mmol/L) and cytokine (pg/mL) analysis.

Analyte	Control female ($n = 15$)	NE female ($n = 38$)	Control male ($n = 30$)	NE male ($n = 190$)	P
HDCL	1.7 ± 0.2	1.2 ± 0.1	1.2 ± 0.2	1.2 ± 0.1	**0.001
Total cholesterol	3.5 ± 0.2	4.5 ± 0.5	3.9 ± 0.2	4.2 ± 0.1	
Triglycerides	1.7 ± 0.2	3.3 ± 0.5	1.6 ± 0.5	3.6 ± 0.2	*0.0001
IL-1Ra	8.0 ± 2.9	68.5 ± 13.7	68.3 ± 13.5	126.5 ± 26.6	**0.001
IL-2Ra	32.9 ± 4.9	101.6 ± 20.4	1.0 ± 0.2	120.1 ± 26.3	**0.0001
IL-3	74.1 ± 4.5	137.5 ± 20.7	45.9 ± 12.6	124.4 ± 10.9	**0.0001; *0.05
IL-4	0.8 ± 0.1	13.2 ± 4.5	1.5 ± 0.2	13.2 ± 1.9	**0.001
IL-5	0.4 ± 0.1	4.0 ± 1.0	1.2 ± 0.2	5.9 ± 0.9	**0.05
IL-6	1.4 ± 0.3	35.2 ± 0.9	3.7 ± 0.7	34.2 ± 8.4	**0.01
IL-7	1.7 ± 0.6	7.4 ± 2.1	2.1 ± 0.4	12.8 ± 2.8	**0.05
IL-8	10.8 ± 2.8	31.1 ± 6.9	44.3 ± 13.9	45.8 ± 9.0	**0.001
IL-9	2.5 ± 0.4	46.9 ± 15.0	4.5 ± 1.4	64.9 ± 16.8	**0.05
IL-10	2.6 ± 1.1	22.4 ± 4.4	4.8 ± 0.9	39.6 ± 6.2	**0.001
IL-12(p40)	110.8 ± 13.8	227.3 ± 43.7	68.0 ± 34.6	186.8 ± 16.2	**0.01; *0.05
IL-12(p70)	5.4 ± 0.7	25.2 ± 8.4	2.0 ± 0.5	29.1 ± 3.9	**0.05; *0.05
IL-13	1.4 ± 0.2	23.5 ± 7.8	1.5 ± 0.2	23.8 ± 4.1	**0.05
IL-15	4.0 ± 0.6	32.5 ± 12.7	8.6 ± 2.7	33.7 ± 9.4	**0.05
IL-17	2.8 ± 1.0	34.7 ± 20.5	1.7 ± 0.4	25.8 ± 4.9	
IL-18	12.4 ± 2.4	25.2 ± 7.2	5.7 ± 1.9	21.9 ± 2.5	**0.0001
CCL2	12.2 ± 1.5	124.1 ± 88.7	16.9 ± 3.6	40.9 ± 4.0	$^{\&}$0.05
CCL4	4.8 ± 0.8	571.6 ± 188.5	18.6 ± 6.7	685.0 ± 109.8	**0.5
CCL5	33.1 ± 3.0	2140.8 ± 720.1	86.8 ± 21.6	2288.8 ± 316.9	**0.01
CCL11	6.4 ± 1.3	67.8 ± 23.1	19.1 ± 4.3	64.3 ± 6.7	**0.5
CCL27	170.9 ± 11.0	74.3 ± 11.7	72.8 ± 11.4	59.1 ± 5.4	**0.0001
CXCL1	54.6 ± 10.3	67.2 ± 29.2	11.5 ± 3.3	23.5 ± 5.4	**0.01; $^{\&}$0.01
CXCL9	131.9 ± 25.2	1646.9 ± 776.2	80.0 ± 18.6	1251.3 ± 124.6	
CXCL10	25.8 ± 3.3	3318.7 ± 806.2	60.4 ± 15.9	2520.8 ± 295.6	**0.001; *0.01
G-CSF	8.2 ± 1.6	19.6 ± 2.6	8.3 ± 1.0	28.7 ± 2.6	**0.001; *0.01
HGF	126.2 ± 19.6	311.6 ± 83.9	64.1 ± 24.1	334.4 ± 33.8	**0.01; *0.05
IFN-α	10.7 ± 0.8	25.1 ± 8.6	6.3 ± 2.3	18.2 ± 2.8	**0.0001
IFN-γ	12.9 ± 2.8	53.0 ± 7.6	57.4 ± 10.3	78.8 ± 8.3	**0.001
MIF	152.2 ± 29.7	262.2 ± 98.6	144.3 ± 63.5	218.4 ± 19.6	**0.05
PDGF	75.9 ± 11.8	1556.2 ± 453.9	213.9 ± 55.1	5871.4 ± 4204.5	**0.001
SCGF-bb	2171.1 ± 405.2	3577.2 ± 532.4	678.8 ± 137.2	5139.9 ± 471.0	**0.0001; *0.001
SDFa	78.2 ± 4.5	74.5 ± 16.5	19.8 ± 6.2	84.8 ± 17.4	**0.0001
TRAIL	19.1 ± 3.6	51.6 ± 11.4	12.9 ± 5.4	33.4 ± 2.6	**0.0001; *0.05; $^{\&}$0.05
VEGF	11.8 ± 1.8	103,2 ± 35.1	22.2 ± 7.5	163.8 ± 28.2	**0.05

$^{**}P$ value between female control and female NE; $^{*}P$ value between male control and male NE; $^{\&}P$ value between male and female NE.

(Table 4). Interestingly, IFN-γ and IL-12(p40) were both upregulated in female NE cases, while only IL-12(p40) was significantly higher in male NE cases. Additionally, female cases had significantly lower scores for disease severity (1.9 ± 0.2 versus 2.0 ± 0.1; $P < 0.05$) and hemorrhagic disturbances (0.2 ± 0.1 versus 0.5 ± 0.1; $P < 0.05$) (Table 5). Although not significant, thrombocyte counts in female patients were higher than in males. IFN-γ and IL-12 are cytokines that play a key role in the activation of Th1 lymphocytes [22, 23]. Upregulation of these cytokines in female NE suggests that there is activation of Th1 lymphocytes. Previously, we have shown an association between the mild form of NE and elevated serum IFN-γ and IL-12 [24]. Therefore, these data suggest that lower severity scores in female cases are associated with activation of Th1 type immune response.

Compared to males, female NE cases had significantly higher levels of TRAIL, CXCL1, and CCL2. These cytokines

Table 5: Clinical presentation of NE patients based on gender.

Criteria	NE female ($n = 38$)	NE male ($n = 190$)	P
Severity score	1.9 ± 0.02	2.0 ± 0.1	<0.05
Hemorrhagic syndrome score	0.2 ± 0.1	0.5 ± 0.1	<0.05
Thrombocytes (×1000 cells/μL)	125.7 ± 6.7	101.2 ± 6.0	<0.1

stimulate mononuclear leukocyte chemotaxis, apoptosis, and angiogenesis. This indicates that differences in clinical presentation between NE in males and females are associated with gender-dependent activation of these cytokines.

3.4. Serum Lipid and Cytokine Activation Analysis Based on Thrombocyte Count. Next, changes in serum lipid and cytokines were analyzed based on thrombocyte counts. Thrombocyte counts below 50,000 cells/μL are considered to be a risk factor for bleeding [25–27]. Recently, a correlation was demonstrated between thrombocyte counts and low density lipids in patients with the severe form of dengue infection [28], suggesting a role for lipid metabolism in the pathogenesis of thrombocytopenia. Therefore, we sought to determine whether low thrombocyte counts were associated with changes in lipid and cytokine profiles of NE cases. NE patients were separated into two groups: low (<50,000 cells/μL) and high (>50,000 cells/μL) thrombocyte counts. Disease severity scores did not differ between patients with high and low thrombocyte counts (Table 6). Neither serum total cholesterol nor triglyceride levels differed between these two groups of patients, although serum levels for each lipid were higher than control (Table 7). However, HDCL was significantly increased in NE cases with low thrombocyte counts, while in NE patients with high thrombocyte counts HDCL was significantly decreased.

Analysis of serum cytokines revealed that 30 cytokines were upregulated in patients with low thrombocyte counts, whereas 27 cytokines were upregulated in NE cases with high thrombocyte counts. Overall the activation pattern was similar between groups, with 24 cytokines being upregulated in both groups (Table 7). However, patients with high thrombocyte counts had significantly lower levels of 18 cytokines compared to patients with low thrombocyte counts. One of the most interesting observations was that serum IFN-γ and IL-12(p40) were significantly higher in patients with high thrombocyte counts relative to those with low numbers of platelets. One possible explanation of these data is that IFN-γ and IL-12(p40) may contribute to thrombocyte protection, preventing hemorrhaging and bleeding. Additionally, significantly higher levels of IL-3 and IFNα were found in patients with high thrombocyte counts. Furthermore, serum levels of IFN-α differed significantly between the two groups of patients with different thrombocyte counts. Since the activity of IFN-α and IL-3 overlaps, targeting dendritic cell proliferation (IL-3) and innate protection (IFN-α), this suggests that activation of dendritic cells may play a role in preventing the drop of thrombocyte counts in NE.

3.5. Analysis of Lipid and Cytokine Serum Profile in NE Patients with High or Low VEGF Levels. Studies have shown a correlation between VEGF serum levels and the concentrations of cholesterol and triglycerides [29, 30], with high triglycerides and cholesterol associated with elevated VEGF, presumably due to endothelial damage. Therefore, we sought to determine whether lipid profiles differed in patients with high or low serum VEGF. NE patients were divided into two groups, with the highest control VEGF concentration (93 pg/mL) used as a separation marker (Table 8). The NE group with low VEGF levels was characterized by significantly lower thrombocyte counts as compared to NE cases with high VEGF. Furthermore, the low VEGF group had higher bleeding severity scores relative to the high VEGF group, although differences were not statistically significant. Interestingly, all 6 DIC cases were grouped in the low VEGF category, suggesting that low VEGF is associated with a higher chance of developing severe bleeding. Both NE groups had high serum triglycerides compared to controls; however, significantly increased concentrations of cholesterol and triglycerides were found in the high VEGF group relative to the low VEGF group. Although HDCL did not differ from that in control in either NE group, they differed significantly between the NE groups, with significantly lower HDCL in the high VEGF group.

Cytokine analyses revealed more cytokines upregulated (32 out of 48) in the high VEGF group than the low VEGF group (26 out of 48) (Table 9). Although the overall pattern of cytokine activation was similar between the two groups, 26 cytokines had significant upregulation in the high VEGF group when compared to the low VEGF group. It appears that patients with high VEGF levels have more pronounced activation of Th1 cytokines, since elevated IFN-γ, a cytokine produced by Th1 lymphocytes, was characteristic of the group. Though serum levels of IL-12(p40) were similar between two groups of patients, high VEGF patients had significantly higher levels of IL-12(p70), a IL-12(p40) precursor.

4. Discussion

Serum triglycerides were elevated in NE cases. These data support previous observations of Mustonen et al., who demonstrated that high triglyceride concentrations were present in NE serum [20]. Interestingly, serum levels of cholesterol remained unchanged in the NE cases enrolled in our study, in contrast to other studies, where cholesterol was lower in NE as compared to controls [20]. Our data suggest that there is an association between increased triglycerides and severe clinical presentation of NE. This observation supports the conclusions of Clement et al., who demonstrated increased fasting triglyceride concentrations in *Hantavirus* patients [21] and further that increased levels of triglycerides were more pronounced in severe cases. Our data confirm high triglyceride concentrations in severe NE cases relative to controls. We have demonstrated that triglycerides were significantly increased in male patients, who also had higher severity scores as compared to female NE patients. In fact, out of 6 patients diagnosed with severe complications, that is,

Table 6: Clinical presentation of NE patients with high (>50,000) and low (<50,000) thrombocyte counts.

Clinical feature	Low thrombocytes	High thrombocytes	P
Severity score	2.1 ± 0.1	2.0 ± 0.01	
Hemorrhagic syndrome score	0.5 ± 0.1	0.5 ± 0.1	
Thrombocyte counts (×1000 cells/μL)	46.5 ± 1.6	123.5 ± 7.4	<0.0001

Table 7: Lipids (mmol/L) and cytokine (pg/mL) analysis based on thrombocytes (×1000 cells/μL) counts (low < 50.000 versus high > 50.000).

Analyte	Control (n = 56)	Thrombocyte low (n = 58)	Thrombocyte high (n = 180)	P
HDCL	1.6 ± 0.1	1.9 ± 0.1	1.1 ± 0.1	*0.05; **0.05
Total cholesterol	3.6 ± 0.1	4.1 ± 0.2	4.1 ± 0.2	*0.05
Triglyceride	1.7 ± 0.2	3.7 ± 0.4	3.4 ± 0.2	*0.05; **0.0001
IL-1a	1.3 ± 0.1	0.1 ± 0.03	0.2 ± 0.03	*0.01; **0.0001
IL-1Ra	29.3 ± 3.1	94.4 ± 14.8	87.7 ± 10.9	*0.01; **0.01
IL-1b	0.8 ± 0.1	25.6 ± 5.0	12.2 ± 2.7	**0.01; $^{\&}$0.05
IL-2	1.8 ± 0.3	29.8 ± 9.6	23.11 ± 10.9	*0.01
IL-3	66.6 ± 5.8	100.5 ± 15.6	117.4 ± 13.4	*0.05; **0.05
IL-4	1.1 ± 0.1	30.6 ± 6.5	12.7 ± 2.3	*0.01; **0.01; $^{\&}$0.01
IL-5	1.0 ± 0.2	12.5 ± 3.0	6.0 ± 0.9	*0.01; **0.001; $^{\&}$0.01
IL-6	2.9 ± 0.6	47.1 ± 13.5	37.5 ± 12.5	*0.01
IL-8	37.9 ± 14.9	102.9 ± 18.7	32.5 ± 4.5	$^{\&}$0.001
IL-9	3.5 ± 0.5	105.9 ± 18.8	77.5 ± 25.9	*0.001
IL-10	3.6 ± 0.6	68.7 ± 12.1	35.7 ± 7.9	*0.001; **0.05; $^{\&}$0.05
IL-12(p40)	88.1 ± 12.1	163.0 ± 23.7	185.8 ± 20.3	*0.05; **0.01
IL-12(p70)	3.7 ± 0.5	54.2 ± 12.9	27.4 ± 4.2	*0.01; **0.001; $^{\&}$0.05
IL-13	1.3 ± 0.1	42.9 ± 10.2	20.1 ± 3.4	*0.001; **0.001; $^{\&}$0.01
IL-15	5.4 ± 0.8	49.7 ± 12.8	38.7 ± 14.5	*0.01
IL-17	2.3 ± 0.5	46.6 ± 7.9	20.7 ± 3.4	*0.001; **0.01; $^{\&}$0.001
IL-18	8.6 ± 1.4	$^{\&}$19.7 ± 4.0	15.9 ± 2.6	
CCL2	13.0 ± 1.3	60.4 ± 9.2	66.8 ± 28.3	*0.01
CCL3	0.9 ± 0.3	94.3 ± 23.8	31.2 ± 6.9	*0.001; **0.01; $^{\&}$0.001
CCL4	10.3 ± 2.0	1946.8 ± 396.7	634.1 ± 108.6	*0.001; **0.001; $^{\&}$0.0001
CCL5	60.9 ± 8.4	5078.9 ± 910.5	2484.4 ± 422.1	*0.001; **0.001; $^{\&}$0.01
CCL11	15.5 ± 2.8	117.3 ± 19.9	55.9 ± 7.8	*0.001; **0.001; $^{\&}$0.001
CCL27	122.7 ± 9.3	$^{\&}$32.9 ± 7.3	55.5 ± 6.1	**0.0001
CXCL9	126.3 ± 18.2	$^{\&}$1720.1 ± 333.7	1334.3 ± 270.4	*0.001; **0.01
CXCL10	49.1 ± 6.9	5912.8 ± 929.9	2797.1 ± 381.1	*0.001; **0.0001; $^{\&}$0.001
G-CSF	8.1 ± 0.9	37.6 ± 4.7	23.6 ± 2.1	*0.01; **0.0001; $^{\&}$0.01
GM-CSF	2.6 ± 0.7	34.6 ± 6.7	16.5 ± 3.7	*0.01; **0.05; $^{\&}$0.05
HGF	95.9 ± 13.1	455.6 ± 122.6	325.3 ± 38.5	*0.01; **0.001
IFN-α	15.2 ± 1.2	$^{\&}$12.8 ± 1.7	20.3 ± 1.5	**0.01; $^{\&}$0.01
IFN-γ	32.6 ± 4.1	62.9 ± 8.3	63.8 ± 5.4	*0.05; **0.001
MIF	145.7 ± 24.6	$^{\&}$235.4 ± 28.6	257.4 ± 38.5	*0.05
PDGF	144.5 ± 22.8	3282.7 ± 500.3	1644.0 ± 230.3	*0.001; **0.0001; $^{\&}$0.01
SCGF	1569.1 ± 234.5	$^{\&}$6499.8 ± 939.4	5149.5 ± 617.8	*0.05; **0.001
TRAIL	15.8 ± 2.4	$^{\&}$33.4 ± 5.2	38.7 ± 4.5	*0.01; **0.01
VEGF	15.2 ± 2.3	249.0 ± 59.6	107.8 ± 18.0	*0.01; **0.01; $^{\&}$0.01

$^{\&}P$ between groups.
$^{*}P$ between thrombocyte counts < 50 patients and control.
$^{**}P$ between thrombocyte counts > 50 patients and control.

Table 8: Severity score based on serum VEGF (high > 93 pg/mL; low < 93 pg/mL).

Analyte	NE low VEGF	NE high VEGF	P
Severity score	2.0 ± 0.01	2.1 ± 0.04	<0.05
Hemorrhagic syndrome score	0.5 ± 0.06	0.3 ± 0.05	
Thrombocytes (×1000 cells/μL)	118.7 ± 6.5	226.6 ± 9.0	<0.01

having DIC, 5 were male. However, unlike the observations of Clement et al., in this study serum cholesterol remained unchanged in NE patients compared to controls. It remains to be determined whether these discrepancies in cholesterol levels are related to regional or national differences in serum lipid level.

We have demonstrated that increased serum IFN-γ and IL-12 are associated with the mild form of NE [24]. These data confirm this observation, with a lower NE severity score being associated with higher levels of these cytokines. It should be noted that triglycerides were lower in patients with the severe form of the disease. Although little is known about triglycerides and cytokine activation, it is generally accepted that hyperlipidemia is associated with activation of proinflammatory cytokines, IL-6 in particular. For example, Göçmen et al. have demonstrated that hyperlipidemia is associated with increased serum IL-6, and furthermore, increases in IL-6 were found in obesity characterized by dyslipidemia [31]. A potential mechanism connecting triglycerides and IL-6 was proposed by Valdearcos et al. [31, 32]. These authors demonstrated that lipin-2 may be involved in regulation of triglyceride concentrations and activation of the proinflammatory cytokines TNF-α and IL-6. Depletion of lipin-2 increased expression of the IL-6 and TNF-α, while its overexpression reduced the release of proinflammatory cytokines. Interestingly, cytokine activation was associated with the ability of lipin-2 to modulate the cellular content of triglyceride, with upregulation of cytokines being associated with high lipid content.

Interestingly, we found significantly higher levels of IL-6 in patients with increased triglycerides in NE cases with higher severity scores. Several studies have shown that the severe form of NE is associated with elevated IL-6 [33–35]. Since IL-6 upregulation could be associated with high serum triglycerides, it could be suggested that metabolic syndrome in NE cases may contribute to proinflammatory cytokine activation, particularly IL-6. High IL-6 could alter lymphocyte differentiation, since IL-6 is known to skew T cell differentiation towards TH2 and TH17 [36–38]. Promoting lymphocyte proliferation in more pathogenic Th17 helpers may have a devastating impact on tissue integrity and explain NE pathogenesis. If this is the case, our data on upregulation of IFN-γ and IL-12 in mild NE cases suggests a protective role for Th1 lymphocytes in NE.

Thrombocyte counts are a predictive marker for bleeding [39, 40]. NE is characterized by low thrombocyte counts, which present clinically with petechia, scleral bleeding, gastrointestinal bleeding, or DIC. Since we found no association between triglyceride concentration and thrombocyte counts, this suggests that hypertriglyceridemia and thrombocytopenia are independent events. However, we found an association between high thrombocyte counts and upregulation of the Th1 type cytokines, IFN-γ and IL-12(p40). Therefore, we suggest that activation of Th1 lymphocytes is protective in NE patients, interfering with thrombocytopenia. Supporting this hypothesis the data of Diehl and Rincón [38] demonstrated a protective role of the Th1 response, with IFN-γ or IL-12 deficient mice being highly susceptible to dengue infection [41]. IFN-$\gamma^{-/-}$ and IL-12$^{-/-}$ animals developed a lethal infection, which was characterized by severe thrombocytopenia. The mechanism of IFN-γ and IL-12 protective roles remains to be investigated.

Significantly higher serum VEGF levels were found in NE patients with increased concentrations of triglycerides. Increased VEGF levels have been demonstrated in hypercholesterolemic patients and in animals fed a high fat diet [29, 30]. Importantly, our data is the first to show an association between hypertriglyceridemia and increased VEGF serum level in NE cases. VEGF can play dual role in regulating endothelial integrity. For example, VEGF can be protective, promoting endothelial cell survival [30, 42]. However, VEGF can also increase endothelial permeability and promote vascular leakage [43]. Studies using animals fed a high fat diet suggest that different VEGF family members vary in their association with hyperlipidemia. It has been demonstrated that VEGF-C, but not VEGF-A, is closely connected to dyslipidemia in animals [30]. Therefore, this suggests that NE cases with high and low triglycerides may differ in levels of different VEGFs. Future studies will determine whether NE cases vary in the upregulation of different VEGFs and how their expression affects disease progression and triglyceride concentrations.

In conclusion, our data confirms previous observation of increased triglycerides in NE cases. This study advances our understanding of the role of triglycerides in NE pathogenesis by demonstrating an association between high triglycerides and patient gender, severity score of the disease, thrombocyte counts, and serum VEGF level. Additionally, our data suggest that low triglycerides are associated with upregulation of Th1 helper cells.

Competing Interests

The authors declare that they have no competing interests.

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Table 9: Lipids (mmol/L) and cytokine (pg/mL) analysis based on high (>93 pg/mL) or low (<93 pg/mL) serum VEGF level.

Analyte	Control	VEGF high	VEGF low	P (between groups)
HDCL	1.6 ± 0.1	0.9 ± 0.3	1.3 ± 0.1	*0.001
Total cholesterol	3.6 ± 0.1	4.7 ± 0.3	3.9 ± 0.1	*0.01
Triglycerides	1.7 ± 0.2	&3.9 ± 0.3	&3.2 ± 0.1	*0.05
IL-1b	0.8 ± 0.1	&39.2 ± 5.2	2.9 ± 0.6	*0.0001
IL-1Ra	31.4 ± 5.5	&296.3 ± 83.3	&87.6 ± 12.5	*0.001
IL-2	1.8 ± 0.3	&56.2 ± 19.1	4.2 ± 0.6	*0.0001
IL-2Ra	29.3 ± 3.2	&203.1 ± 73.8	&101.7 ± 9.2	*0.05
IL-3	66.6 ± 6.0	&166.8 ± 38.6	&154.7 ± 15.9	
IL-4	1.1 ± 0.1	&36.9 ± 4.5	2.7 ± 0.4	*0.0001
IL-5	1.0 ± 0.2	&13.8 ± 2.1	&2.3 ± 0.2	*0.0001
IL-6	2.9 ± 0.7	&96.5 ± 22.6	&11.0 ± 1.1	*0.0001
IL-7	2.1 ± 0.4	&31.6 ± 7.2	&3.93 ± 0.5	*0.0001
IL-8	37.9 ± 15.7	&100.7 ± 23.3	24.0 ± 2.4	*0.0001
IL-9	3.5 ± 0.5	&173.7 ± 43.8	&19.5 ± 3.0	*0.0001
IL-10	3.6 ± 0.7	&99.2 ± 21.5	&20.7 ± 3.9	*0.0001
IL-12(p40)	88.1 ± 12.7	&233.2 ± 41.6	&228.5 ± 17.6	
IL-12(p70)	3.7 ± 0.5	&75.7 ± 9.4	9.5 ± 1.8	*0.0001
IL-13	1.3 ± 0.1	&6.5 ± 10.2	&6.6 ± 0.7	*0.0001
IL-15	5.4 ± 0.9	&91.8 ± 25.2	&10.9 ± 1.2	*0.0001
IL-17	2.3 ± 0.6	&73.9 ± 15.4	6.4 ± 1.8	*0.0001
IL-18	8.6 ± 1.5	&25.2 ± 5.5	&23.4 ± 2.3	
CCL2	13.0 ± 1.3	&142.5 ± 48.8	&25.2 ± 2.7	*0.0001
CCL5	60.9 ± 8.8	&6584.9 ± 658.2	412.7 ± 107.6	*0.0001
CCL11	15.5 ± 2.9	&157.5 ± 18.5	&33.9 ± 3.5	*0.0001
CCL27	122.7 ± 9.8	&39.3 ± 6.6	&65.4 ± 5.2	*0.01
CXCL9	126.3 ± 19.1	&1844.2 ± 476.7	&1331.7 ± 115.8	
CXCL10	49.1 ± 7.2	&6741.3 ± 652.5	&1058.7 ± 120.4	*0.0001
G-CSF	8.1 ± 0.9	&50.9 ± 4.7	17.7 ± 1.0	*0.0001
G-MCSF	2.6 ± 0.7	&474 ± 7.8	&6.7 ± 0.8	*0.0001
HGF	95.9 ± 13.7	&433.8 ± 59.4	&283.0 ± 2.4	*0.05
IFN-γ	32.6 ± 4.4	&139.8 ± 32.2	&76.4 ± 6.7	*0.01
M-CSF	1.5 ± 0.1	11.9 ± 4.9	&3.0 ± 0.3	
MIF	145.7 ± 25.9	&393.1 ± 71.4	&342.1 ± 45.1	
PDGF-b	144.6 ± 24.0	15675.3 ± 11482.3	&626.6 ± 73.4	*0.05
SCGF	1569.1 ± 246.8	&7425.2 ± 1016.7	&5874.4 ± 605.3	
TRAIL	15.8 ± 2.5	&41.3 ± 6.9	&42.2 ± 3.5	
VEGF	15.2 ± 2.5	&452.3 ± 67.7	&28.2 ± 1.8	*0.0001

&Significant differences between group and control.

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