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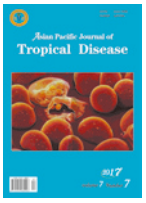

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Serum interferon-gamma and interleukin-4 in patients with brucellosis before and after treatment

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ABSTRACT

Objective: To evaluate serum levels of interferon-gamma (IFN-γ) and interleukin-4 (IL-4) in patients with brucellosis before and after treatment.
Methods: In this cross-sectional study 30 patients (18 males and 12 females) were studied. Serum levels of IFN-γ and IL-4 were measured before and after treatment by ELISA. Inclusion criteria were 2-mercaptoethanol ⩾ 1/40 and Wright ⩾ 1/80.
Results: The mean age of patients was (37.20 ± 16.15) years. Patients with the positive history of non-pasteurized dairy consumption and contact with animals were 25 (83.3%) and 19 patients (63.3%), respectively. The average serum levels of IFN-γ and IL-4 before treatment were (1 109.34 ± 322.51) pg/mL and (385.15 ± 115.68) pg/mL and after treatment were (253.00 ± 132.45) pg/mL and (228.01 ± 71.17) pg/mL, respectively ($P < 0.001$). Increased levels only observed in the case of IFN-γ before treatment (60%). Decrement of IL-4 level was significantly higher in patients with a history of contact with livestock. There was no significant correlation between these two parameters and age, sex, and dairy consumption.
Conclusions: Based on the findings of this study, IFN-γ and IL-4 serum levels significantly reduced after treatment. A significant reduction in the level of serum IL-4 after treatment seem to rise the importance of the individuals' sensitizing history due to previous contact.

1. Introduction

Brucellosis is a common disease between humans and animals and is one of the main reasons of morbidity in these two groups. About 15 million new cases are diagnosed yearly in the world. Some countries such as Peru and Mexico in Latin America, Spain and Greece in Europe, Iraq, Iran, Jordan and Kuwait in the Middle East, are known as hyper endemic areas which have more than 4 000 newly diagnosed cases each year. Most prevalent type of *Brucella* is *Brucella melitensis*[1].

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The study was carried out after getting the approval of Ethic Committee of Zanjan University of Medical Science, and written consent of patients was obtained for participating in the investigation.

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Brucellosis can involve different types of domestic animals. Four species mostly can cause disease in humankind which include *Brucella abortus*, *Brucella melitensis*, *Brucella suis* and *Brucella canis*[2].

Brucellosis disease is seen in three forms: acute, sub-acute and chronic. Due to nonspecific signs and symptoms of brucellosis, Para clinic can be a big help for diagnosis. Definite diagnosis is by obtaining microorganism but it needs 30–40 days. Serum agglutination test is the simplest and the most common test for diagnosis. Both IgM and IgG are measured in this test. Polymerase chain reaction test is one of the most precise, sensitive and rapid tests, but it has not been standardized and is not common yet[3-5].

Interferon-gamma (IFN-γ) is a dissolved dimerized cytokine that is the only member of type II interferon's family. IFN-γ is an important macrophage activator and MHC I inducer. This cytokine is mostly produced by natural killer cells (NK cells) and natural killer T cells (NKT cells) as one of the natural immunity steps.

It is important in acquired immunity, too, and is produced by T helper (Th) CD4+ cells and cytotoxic T lymphocytes. Functions of IFN-γ can be summarized as below: NK cells activity progression, enhancement of antigen presenting and lysosomal activity of macrophages, activation of nitric oxide synthase enzyme, induction of IgG2a and IgG3 production by B cells, increment of T helper 1 cells differentiation, induction of MHC I and II expression in NK cells and antigen presenting cells (APCs), respectively, enhancement of required connecting and sticking abilities of leukocytes for migration and internal defensive factors induction[6].

Interleukin 4 (IL-4) induces differentiation of T helper 0 cells to T helper 2 cells. Th2 cells produce more IL-4 after stimulation by IL-4 and create a positive feedback loop. So, the cell which primarily produces IL-4 is not recognized correctly, however, some studies have introduced basophils as the cornerstone of this defect[7,8].

IL-4 stimulates B cells for producing IgE and enhances production of MHC class II cells. Studies have shown that IL-4 reduces or inhibits production of Th1 cells, macrophages, IFN-γ and dendritic cells' IL-12[9].

Basal mechanisms of APCs in brucellosis are phagocytosis and autophagy, anti-microbial cationic peptides, oxidative burst, IFN-γ and IL-12 production and chemokine releases. Th1 role in producing acquired immunity in brucellosis is approved by these findings: inability of mice without main Th1 constituents such as IFN-γ and IL-12, for defense against brucellosis, creating immunity against brucellosis even after injection of Th1 constituents such as IFN-γ and IL-12 to them, predominantly secretion of IFN-γ by mouse splenocytes, T CD4+ cells, human T and mononuclear cells in facing with *Brucella* antigens, responses of Th1 in patients with brucellosis and correlation between responses of Th1 and chronic or relapsing disease, and hypersensitivity of humans which have polymorphisms or mutations of Th1 cell immunity mediated molecules[10].

Immune response of Th1 to *Brucella* causes IFN-γ secretion by antigen specific T cells. Measurement of IFN-γ and IL-4 level before and after treatment can help us better understand mechanisms of cell survival and immune system response to these microorganisms, and it can also be helpful for determining the responses of the patients with brucellosis to drug treatment. So, measuring the producing level of these two cytokines can be a great tool for assessment of immune responses to pathogens, vaccines and immunity challenges. For the sake of *Brucella*'s high prevalence, identification of intercellular survival mechanism of *Brucella* can help us for better treatment and eradication.

2. Materials and methods

2.1. Study design

This study has designed as a cross-sectional study. The study was carried out after getting the approval of Ethic Committee of Zanjan University of Medical Science, and written consent of patients was obtained for participating in the investigation.

2.2. Participants

All patients who recoursed to Zanjan University of Medical Science's hospitals with brucellosis were asked to sign a written consent if they wished and then were engaged in study. Criteria for approving active brucellosis in patients were elevation of brucellosis agglutination titer by 4 folds or more, 2 weeks after primary test, or titer of 2-mercaptoethanol (2ME) test $\geqslant$ 1:160. Patients should have had one below conditions to be engaged: age between 12 and 65 years old, titer of Right test $\geqslant$ 1:160 (it is considered 1:80 in Iran for its endemicity), titer of Coombs-Right test $\geqslant$ 1:40, positive result for blood or bone marrow *Brucella* culture, concomitant clinical symptoms with one of the previous criteria plus first time brucellosis. They all should have signed written consent. Immune suppressed patients and who had used immunosuppressive drugs plus patients who did not have aforementioned criteria were all excluded.

2.3. Measurement

According to the statistical formulas, study population was calculated as 30 individuals. We obtained venous blood samples of all patients in fasting mode at the beginning and end of the treatment. The sample size was 5 mL and stored in pipes without anticoagulant agents. We stockpiled the samples at –70 ℃ until the laboratory protocols carried out. IL-4 and IFN-γ were measured in blood samples, concomitantly, by the Crystal Day Biotech laboratory kit which was made in china. We gathered demographic and clinical properties by a designed questionnaire. Sensitivity for IFN-γ measurement was 0.49 ng/mL and for IL-4 was 1.13 ng/mL. Normal serum level for both was considered in the range of 15.6–1 000 pg/mL.

2.4. Bias

There were two sources of bias included patients' lack of cooperation in giving blood sample, and patients' lack of punctuality in referral times through therapeutic period. We tried to inform patients and describe their duty for overcoming to these biases.

2.5. Study size

Study population size was calculated by below formula, in which a = 0.05 and B = 0.20. According to Akbulut *et al*., m_1 was average level of IL-4 before treatment which was equal to 1.6 and m_2 was for after treatment and was equal with 2.2[11].

$$N = [Z_{1-a/2} - Z_{1-B}]^2 [s_1^2 + s_2^2]/(m_1 - m_2)^2$$

2.6. Statistical analysis

Data were analyzed by frequency tables, central indicators, dispersion, Paired T-test and Pearson correlation test by SPSS version 16.0 software. Normal distribution of data was approved by Kolmogorov-Smirnov test and QQ curve. $P \leqslant 0.05$ was considered statistically meaningful.

3. Results

A total of 30 patients including 18 males and 12 females with average age of (37.2 ± 16.1) years old (16–84) participated in the study. Dairy consumption history in 25 patients (83.3%) was positive and was negative in 5 patients (16.7%). Contact with livestock was positive in 19 (63.3%) and negative in 11 patients (36.7%).

Average base level of IFN-γ was (1 109.3 ± 322.5) pg/mL (453.8–1 806.5) with 1 029.6 as median and 25% to 75% were 899.8 and 1 366.3. Study's end measures were as follows, average IFN-γ level was (253.00 ± 132.50) pg/mL (56.6–601.5), median was 248.5, 25% to 75% were 144.3 and 341.1.

Average diminishing in IFN-γ level after treatment was (856.3 ± 299.9) pg/mL (397.2–1 579.8). According to Paired T-test result, decreasing in IFN-γ level after treatment was statistically meaningful ($P < 0.001$) (Table 1).

The average base IL-4 level was (385.15 ± 115.70) pg/mL (173.4 to 632.1) with 410.2 as median and 25% to 75% were 312.5 and 474.6. Study's end measures were as follows, average IL-4 level was (228.01 ± 71.10) pg/mL (76.0 to 361.5), median was 233.4, 25% to 75% were 191.2 and 280.6.

Average diminishing in IL-4 level after treatment was (156.2 ± 137.4) pg/mL (110.4 to 434.1). According to Paired T-test result, decreasing in IL-4 level after treatment was statistically meaningful. ($P < 0.001$) (Table 1).

The abnormal base level of IFN-γ (increased) recorded in 18 patients, while, there was not any abnormal increment in base level of IL-4 (Figure 1).

Final amounts of both parameters were in normal range. Correlation between patients' age and changes of IFN-γ and IL-4 level was evaluated by Paired T-test. Although differences were meaningful in each sex, there was not any meaningful difference between two sexes (Table 2).

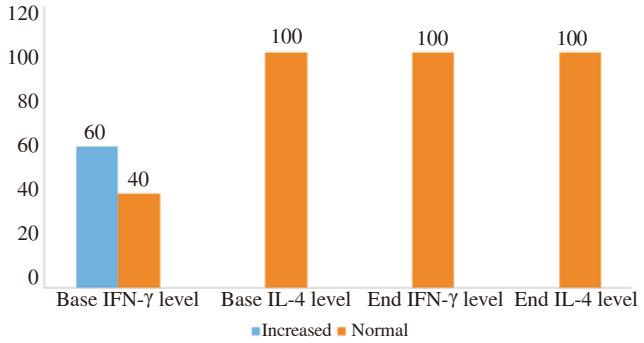

Figure 1. Percentage of patients with normal and abnormal IFN-γ and IL-4 level in different sections.

Table 2
IFN-γ and IL-4 levels at beginning and end of study and their changes in males and females.

Serum variables (pg/mL)	Female patients	Male patients
IFN-γ		
Base	1 095.97 ± 346.69	1 118.26 ± 315.39
End	276.43 ± 158.50	237.39 ± 114.13
P-value	$P < 0.001$	$P < 0.001$
IL-4		
Base	388.08 ± 92.18	383.08 ± 132.53
End	245.96 ± 67.91	216.04 ± 72.65
P-value	$P < 0.001$	$P < 0.001$

According to Paired T-test for independent groups, none of the variables had statistically meaningful difference between two groups. Medians of serum variables' level at beginning and after the treatment and their changes, were evaluated individually for each patient according to their dairy consumption history by Paired T-test and there was no statistically meaningful difference. Also, correlation of patients' age was assessed by Pierson correlation test but there was not any statistically meaningful correlation, either.

4. Discussion

According to our study results, IFN-γ (Th1 activity indicator) and IL-4 (Th2 activity indicator) levels were decreased meaningfully after treatment. While there was not any correlation between these two variables and patients' age, sex and dairy consumption history.

Although there are many studies about role of IFN-γ in pathogenesis, progression and result of brucellosis, that they all

Table 1
Comparison of studied variables before and after treatment.

Serum variables (pg/mL)	After treatment			Before treatment		
	25%–75%	Median	Average	25%–75%	Median	Average
IFN-γ	144.3 and 341.1	248.5	253.00 ± 132.50	899.8 and 1 366.3	1 029.6	1 109.34 ± 322.50
IL-4	191.2 and 280.6	233.4	228.01 ± 71.10	312.5 and 474.6	410.2	385.15 ± 115.70

partially have same results, there are few pieces of evidence of IL-4 role in pathogenesis of brucellosis and there are few studies about that[12].

The study of Yin *et al*. showed that after injection of recombinant multi-epitope antigen vaccine to BALB/c mice, there was increment of IgG, IFN-γ, IL-6, CD3, CD4 and CD8 in vaccinated groups compared with control group mice[13]. They concluded that these changes can elevate immunity against *B. mellitensis*[13]. In another study by Mambres *et al.*, they showed that two of most important agents in controlling mucosal infection by *Brucella* in mice with genetically defects of identifying the lymphocytes and the main pathways that are used in primary and secondary control of intranasal *B. mellitensis*, are IFN-γ and IL-17RA-mediated responses[14]. In another similar study, Murphy *et al.* showed that mice without any amounts of IFN-γ died with brucellosis[15]. We also showed that most of the patients (60%) had high IFN-γ level before treatment and this amount decreased to zero with appropriate treatment. Yingst *et al.* showed that IFN-γ may directly induce producing of reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs) in active macrophages, and it may cause apoptosis in contaminated cells[16].

In a study by Rafiei *et al.* on 27 patients with brucellosis, they showed that intra and extracellular levels of IFN-γ in acute disease in comparison of control group, show significant increase. While producing of IL-6 in these patients is opposite[17].

In a study by Sheikh F *et al.*[18], they found that IL-4 and IL-13 do their tasks by the same receptor. They showed that IL-4 and IL-13 both bind to type II IL-4 receptor complexes and induce STAT-6 downstream gene expression pathway[18]. So they have the same role. According to these studies, it can be concluded that IL-4 has the same condition with IL-13 in brucellosis.

Akbulut *et al*. measures intracellular IFN-γ and IL-4 level in 25 patients with brucellosis and 11 healthy individuals[19]. Changes of these variables were assessed before and after treatment. It was shown that CD3 cells which had IFN-γ component level were meaningfully more in patients group, but there was no statistically meaningful difference in abundance of CD4 cells that had IFN-γ and IL-4 component and CD4 cells which had IL-4 component between control and case groups. CD3 cells with IL-4 component, were fewer in patients group. The abundance of CD3 cells with IFN-γ component was higher in patients who had better response to treatment before the treatment. Changes of this type of cells felt down after treatment in patients with response to treatment but it was not meaningful in patients who had not any response to treatment. So, they concluded that adding IFN-γ to brucellosis treatment regimen can make it more effective[19]. Although, this study's results confirm the role of our study's results and changes of IFN-γ, it is opposite in results of IL-4 serum level assessment. It should be mentioned that none of our patients had increased IL-4 before and after treatment and it was within normal range, but because of its decrement after treatment, it can be placed on the opposite side of other studies.

As we mentioned previously, studies about role of IL-4 in brucellosis are in limited numbers and also are inconsistent. For example in the study of Rasouli *et al*.[20], they evaluated correlation between IFN-γ and IL-4 gene polymorphisms and sensitivity to brucellosis. It was shown that these cytokines frequency had statistically meaningful differences between control and groups. According to this, these two cytokines' significant role in resistance and sensitivity to brucellosis was emphasized.

According to Galanakis *et al*., IL-4 serum level was meaningfully more in acute phase in patients group and on the base of this, they concluded that Th2 plays role in children brucellosis[21]. On the contrary of other studies, this study confirms our result about relevance of IL-4 with brucellosis and its treatment.

Reasons for these differences can be one of the bellows. Th1 cells are necessary for producing resistance against intracellular pathogens, while Th2 cells make patient's condition worse by suppressing active macrophages defense mechanisms[22]. It seems that in brucellosis, balance between Th1 and Th2 play major role in disease pathogenesis. According to various studies, Th1's cytokines (IFN-γ, …) produce resistance against this disease and Th2's cytokines (IL-4, …) make individuals predisposed for brucellosis[21,23,24]. Studies have shown that this role cannot be allocated to all community and patients and it cannot describe oppositions about immunity role against brucellosis[25]. In addition to this, outcomes of individuals exposure to pathogens are relevant to some other factors such as numbers of invader pathogens, immunologic memory due to past exposure, genetic factors, invaders' virulence, *etc*.[19].

Although it has been shown that IL-4 inhibits some immunity functions of phagocytes, it can play an important role in immunity against *Brucella* with augmentation of antibody relevant responses and aggregation of eosinophil. On the other hand, genetic and environmental parameters both have their roles in determining superiority of Th1 or Th2 immunity role[26,27].

This study showed that, although demographic variants and dairy consumption history had not significant role in changes of IFN-γ and IL-4 serum level, IL-4 level decrement was meaningfully more in patients with history of contact with livestock. This finding verified the importance of previous sensitization and confirmed previous similar studies[20].

In this study, it has been observed that IFN-γ level had risen

in 60% of patients which decreased to zero after the treatment. However IL-4 levels before and after treatment was within normal range, but there was a meaningful decrement in its serum level after treatment. This increment was more in the patients with the history of contact with livestock than patients without that. This finding shows the importance of previous sensitization in this context.

Conflict of interest statement

We declare that we have no conflict of interest.

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