Detection of TNF Alpha Level as Biomarker in Different Stages of Cutaneous Leishmaniasis Infection

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Abstract:

Leishmaniasis is a global illness that is endemic in many countries, including Iraq. The characteristic of cutaneous leishmaniasis (CL) is the development of skin ulcers that are controlled by the immune system. Tumor necrosis factor-alpha (TNF-α), a cytokine generated by the innate immune response to CL infection, can influence disease clearance in the human host. The effect of this pro-inflammatory cytokine in CL ulcer development during the infection is not well established. In this study TNF-α level was detected in the patients who suffered from cutaneous leishmaniasis. This level was also assessed in the newly diagnosed patients and others who were undergoing different stages of pentostam treatment. Notably the results revealed a significant increment in TNF-α serum levels in the test groups of newly infected patients, as well as, in the patients enduring second and third trials of pentostam treatment, which was (1125.49, 838.75, 1264.26) ng/ml accordingly, in comparison with healthy control group which was 235.35 ng/ml. Furthermore, there was no significant observation in TNF-α among the three patient groups. The perceived rise of TNF-α serum levels may give insights into this pro-inflammatory cytokine as a biomarker in the prognosis and tracking the disease progression.

Keywords: Pro-inflammatory cytokine, Leishmaniasis, Immune response, Markers.

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التحري عن مستوىTNF–alphaكمؤشر حيوي خلال فترات مختلفة من الاصابة بداء اللشمانيات الجلدي

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الخلاصة:

داء الليشمانيات الجلدي هو مرض منتشر في دول عدة من انحاء العالم بما في ذلك العراق. السمة المميزة لداء اللشمانيات الجلدي هي تقرحات الجلد التي من المحتمل ان تكون معززة بفعالية الجهاز المناعي للسيطرة على نموالافة و تطور الطفيلي. للسايتوكينات التي تفرز عن طريق الاستجابة المناعية الفطرية لعدوى اللشمانيا الجلدي مثل عامل نخر الورم(TNF–α) دورا في القضاء على المرض في الانسان المضيف. لم يتم اثبات تاثير هذا السايتوكين المضاد للالتهابات في تطور القرحة CLاثناء العدوى. في هذه الدراسة تم الكشف عن مستوى عامل نخر الورم الفا في المرضى الذين يعانون من داء الليشمانيات الجلدي. تم تقييم ذلك في

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المرضى الذين تم تشخيصهم حديثا وغيرهم ممن يخضعون للعلاج بالبنتوستام في مراحل مختلفة. أظهرت
النتائج زيادة معنوية في تركيز عامل نخر الورم في مجموعات الاختبار للمرضى المصابين الجدد وكذلك في
المرضى الذين خضعوا الى العلاج بالبنتوستام من الجرعة الثانية والثالثة والتي بلغت 1264,26، 83875،
1125,49 نانوغرام/مل على التوالي، بالمقارنه مع مجموعه السيطرة والتي كانت 235,35 نانوغرام/مل.علاوة
على ذلك لم يكن هناك تغيير معنوي ملحوظ في عامل نخر الورم بين مجموعات المصابين الثلاثة. قد يعطي
الارتفاع الملحوظ لمستوى عامل نخر الورم نظرة مهمة لهذا السايتوكين المضاد اللالتهابات كمؤشر حيوي في
التشخيص وتتبع تطور هذا المرض.

Introduction:

Leishmaniasis is a parasitic disease spread by mosquitoes and caused by an obligatory intra-macrophage protozoan *Leishmania.* There are at least 20 species of the genus *Leishmania* that belong to Trypanosomatida [1, 2]. The parasite is transmitted to humans by the bite of a female sand fly vector of the genus *Phlebotomus* in the old world and *Lutzomyia* in the new world [3]. Leishmaniasis is endemic in more than 98 nations in the tropics, subtropics and the Mediterranean region [4, 5]

The clinical signs vary according to the *Leishmania* species, vector virulence factors and the host immunological response. In cutaneous leishmaniasis (CL) the patient usually has one or more of lesions and/or nodules in the fly-bitten skin area that may range from a solitary spontaneous healing ulcer to acute ulceration with partial or total tissue destruction [6, 7]. The predominant effector cells in infection-induced acute inflammatory reactions are polymorpho-nuclear leukocytes (PMN) that are responsible for phagocytosing *Leishmania* foreign particles and get destroyed by proteolytic enzymes stored in specific granules, in addition to the reactive oxygen species generation after they have been ingested [8]. After *Leishmania* are phagocytosed by neutrophils, they begin to secrete chemokines such as IL-8 which is critical to get more neutrophils to the infection site [9]. T-cell responses, CD4+ and CD8+ T cells operate as a source of biologically relevant cytokines for monocytes activation and macrophages in all CL clinical manifestations, hence determining susceptibility or resistance to the disease [10]. Cytokines are primarily associated with the cellular immunological response mediated by CD4+ T cells. The Th1-mediated response is linked to macrophage activation, host resistance and protection against *Leishmania* parasites, all of which lead to the disease control [11].

Th2-mediated response is related to the downregulation of macrophage activation and eventually progression of the disease [12]. TNF-α is secreted by macrophages and lymphocytes. It is considered a crucial mediator of many inflammatory reactions and plays a major role in innate response in pathogenesis in relevant human diseases to provide synthase stimulation oxygenated products and nitric oxide, components important for killing of *Leishmania*, via encounter *Leishmania* invasion, synergistically with other cytokines including IFN-γ [13, 14]. Some macrophage-secreted cytokines are considered indirect biomarkers for leishmaniasis infection, including IFN-γ, TNF-α and IL-10 [15]. IL-10 was first described as a Th2 specific cytokine with the power to inhibit cytokine synthesis. This activity largely reflects indirect actions on antigens present in cells instead of directly effecting Th1 cells [16].

Previous studies have identified a central role of IL-10 in susceptibility, immunopathology and parasite persistence. However, IL-10 inhibits cytokine production and cytotoxic activity of macrophages [17]. Gene expression of TNF-α has proved its correlation with the lesion size. Furthermore, the TNF-α serum concentration is strikingly raised in muco-cutaneous leishmaniasis [18]. In addition, similar studies demonstrated that TNF-α was found to be increasing during infection and decreasing during the treatment back to its healthy level [19].

TNF-α can be used as a biomarker that indicates the severity of leishmaniasis disease and could be employed to help with therapeutic prognosis and, if needed, treatment modifications [20]. Despite the fact that circulating antibodies can and have been used to control entrance into clinical trials, many lines of the research have showed that anti-leishmanial antibodies can stay longer even after effective chemotherapy treatment and are potentially harmful [21, 22, 23]. IFN- and TNF- levels in supernatants of *Leishmania* antigen-stimulated lymphocyte cells were measured before and after antimonial therapy to see whether the release of these cytokines can be employed as therapeutic response markers [24]. This study was aimed to investigate TNF-α serum level in newly infected CL patients and patients undertaking pentostam treatment in different stages of intake and the possible consideration of TNF-α as a biomarker in cutaneous leishmaniasis disease.

Materials and Methods:

Patient's Collection:

The participants in this study had cutaneous leishmaniasis assembled between October 2020 to February 2021 from Baqubah General Hospital, Diyala provenance north of Baghdad. All 52 patients were suffering from cutaneous ulcers and were diagnosed by the resident dermatologist depending on clinical manifestations and laboratory diagnosis through Giemsa stain preparation of suspected ulcers; amastigotes were screened under light microscope 100x oil immersion [25].

Blood Samples:

5 ml venous blood samples from each patient were collected in a gel tube. In addition, personal information was documented including: age, sex, the number of lesions and the location of the lesions of family, duration of infection and number of Pentostam treatment. Blood samples were centrifuged and the serum was divided into an Eppendorf tube, each containing at least 500 µl of pure serum and were then stored at -20°C for later investigation [26].

Experimental Design:

Of the total 52 patients, the experimental groups were divided according to pentostam treatment, as the following:

Group-1: 20 CL patients of a new infection, no pentostam treatment.

Group-2: 17 CL patients with 2nd trial-treatment of pentostam.

Group-3: 15 CL patients with 3rd trial-treatment of pentostam.

In addition, 15 blood samples were taken from healthy people in the area.

Tumor Necrosis Factor-alpha (TNF-α) Detection:

Pro-inflammatory cytokine TNF-α ELISA kit was purchased from Abcam company, USA. Sandwich ELISA was processed for all samples according to the manufacturer's instructions at 450 nm wavelength ELISA reader, Glomax, USA, in a 96 well-plate ELISA plates Promega.

Results and Discussion:

Parasite Infection and Ulcers:

All suspected CL patients involved in this study were pre-diagnosed in the laboratory before being processed for TNF-α ELISA detection. Clinical features of cutaneous ulcers were first examined for each patient. In addition, Giemsa-stained slides from samples of at least one visible ulcer were investigated under microscope oil immersion and the invaded amastigotes were seen (Figures 1 and 2).
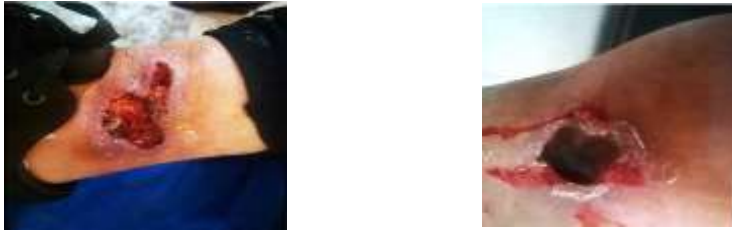
Figure 1- Patient with multiple cutaneous leishmaniasis lesions

Figure 2-Macrophage showing intra-cellular amastigotes from ulcer swab under light microscope 100X.

TNF-α Investigation in New CL Infection Patients (no treatment group):
Results of patients with new infection and with no-treatment trials showed that the average TNF-α concentration was significantly higher than that recorded values of the control group, which was 1125.496055 ng/ml compared with the mean of 235.3496 ng/ml control subjects (Figure 3).
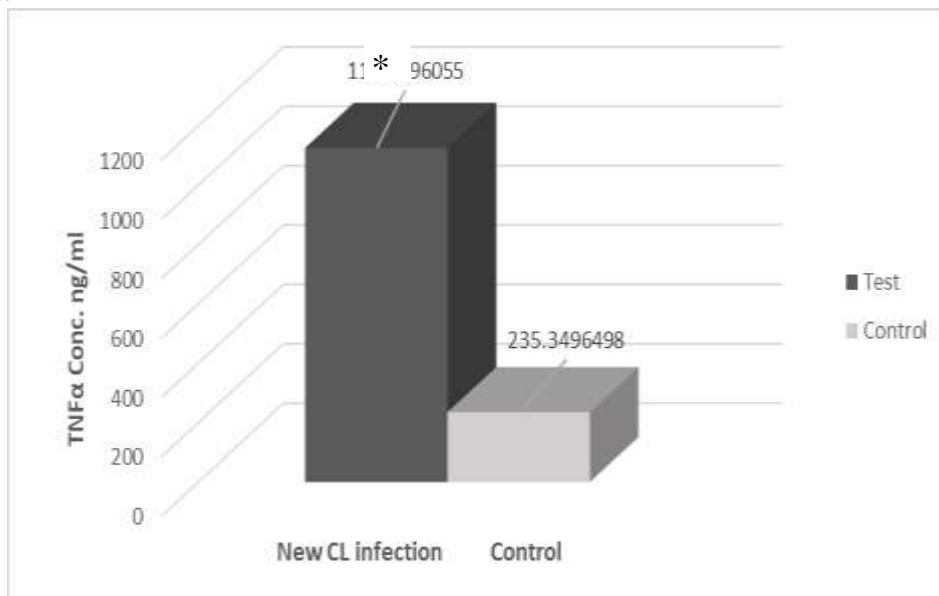

Figure 3-TNF-α mean concentration in CL patients with no treatment* = significance (p value ≤0.05).

TNF-α Investigation in 2nd and 3rd Trials Treatment:
The result of both patient groups who received two or three doses of pentostam also revealed raised TNF-α concentrations (838.7557633, 1264.2602) ng/ml, respectively, when correlated to the mean of the control group which was equal to 235.3496 ng/ml (Figures 4 and 5).
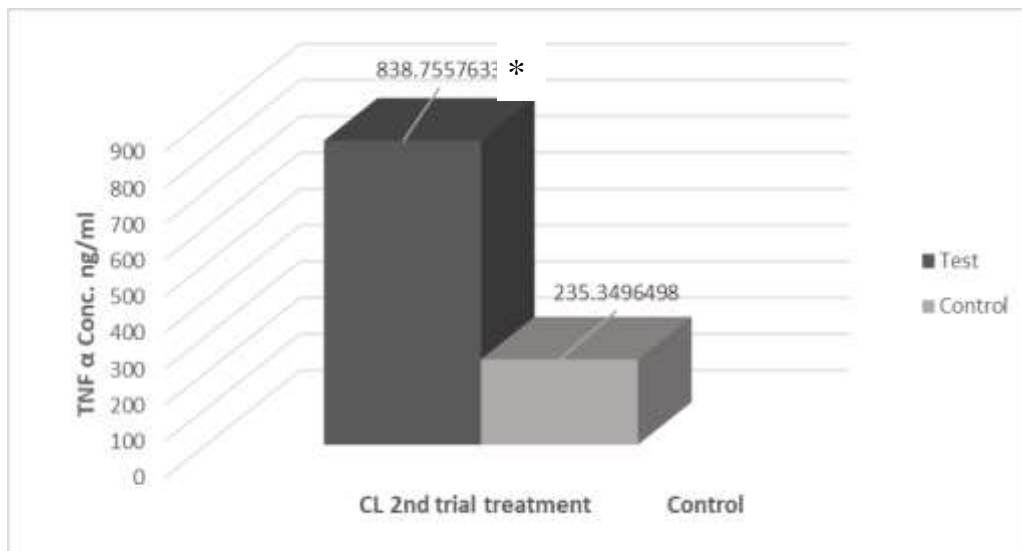
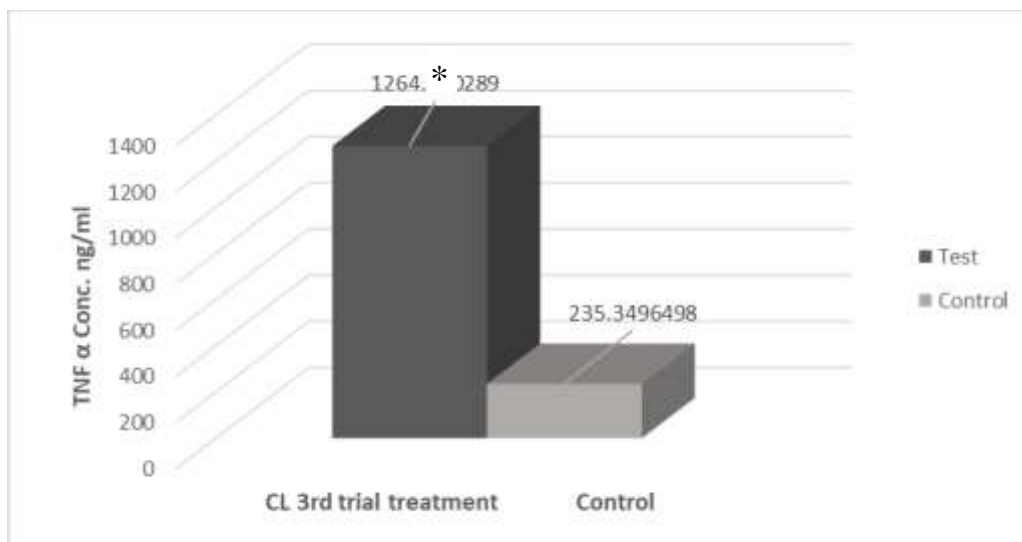**Figure 4-**TNF-α level in the 2nd trail pentostam treatment patients * = significance (p value ≤0.05)

Figure 5-TNF-α level in the 3rd trail pentostam treatment patients * = significance (p value ≤0.05)

Furthermore, there was no significant difference between the three test groups of new infection, 2nd and 3rd trial-treatment where p-value was > 0.05, according to ANOVA data analysis (Figure 6).
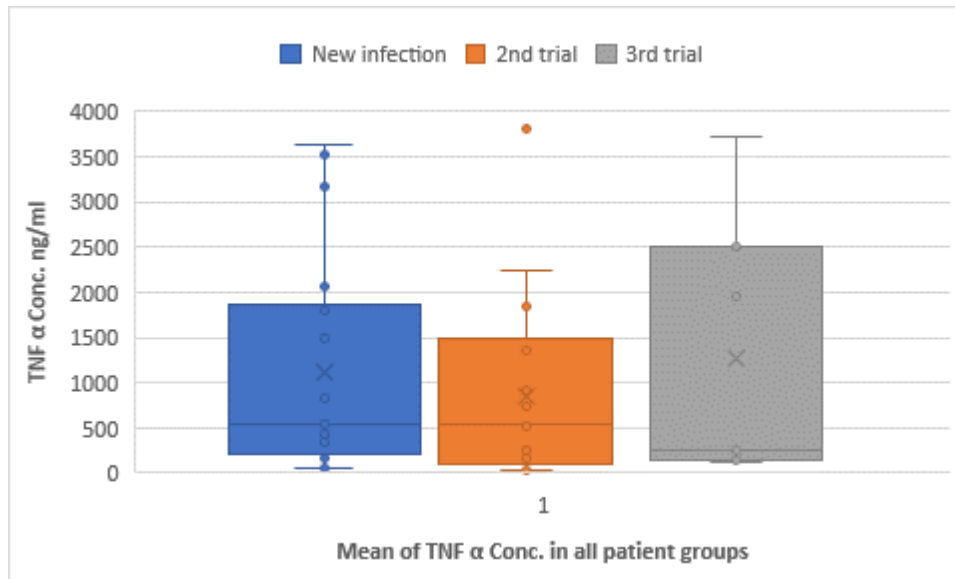
Figure 6-Comparison of the mean concentration levels of TNF- α among the three test groups, not significant.

Discussion:

In cutaneous leishmaniasis, the size of the immune response is determined by the duration of the illness, clinical type of the disease, parasite species and vector species [27]. TNF-α is a proinflammatory cytokine that is released by macrophages and lymphocytes and is significant in *Leishmania* spp. infections treatment because it activates type 1 T helper (Th1) immune response [28, 29]. Previous research has revealed that action of both TNF-α and IFN-γ has proved to kill *Leishmania* parasite by activating macrophages to produce oxygen and nitric oxide. However, the patients were found to have a diverse prone to produce such cytokines and eventually control the disease [30].

TNF-α has been demonstrated to have importance in leishmaniasis development. Very high serum levels of such cytokines were reported in active leishmaniasis. High TNF-α levels, on the other hand can cause tissue damage and as a result skin sores in CL [31]. A similar study showed that CL patients who had failed treatment revealed a decreased TNF-α serum level, while the level of this cytokine had significantly increased before the therapy [32]. Another similar study had proved the ability to use TNF-α level as a biomarker in diagnostics and progression in monitoring CL disease caused by cutaneous *Leishmania spp.* [33]. Some pro-inflammatory cytokines were chosen to be indirect biomarkers in CL patients, for example IL-10 which was used as a biomarker for CL failure therapy [34]. In addition, elevated TNF-α and IFN-γ level is correlated with the severity of infection and lesion size [35].

During the active phase of the disease, TNF- α is usually higher. Furthermore, these levels could be used as a marker of CL activity after or during the treatment [36]. Studies have suggested that some cytokines can be used as markers in epidemiological studies conducted in endemic areas to distinguish between the different clinical forms of CL, and that the immune response is effective in controlling the disease, followed by the establishment of protective mechanisms similar to those found in asymptomatic individuals [37]. They showed that serum TNF-α levels will be used as a sign of disease activity and their fall as a marker of effective therapeutic response. This elevation in cytokine levels agrees with the results of other studies [38]. Innate TNF-α dependent mechanisms drives cell mediated immunity by CD4+ and CD8+ T cells activation in patients where the ulcer had remained active [39].

A previous study proved that host immune response against leishmaniasis can be evaluated by measuring the cytokines serum level that corresponded to Th1 (TNF-α IL-12 and IFN-γ) and Treg (IL-10) cells. These cytokines had significantly increased in the patients, with the

exception of IL-12 [40]. Growing efforts are focused on the identification of biomarkers to predict different species of *Leishmania* and reflect the altered systemic and skin immune response, also, curing progression for CL in comparison with other types of visceral leishmaniasis (VL) or PKDL [41]. Similar research concluded the possibility of considering the difference in gene expression of spleen infected mice as biomarkers for curing disease progression in VL [42].

In addition, the biomarkers can give better understanding of the therapy response , which in turn prevents relapses. In addition, the biomarkers of CL infection are necessary for the detection of prevalent infection of asymptomatic patients in endemic areas and/or in immune compromised patients [43].It was found that individuals living in L. endemic areas showed an increase in the level of TNF-α with no evidence of IFN-γ change in healthy individuals, in association with increased level of IL-5[44].

In this research, increased level of TNF-α was found in new infection group and patients undergoing different trials of pentostam treatment. It can be concluded that this cytokine gives an insight of CL infection in the studied groups. Although there is little known about this concept, more studies are needed to investigate TNF-α level and more parameters in CL patients and completely cured individuals as markers for disease progression.

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