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(RESEARCH ARTICLE)

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Proinflammatory cytokines (TNF-a, IL-1B, IL-6) and antioxidant in different stages of patients with cutaneous Leishmaniasis

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Abstract

Introduction: Leishmania is a significant health problem in many parts of world. TNF-α is an essential player in infections with leishmania major, contributing to the control of the inflammatory lesion, a lesser degree and parasite killing. Inflammatory molecules (TNF-a, IL-1B and IL-6) were probably responsible for the stronger cellular recruitment and mainly seen at the site of infections. The aim of this study was evaluated of pro inflammatory cytokines and (antioxidant) in four groups of cutaneous leishmaniasis patients compared with together and normal group. These cytokines measured in 39 patients were divided into four groups of: 1) active (acute phase of treatment); 2) non-healing (received treatment for almost two years without recovery);3) healing (recovered upon treatment); and 4) healed (treatment previously received and achieved complete remission). Pro inflammatory cytokines and antioxidant about 3 of these patients from active stage to heal were specifically evaluated also.

Material & Methods: IL-1β, TNFα, IL-6 in serum of patients measured by ELISA and antioxidant determined by FRAP method.

Results: Antioxidant mostly decreased in groups near to normal group, but increased in non -healing group. The serum level of pro inflammatory cytokines: TNFα, increased in three groups of patients compared to normal group. Highest level of IL-1β belong to non- healing average, whether, others and normal group had zero value of IL-1β. IL-6 had highest level in non- healing, but had not significant difference in other groups. In three of these active patients which leading to health, TNFα, IL6& IL1β strongly decreased.

Discussion: This Study suggestion, there is direct correlation between of pro inflammatory cytokines (IL-1B, TNF α, IL-6) and progressive of disease. Another finding shows that antioxidant level had extremely strong correlation with progresses response to drug and lead to healthy of disease.

Keywords: Cutaneous leishmaniasis; Proinflammatory cytokine; TNF-a; IL-1B; IL-6; Antioxidant

1. Introduction

Leishmania infection involving the intracellular protozoan parasite which estimated to affect more than 15 million people worldwide, with 400, 000new cases each year.[1] Human infected with this pathogen develops species –specific pathologies (e.g. cutaneous, mucocutaneous, and visceral infections).In its mammalian host, Leishmania can promote numerous phagocyte dysfunctions leading to the inability of these cells to elicit an effective innate and cell-mediated immune response, which followed by presence of the organism , creation of infection and progressive infection.[2] Tumor necrosis factor TNF- α secretion is a major effect or mechanism of memory CD8+ T cells that are believed to be required

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for immunological protection which has an essential player in infections with leishmania major, contributing to the control of the inflammatory lesion and, parasite killing.[3]Using an air pouch model, showed that viable motheaten SHP-1deficient mice .Harbored have a stronger inflammatory response against leishmania infection than wild –Type mice. This response was portrayed by higher proinflammatory cytokine (TNF- α, IL-1β, and IL-6) and chemokine expression and secretion and greater chemokine and chemokine receptor expression. These inflammatory molecules were probably responsible for the stronger cellular recruitment (mainly neutrophil) seen at the site of infection in viable motheaten mice within 6 h post inoculation.[4]Leishmanial lipid is a strong immune suppressor of host cells. Inhibition of the inflammatory responses of synovial cells through induction of apoptosis is one of the main targets of therapeutic intervention in rheumatoid arthritis (RA). [5] It is known that TNF- α is an important inflammatory mediator recognized for modulation of cell recruitment [6, 7] and healing of pathogen-induced lesions [8].It was essential to evaluate its expression in the footpads during leishmania infection. Pro-inflammatory cytokines such as TNF α, IL-1β and IL-6 are recognized for their role in the inflammatory response.[4] In other study, [5] observed that leishmanial lipid treatment in normal rats causes that serum's TNF α and IL-1β elevated. Another finding showed macrophages had deficiency for the gp91phox subunit of NADPH oxidase or TNF-α, because both TNF α and the oxidative burst have been described as contributing to the control of L. major in vitro and/or in vivo.[12-16]. Lipophosphogly can suppressed TN- α, IL-1 β and NO production by lipopolysaccharide-stimulated or PMA-stimulated macrophages. TNF-**α** induces mononuclear phagocytes and neutrophils to produce reactive oxygen intermediates (ROIs**).**TNF- **α** is an in vitro inducer of ROIs that directly allows infected myeloid cells to kill bacteria.[17, 18] It has been shown that inflammatory response is presently concurrent with high production of free radicals. These prompt us to evaluate the level of total antioxidant as an indicator for level of free radicals. This study was undertaken to examine the innate inflammatory cytokines (IL-β, TNF-α, IL-6) and antioxidant levels evaluate in different group of patients. This study evaluated pro-inflammatory cytokines and antioxidants in39 patients were divided into four groups of cutaneous leishmaniasis patients contain: active, non-healing, healing (recovered upon treatment); and, healed. Serum levels of pro-inflammatory cytokines (IL-1B, TNF-α, IL-6 measured by ELISA method and serum antioxidant levels by FRAP assay.

2. Material and methods

In this study four group of patients (Active, healing, healed and non- healing) compared with each other and normal group. For this purpose, innate proinflammatory cytokines measured by enzyme linked immunosorbent assay (ELISA). The antioxidant level as eradication of free radicals was determined using FRAP method.

2.1. Characteristics of the patients with Leishmaniasis

Serum sample were obtained from the clothing blood of 39 patients who were divided in four groups:

- active infection,
- non healing,
- healing
- Healed leishmaniasis and a normal individuals as control group. Patients who had criteria for leishmaniasis and referred to the Leprosy and Dermal disease Center, Tehran University of Medical Sciences, Tehran, Islamic Republic of Iran.

2.1.1. Patient groups

- active, (had latest sever leishmaniasis disease),
- Non-healing, (with disease for two years and had not response to the relevant medicine.)
- Healing, (had leishmaniasis that received drugs and responses to treatment.
- Healed, (had leishmaniasis, and received treatment and were recovered of infection) .The serum of three patients that were healed, compared with their own serum when they were in stage of active infection. All of the patients compared with that of normal group (control).

2.2. Measurement of cytokine production by enzyme linked immunosorbent assay (ELISA)

Levels of TNFα, IL-β, and IL-6 in the patients and normal group were determined by sandwich ELISA, according to the recommendations of manufacturer. Human serum Levels of TNFα, IL-β, and IL-6 in the subjects measured by ELISA, using an automated microplate reader, set at 405 nm. The sensitivity limit was 20 pq/ml for TNFα, IL-β, and IL-6.

2.3. Measurement of antioxidant

A simple, automated test measuring the ferric reducing ability of plasma, the FRAP assay, is a recently developed /direct test of "total antioxidant power," which presented as a novel method for assessing "antioxidant power." Ferric to ferrous ion reduction at low pH causes a colored ferrous-tripyridyltriazine complex to form. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration. Absorbance changes are linear over a wide concentration range with antioxidant mixtures, including plasma, and with solutions containing one antioxidant in purified form. There is no apparent interaction between antioxidants. Measured stoichiometric factors of Trolox, α-tocopherol, ascorbic acid, and uric acid are all 2.0; that of bilirubin is 4.0. Activity of albumin is very low. Within- and between-run CVs are <1.0 and <3.0%, respectively, at 100–1000 μmol/liter. FRAP values of fresh plasma of healthy Chinese adults: 612–1634 μmol/liter (mean, 1017; SD, 206; n= 141). The FRAP assay is inexpensive, Reagents are simple to prepare, results are highly reproducible, and the procedure is straightforward and speedy. The FRAP assay offers a putative index of antioxidant, or reducing, potential of biological fluids within the technological reach of every laboratory and researcher interested in oxidative stress and its effects.[9-11]

2.4. Statistical analysis

First we calculated sampling parameters include: arithmetic mean, standard error of mean, minimum and maximum for every dependent variable, then we used one way ANOVA for testing the hypothesis about the difference between groups for every variable.

3. Results

Results of the innate pro inflammatory cytokines (TNF α, IL-6, IL-1β) and total antioxidant in four groups of patients: Active, non-healing, healing, healed compared with normal group.

In active patients, TNF-α level showed highest value compared with other groups. Healing and healed group had second and third highest TNF-α level, while non-healing group had lowest value compare to normal group (Figure -1). But statistical Analysis (ANOVAs) did not show significant differences between four groups together and with normal (Table- 1).

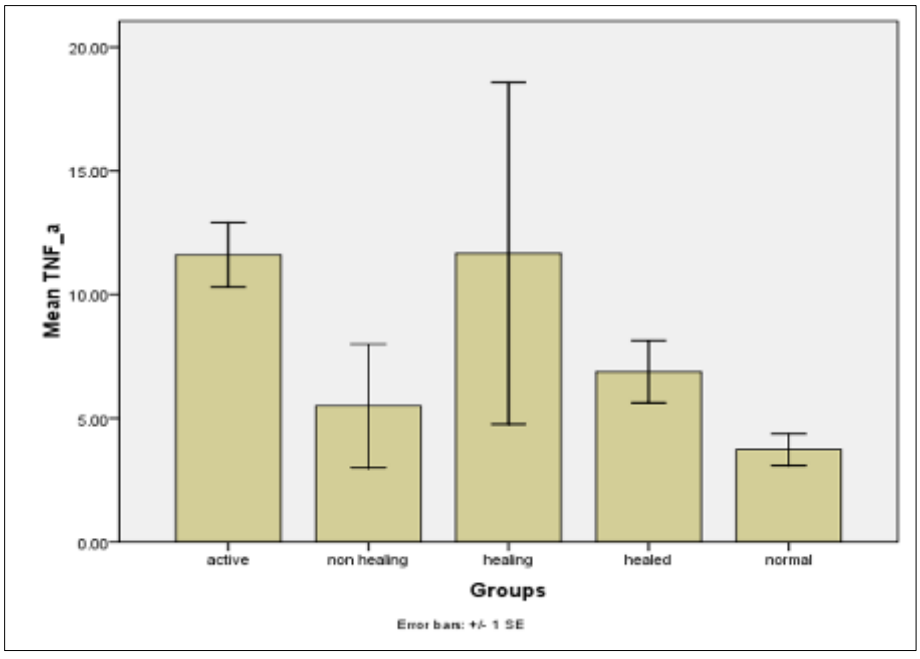

Figure 1 Average of Innate proinflammatory cytokines (TNF-α) in four groups of patients: 1-Active 2- nonhealing 3-healing 4- healed compared with together and normal group

Non-healing patients produced highly IL-6 in compare with others and normal groups, while in healing and active groups production of it was decreased but those differences statistically were not significant (Fig.1, table- 1). Although non-healing had high production of IL-1ß in compare with normal and other groups, it was not statistically significant (Table- 1).

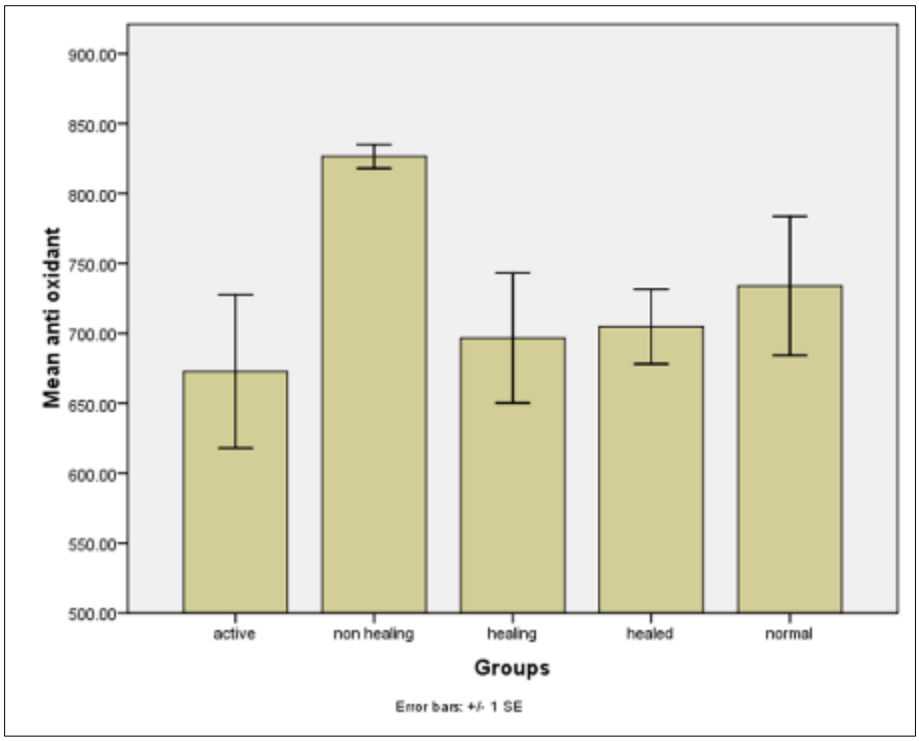

Figure 2 Average of Innate proinflammatory cytokines (IL-6) in four groups of patients: 1-Active 2- nonhealing 3-healing 4- healed compared with together and normal group

Non-healing patients have highest total antioxidant in sera while in other groups this value was lower than normal however this difference was not significant, also (Fig.4, table 1). Results of the innate pro inflammatory cytokines in serum of patient showed that:

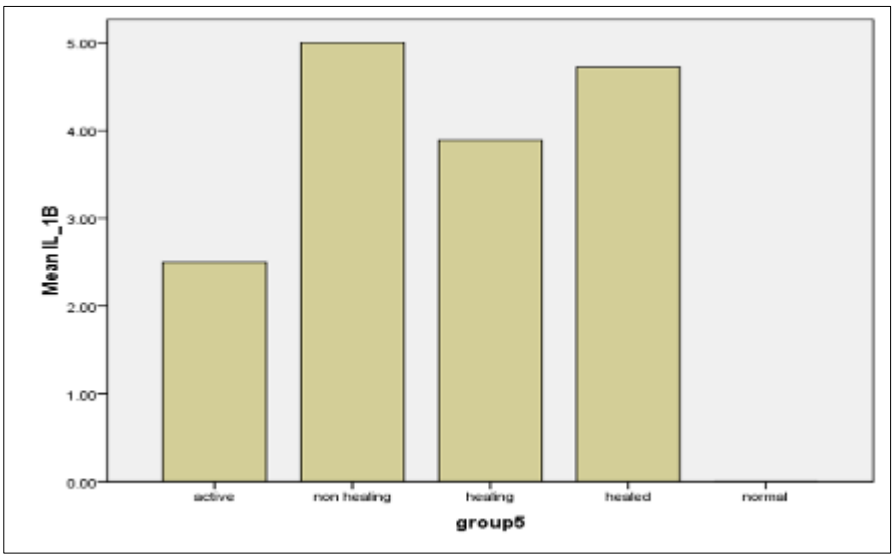

Figure 3 Average of Innate proinflammatory cytokines (IL-1β) in four groups of patients: 1-Active 2- nonhealing 3-healing 4- healed compared with together and normal group

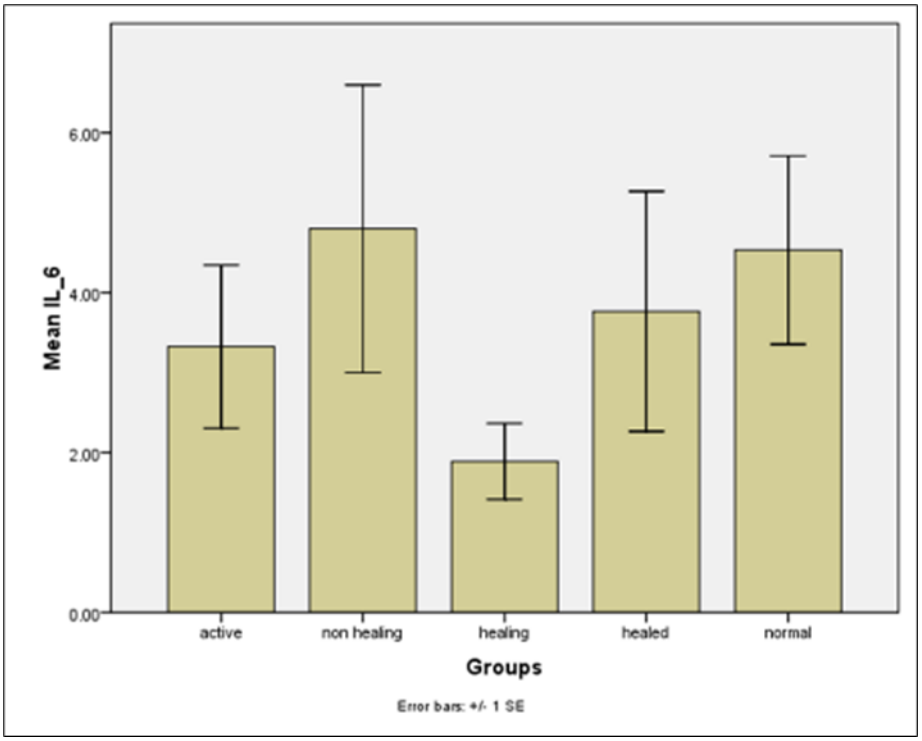

Figure 4 Average of antioxidant in four groups of patients: 1-Active 2- nonhealing 3-healing 4- healed compared with together and normal group

TNF- α had highest value compared with other groups. Secondly healing was higher than healed group, while non-healing had lowest value of TNF- α compare to normal group (Figure -1). But statistical Analysis (ANOVAs) did not show significant differences between together and normal group (Table- 1).

IL-6 had highly production non-healing patients in compare to others and normal group (fig.2). While in healing and active group production of it was decreased but those differences statistically were not significant (table 1)

IL-1ß Production was high in non-healing in compare with another and normal group although this was not statistically significant also (Table- 1).

Antioxidant presence in sera showed that total antioxidant in non-healing patients was highest while in other groups this value was lower than normal (Figure 4). However, this difference same was not significant (Table-1).

In this study we evaluated innate inflammatory cytokines (IL-β, TNF-α, IL-6) , antioxidant levels in specifically relationship between level of these factors and progression of the infection in three active patients that leading to health. In three patients that cured of leishmaniasis infection, all of the pro inflammatory cytokines and antioxidant were measured in their serum, compared with own their serum when they were in stage of active leishmaniasis infection. Results showed TNF- α and IL-6 average was greatly high in active period for all of them, but when they healed, these values highly decreased. IL-1β had not difference (Table 2, figs.5-7).

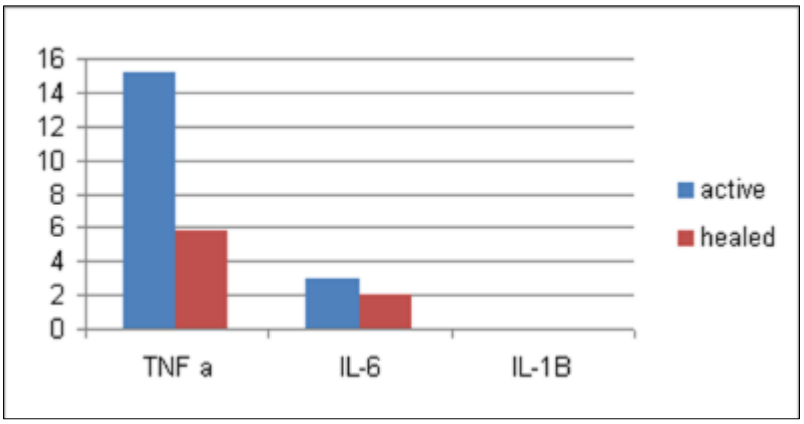

Figure 5 Compration of TNF- α , IL-6, IL-1β average value in one patient :N.12withN.10 (active and healed period.)

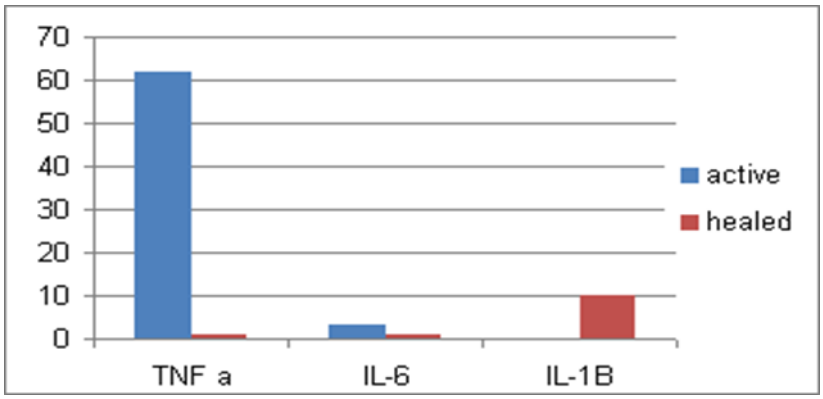

Figure 6 Compration of TNF- α , IL-6, IL-1β average value in one patient :N.13 with N:29(active and healed period.)

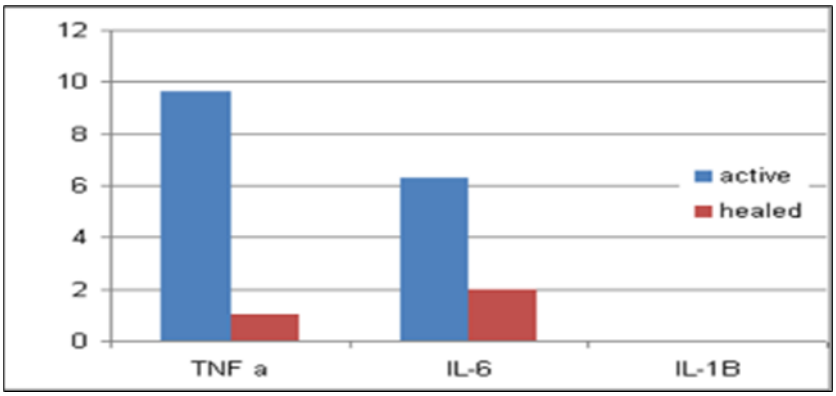

Figure 7 *C*ompration of TNF- α , IL-6, IL-1β average value in one patient :N.9with N:11(active and healed period.)

Table 1 Statistical analysis of the means of serum innate pro inflammatory cytokines (TNFα IL-6,IL-1β) and antioxidant level in four groups of cutancous leishmaniasis patients

	N	Mean	Std. Deviation	. Std Error	95% Confidence Interval for Mean Minimum Maximum Lower Bound	Upper Bound	Minimum	Maximum
TNF-α								
Active	4	11.6000	2.59743	1.22.50000	7.4669	15.7331	9.60	15.20
non healing	25	5000	320.72318	2.50000	-26.2655	37.2655	3.00	8.00
Healing	9	11.6667	20.72318	6.90773	-4.2626	27.5959	3.00	66.00
Healed	18	6.8778	5.35389	1.26192	4.2153	9.5402	1.00	18.00
Normal	3	3.7333	1.10151	0.63596	.9970	6.4696	3.00	5.00
Total	36	8.2611	10.97819	1.82970	4.5466	11.9756	1.00	66.00
IL-6								
Active	4	3.3250	2.03859	1.01929	0812	6.5688	2.00	6.30
non healing	2	4.8000	2.54558	1.80000	-18.071	27.6712	3.00	6.6
Healing	9	1.8889	1.42517	0.42506	.7934	2.9844	0.00	4.40
Healed1	18	3.7667	6.37172	1.50183	.598	6.9352	0.00	28.00
Normal	3	4.5333	2.04287	1.17954	-.5414	9.6081	2.20	6.00
Total	36	3.3694	4.67350	0.77892	1.7882	4.9507	0.00	28.00
IL-1β								
Active	4	2.5000	5.00000	2.50000	-5 .4561	10.4561	0.00	10.00
non healing	2	5.0000	7.07107	5.00000	-58.5310	68.5310	0.00	10.00
Healing	9	3.8889	4.85913	1.61971	.1538	7.6239	0.00	10.00
Healed1	18	4.7222	4.99182	1.17658	2.2398	7.2046	0.00	15.00
Normal	3	0.0000	0.00000	0.00000	0.0000	0.0000	0.00	0.00
Total	36	3.8889	4.79749	0.79958	2.2657	5.5121	0.00	15.00
Anti-oxidant								
Active	4	6.7275E2	109.61866	54.80933	498.3223	847.1777	567.00	823.00
non healing	2	8.2650E2	12.02082	8.50000	718.4973	934.5027	818.00	835.00
Healing	9	6.9667E2	139.51613	46.50538	589.425	803.9083	531.00	883.00
Healed1	18	7.0483E2	112.98790	26.63150	648.6458	761.0209	548.00	897.00
Normal	3	7.3400E2	86.12201	49.72256	520.0611	947.9389	641.00	811.00
Total	36	7.0842E2	114.66532	19.11089	669.6195	747.2138	531.00	897.00

4. Discussion

Leishmania is a significant health problem in many parts of the world. Tumor necrosis factor- alfa (TNF- α) plays an essential role in *Leishmania major* infections. TNF-a, IL-1B and IL-6 inflammatory molecules were probably responsible for the stronger cellular immunity. Neutrophils mainly seen at the site of infections .Leishmaniasis is caused by an obligate intracellular protozoan of the genus Leishmani**a** and parasitic infection**.** Current treatment is based on chemotherapy which is difficult to administer, expensive and is effective due to the emergence of drug resistance. In its mammalian host, leishmania can promote numerous phagocytes dysfunctions leading to the inability of these cells to elicit effective innate immune response which cause persistence and progression of the infection. It has been shown that innate inflammatory response would be harmful to leishmaniasis survival and progression of the disease .Our results show that TNF-α and IL-1β increase in active and healing patients (Table-1& Fig 1-3). In our patients that were in active and healing stages of leishmania infection which received treatment and led to recovering state had lowest value of antioxidant which was nearly average of healed patients, whereas, highest value of antioxidant related to average of non-healing patients. It could be resulted that there is high value of reactive oxygen intermediates (RIOs); in our patients that were in active and healing stages of leishmania infection which received treatment and led to recovering. And conversely, highest value of antioxidant related to average of non-healing patients It could be resulted that there is low value of reactive oxygen intermediates (RIOs); in our patients that were in non- healing stages of which received treatment but did not lead to recovering of infection.Our hypothesis is high level of antioxidant causes decrease of (RIOs) which it is requires to eliminate intracellular pathogens subsequently inhibit disease, and when antioxidant level is high, level of (RIOs) decline and subsequently infection progress and resistance to treatment increases. Our finding showed that non- healing patients that resistance to treatment, had higher antioxidant level than of another four groups (fig.4), and IL-1 β in active and healing patients was very low. It seems active and healing groups had good response to treatment, IL-1 β had inhibited by leishmania lipid. But highest value of IL-1 β belong to non- healing group that had chronic leishmaniasis and resistance to treatment (fig.1). Another result of this study, show that, there is direct

correlation between proinflammatory cytokine (TNF-α) and accuracy of leishmania disease. So that in active and healing patients it is higher than non healing because leaded to accuracy of disease (fig.1). Non healing patients although give drug but have not accuracy. We can conclude that in non-healing group leishmania antigen cause suppresses producing TNF-α.(fig.1). Another finding show antioxidant in non-healing is higher than others, which can result in the reduction of RIOs in this group. Others has lower antioxidant, which can conclude high RIOs, which can be useful for curing Leishmania disease. (fig.4). One study was shown macrophages deficient for both gene were able to clear an infection with L.major strains in a manner similar to that of wild-type macrophages and released comparable amounts of nitrit after stimulation with IFN-c alone or in combination with TNF or LPS.[19]TNF has also been reported to be essential in the healing process of leishmania induced lesions.[4]Our finding showed same results in human lower than healing and higher than non healing, whereas lowest in fourth group is related to healed .Of course, lowest of them belong to normal group. Whether high value of IL-1β which is belongs to non healing patients, is correlated to Th2 and stimulated by (IL-4, IL-10).The highest TNF-α.was related to the active group, and then healing with the highest rate, and healed group was lower than it, but, non- healing is lowest(fig.1).Our results confirm previous studies (Muler and Goosseens), that finded lipophosphoglycan suppress TNF-α**.** A previous study has shown that lipophosphoglycan obtained from an infective strain of leishmania suppresses host immune responses and promotes the progress of leishmaniasis following infection.[20] Our results also showed the same finding about TNF- **α**, which is in active and healing average was high, while antioxidant is low and as a result ROI was high. The result of another study showed that rapid release of TNF- **α** by MPCs may be critical for the production of high levels of ROI and subsequent clearance of bacteria.[21] Our finding IL1-β, had highest value for non- healing. It was accompanied by increasing antioxidant value which subsequently decrease free radicals, that is essential for killing of intracellular parasite or bacteria. This finding confirmed by other researches that demonstrated this pV-mediated protection against leishmaniasis in part due to increasing production of nitric oxide and proinflammatory molecules (such as IL-6, IL-1b, MCP- 1/CCL2 and MIP-2/CXCL1), which hence by accompanied by increasing leukocyte Recruitment.4TNF- α secretion of by MPCs, phosphorylation of p47phox is upon binding of TNF-α to its surface receptor, activate of NADPH oxidase, and ROI production. The ability of TNF- α to induce both MPCs and neutrophils to produce ROIs results in an amplification loop that allows rapid clearance of high doses of bacteria, a mechanism that does not occur in naive mice because of the lack of memory CD8+ T cells. [21] Regard to our previous study's results, we think that difference of DTH response and expansion and increasing in spleen white pulp size results belong to various cytokines amount (26, 27, and 28). Scott et al, suggested that low antigen doses may preferably promote a CD4+ Th2 response in vivo, whereas high doses may favor Th_1 cells develop [29, 30, 31, 32.] The disease is usually self-limiting, but time to induce lesion manner is various between species and individuals. Recent evidence has also shown that Leishmania and virus co-infections in human with immunodeficiency is a major health problem in affected areas.[34] The pathology of Leishmania infection is determined not only by parasite species, but also host genetics and immune factors affects it. Most of the experimental immunological data comes from mouse models, and few has known about the immunology of human Leishmaniasis.

Although mouse models have been used for the study of both cutaneous and visceral Leishmaniasis, they more closely reflect the situation in human cutaneous Leishmaniasis than visceral disease. Our finding show same results in human. These results show TNF-α has high average in active patients, but lower than healing and higher than non-healing, whereas lowest in fourth group is healed group. Of course, lowest of all of them belong to normal group. Whether high value of IL-1β which is belongs to non healing patients has correlated to Th2 and stimulated by (IL-4, IL-10). Healed group average has lowest value of TNF- α which was greater than over other three groups that also had low IL-1 β, which leading to Th1 immune response. Our hypothesis is: in healing & active patients average of TNF- α has been increased stimulated IL-4 and subsequently Th2 that we didn't know (he or she) cured and will have been good response to drugs or no and vice versa. Leishmaniasis in general, and especially cutaneous Leishmaniasis, is one of several parasitic diseases that can have a vaccine to control. In author recent researches had shown greatest increase in spleen white pulp size expansion in mice may be related to IL-4 and IL-10 production which resultd in humural immune responses. Both IL- 4 and IL-10 are associated with systemic disease without long term immunity to cutaneous Leishmaniasis, as suggested by her results in type I mice (susceptible) in injection group 3: include leishmania antigen plus BCG and booster dose of antigen at doses of 400– 500 µg/ml. A cure was associated with a fall in IL-10 mRNA levels. Spleen White Pulp (SWP) size expansion in mice is related to the production of cell mediated immune responses, IFN-γ and mostly IL-4suppression, which may lead to long term immunity to cutaneous Leishmaniasis, similar to our results in type

II mice (resistant), in injection group 1: include inoculation leishmania antigen plus booster dose at doses of 100–200 µg/ml [35] .Another animal model study of cutaneous leishmaniasis indicates that Th1 responses are essential for protection by vaccination. This has been usually predicated on the induction of high levels of IFN-γ and low levels of IL-4. However, recent studies indicate even vaccines triggering high levels of IFN- γ do not protect in the presence of high levels of the regulatory cytokine IL-10. And also, author previous results in experiments shown that pro inflammatory

cytokines (IL17 and&IL23), Th1, Th2 cytokines (IL-4,10.12, IFN- γ) have an effective relationship between survival of vaccinated mice with new leshmania major vaccine prepared by author and her collaborations [37,38,39,40,41].

5. Conclusion

In this research TNF-α and antioxidants and subsequently RIOs have correlation with exist or inhibitor of intracellular parasite and bacterial.IL1-β has related with non-healing patient and increasing antioxidant, which we guess decreased RIOs. To this regard our research suggestion that there is direct correlation between excess of IL1-β and humural immune system.

IL-6 is also higher than for non-healing group between another three groups. Although, all of the reported results in this research have not significant differences, used by ANOVAs statistical analysis, but those average which showed in four graphs, are various. This research requires more study in future. Pro-inflammatory cytokines IL-1β, TNF-α, IL-6 may be related to the progression of leishmaniasis. Serum antioxidant levels maybe correlated with patient response to drug treatment.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that there is no conflict of interest.

Statement of ethical approval

The present research work does not contain any studies performed on animals/humans subjects by any of the authors.

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