A comparative study between single ulcers and multiple ulcers of the cutaneous leishmaniasis parasite from an immunological point of view

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Abstract

This study deals with the comparison between single ulcers and multiple ulcers of the cutaneous leishmaniasis parasite from an immunological point of viewThis study included (90) people infected with cutaneous leishmaniasis from Samarra General Hospital in Samarra city in Salah El-Din Governorate for the period from August 2021 to April 2022. (54) a person with multiple ulcersThe results of the study showed a significant increase (P≤ 0.05) in the level of Interleukin-6 (IL-6) in the single ulcer group (pg/ml 46.04 ± 15.0) and the multi-ulcer group (pg/ml 59.16 ± 18.3) compared to the control group (pg). /ml 25.42 ± 6.36), it was also observed that the increase of IL-6 in the multi-ulcer group (pg/ml 59.16 ± 18.3) compared to the single ulcer group (pg/ml 46.04 ± 15.0.(The level of Interleukin-17 (IL-17) was also increased in the single ulcer group (pg/ml 47.17 ± 27.8) and the multi-ulcer group (pg/ml 87.41 ± 30.5) compared to the control group (pg/ml 38.14 ± 17.9), as it was noted. The elevation of IL-17 in the multi-ulcer group (87.41 ± 30.5 pg/ml) compared with the single ulcer group (47.17 ± 27.8 pg/ml). When comparing the immunoglobulins, the results showed that (15.6)% of the patients showed IgG specific antibodies. And (84.4%) showed that they had IgM antibodies, and the results showed that most infections were severe.The objectives of the research were represented i1 -Calculating the concentrations of cellular kinetics (IL-6, IL-17) in people infected with the cutaneous leishmaniasis parasite in comparison with the control group.2 -Calculating the concentrations of immunoglobulins (IgG, IgM) in people infected with the cutaneous leishmaniasis parasite compared to the control group

Kewords:- Cutaneous leishmaniasis, interleukin-6, interleukin-17,immunoglobulin IgG, IgM

1. Introduction

Leishmaniasis is a widespread parasitic disease that is endemic in more than 92 countries, including Iraq, Iran, Brazil, Afghanistan, Syria, India, Bangladesh and Sudan, and more than one million cases of leishmaniasis are recorded annually (WHO, 2020) Leishmaniasis has three clinical forms: Cutaneous Leishmaniasis, Mucocutanous Leishmaniasis, and Visceral Leishmaniasis. Cutaneous leishmaniasis has many names, including: Oriental sore, Baghdad boil, Aleppo boil, as well as other names depending on the areas In which; In Iraq, there are two types that cause cutaneous leishmaniasis, Leishmania tropica and Leishmania major, and they are similar in the life cycle. The parasite lives in the cells of the reticulum-endothelial system in the skin, especially the phagocytes, which grow and multiply inside, causing the appearance of skin ulcers (Schmidt et al, 2005; Ghosh, 2018)The parasite of the genus Leishmania goes through an indirect life cycle, as its life cycle is completed by two phases, the first phase is called the amastigote phase, which parasitizes inside the cells of the macrophages of the vertebral host represented by humans and mammals (rodents, canine family, cats), and the second phase is represented by the promastigote phase, which It is found in the invertebrate host represented by the vector insect (the female sand fly) of the genus Phlebotomus (Jamal et al., 2020). The life cycle of the parasite begins when the sandfly takes phagocytes loaded with flagellaless parasites with blood meal while feeding on infected vertebral hosts. The other during feeding the infected insect on the blood of these hosts (Al-Alusi, 2021Cutaneous leishmaniasis is a global health problem that causes permanent skin deformities and scars in most affected cases. It is the most common type and occurs after a sandfly bite. It usually affects exposed areas of the skin, which develop into a small red papule, then nodules or plaques, and eventually form a ulcer ulcer and two ulcers may fuse to form a large ulcer (Karami et al., 2013). This process is painless and is characterized by slight itching (De silva et al., 2016). Upon penetration of the anterior flagella, the host's autoimmune defenses are ingested by macrophages. The process of phagocytosis takes (4-8) hours, as the anterior protozoan adheres to the surface of the phagocytic cell. Then it becomes completely surrounded and devoured by the pseudopodia and thus is surrounded by a phagosome and turns into a flagellumless phase (Parham, 2014It has been proven that the phagosomes containing Leishmania parasites fuse with the lysosome, and this process does not seem to affect the parasite, which continues to multiply inside the phagolysosomes resulting from this fusion (Pinkovich et al., 2019).The infection with Leishmania parasite stimulates both the cellular and humoral immune response, as the host body's resistance to the Leishmania parasite depends on the cellular immunity of the T-helper1-T lymphocytes, while the susceptibility to infection with the parasite depends on the secretions of the T-helper 2T-helper cells. During differentiation affects T lymphocyte differentiation, IL-6 acts as a growth factor for B cells, transforming growth factor TGF stimulates cytokinesis IL-6 to develop Th17 T-cell responses and production of cytokines (IL-10,17) and suppresses the pathogenic function of Th17 cells. (Barbosa et al., 2018)IL-6 enhances Th2-cell responses in cutaneous leishmaniasis (Hu et al., 2018), IL-6 promotes inflammation through lymphocyte activation and proliferation, B-cell differentiation, and acute phase protein response stimulation in the liver. 6- An important signaling pathway closely involved in the transition between early and late phases of the inflammatory response, the main effects of cytokinetic IL-6, as a result of its presence in the

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circulation, can occur in distinct and distant sites from its origin, IL-6 is dominated by TNF-a and IL- 1 influences the acute inflammatory response, and IL-6 is almost solely responsible for the fever and acute phase response in the liver (Geraldo et al., 2016).

IL-17 plays an important role in acute phase interaction, inflammation, its role in host defenses against bacterial infection and in the development of allergic and autoimmune diseases. It affects the functions of neutrophils and macrophage cells, and it also induces some immune and non-immune cells to produce the cellular kinetics that initiate inflammation (Cardoso et al., 2009). The helper T cell represents 17 subsets of CD4 + T cells, and IL-17A is the most important Secretions of this cell, which has an important impact in the regulation of immune inflammation and reveals strong potential for autoimmunity (Rohn et al., 2006), and the availability of IL-17A at a high level is associated with chronic inflammatory diseases (Katara et al., 2013

2. Working Methods

blood samples collection

Blood samples were drawn using a sterile (5) ml syringe. Divide the blood into two parts, and put (3) ml inside sterile test tubes free of any anticoagulant to obtain the serum, as it was incubated at a temperature of (37) C for half an hour until coagulation occurred, then separated the serum using a centrifuge quickly (3000).) cycle/minute for a period of (15) minutes, and the serum was kept at a temperature of (-20) C until it is used in the necessary tests. As for the other section of blood (1) ml was placed in tubes containing an anticoagulant substance Ethylene diamine tetra acetic acid (EDTADetection of specific antibodyA quantity of serum was withdrawn by means of a micropipette of 20 microliters, and placed in the place designated for placing the sample in the test strip designated for the examination of specific antibodies of type IgM or IgG. Then two drops of Diluent Solution were added to it as in Figure (3-). Then I left 15 minutes for the result to appear in the test strip. When the indicator (red) appears on the (C) Control line, this indicates that the sample is negative. When the indicator appears on (G), this indicates that the sample is positive for IgG specific antibodies. When the indicator appears on (M), this indicates that the sample is positive for IgM specific antibodies.Determination of the concentration of interleukin-6 (IL-6) in the serumEstimation of interleukin-6 (IL-6) concentration in the serum The level of interleukin-6 in the blood serum was determined by following the steps attached to the special analysis kit and according to the manufacturer's instructions for the ELISA techniqueEnzyme-linked immunosorbent adsorption (ELISA) technique was used to measure the level of cytokinetic IL-6 in human serum, which was prepared by RayBio of the United States, which contains special specific antibodies needed to quantitatively measure the level of cytokinetic IL-6 in human serum, which is present on the surface of the pits in the micro-calibration plate. Adding the model and standard solutions to these antibodies, some of them are bound and others are not, and through the second incubation with the addition of secondary antibodies, any unbound antibody is removed and then comes the role of the paired enzyme, which is added to increase the binding strength of the antibodies against the antibody. At the end of the method, the pits are washed to remove the completely unbound antibodies, then the substrate is added, which leads to the formation of a blue color, which depends on the amount and intensity of the reaction of the antibodies with the antibody. Through its transmittance in the ELISA device using a wavelength of 450 nm.Test materialsThe measurement kit consists of the following material .The 96-hole micro-calibration plate is well coated with human interleukin-6 antibody2 .Concentrated Wash Buffer Solution (20X)) 25 m3 .Standard solutions of Recombinant human IL-64 .Assay Diluent A) 30% Sodium azide 0.09 ml It is used as a preservative for the sample and standard solutions5 .Assay Diluent B: 15 ml (5x), which is a concentrated buffer.6 . Antibodies detecting IL-6 formed from a solution of human IL-8 antibodies bound to biotin7 .Conjugated Conjugated Enzyme: 6µl x 30,000 HRP-Conjugated streptavidin8 .TMB matrix reagent: 12 ml of Teyramethylbenzidine.9 .Stop solution: 8 ml at a concentration of 2 M. Sulfuric acid • Reagent preparationWhen starting work, the solutions and samples should be at room temperature (18-25) degrees Celsius •Washing SolutionDilute the washing solution prepared by the company in a ratio of 1:20, adding 950 ml of distilled water to 50 ml of the washing solution prepared from the company to obtain 1000 ml of washing solution.

Standard solution (800) µl of Assay Diluent solution (A) was added to the standard solution vial to prepare a concentration of 50 ng/ml and it was dissolved by mixing, 10 µl of the prepared standard solution was taken in the vial and added in a te tube with the addition of 823.3 µl of Assay Diluent solution A)) To obtain a concentration of 600 pg/ml, and using this concentration, dilutions of the standard solutions **were prepared as shown in Table (3-3 .(Standard curve dilutions for th measurement of interleukin-6**

823.3 µl of incubation solution	10 µl of a concentration of 50 ng/ml	600 pg/ml
800 microliter of incubation solution	200 µl of a concentration of 600 pg/mL	200 pg/ml
800 microliter of incubation solution	200 µl at a concentration of 200 pg/mL	66.7 pg/ml
800 microliter of incubation solution	200 µL of a concentration of 66.7 pg/mL	22.2 pg/ml
800 microliter of incubation solution	200 µl at a concentration of 22.2 pg/mL	7.4 pg/ml
800 microliter of incubation solution	200 µl at a concentration of 7.4 pg/mL	2.5 pg/ml
800 microliter of incubation solution	200 µl at a concentration of 0.8 pg/mL	0.8 pg/ml
800 microliter of incubation solution	200 µl at a concentration of 0.8 pg/mL	0 pg/ml

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Detection antibodies

We add 100 µl of Assay Diluent B (assay diluent B) which was prepared by dissolving 10 mL of the concentrated solution with 40 mL of distilled water to be (B1x) to the vial of the concentrated antibody reagent, and it was mixed by withdrawing and returning with the micropipette, then diluting the concentrate 80 times by adding 100 µl of Antibody reagent to 7.9 mL of (B1x •Conjugate solutionThe conjugated enzyme HRP-Streptavidin is prepared by adding 2 µl of the conjugated enzyme concentrate to a test tube with 198 µl of (B1x) diluted 100 times, diluting it to 30,000 times and taking 50 µl of the diluted 100 times in a test tube with the addition of 15 ml of (B1x). To prepare the conjugated enzyme diluted 30,000 times

• The method of work 1 .Add 100 microliters of each dilution of Standard Solution A or sample serum to the holes designated for them in the Microtiter plate and cover them.2 .Incubate the plate for 2.5 hours at room temperature 25°C using a Microtiter plate shaker3 .The washing process was carried out using the washing solution using the Microtiter plate washer device. The plate was washed 4 times with a volume of 300 microliters for each hole4 .Add 100 µL of Biotinylated antibodies to each pit5 . Incubate the dish for 1 hour at room temperature 25°C using a vibrator.6 .The washing process was carried out as in the third step7 100 .µL of HRP-Streptavidin conjugate solution was added to each hole.8 .Incubate the dish for 45 minutes at room temperature 25°C using a vibrator9 .The washing process was carried out as in the third step10 .Add 100 microliters of Substrate solution (TMB) to each hole11 . Incubate the plate for 30 minutes at room temperature 25°C and in a dark medium using a vibrator12 .Add 50 microliters to each pit of the stop solution.13 .The optical density was read using a read-out device at a wavelength of 450 nm14 . The optical density readings were used for each of the dilutions of the standard solution in drawing the standard curve, and from the curve, the concentrations of interleukin-6 in the unknown samples were known Determination of the concentration of interleukin-17 (IL-17) in the serum

Estimation of interleukin-17 (IL-17) concentration in the serumThe level of interleukin-17 in the blood serum was determined by following the steps included with the special analysis kit and according to the manufacturer's instructions for the ELISA technique The same principle was used to measure the level of IL-6 cytokinesisTest materialsThe measurement kit consists of the following materials The 96-hole micro-calibration plate is coated with anti-human interleukin-172 .Concentrated Wash Buffer Solution (20X)) 25 ml3 .Recombinant human IL-10 standard solutions4 .Assay Diluent A) 0.09 30% Sodium azide ml is used as a preservative for the sample and standard solutions5 .Assay Diluent B: 15 ml (5x), which is a concentrated buffer6 .Antibodies detecting IL-17 formed from a solution of human IL-10 antibodies bound to biotin7 .Conjugated enzyme concentrate: 8 µl x 30,000 HRP-Conjugated streptavidin8 .TMB Base Reagent: 12 ml Teyramethylbenzidine9. Stop solution: 8 ml at a concentration of 2 molar sulfuric acid •Reagent preparatio Whenstarting work, the solutions and samples should be at room temperature 18-25 degrees Celsius •Washing Solution The washing solution supplied by the company was diluted in a ratio of 1:20, as 950 ml of distilled water was added to 50 ml of the washing solution prepared from the company to obtain 1000 ml of washing solution •Standard solution400 µl of Assay Diluent A solution was added to the standard solution vial to prepare a concentration of 22 ng/ml and dissolved by mixing, 5 µl of the prepared standard solution was taken in the vial and added in a test tube with the addition of 728.3 µl of Assay Diluent A solution to obtain At a concentration of 150 pg/ml, and using this concentration, dilutions of the standard solutions were prepared as shown in the table below

Standard curve dilutions for the measurement of IL-17

dilution solution	Interleukin-17 (IL-17)	Standard concetration
728. 3 µl of incubation solution	5µl of concentration 22nan/ml	150 pg/ml
400 µl of incubation solution	300µl of concentration150 pg/ml	75 pg/ml
400 µl of incubation solution	300µl of concentration75 pg/ml	37.5 pg/ml
400 µl of incubation solution	300µl of concentration37.5 pg/ml	18.75 pg/ml
400 µl of incubation solution	300µl of concentration18.75 pg/ml	9.38 pg/ml
400 µl of incubation solution	300µl of concentration9.38 pg/ml	4.69 pg/ml
400 µl of incubation solution	300µl of concentration4.69 pg/ml	2.34 pg/ml
400 µl of incubation solution	300µl of concentration2.34 pg/ml	0 pg/ml

3. Results and Discussion

Immunoglobulin assay

The results of the current study showed that when collecting 90blood samples for people with cutaneous leishmaniasis and conducting serological tests, (15.6)% of the patients showed IgG specific antibodies and (%84.4showed IgM antibodies as in Table (1-4) and Figure 4) -.(8

Table (4-1): The concentration of immunoglobulins (IgG, IgM) in the serum of leishmaniasis infected and the control group.

Percentage	number	Antibodies
15.6%	14	IgG
84.4%	76	IgM
100%	90	Group

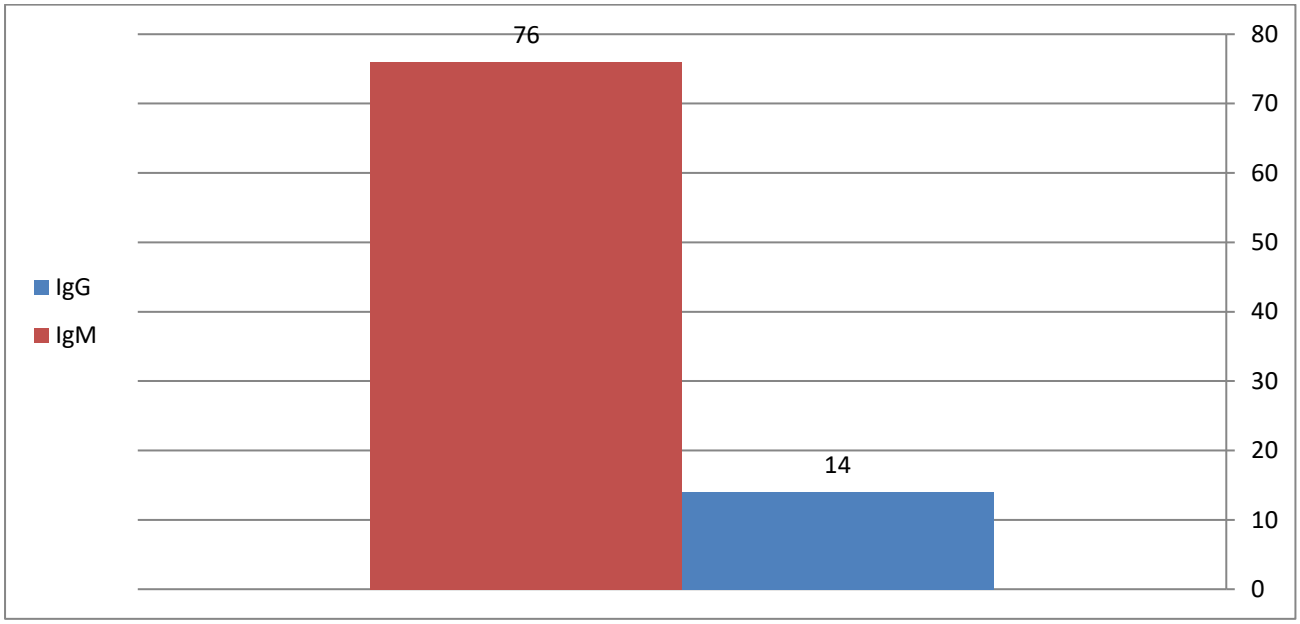

Figure (8-4): Immunoglobulin concentrations (IgG, IgM) in the serum of leishmaniasis infected and the control group.

The results of our current study showed an increase in the level of IgM and IgG immunoglobulins in patients with cutaneous leishmaniasis. In cutaneous leishmaniasis, it leads to high levels of immunoglobulins in general and IgG in particular. On the presence of infection, IgG represents the chronic infection, while the appearance of IgM is the most severe infection (Roitt et al., 2001From the results of measuring the levels of immunoglobulins, we notice a significant increase in the levels of IgG and IgM during infection, and this rise increases with the progression of the disease. Increasing the number of parasites as a result of stimulating B cells, which leads to an increase in the production and construction of immune globulins, and consequently their levels rise during infection. The presence of IgG-specific antibodies represents an important diagnostic characteristic of the sera of patients with cutaneous leishmaniasis, as these antibodies are one of the most important mechanisms of immune defense in the host. Against cutaneous leishmaniasis. The detection of parasite-specific IgG antibodies is evidence of infection (Ravanbod, 2000It was also noted that there is a relationship between infection with Leishmania parasite and cases of autoimmune diseases, where the humoral response of patients with leishmaniasis is an indication of increased levels of immune globulins and antibody specific to leishmaniasis, despite the presence of some immune globulins that are not affected by this infection, but there are large numbers of those globulins

Immunity levels are elevated in the blood serum of people infected with Leishmania parasite. A key component of innate immunity is the ability of macrophages to differentiate into cells with a wide range of functions. These cells are able to respond to various stimuli such as microbial molecules, components of damaged cells, co-stimulatory molecules, cytokines and chemokines by changing its phenotypes. Studies have also confirmed that cutaneous leishmaniasis infection can lead to the production of large numbers of IgG-IgM-IgA antibodies after activating lymphocyte immune B cells to produce polyclomal antibody to B lymphocytes, generating specialized antibodies to infection. It is associated with the formation and activation of T-cytoxicty cells by activating them through another pathway through the formation of first helper T-helper-1 cells and some other cytokines in response to infection with Leishmania parasite (Malla and Mahajan, 2006IL-6 concentration in serumThe results of the current study showed an increase in the rate of interleukin-6 in the group of patients with leishmaniasis compared to the control group, and this increase formed a significant difference with statistical significance at a level of probability less than (Po ≤ 0.05), where the rate of interleukin-6 was in patients with cutaneous leishmaniasis. Single-ulcer (46.04 ± 15.0 pg/ml) and multi-ulcer (59.16 ± 18.3 pg/ml) compared with the control group (25.42 ± 6.36 pg/ml), as shown in Table (4-2) and Figure (4-9

Table (2-4): IL-6 concentration (pg/ml) in serum of leishmaniasis infected and control group			
P value	mean±SD	Number	Study samples
0.05 a	25.42±6.36	40	Control group
0.05 b	46.04±15.0	36	One ulcer group
C 0.05	59.16±18.3	54	Multi ulcer group

The different English letters indicate the presence of significant differences at the level of Pp ≤0.5))

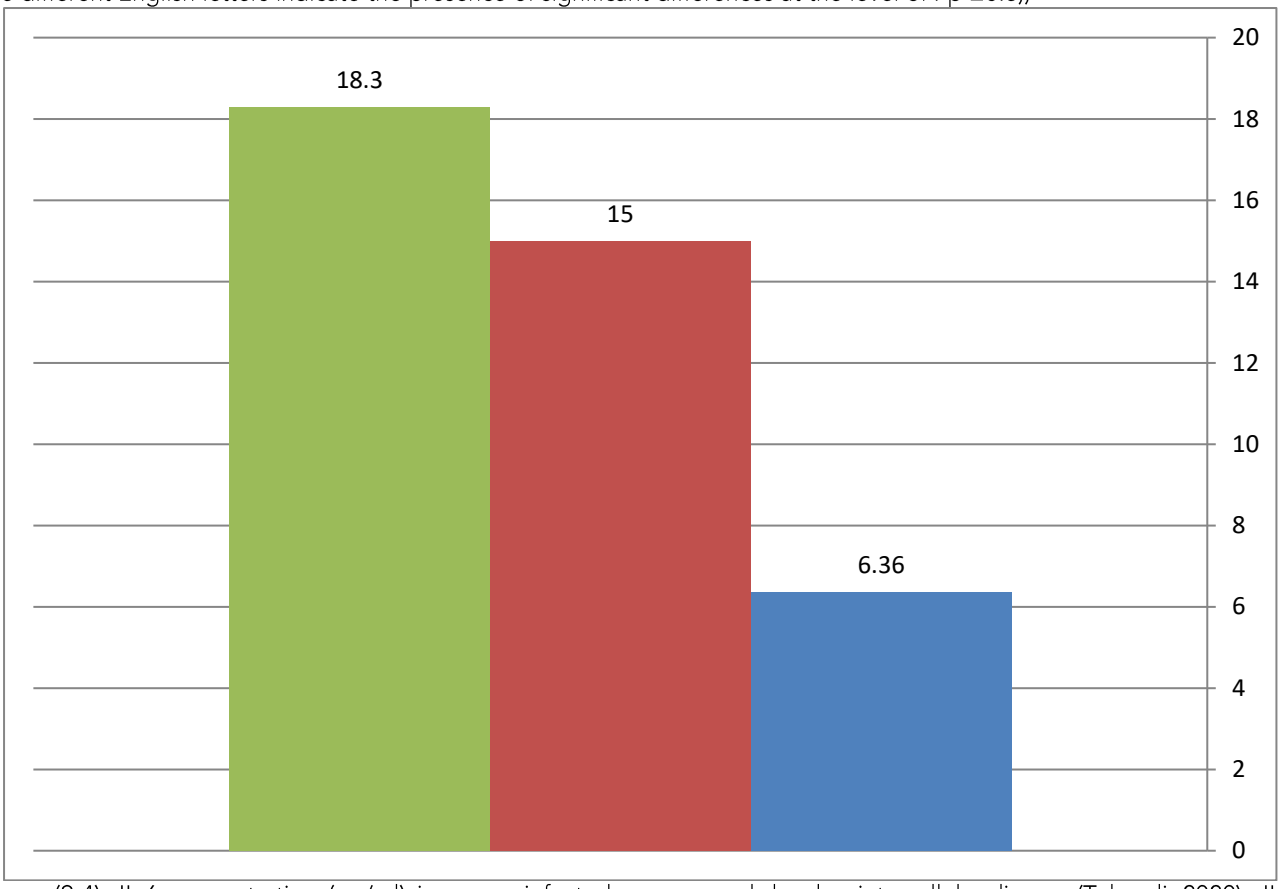

Figure (9-4): IL-6 concentration (pg/ml) in serum infected with leishmaniasis and control groupThe results of this study agree with the results of the study (Al- Aubaidi, 2010 Rohani and Parks, 2015;) that there was a significant increase in IL-6 for the cutaneous leishmaniasis group, and the results of the current study were in agreement with the results of Rubio and his group (2019), which recorded an increase The level of IL-6 in cutaneous leishmaniasis infection before treatment (pg/ml 12.85 ±.1.28) and began to decrease after treatment to reach (pg/ml 5.08 ± 0.70). The present results are in agreement with the results of the study of Al-Hassani (2020) and Taher Ali (2020), which recorded an increase in the IL-6 rate in people with cutaneous leishmaniasis compared to the control group, where it reached (0.017 pg/ml ± 0.035) in comparison with the control group. Control (0.017 ± 0.028 pg/ml) (Shakir *et al.,2019)*The reason for the increase of IL-6 in the serum of patients with cutaneous leishmaniasis is due to the response of the host to antigens of the parasite and the activation of macrophages that produce IL-6, which led to an increase in it in people with cutaneous leishmaniasis. IL-6 increased more in the multi-ulcer group, and this is attributed to the fact that the parasite numbers are more than the one ulcer group. IL-6 is almost solely responsible for the fever and acute phase response in the liver, and it is produced by Th2 helper cells, which act against Th1 cells by inhibiting the activity of helper macrophages, thus allowing parasites to grow and develop intracellular disease (Taherali, 2020). .IL-6 is one of the most important cellular kinetics secreted by macrophages, dendritic cells, and T cells and regulates B lymphocyte function and acute phase response. 6 and activated TNF-α in cutaneous leishmaniasis by increasing the synthesis of acute phase proteins, hypergammaglobulinemia, and induction of fever, and that these changes may not be the result of a specific deficiency of nutritional imbalances but are part of the defense strategies of the organism regulated by immune cytokines. The autoimmune system has a role Essentially when infection occurs effectively against pathogens in addition to macrophages that have a repertoire of IL-6 cytokinesis, host response to parasite antigens and activation of macrophages that produce IL-6, which led to its increase in people with cutaneous leishmaniasis (Narazaki et al. ., 2017The concentration of interleukin-17 IL in the serumIL-17 concentration in serum

The results of the current study showed an increase in the IL-17 rate in the group of patients with leishmaniasis compared to the control group, and this increase formed a significant difference with statistical significance at a level of probability less than (Po ≤ 0.5), where the IL-17 rate was in patients with leishmaniasis. Skin with single ulcers (pg/ml 47.17 ± 27.8) and with multiple ulcers (pg/ml 87.41 ± 30.5) compared with the control group (38.14 ± 17.9 pg/ml) as shown in Table (4-3) and Figure (10-4)

Table 3-4: The concentration of IL-17 (pg/ml) in the serum of patients with leishmaniasis and the control group			
P value	mean±SD	count	Study samples
0.05 a	38.14±17.9	40	Control group
0.05 b	47.17±27.8	36	one ulcer group
0.05 c	87.41±30.5	54	multi ulcer group

The different English letters indicate the presence of significant differences at the level of Pp ≤((0.5

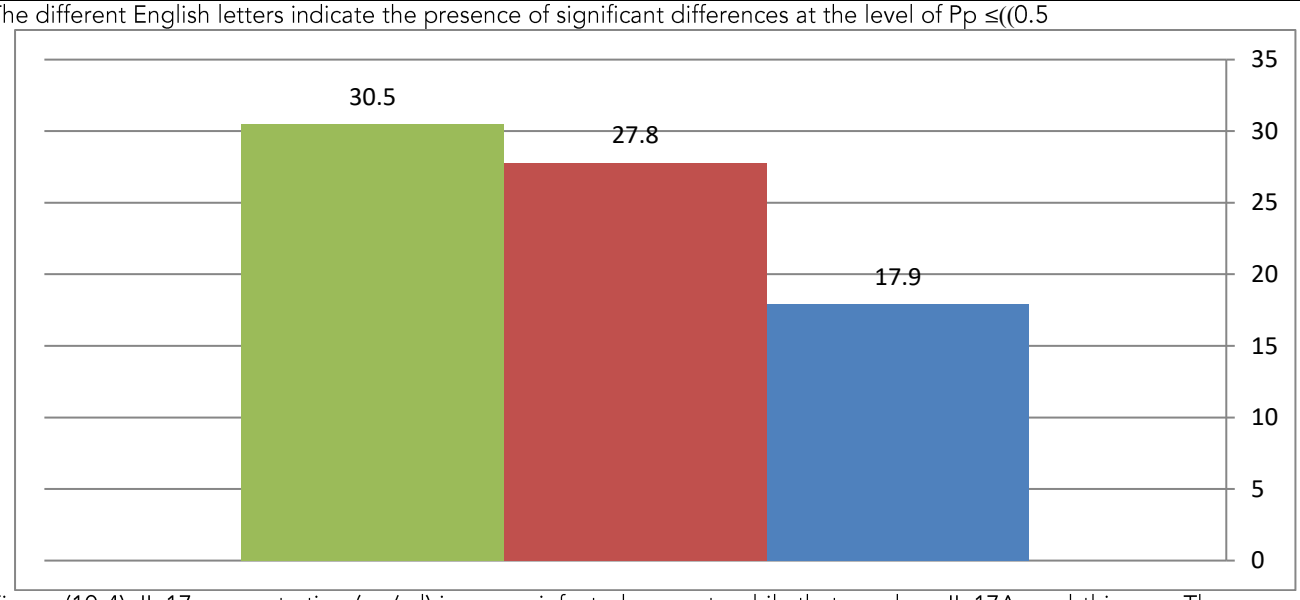

Figure (10-4): IL-17 concentration (pg/ml) in serum infected with leishmaniasis and control groupThe results were consistent with a study conducted by Bacellar et al. (2009), where the results of the current study showed an increase in the rate of IL-17 in the serum of patients with cutaneous leishmaniasis, and this is consistent with what was found (Novoa et al., 2011; Katara et al., 2013).The results also agreed with Al-Nasiri (2009), Al-Daham and Al-Alusi (2011) and Baraa (2014), where multiple ulcers were higher than the incidence of single ulcers, where multiple ulcers occur as a result of repeated stings of the same vector at the same time or other or at different times because The insect does not go far from its breeding areasOur study differed with the study of Al-Saadi (2014), where his study showed that there is a non-significant increase among multiple ulcers compared to patients with single ulcers, and this is attributed to the fact that infection with cutaneous leishmaniasis leads to inflammatory reactions and that these reactions occur in the blood leading to the activation of IL-producing cells. 17 And that the production of these cellular kinetics reaches a certain level, whether it is a single ulcer or multiple ulcers, and these results differ with what we have found, as the number of infections with one ulcer was close to injuries to several ulcers, so there was no significant difference, while in the results we obtained The number of infections with multiple ulcers more than one ulcerIL-17 has diverse biological functions, enhancing protective immunity against many pathogens. IL-17A and IL-17F are produced by CD4, CD8, repentant cells, and several populations of innate immune cells in response to IL-1β and IL-23, and they mediate protective immunity against fungi and bacteria by enhancing neutrophil recruitment, promoting antimicrobial peptide production and enhancing antimicrobial peptide production. The barrier function, the reason for the increase is due to the response of the host to parasite antigens and the activation of the helper T cell Th17 that works to produce IL-17A, as there are many cells that accumulate in the area of leishmaniasis infection, including Th17 cells and neutrophils that produce IL-17A, and this may The reason for its increase in the serum of the infected group is that Th17 helper T cells increase when exposed to most parasitic infections and allergies, and IL-17A is one of the most important cellular initiating kinetics of inflammation produced by Th17 (Nylen, 2012 and EiedsmoAlthough a vaccine against leishmaniasis has not yet been developed, individuals who have recovered from cutaneous leishmaniasis are able to protect themselves for a long time against the same disease and this protection is associated with increased IL-17 and IL-22, as IL-17 despite Although it has the ability to increase the symptoms of disease in the host, it has a role in the protective immune response as it is able to stimulate memory CD4 and CD8 cells against Leishmania infection (Banerjee et al., 2016).

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