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## Detection of some CC Chemokine Ligands in Patients with Cutaneous Leishmaniasis

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### Abstract

Cutaneous leishmaniasis (CL) is an endemic parasitic disease found in many provinces of Iraq. The immune system plays a crucial role in the development or healing of lesions through chemotactic cytokine activity. This study was aimed to detect the levels of two chemokine ligands (CCL2 and CCL5) in Iraqi patients suffering from dermal ulcers, caused by cutaneous leishmaniasis. It was measured in pre and post-treatment state of Pentostam (Pentavalent Antimony 100 mg). Blood serum concentrations of CCL2, CCL5 were measured by enzyme-linked immunosorbent assay among newly infected patients, two-trial treatment patients and three-trial treatment patients, in comparison with the control group. The result indicated a significant difference in CCL5 level for the three groups of CL patients. Whereas the control ( $p < 0.5$ ), CCL2 level counterparts showed a significant difference only in newly infected and the three-trial treatment groups. Moreover, there was a significant difference between all CCL5 patient groups, while no observed difference was detected within patient groups of CCL2. Thus altering the chemokine levels before and after treatment gives insights for parasite role in chemokine expression which may help in new therapeutic approaches for dry or wet CL.

**Keywords:** CCL5, CCL2, Pentostam and Cutaneous Leishmaniasis.

### التحري عن بعض الروابط الكيميائية في المرضى المصابين بداء اللشمانيات الجلدي

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### الخلاصة

يعتبر داء اللشمانيات الجلدي احد الامراض المتوطنة في مناطق مختلفة من العراق. الجهاز المناعي للجسم له دور فعال في تطور الافة الجلدية او شفائها للأشخاص المصابين من خلال فعالية السايبتوكينات الكيميائية. هدفت الدراسة الحالية الى التحري عن اثنين من الروابط الكيميائية (CCL2, CCL5) في مرضى عراقيين يعانون من القرحة الجلدية الناجمة عن الإصابة بطفيلي اللشمانيا الجلدي و قد تم الكشف عن ذلك لدى المصابين في مرحلة قبل وبعد العلاج بعقار البنتوستام. تم قياس تراكيز الـ CCL2 و CCL5 في مصل الدم باستخدام تقنية الاليزا لمرضى العدوى الجديدة و المرضى الذين خضعوا مرتين للعلاج و المرضى

الذين خضعوا ثلاث مرات للعلاج والمقارنة مع مجموعة السيطرة من الاصحاء. أوضحت النتائج ان هنالك فرق معنوي في مستوى الـ CCL5 لمجموعات الإصابة الثلاث مقارنة مع مجموعة السيطرة ( $p < 0.5$ ). بينما سجلت نتائج الـ CCL2 فرق معنوي واضح في مجموعة الإصابة الجديدة التي لم تخضع الى العلاج فقط. من جانب اخر بينت نتائج المقارنة فيما بين مجموعات الإصابة لكل رابط كيميائي وجود اختلاف معنوي بين مجموعات الإصابة للـ CCL5 بينما لم يسجل فرق واضح بين مجموعات الإصابة للـ CCL2. توضح هذه النتائج ان التغيير الحاصل في مستويات السايبتوكينات المدروسة قبل و بعد اخذ العلاج تعطي فكره مهمه عن دور الطفيلي في احداث هذا التغيير و الذي يساعد في إيجاد أساليب علاجية جديدة لقرح الشمانيا الجافة و الرطبة.

## Introduction:

Leishmaniasis is one of the most important infectious disease that causes a serious health concern around the world [1]. *Leishmania* parasite has several types of infections fluctuating between cutaneous and visceral, in which the epidemiological state, clinical features, prognosis and treatment response varies depending on the species [2,3]. In the Middle East, the most prominent species of skin ulcers are caused by *L. aethiopica*, *L. major* and *L. tropica* which is usually referred as old-world [4].

The progression of skin lesion is controlled, in part, by chemokines and chemokine receptors. Chemokines are a group of small proteins composing of single polypeptide chain which ranges in length between 67 and 127 amino acid residues [5]. They vary by the location of cysteine motifs in the N-terminus of biochemistry as CXC, CX3C, CC. The first two cysteines adjacent are to each other in chemokines known as CC ( $\beta$ ), while the first two cysteines are separated by an amino acid in chemokines CXC ( $\alpha$ ). Leukocyte's recruitment was interfered by chemokines which is involved in homeostasis, adaptive plus innate responses of immune system [6].

During *Leishmania* infection, the host induced the expression of genes which are potentially important to the parasite through recruitment invaded cells (macrophage) [7,9]. In *Leishmania major* infection, the chemokine level varies actively and thus, recruiting cells which are in service in the development of infection and persistent [10]. Many species of *Leishmania* like *Leishmania amazonensis* have their virulence factor that able to inhibit production of pro-inflammatory cytokines and chemokine expression, and shading their entry in host [11,12]. The surface of *Leishmania* promastigotes contains a glycolipid called lipophosphoglycan (LPG) which has effects on the migration of monocyte. Cytokines and chemokines synergetic action has a major role in the pathogenesis of cutaneous and diffused cutaneous disease. The effect of chemotactic cytokines is essential for the recruitment of lymphocytes, tissues and trans-endothelial migration of monocyte trans-endothelial by the suppress production of CCL2 in endothelial cells [13].

Immunological response towards *Leishmania* infection is well characterized in terms of Th1 trigger. The parallel involvement of IL-12 driven Th1 response and IFN- $\gamma$  production, in addition to the enrolment of effector cells, macrophages, NK cells, CD4+ and CD8+ cells to the bitten-skin area, has an important role in lesion progression [14,15]. Notably, further initiation of both innate and acquired immune response was found to be rolled out by chemokine action [16]. Chemokines assembly during the early phase of infection, determines the composition of migrating cells which boosts inflammatory response [17]. Additionally, the intracellular amastigotes were found to suppress the chemokine receptor CCR1 on human macrophages which reduced macrophage recruitment to the infected tissues and, hence, resulted in parasite progression [18]. The expression of chemokine and chemokine receptor by *Leishmania* infected host cells may potentially be important in facilitating the ability of host to restrain the parasite to the site of inoculation [11]. Further to the mediating cellular

recruitment, chemokines can activate various cell populations, participate in cell mediated immunity and possess antileishmanial properties. According to immunological roles of CCL5 and CCL2, the present study was aimed to estimate the level of these chemokines in serum of Iraqi patients with cutaneous leishmaniasis in both pre and post pentostam treatment.

#### **Materials and Methods:**

Samples of 78 male and female participants aged between 10 and 55 years were involved in this study during their attendance to Baqubah General Hospital in Diyala province during October 2020 to February 2021. All patients were positively diagnosed and confirmed infected with cutaneous leishmaniasis by the dermatologists and laboratory diagnosis. The patient's lesions appeared as papules or nodules, wet or dry with acute ulceration in some cases. Most lesions were seen at face and/or arms. Ulcers have higher borders with faint centers. Controlled healthy subjects were collected from the same area.

**Serum samples:** A minimum of 2 milliliters of veinous blood was collected from each subject using gel tube and allowed to clot at 37°C for 10 minutes. Blood samples were centrifuged for 5 minutes at 3000 rpm until serum was separated. Pure serum samples were stored at -20°C until use.

**Study design:** Patients samples were divided according to the number of treatments as following: 40 patients with new infection and no treatment, 23 patients with 2<sup>nd</sup> trial-treatment and 15 patients with 3<sup>rd</sup> trial-treatment of Pentostam (Sodium stibogluconate) as an Anti-leishmaniasis Agents, and 15 of healthy non-affected controlled subjects. Sodium stibogluconate was administered through the intradermal injections. The dose of Pentostam used in the hospital was 1-2 ml of 100 mg Pentostam stock (India). Injection was repeated every 3–7 days until healed.

#### **Measurement of serum chemokines levels:**

**CCL2 and CCL5** serum ELISA kits were purchased from Bioassay Technology Laboratory, Cat.No. E5009 Hu, Shanghai, China. Both CCL ligands were detected in the patients with cutaneous leishmaniasis infection, pre and posttreatment, in addition to the controlled group by indirect ELISA according to the manufacturer's procedure. The sensitivity of ELISA kits are 3.09ng/L and 5.26ng/L for CCL2 and CCL5 respectively.

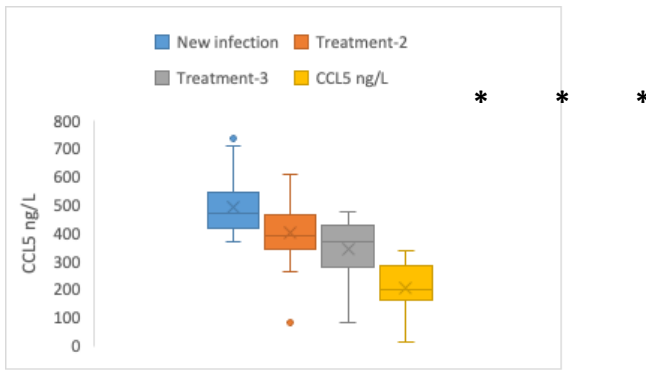
**Statistical Analysis:** Data was analyzed by GraphPad Prism v7.0; t test was used to compare each patient group against the healthy control. While ANOVA test was used to detect differences between all patient groups for each chemokine ligand (Differences were considered significant if  $P < 0.05$ ).

#### **Results and Discussion**

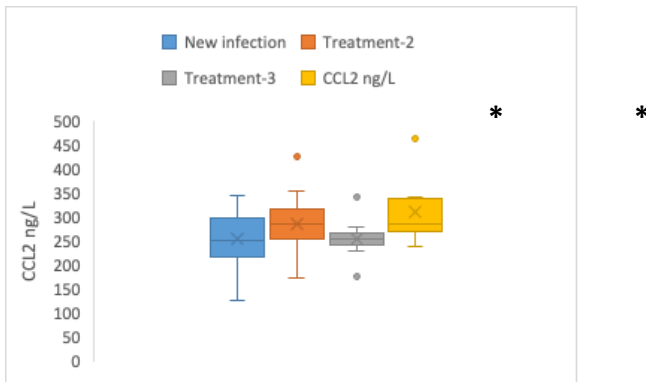
Three groups of cutaneous leishmaniasis patients were studied at different therapeutic stages with pentostam (P: newly infected patients with no Pentostam administration,  $n = 40$ ; Patients after two doses of pentostam,  $n = 23$ ; Patients after three doses of pentostam,  $n = 15$ ) in addition to 15 samples of healthy control.

Results revealed that the mean of CCL5 serum levels in new CL patients, patients after two-trail treatment and patients after three-trail treatment were 496.1607143, 405.4751553 and 346.3351648 ng/L respectively, with maximum significant at  $p < 0.0001$  for new infection group; in comparison with the control group, which was 209.2766162 ng/L (Figure 1). While the mean of CCL2 serum levels were 255.9218559, 287.3593074 and 256.4285714 ng/L respectively, in comparison with the control group, which was 310.1428571 ng/L, given no significance was observed in patients only with two-trail treatment, Figure 2.

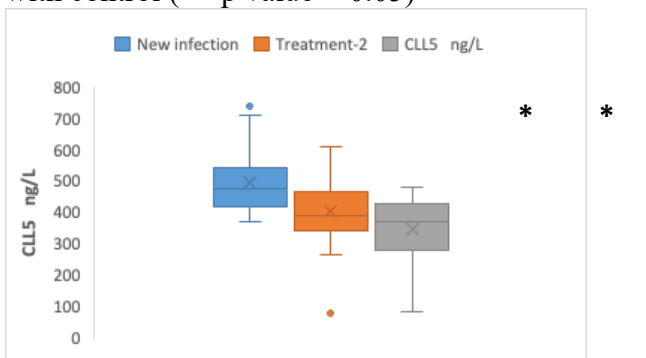
Additionally, the results indicated existence of significant difference in CCL5 level among the three groups of CL patients, whether they received Pentostam treatment or not, at any stage (Figure 3). Furthermore, there was no significant difference among the three patient groups in CCL2 level (Figure 4).



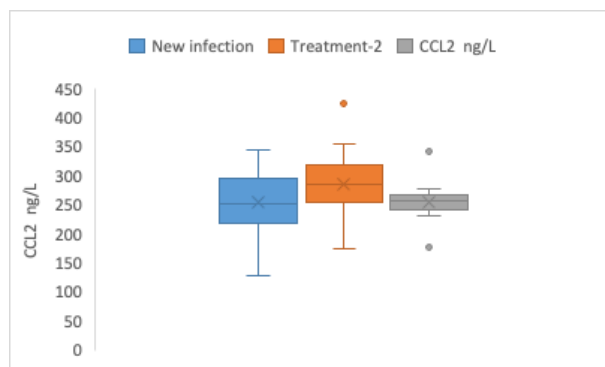
**Figure 1-**The levels of CCL5 chemokines in serum of three CL patients groups in comparison with control (\*= p value < 0.05)



**Figure 2-**The levels of CCL2 chemokines in serum of three CL patients groups in comparison with control (\*= p value < 0.05)



**Figure 3-**A comparison of CCL5 chemokine level in the 3 groups of patients (\* = p value < 0.05)



**Figure 4-**A comparison of CCL2 chemokine level in the 3 groups of patients, no significance

As shown in the results above, serum concentration of CCL5 was increased in CL patients before and after the treatment, in all patient groups, when compared with healthy subjects. Moreover, an interesting gradual decrease was observed after Pentostam treatment, in which p values were equal to 0.0001, 0.001 and 0.0011 for new infection, two-trial treatment and three-trial treatment respectively. However, the CCL2 concentration declined in CL patients before and after the treatment when compared with healthy subjects, in a reduced way, in which no significance was observed in the two-trial treatment.

CCL2 is an angiogenic chemokine that effects the integument scar and wound healing. In humans leishmaniasis, CCL2 and macrophage inflammatory protein 1a (MIP-1a) have an important mutual activity for macrophage stimulation in skin lesions. A high CCL2 expression and moderate levels of macrophage inflammatory protein (1a) were explored in biopsy samples isolated from patient with localized CL. In contrast, high levels of MIP-1a and low levels of CCL2 were discovered in the blistered DCL lesions [19]. A comparable study demonstrated that motivation of macrophages by synergistic accomplishment of IFN- $\gamma$  and CCL2 can kill the intracellular amastigotes of *Leishmania* in localized CL. While IL-4 activity in DCL lesions may inhibit the expression of CCL2 in addition to continuation of disease [2].

Numerous studies verified the rapid variation of chemokine expression in cutaneous leishmaniasis infection. This may explain the possible association of chemokines in the disease pathogenesis in addition to etiology [17,21]. A related study exposed the problem in *Leishmania* parasites eradication in the presence of low level of CCL2 concentration. CCL2 has a critical role in mechanism of pathogenesis. The main constituent of immunity against leishmaniasis is cellular immunity. Moreover, the chronic state of the disease has incompatible levels of cellular immunity to eradicate the parasite [22].

In *Leishmania* infection, chemokines have different roles that includes the aspect in adaptive immunity, stimulation of macrophage and killing of the parasite. Nevertheless, the most evident function is the recruitment of immune cells to the site of parasite delivery [23]. In the bite-site of entry, sentinel cells, including antigen-presenting cells (DCs), T lymphocyte and macrophages trigger the immune response. These cells were found to be well labeled with Toll Like Receptors (TLRs) [20], in addition to receptors of phagocytosis, thus allowing their cognition of pathogen associated molecular patterns and engulf of pathogens plus opsonized particles [24]. The sentinel cells also express various cytokines and, together with tissue cells, yield a several chemokines originating the innate responses cascade. Mice infected by *Leishmania major* were found to undergo induce expression of CCL5, CXCL10, MIP-1a and CCL2 in the footpad [25].

In case of CCL5, this chemokine up-regulates IFN- $\gamma$  [1], IL-12 [27], and migration of T helper 1 cells; mainly memory T cells [24]. A related study demonstrated that treatment of BALB/c mice by Met-RANTES or anti-CCL5 reduced and altered the immune response from type 1 to type 2 by diminishing the production of IFN- $\gamma$  with draining lymph nodes and increased the expression of IL-4 mRNA in the lesions. This may explain why the increase of susceptibility to infection is promoted by treatment with Met-RANTES or anti-CCL5. Furthermore, a previous study proved the attracting of inflammatory lymphocytes by CCL5 and CCL4, mainly T helper1 cells; eosinophils attracted by CCL24; while lymphocytes, monocytes, neutrophils were attracted by CXCL8. Also, chemokine CCL17 service in establishing the inflammatory infiltrate, a characteristic feature of various inflammatory skin conditions, by attracting CCR4-bearing cells, which are especially polarized to Th2-type cells and regulatory T cells [28].

A study by [29] reported that IFN- $\gamma$  induced chemokines as a response to pentostam treatment and showed a killing activity of *Leishmania donovani* parasite in livers of C57BL/6 mice. This knockout study found that a group of mice suffering from chemokine receptor deficiency were normally responding to pentostam treatment. However, some chemokines CCL5, CXCL10, CCL2, and CXCR3 had a minor tissue inflammation during treatment administration in mice. Curiously, mice endured gene deficiency exhibited that killing of the intracellular parasite was unaffected by CXCL16 plus CXCL13, suppressed not only by CXCL10 but also by CXCL9, and enhanced by CCL2 and especially by CCL5 after Pentostam treatment.

**Conclusion:** Fluctuating levels of CCL5 and, in lesser rate, CCL2 may help in new therapeutic approaches by understanding immuno-pathogenesis of the parasite. Furthermore, identifying target chemokines roles may be helpful in the novel vaccination against leishmaniasis.

**Conflict of Interests:** The authors have no conflict of interests to declare.

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