

Role of some Cytokines and Oxidative Stress enzymes in Iraqi Children infected with Visceral Leishmaniasis

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Abstract

A totals of 66 children whose ages range from seven month to six years, suspected to be infected with Visceral Leishmaniasis (VL), they were compared to fifty healthy subjects in the control group. The diagnosis was done by rKE16 dipstick test, and evaluating the level of cytokine IL-6, Inter cellular Adhesion Molecule (ICAM-1) and Monokine induced by INF- γ (MIG-1), was evaluated. Stress biomarkers estimate including the level of malondialdehyde (MDA) as an index of free radicals induced lipid peroxidation and catalase as an index of antioxidant enzyme in the serum of patients infected with VL.

The rKE16 antigen strip test shows valid highly in the diagnosis (N=65) of suspected children with VL. The test rapid, sensitive, and specific. The results show an increase ($P \leq 0.05$) in the serum levels of IL-6, ICAM and MIG-1 in patients than in healthy subject. Result of oxidative stress showed a significant ($P \leq 0.005$) elevated level of MDA and a decrease CAT in patients with VL as compared to the cases in the control.

These results showed points in the pathology of VL and might open new treatment perspectives associated with antioxidant.

Keywords: *Leishmania donovani* diagnosis, Cytokine, MDA, CAT

الخلاصة

شملت الدراسة 66 طفلاً مشوكاً بإصابتهم بداء الليشمينيا الاحشائية و 50 طفلاً كمجموعة مقارنة بمعدل عمر تراوح بين 7 اشهر الى 6 سنوات , تم اختبار عينات المصل باستخدام rKE16 dipstick الكرموتوغرافي المناعي للتحري عن وجود الاجسام المضادة لطفيلي داء الليشمينيا الاحشائية , والتقييم المصلي لمستوى الحركي الخلوي IL-6 , وجزيئات الالتصاق الداخلي ICAM-1 و MIG-1. اظهر اختبار rKE16 سرعة مع حساسية وخصوصية عالية في تشخيص الاطفال (N=65) المشبهة باصابتهم بالليشمينيا الاحشائية مقارنة بالاعراض السريرية والفحوصات الاخرى وظهرت النتائج ارتفاع معنوي ($P \leq 0.05$) في مستوى IL-6 , ICAM-1 وكذلك MIG-1. نتائج الجهد التأكسدي اوضحت ارتفاع معنوي ($P < 0.05$) في مستوى MDA وانخفاض كبير CAT في المرضى الذين يعانون من VL بالمقارنة مع مجموعة الاصحاء .

اعطت النتائج مؤشراً حول امراضية الليشمينيا الاحشائية , لربما تفتح الآفاق لعلاج مرتبط بمضادة للأكسدة.

الكلمات المفتاحية: تشخيص داء الليشمينيا الاحشائية، Cytokine، مستوى MDA، مستوى CAT .

Introduction

Leishmaniasis is a vector –born disease, also known as kalazar caused by obligate intra macrophage, it is endemic in less developed countries. Visceral Leishmaniasis (VL) is a potentially fatal human disease with estimated incidence of at least 0.2 to 0.4 million case worldwide, causing 20,000- 40,000 death each year (Alvar *et al.*, 2012). Dipstick is an immune chromatographic test for the qualitative determination of anti- Visceral Leishmaniasis(VL) antibodies, using a patented recombinant antigen (rKE16) is highly specific for VL caused by parasite members of *L. donovani* complex of old world (Sivakumar *et al.*, 2006).

The beginning of infection is marked by recruitment of innate immune cell such as, Neutrophils, Macrophages, Natural killer cells, dendritic cells and Monocytes. VL was initially thought to be associated with a dominant Th2-type immune response (López *et al.*, 1988) and Th17 cell on Th1/Th2 signaling pathway and imbalance in immune responses could lead to disease progression (Asad and Ali, 2014). At the side of infection due to the secretion of different chemo-attractant proteins fore generation

innate immune responses are significant in bringing innate and adaptive responses side by side for combating against VL infection.

Later this recruitment generates a mixed regulatory and inflammatory responses by secreting chemokines and cytokines and recruits an early inflammatory reaction (Matt and Oliver, 2002; Rabhi *et al.*, 2012). Anti-inflammatory cytokines are a series of immunoregulatory molecules that cytokines counteract the effects of pro-inflammatory cytokines to limit the inflammation present. The major Anti-inflammatory cytokines are IL-6, IL-4, IL-10, IL-13 and TGF- β (Owen, *et al.*, 2013). Interleukin-6 is a pleiotropic cytokine elaborated in response to a wide range of inflammatory stimuli, and is ordinarily considered as a pro-inflammatory cytokine. However, it has also inflammatory suppressive activity and impair macrophage activation via inhibition its production ability of TNF- α and IL-1 (Ansari *et al.*, 2011). ICAM-1, known as CD54, is a cell surface glycoprotein that contributes to the interactions between leukocytes and several other cell types, including fibroblasts, keratinocytes, and monocytes/macrophages, lymphocytes. The presence of ICAM-1 may reflect the presence of inflammation and damage to vascular endothelium (Bradley *et al.*, 1994). MIG/CXCL9 is a small cytokine belonging to the CXC chemokine family that is also known as Monokine induced by INF- γ , MIG-1. It is a strong T-cell chemottractant to the site of inflammation and it repairs tissue damage (Rosenblum *et al.*, 2010; Comerford and McColl, 2011). Normal cellular metabolism involved the production of Reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radicals are produced during normal cellular function, which leads to the oxidation of lipids, proteins, and nucleic acids. ROS generation is controlled by the cellular antioxidant defense system (Limon-Pacheco and Consebatt, 2009).

Low levels of ROS are vital for proper cell functioning, while excessive *In vivo* generating from these products can diversely affect cell lipid oxidation production (LOP) occurs in clinical setting shock, and parasite infection (Kocyigi *et al.*, 2005). MDA is one of the final products of LOP in human cells, and an increase in ROS causes overproduction of MDA. Accordingly, the MDA level is considered a surrogate marker of oxidative Stress (Horoz *et al.*, 2006; Khoubnasabjafari *et al.*, 2015).

Materials and Methods

Subjects

Sixty six patients of visceral Leishmaniasis (VL), were diagnosed by Crystal – KA (rKE16), and their ages range from seven month to six years attending Central Public Health Laboratory, AL-Karama Teaching and Central Teaching Hospital of Pediatric in Baghdad, during the period from 1st of December 2014 to 30th of May 2015 (seasons of the disease in Iraq). The presumptive diagnosis was made by pediatrician on the basis of the classic clinical presentation of prolonged fever (more than 10 days), pallor, splenomegaly, hepatomegaly or hepatosplenomegaly. The Fifty subjects (the control group) were also included in this study all the suspected children and healthy ones were tested serologically by rKE16.

Blood sample collection: Ten ml of venous blood were collected from each child. The blood was put in 10ml tube and the tubes left to stand for one hour at room temperature for clot formation. For serum collection, the tube was cooled centrifugation at 300 rpm for 10 minutes, then it was dispensed into a sterile Eppendorf tubes and stored frozen at -20°C until used.

Assay procedure:

- 1- All reagents and samples were cooled to 25°C for use.

- 2- Four drops of reaction buffer were dispensed into the test tube.
- 3- Serum sample (20µl) was added 20 µl was mixed properly by agitation. Dipstick rKE16 (Span, India) was brought from blister pack and labeled with patient's identification code and was placed vertically into the tube containing diluted sample, until the sample liquid reached the arrow mark. The dipstick was removed from the sample after 15 minute, and the result was then read.

Positive result

The presence of two pinkish red bands, one at T and another at C region indicate that the sample is reactive for Leishmaniasis.

Negative result

The presence of only one pinkish red band at control C that the sample is non-reactive for Leishmaniasis.

Invalid result

The absence of pinkish red band at control C region with presence or absence of a band at T region indicates invalid results.

The presence or absence of a particulate matter in sample can be due to deterioration of the sample \reagent. In such case the test using new dipstick and fresh sample.

The sensitivity and specificity are two measures of the validity of a screening rKE16 test according to clinical diagnosis (with other tests) by Grimm (2001).

Serum Level of Cytokine: Sera of VL patients were assessed for a level of 3 cytokines (IL-6, ICAM-1 and MIG-1) by kit (PeproTech; USA) which is a sandwich Enzyme-linked Immunosorbent Assay (ELISA).

Stress biomarkers: Stress biomarkers estimate including MDA by Ohkaw *et al.*, (1979) and CAT according Aebi, *et al.*, (1984).

Statistics: Statistical analysis was achieved with the help of the computer through using computer assisted using Statistical Package for Social Science (SPSS) Version 13.0 was applied and there was the least significant difference (LSD) between groups.

Results

This study included sixty six children suspected of having VL and fifty healthy children as a control group, which were tested by using rKE16 dipstick assays to detect antibodies to *L. donovani* complex. The results showed that sixty five samples were positive to have infection by rKE16 dipstick test. None of the samples taken from healthy control showed reactivity in any of this tests (Table-1).

Table -1: Sera-prevalence of Visceral Leishmaniasis in children.

Subject	No. of cases	rKE16	
		-Ve No.	+Ve
Suspected group	66	1(1.51%)	65(98.48%)
Control group	52	52 (%100)	0 (0%)

The data in table-2 demonstrated the sensitivity, specificity and accuracy of strip rKE16 are 98.48%, 100% and 99.13% respectively in contrast to clinical diagnosis.

Table-2: Validity of strip rKE16 compared with clinical diagnosis of VL.

		Clinical diagnosis		Total
		Positive	Negative	
Serum rKE16 strip	Positive	65	0	65
	Negative	1	50	51
Total		66	50	116
Sensitivity		98.48%		
Specificity		100%		
PPV.		100%		
NPV.		98.03%		
Accuracy		99.13%		

PPV: Positive predictive value NPV: Negative predictive value

All the 65 studied children proved that they have VL, their ages ranged from 5 months to 4 years and the infection rate was significantly higher in age more than one year 39 (60%) of them were males and females, the percentage of females was significantly higher ($P < 0.01$) than that of males (61.54%), (38.46%) respectively (Table-3).

Table-3: The percentage distribution of Visceral Leishmaniasis according to age and sex.

parameter		No. of cases (%)	Total No. (%)	Chi-square
Age	Less than one year	26(40)	65(100)	9.52*
	More than one year	39(60)		
Sex	Female	40(61.54)	65(100)	7.23*
	male	25(38.46)		

*($P < 0.01$)

The data in Table-4 demonstrated the mean level of IL-6 in patients group was significantly higher than that in controls group (13.00 ± 1.82 pg/ml, 6.38 ± 0.78 pg/ml; respectively, $P < 0.01$). The value of mean \pm SE level of ICAM-1 and MIG-1 in patients group was significantly higher (11.9 ± 1.77 pg/ml, 0.03 ± 0.088 pg/ml; respectively, ($P < 0.01$) than that in control group.

Table- 4: The Mean level of IL-6, ICAM-1 and MIG-1 in sera of Visceral Leishmaniasis patients in comparison to control group.

Cytokine	Serum Level (Mean \pm S.E.; p g/ml)		LSD value
	Controls (No. = 50)	patients (No. = 65)	
IL-6	6.38 ± 0.78	13.0 ± 1.82	3.45**
ICAM-1	5.28 ± 0.61	11.96 ± 1.77	3.15**
MIG-1	$.0047 \pm 0.000040$	0.03 ± 0.088	0.048**

** $P < 0.01$

In the current study we observed a significant increase ($p < 0.05$) in the MDA in sera with VL (4.60 ± 2.15 nM/ml) compared with the control group (1.35 ± 0.31 nM/ml). Catalase which is an oxidant enzyme activity has shown significant decrease in

patients with VL ($0.75 \pm 0.58 \mu\text{mol/mol}$ of protein) in comparison with control group, Table-5.

Table-5: The mean serum Malonaldehyde and Catalase of Visceral Leishmaniasis patients in comparison to control group.

Group	NO.	MDA((nM/ml \pm SD)	CAT($\mu\text{mol/mol}$ \pm SD)
Patients	65	4.60 ± 2.15	0.75 ± 0.58
control	50	1.35 ± 0.31	9.81 ± 1.16

MDA: Malonaldehyde P <0.005 Significant CAT: Catalase P <0.005 Significant

Discussion

The study indicated that rKE16 test was sensitive (98.48%) to diagnosis of VL. The study by (Sulaiman, *et al.*, 2014, Gani *et al.*, 2010) showed that the antigen (rKE16) is its found to be 100 percent sensitive and specific. The result of the current study was nearly similar to the above studies. This diagnostic kit (rKE16) has several major advantages when compared with other testes in the field setting, the simplicity and ease of use, less cost, and rapidity of rKE16 dipstick are especially important in a setting such as rural areas in Iraq, where bone marrow can be performed by only few expert practitioners. While, the only disadvantage of the rKE16 test is inability to differentiate between recent and old infection. The (rKE16) antigen has now been commercialized and has a tremendous potential for the serological diagnosis of VL worldwide (Sivakumar *et al.*, 2006).

This study disagrees with the previous studies which showed that there most infection was in children group less than 2 years (AL-Kassar, 2005). The highest incidence recorded in more than one year of age group (60.0%), the increase of infection among this group may be due to the movement and activity of children leading of possibility to contact with vector sand fly that spread in the environment beside their immune system which is not well developed.

In the current study the distribution of VL according to the gender shows significant different percentage between females and males which were (61.54%) and (38.46%) respectively, this results disagrees with previous results of Al-Saqur *et al.* (2008) who showed the distribution of VL according to gender in Baghdad was nearly similar in males and females which was 52.2% and 47.8% respectively. On the other hand the result of the current study is similar with the reported works (Sulaiman *et al.*, 2014; Akbarpour *et al.*, 2012), which revealed that males infected with VL were less than female cases in percent.

IL-6 is a pleiotropic cytokine elaborated in response to a wide range of inflammatory stimuli, and it is ordinary considered as a pro-inflammatory cytokine produced by many cell types and is involved in acute –phase response in B cell maturation and differentiation of macrophages. IL-6 promotes TH2 differentiation and simultaneously inhibits TH1 via independent molecular mechanisms (Ewunetu *et al.*, 2015)). In this work the patients had high serum concentration of IL-6 when compared to control group (P<001); the same study in human infection demonstrates that production of IL-6 is associated with progressive nature of VL and is responsible for polyclonal activation B cells with increased level of circulating immune complex (Hailu *et al.*, 2004).

The CXCL9, also known as monokine induced by gamma interferon (MIG-1), is a strong T cell chemottractant to the site of inflammation (Rosenblum *et al.*, 2010; Comerford and McColl, 2011). The study shows that there is a significant increase of MIG in the serum of patients compared that of the subject in the with control group ,

this increase is the result of the continuation of the stimulus antigens parasite, leading thus to increase the activation of immune cells producing MIG-1 leading to high level in the serum of infected compared to serum of the subjects in the control group and this is consistent with the findings that MIG-1 were elevated in sera from *L. tropica* and *L. braziliensis* infected (Vargas –Inchaustegui *et al.*, 2010; Hubbaed and Rothlein, 2000), the chemokine CXCL9 and CXCL10 are critical in both innate and adaptive immune responses to *leishmania* infection.

The ICAM-1 is expressed constitutively at low levels by vascular endothelial cells under normal conditions, but expressed at large quantity upon stimulation by inflammatory cytokines such as TNF- α , increased ICAM-1 allows the attachment of leukocytes to the endothelium and subsequent migration to peripheral tissue (Hubbaed and Rothlein, 2000). It has been demonstrated that patients infected VL had higher ICAM-1 levels ($P < 0.001$) when compared to in the subject in the control.

Leishmania are obligate intracellular protozoa that infect and replicate within mammalian macrophages. Macrophage, neutrophils and other phagocytic cell are key components of antimicrobial and tumoricidal immune responses. These cells are capable of generating large amount of reactive oxygen and reactive nitrogen species (RNS) (Hubbaed and Rothlein, 2000). Highly reactive oxygen free radicals have been indicated in the pathogenesis of various parasitic infections including cutaneous Leishmaniasis (Kocyigit *et al.*, 2003). Lipid peroxidation caused by ROS results in the disarrangement and ultimately, disruption of cell membranes, which leads to necrotic death. There are several reports indicating that infection with various parasites is associated with a marked elevation in lipid peroxidation. In the present study, increased levels of lipid peroxidation are detected in humans infected with VL; this may be considered as an indication of cell injury caused by *L. donovani*. Lipid peroxidation (MDA) which is a well- established mechanism of cellular injury in human, and used as an indicator of oxidative stress in cells and tissues.

Levels of MDA were significantly increased in *L. donovani* seropositive patients. MDA is an endogenous genotoxic product of enzymatic and oxygen radical-induced lipid peroxidation. The significant increase of MDA level in the present study strongly reflects an increased lipid peroxidation initiated by reaction of free radicals to polyunsaturated fatty acids in biological membranes. Lipid peroxidation is produced by oxidative stress resulting from the over-production of ROS and nitrogen species (RNS) in cutaneous Leishmaniasis (Kocyigit *et al.*, 2003).

The high serum MDA value in CL reflects the host defense against the parasite infection. Moreover, the rapid production of oxygen free radicals depletes the protective antioxidant enzymes, which is attributed to cell injury caused by *Leishmania* (Ozbilge *et al.*, 2005). A significant decrease in catalase activity and the increase MDA levels in patient with cutaneous leishmaniasis have been reported. Several antioxidant enzymes such as SOD and CAT exist that convert ROS into harmless products (Limon-Pacheco *et al.*, 2009).

Catalyzes the dismutation of superoxide anion to hydrogen peroxide (H_2O_2). H_2O_2 can be transformed into H_2O and O_2 by CAT. The activities of antioxidant enzymes may be increased or inhibited under infection depending on the duration of the infection (Limon-Pacheco, *et al.*, 2009; Oter *et al.*, 2012).

Conclusion

The study indicated that rKE16 dipstick test could be more sensitive in the diagnosis of VL according to clinical diagnosis.

A highly significant increase in IL-6, ICAM-1 and MIG-1 was shown in comparison to their counter parts in VL and control group. The high MDA level strongly indicated the event of oxidative stress and lipid peroxidation as a mechanism of tissue damage during infection with VL and associated it with the decreased level of antioxidant enzyme (CAT). These results showed points in the pathology of VL and might open new treatment perspectives associated with antioxidant.

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