

SERUM PRO-INFLAMMATORY CYTOKINES LEVEL AND ADENOSINE DEAMINASE ENZYME ACTIVITY IN PATIENTS WITH TYPHOID FEVER IN NAJAF/IRAQ

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ABSTRACT

In the current study, 60 serum samples from patients with typhoid fever and control (50 healthy individuals) were collected during 2014 years and examined for the presence of ADA enzyme, IL-6, and IFN- α . The mean values of ADA enzyme in the serum of typhoid fever patients were significantly higher (52.2 ± 4.32) than that of the control group (12.3 ± 6.03). The mean concentration of serum IL-6 and TNF- α were also higher significant ($P < 0.001$) in typhoidal patients than healthy control (30.41 ± 1.03 and 6.73 ± 3.62) and (72.35 ± 2.03 and 42.71 ± 4.12). According to the gender, ADA enzyme in male showed high (49.3 L/U) than female (42.5 L/U) with no significant variation ($P > 0.05$). Male appeared significant raised in the concentration of IL-6 and TNF- α (62.14, 52.45) pg/ml than female (57.3, 45.32) pg/ml.

KEYWORDS: ADA enzyme, Typhoid fever, Pro-inflammatory cytokines, IL-6, IFN- α .

INTRODUCTION

Typhoid fever is an important public health problem in many developing countries. It is a life-threatening systemic infection which is caused by the bacterium, *Salmonella enterica* serotype typhi. It is a highly adapted, human-specific pathogen which occurs more frequently in the under developed regions of the world, where overcrowding and poor sanitation are prevalent (WHO, 2003 and Bhuiyan *et al.*, 2014). Typhoid is usually acquired through the ingestion of water or food which is contaminated by the urine or the faeces of infected carriers and, as such, it is a common illness in the areas where the sanitation is poor (Pang *et al.*, 1998 and Kidgell *et al.*, 2002). Typhoid is a disease with high clinical toxicity and possible evolution to death. The symptoms and clinical signs in patients with typhoid fever are related

to cellular microbiological phenomena. The bacterial invasion of several host cells and the inflammatory response (neutrophils, monocytes-macrophages, T and B lymphocytes) with high cytokines production are important elements causing the clinical manifestations (Balows *et al.*, 1991, Andrade & Andrade –Jonio, 2003).

Adenosine deaminase, an enzyme, which is present in the red cells and the vessel wall, catalyses the irreversible hydrolytic deamination of adenosine to inosine and 2'-deoxyadenosine to 2'-deoxyinosine. Inosine and 2'-deoxyinosine are converted to hypoxanthine, xanthine and finally, to uric acid. ADA is considered as a good marker of the cell mediated immunity. The Adenosine deaminase (ADA) enzyme was required for lymphocyte proliferation and differentiation (Sullivan *et al.*, 1977 & Hoshino *et al.*, 1994).

Clinical studies demonstrated that *S. typhi* infection stimulates both an intestinal mucosal and systemic humoral and cellular immune response, which play roles in controlling and clearing *S. typhi* infection. Generally, the effective of immune responses against intracellular pathogens are based on the cellular arm (T cells), not on the humoral arm (antibodies) of immune responses. Circulating pro inflammatory and anti-inflammatory cytokines in patients with typhoid is increasing levels compared to patients with other severe disease. Immune response to infection *Salmonella* which activated macrophages and CD4+ T cells produce and secrete proinflammatory cytokines (Salerno-Goncalves *et al.*, 2003 & Wahid *et al.*, 2007)

Proinflammatory cytokines, such as interleukin-1 β (IL-1 β), IL-6, and tumor necrosis factor alpha (TNF- α) have been shown to play an important role in the development of fever and other symptoms. The pro-inflammatory cytokines (IL-6, TNF- α , IL-8) have been implicated in the pathogenesis of sepsis caused by gram negative microorganism (Keuiter *et al.*, 1994). These cytokines are released in response to gram-negative and other bacterial infections (Watking *et al.*, 1995). Lipopolysaccharide (LPS) has been demonstrated to elicit the production of IL-1 β , TNF- α , IL-6, and other cytokines (Engervall *et al.*, 1997). LPS is considered a powerful agent for the inflammatory cells activation, and it acts through CD14, Toll R2 and Toll R4 receptors. When LPS is injected intravenously into animals or human volunteers, elevated concentrations and signs of sepsis are mimicked (Van Amsterdam *et al.*, 2004).

The present study was taken up to evaluate the level of serum ADA activity and the pro-inflammatory cytokines in patients of typhoid fever in Najaf city.

PATIENTS AND METHODS

Patients

This case control study was conducted during the year 2014. The subjects who were included in this study were 60 outpatients (35 males, 25 females) aging from 11-55 years. Admitted to the clinical laboratory in Al-Sadder Medical City in Najaf /Iraq. All patients included in this study have symptoms of acute typhoid fever (a temperature of $\geq 38^{\circ}\text{C}$, diarrhea, toxic appearance, and rose spots) with positive result of widal (the routine test used in laboratory for diagnosis of typhoid fever). A group of 50 normal healthy individuals who were age and gender matched, served as the controls.

Sample Collection

Five ml of venous blood were collected from typhoidal patients and control by vein puncture using sterile syringes, and allowed to clot at room temperature for 30 min. then centrifuged for 20 minutes at approximately $1,000 \times g$. The serum separated into two tubes: one for ADA level detection and the other for pro-inflammatory cytokines detection.

ADA level detection (ELISA)

The serum ADA levels were estimated by using human ADA ELISA KIT (Cat.No.KT-5788) from KAMIYA Biomedical company/seattle WA). The principle of this test includes:

The microtiter plate provided in this kit has been pre-coated with an antibody specific to ADA. Calibrator or samples were then added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific for ADA. Next, Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. Then a TMB substrate solution is added to each well. Only those wells that contain ADA, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulfuric acid solution and the color change is measured spectrophotometrically at a wavelength of $450 \text{ nm} \pm 10 \text{ nm}$. The concentration of ADA in the samples is then determined by comparing the O.D. of the samples to the calibration curve.

Cytokine detection (ELISA)

IL-6 and INF- α level were measured by enzyme-linked immune assay according to the manufacturer instructions (Sigma/USA). All procedures were performed at ambient temperature. **The principle of the two cytokines include:** An antibody specific for human

cytokine is coated onto the wells of the micro titer plate. Samples and standards of human cytokine are pipette into the wells for binding to the coated anti-body. After washing procedure to remove unbound compounds, an enzyme-linked antibody specific for human cytokine was added to the wells. Following a wash to remove any un-bound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of human cytokine_γ bound in the initial step. The color development is stopped and the intensity of the color is measured. The magnitude of the absorbance for this developed color is proportional to the amount of human cytokine.

Reagents preparation

(All preparations mixed thoroughly and warmed up at room temperature prior to use). standard of IL-6 was prepared by Fifty five µl sterile water was reconstituted for lyophilized Human IL-6 Standard (55ng) to become final concentration of 1ug/ml. A 275 µl sterile water was reconstituted for lyophilized detection antibody (2.75µg) to become final concentration of 5ug/ml. Interleukin -6. Reagent preparation was occurred by 1 Liter sterile water was resolved the PBS powder (1 pouch) and 1 ml Tween-20 (50%) was added to this solution and mix well. the standards and samples were diluted in assay diluents at 1:2 serial dilutions. The reconstituted detection antibody in Assay Diluents to a concentration of 0.5 ug/ml was diluted (1:20 dilution). the Streptavidin-HRP conjugate was diluted 1:20 in assay diluents. 1 volume of color development reagent A was Mixed with 2 volume of reagent B (1:2) prior to use.

Interferon -α (IFN- α)

Standard preparation : the standard vial was centrifuged, at 10,000rpm for 30s. And reconstitute with 1.0 ml of sample diluents as concentration (500 pg/ml) and the Sample Diluents serves as the zero standard (0 pg/ml). The vial was centrifuged before opening, a 100µ from biotin antibody was diluted with 9.900µ of D.W. to the working concentration using Biotin-antibody diluents (1:100), respectively. the vial was centrifuged before opening. To make working concentration (1:100), a 110 µl HRP-avidin (100x) was added to the (10890 µ) HRP-avidin Diluents. A 9900µ was added from TMB solution (A) to 1100µ TMB Solution (B) mixed together to become a ratio of 9:1. A 25ml from 25× wash buffer was mixed with 600ml deionized water. - the standards were diluted in assay diluents at 1:2 serial dilutions. TMB stop solution was added to each well to stop the color reaction. The optical

density was determined of each well within 30 minutes, using a micro plate reader set to 450 nm.

ELISA Protocol

A 200 μ l of Washing Solution was added to each well. And the wells were aspirated to remove liquid and the plate was washed 3 times by using 300 μ l of Washing Solution per well. 100 μ l of standard or sample were added to each well, and the plate was covered with sealer provided, and incubated at room temperature for at Least 2 hours. The wells were aspirated to remove liquid and the plate washed 4 times. A 100 μ l of the diluted detection antibody (0.5 μ g/ml) were added to each well. The plate were covered by plate sealer provided and incubated at room temperature for 2 hours. The wells were aspirated to remove liquid and the plate washed 4 times like. A 100 μ l of the diluted color development Enzyme (1:20 dilute) was added to each well. The plate was covered by plate sealer provided and incubated at room temperature for 30 minutes. . The plate washed 4 times like as step 1. A 100 μ l of color development solution was added to each well, and incubated at room temperature for a proper color development. (15 minutes).After that 100 μ l of the stop solution were added to each well to stop the color reaction.micro titer plate reader were used to read the plate at 450 nm wavelength.

Calculation of results

The standard curve was used to determine the amount of IL-6 & IFN- α in an unknown sample. The standard curve is generated by plotting the average O.D. (450 nm) obtained for each of the standard concentrations on the vertical (Y) axis versus the corresponding IL-6 or IFN- α concentration (pg/ml) on the horizontal (X) axis, . Create a standard curve by reducing the data using ELIZA readers computer software capable of generating standard curve –fit. standard curve generated for each set of samples.

Statistical analysis

All values were expressed as means \pm SD. The data were analyzed by using of SPSS (T test) version 17 and Microsoft Excel computerized programs and taking P value less than 0.05 ($p < 0.05$) and 0.001 ($P < 0.001$).

RESULTS

The mean values of ADA enzyme in the serum of typhoid fever patients were significantly higher (52.2 ± 4.32) as compared to the control group (12.3 ± 6.03) as shown in table (1).

The mean concentration of serum proinflammatory cytokines (IL-6 and TNF- α) were highly significant ($P < 0.001$) in typhoidal patients than healthy control (30.41 ± 1.03 and 6.73 ± 3.62) and (72.35 ± 2.03 and 42.71 ± 4.12), respectively (table 1).

Table (1): The study groups difference in mean serum concentration of ADA ,IL-6,and INF- α .

Parameters	Study group		P value
	Control n :50	Patients n :60	
	Mean \pm SD	Mean \pm SD	
ADA (L/U)	12.3 ± 6.03	52.2 ± 4.32	$P < 0.001$
IL-6(PG/ML)	30.41 ± 1.03	72.35 ± 2.03	$P < 0.001$
TNF- α	6.73 ± 3.62	42.71 ± 4.12	$P < 0.001$

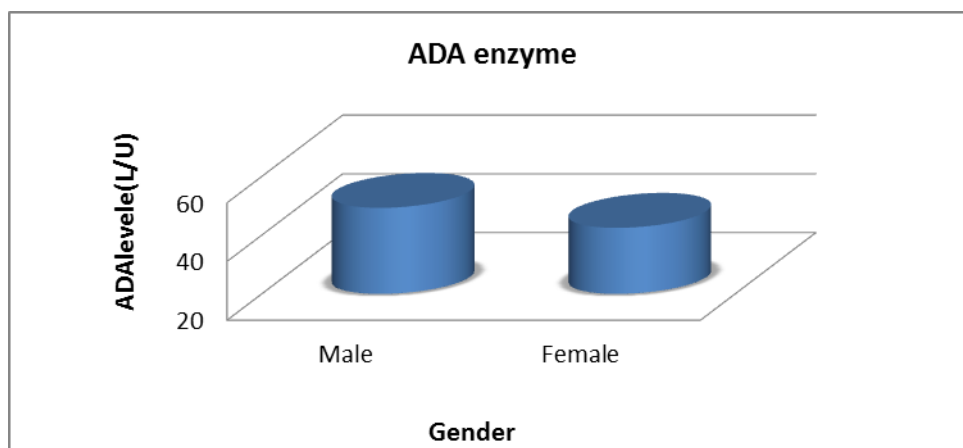
Table (2) show the distribution of mean and SD value of serum ADA ,IL-6,and INF- α of patients with typhoid fever according to age groups, in which patient in (5-15) age group appeared the higher ADA level (60.41 ± 1.22) followed by (16-30),(31-45),and ≥ 46 years age groups with ADA level 58.50 ± 4.86 , 52.3 ± 1.34 ,and 39.54 ± 3.45 , respectively. i.e in patients with typhoid fever ,ADA level increase with increase of age .

The mean of IL-6 and TNF- α concentration revealed a significant ($P < 0.05$) increase in 31-45 age group (89.54 ± 1.25 ; 64.51 ± 4.53) followed by elderly group (≥ 46), 5-15, and 16-30 years as show in table (2).

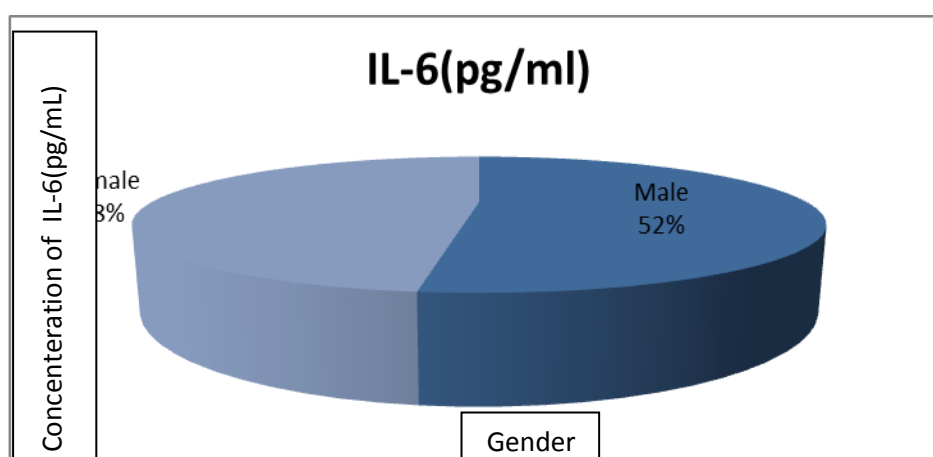
Table(2):Distribution of mean and SD value of serum ADA ,IL-6,and INF- α of patients with typhoid fever according to age groups.

Age group	Mean \pm SD		
	ADA	IL-6	TNF- α
5-----15	60.41 ± 1.22	72.58 ± 0.12	57.81 ± 2.72
16----30	58.50 ± 4.86	59.43 ± 2.67	41.32 ± 4.01
31----45	52.3 ± 1.34	89.54 ± 1.25	64.51 ± 4.53
----- ≥ 46	39.54 ± 3.45	79.53 ± 2.46	60.41 ± 1.36
P value	$P < 0.05$	$P < 0.05$	$P < 0.05$

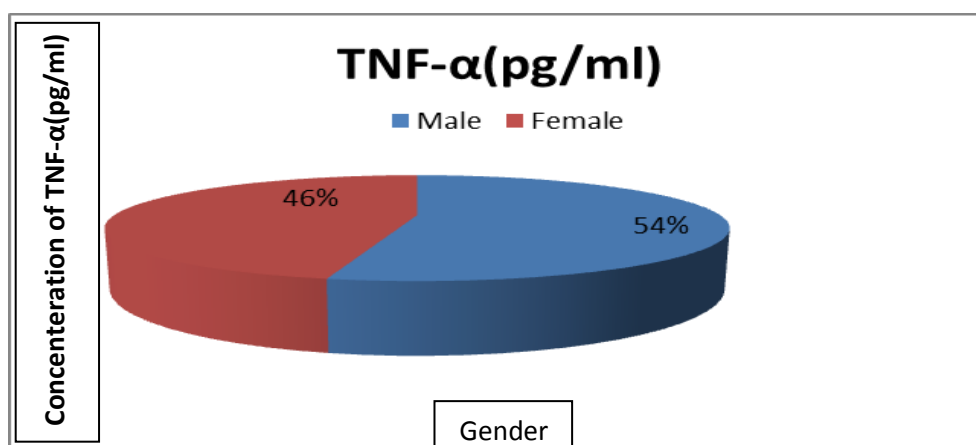
In this study ,the level of ADA enzyme in male showed high (49.3 L/U) than female (42.5 L/U) with no significant variation ($P > 0.05$). Male appeared significant raised in the concentration of IL-6 and TNF- α (62.14 , 52.45) pg/ml than female (57.3 , 45.32) pg/ml.



Figure(1): Distribution of ADA level (L/U) in typhoidal patients according to the gender.



Figure(2): Distribution of IL-6(pg/ml) in typhoidal patients according to the gender.



Figure(3): Distribution of TNF- α (pg/ml) in typhoidal patients according to the gender .

DISCUSSION

This result supports that INF- α and IL-6 are implicated in the pathogenesis of typhoid fever and agrees with result of Mizunio *et al.*, 2003 and Abbas *et al.*, 2014) whom reported that the levels of pro inflammatory cytokines (IL-6 and TNF- α) are elevated in typhoid patients compared to control. This results incompatible with Keuter *et al.*, (1994) who reported that inhibited production of cytokines during the acute phase may be due to a switch from a proinflammatory to an anti-inflammatory mode.

The role of proinflammatory cytokines in the pathogenesis of fever (the most important feature of typhoid fever) has been discovered at the end of the 1970s and the beginning of the 1980s, with the purification of IL-1 and demonstration of its pyrogenic properties (Dinarello *et al.*, 1999). Butler *et al.* (1993) indicate that the concentration can be useful in predicting outcome.

IL-6 is a cytokine initially described as a potent acute-phase protein inducer. It has been shown that IL-6 elicited fever when injected into rabbits and that IL-6 concentrations were correlated with fever in human patients with burns. IL-6 induction of fever in rabbits requires 50- to 100-fold higher amounts of IL-6 than of IL-1 (Netea *et al.*, 2000).

TNF is a proinflammatory cytokine that shares many biologic properties with IL-1. TNF injection induces a typical fever in rabbits that is indistinguishable from IL-1. Recombinant human TNF is highly pyrogenic in humans, and the fever induced is rapid and associated with generalized malaise and joint pain (Chai *et al.*, 1996). Netea *et al.* (2000) reported that The febrile response is thought to be mediated by endogenous mediators, generically called "endogenous pyrogens. These mediators act at the level of the organum vasculosum of the lamina terminalis in the central nervous system (CNS), inducing synthesis of prostaglandins, which are the central mediators of the coordinated responses leading to fever.

Lipopolysaccharide (LPS), a constituent of gram-negative bacterial cell walls, has been demonstrated to elicit the production of IL-1b, TNF- α , IL-6, and other cytokines (Engvall *et al.*, 1997). It has been shown that IL-6, as well as the proinflammatory cytokines IL-1b and TNF- α , are involved in the development of SIRS (Dinarello *et al.*, 1993). However, it is unlikely that fever and other symptoms of typhoid fever are caused by LPS, since most patients with typhoid do not exhibit increases in circulating levels of endotoxin and volunteers made LPS tolerant by repeated injections of endotoxin developed typhoid fever

when challenged with virulent *S. typhi* (Hoffman *et al.*,1991). House *et al.*,(2002) found that TNF- α release by LPS stimulated blood was found to be lower during acute typhoid fever than after a course of antimicrobial therapy.

The elevated level of ADA enzyme in this study was compatible with the result of Ketavarapu *et al.* (2013) who concluded that there was a statically significant increase in the serum ADA level in the patients with typhoid fever.

The result in this study disagreement with Bhutta *et al.*, (1997) reported an association between high plasma TNF- α level and the severity of disease has been described in children infected with *S.typhi*.since in this study the age group 31-45 years showed high concentration of of proinflammatory cytokines than other age groups. Results also disagreement with other studies, documented by Alixe *et al.*,(2013), who found that levels of IL-6 appear to increase with age, increasing age that associated with latent low grade inflammation, also the results similar with results documented by (David, *et al.*,2004; Sawako ,*et al.*,2008). Muhammed Ali (2015) reported that concentration of IL-6 was significantly raised in all groups of patients suffering from typhoid fever in comparison to the healthy control group. The present study showed that elevated circulating IL-6 concentration were found in all *S typhi* patients groups compared with control group ,in age group (41-60) years recorded higher percentage (90.40 pg/ml).

The results of Muhammed Ali (2015) also showed high significance increment between the concentration of IL-6 in both sexes,she showed increasingly recognized in IL-6 levels with gender groups females and males about (82.67pg/ml), (79.36pg/ml) respectively,thes result differ from of our result,male show high concentration of cytokines than females with no significant variation.

In conclusion,the induction of proinflammatory cytokines by som bacterial substances that causes typhoid fever,leads to alterations in the contrations of many biological parameters in the body fluids like ADA. So that ADA is considered as marker of the cell mediated immunity.The raised level of the ADA activity under antigenic stimulation and increase the concentration of pro-nflammatory cytokinesshows the importance of this enzyme in the rapid proliferation of cells, in order to prevent the accumulation of toxic metabolites. Typhoid fever is associated with the development of the cell mediated immunity and thus, has increased values of serum ADA.

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