Correlation between interleukin-6 and endotoxin an in vitro study

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Abstract: Endotoxin, a component of the outer membrane of Gram-negative bacteria, has been extensively studied as a stimulator of the innate immune response. However, the temporal aspects and exposure-response relationship of endotoxin and resulting cytokine induction. IL-6 is a pro-inflammatory cytokine that plays a crucial role in the immune response and inflammation. This in vitro study aimed to investigate the factors influencing the interleukin-6 (IL-6) response to bacterial endotoxin stimulation, we utilized an in vitro model to examine the factors influencing IL-6 production in response to bacterial endotoxin this experimental study was performed to find out the effect of healthy person on peripheral blood mononuclear cells(PBMCs) namely T-cells and natural killer cells(NK cells) function through challenging their ability for cytokines production in a comparative study by using of standardized inoculum of Bacterial endotoxins.

Keywords: Interleukin-6 (IL-6), Bacterial endotoxin, In vitro study, Immune response, Inflammation, Peripheral blood mononuclear cells, Cell culture, Elisa

1. Introduction

Septic shock (SS) is the last and most severe stage of sepsis. Sepsis occurs when your immune system has an extreme reaction to an infection. The inflammation throughout your body can cause dangerously low blood pressure[1]. Any type of bacteria can cause the infection. Fungi such as candida and viruses can also be a cause, although this is rare. At first the infection can lead to a reaction called sepsis. Pathogenesis refers to the biological processes that lead to the development of a disease or a pathological condition. Disease can arise from pathogens that secrete toxins, from dysregulation of the immune system. Organisms are constantly exposed to microbial pathogens in their environments[2]. The immune system encompasses the protection of the body from invasion by microorganisms (bacteria, viruses, toxins and fungi) and other internal and external threats that may lead to abysmal functioning of the body and disease conditions[3]. Human peripheral blood mononuclear cells (PBMCs) are used to investigate the effect of immune modulatory effects on various immune cells[4]. Cytokines are small proteins secreted by immune cells that mediate communication between different cells of the immune system and regulate immune responses. They are produced by cells of the immune system, such as T cells, B cells, and macrophages, and can act on others cells to stimulate an immune response. Cytokines can be classified into different groups based on their functions, such as pro-inflammatory cytokines, which promote inflammation and the activation of the immune system, and anti-inflammatory cytokines, which help to down regulate the immune response and reduce inflammation[5]. The immune system is basically composed of two compartments, innate and adaptive immunity. The innate immunity provides immediate but less-specific responses, while the adaptive immunity, which is activated by the innate response, gives stronger and very specific responses and develops a memory, by which it remembers specific invaders and mounts a faster and stronger response in later challenges[6]. Exposure to LPS can stimulate the production of pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1(IL-1), and interleukin-6(IL-6). Theses cytokines play a role in activating the immune system and promoting inflammation. In addition to the production of pro-inflammatory cytokines, exposure to LPS can also stimulate production of anti-inflammatory cytokines, such as interleukin-10(IL-10)[7]. Lipopolysaccharide (LPS) is a large molecule composed of lipid and polysaccharide portions. It is a major component of the outer membrane of gram-negative bacteria. LPS is also referred to as endotoxin because its release from bacterial cells during infection can trigger strong immune responses in mammals[8]. In most Gram-negative bacteria, the outer membrane is composed predominantly of the glycolipid known as lipopolysaccharide (LPS). In E. coli, this complex molecule is composed of three structurally distinct regions; the hydrophobic anchor called lipid A, a core oligosaccharide, and the long-chain polysaccharide called O antigen (or O-polysaccharide; O-PS).

When gram-negative bacteria replicate or undergo lysis, LPS is released into the surrounding environment, such as the bloodstream during an infection.

Aim of study **:** An attempt to investigate the underlining immunopathogenesis of septic shock.

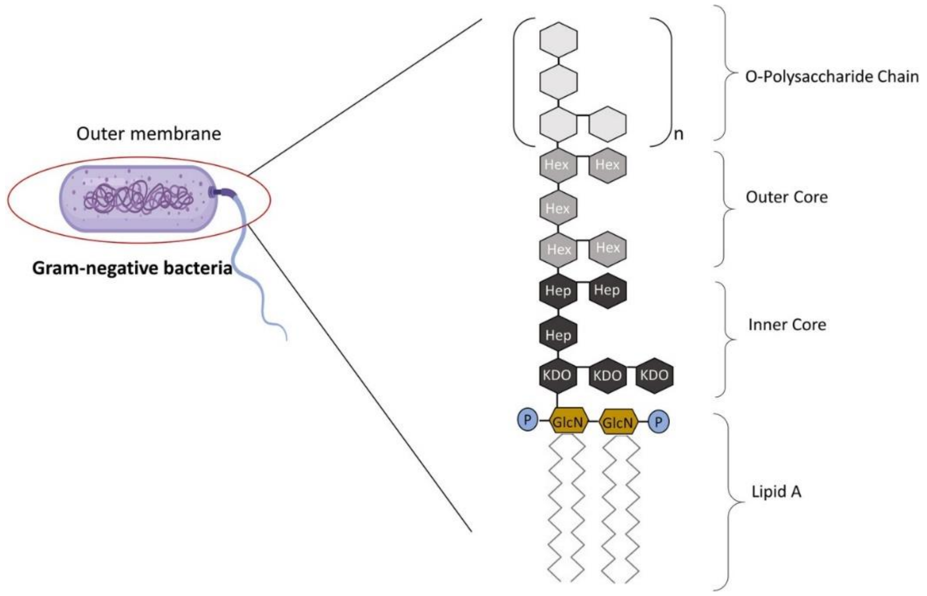

Figure 1 : Lipopolysaccharide (LPS) Structure[9].

2. Materials and Methods

2.1 Samples collection

40 Blood Samples were collected from Healthy persons, aged 20–30 years male and female, which was carried out in the Department of Microbiology at the College of

Medicine/ Wasit university. Five ml of venous blood was collected from each participant . Take 2 ml from blood in EDTA tubes and soon treated for peripheral blood mononuclear cells (PBMCs) separation.

2.2 Peripheral Blood Mononuclear Cells (PBMCs) separation from Whole Blood:

All the specimens of 2 ml fresh anticoagulant venous blood were treated first to separate PBMCs by density-gradient centrifugation. Gently layer the blood sample 2 ml on the top of lymphocytes separation medium 2 ml. Take care not to mix the two layers [10]. The separation process is performed by Centrifuge at 1000 x g for 20 minutes without brake.

The separated PBMCs from each blood specimens were then transferred to a sterile conical tube and volume corrected into 2 ml by adding of phosphate buffer saline (PBS). Centrifuged at 1000 x g for 10 minutes for cell washing, supernatant removed and the procedure repeated twice. The final cell count for each PBMCs pool was about 400 000 cell /ml cRPMI ensured by slide chamber method of cell counting.

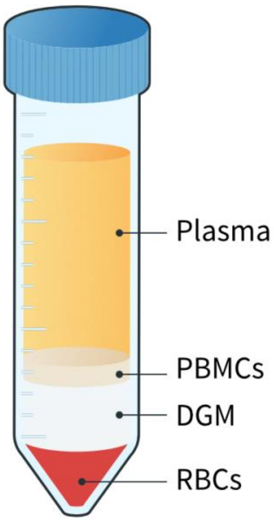

Figure 2 : Peripheral Blood Mononuclear Cells (PBMCs) separation from Whole Blood [11].

2.3 Cell culture:

Human peripheral blood mononuclear cells (PBMCs) were isolated, the cell pellet were then divided into two equal volume and cultured in appropriate media Roswell park Memorial Institute Media(RPMI -1640) incubate both glass tube for 3–5 days in an incubator at 37°C.

2.4 Bacterial endotoxin:

Source : Escherichia coli 055:b5 (Lipopolysaccharide 10 mg) Solubility:
Stimulation: PBMCs were stimulated with varying concentrations of bacterial endotoxin for different time intervals.

2.5 Laboratory Experiments

2.5.1 Estimation of the base level interleukin-6 (that expressed by PBMCs in complete RPMI medium in absence of the LPS antigen):

To detect the base level of interleukin-6 (IL-6) the first 40 samples of PBMCs that prepared from the previous step were incubated in cRPMI medium at 37 degree centigrade for 5 days in the fifth day the tubes of cRPMI were centrifuged at 1000 x g for 10 minutes and then (40 micro litters) of the sample supernatant was aspirated to be tested by ELISA method to determined IL-6 level (which was regarded as the base level) for each member of the test group[12].

2.5.2 Detection of the stimulation level of interleukin-6 (that expressed in response to LPS along in cRPMI medium as stimulating antigen):-

To detect the stimulation level of interleukin-6 (IL-6), the second 40 samples of PBMCs that prepared from the previous step were incubated in cRPMI medium at 37 degree centigrade along after adding of 50 µL of LPS for each cRPMI tube. This incubated for five days .Thereafter, each sample was centrifuged at 1000 x g for 10 minutes and the supernatant (40 micro litters) was aspirated to be tested by ELISA method for detecting the level of TLR4, IL-6, secreted in culture media in response to the stimulating Antigen (i.e. LPS). And this level was regarded as the stimulation level IL-6 one for each member of the test group[13]

2.6 The ELISA Method

Enzyme- linked immunosorbent assay kit were used to measure the levels of human interleukin 6 (IL-6) in the both group with & without bacteria endotoxin lipopolysaccharide(LPS).

2.6.1. Human Interleukin 6(IL-6) ELISA Kit:

The kit is a sandwich enzyme immunoassay for in vitro quantitative measurement of IL-6 in human serum, plasma, Urine ,tissue homogenates and other biological fluid[14].

2.6.1.a Test principle

This kit uses enzyme-linked immune sorbent assay (ELISA) based on the Biotin double antibody sandwich technology to assay the Human Interleukin 6 (IL-6). Add Interleukin 6(IL-6)to the wells, which are pre-coated with Interleukin 6(IL-6)monoclonal antibody and then incubate. After that, add anti-IL-6 antibodies labeled with biotin to unite with streptavidin-HRP, which forms immune complex. Remove unbound enzymes after incubation and washing. Add substrate A and B. Then the solution will turn blue and change into yellow with the effect of acid. The shades of solution and the concentration of Human Interleukin 6 (IL-6) are positively correlated.

2.6.1.b.Assay procedure:

1. Dilution of standard solutions: (This kit has a standard of original which could be diluted in small tubes by user independently following the instruction) the below chart as follows in figure (3):

320 ng/L	Standard No.5	120µl Original Standard + 120µl Standard diluents
160 ng/L	Standard No.4	120µl Standard No.5 + 120µl Standard diluents
80 ng/L	Standard No.3	120µl Standard No.4 + 120µl Standard diluent
40 ng/L	Standard No.2	120µl Standard No.3 + 120µl Standard diluent
20 ng/L	Standard No.1	120µl Standard No.2 + 120µl Standard diluent

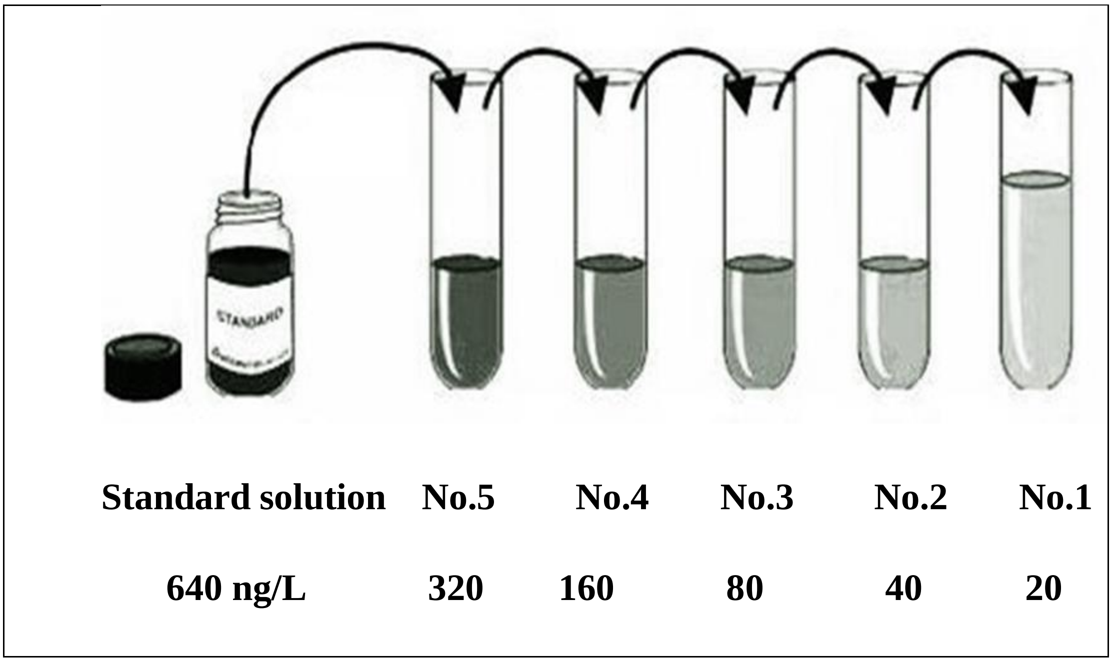

Figure 3: Serial dilution of IL-6 standard solution.

2. The number of stripes needed is determined by that of samples to be tested added by that of standards. It is suggested that each standard solution and each blank well should be arranged with three or more wells as much as possible.

3. Sample injection:

1) Blank well: Add only Chromogen solution A and B, and stop solution.

2) Standard solution well: Add 50µl standard and streptavidin-HRP 50µl.

3) Sample well to be tested: Add 40µl sample and then 10µl IL-6 antibodies and 50µl streptavidin-HRP. Then cover it with seal plate membrane. Shake gently to mix them up. Incubate at 37°C for 60 minutes

4. Preparation of washing solution: Dilute the washing concentration (30X) with distilled water for later use.

5. Washing: Remove the seal plate membrane carefully, drain the liquid and shake off the remaining liquid. Fill each well with washing solution. Drain the liquid after 30 seconds' standing. Then repeat this procedure five times and blot the plate.

6. Color development: Add 50µl chromogen solution A firstly to each well and then add 50µl chromogen solution B to each well as well. Shake gently to mix them up. Incubate for 10 minutes at 37°C away from light for color development.

7.Stop: Add 50µl Stop Solution to each well to stop the reaction (the blue

color changes into yellow immediately at that moment).

8.Assay: Take blank well as zero, measure the absorbance (OD) of each well one by one under 450nm wavelength, which should be carried out within the 10 minutes after having added the stop solution.

2.6.1.c. Calculation of results:

Make concentration of standards the abscissa and OD value the ordinate. Draw the standard curve on the coordinate paper. According to the OD value of the sample, locate its corresponding concentration (which is the concentration of the sample); or calculate the linear regression equation of standard curve according to the concentration of the standard and the OD value. Then substitute with the OD value of the sample to calculate its concentration in figure(4).

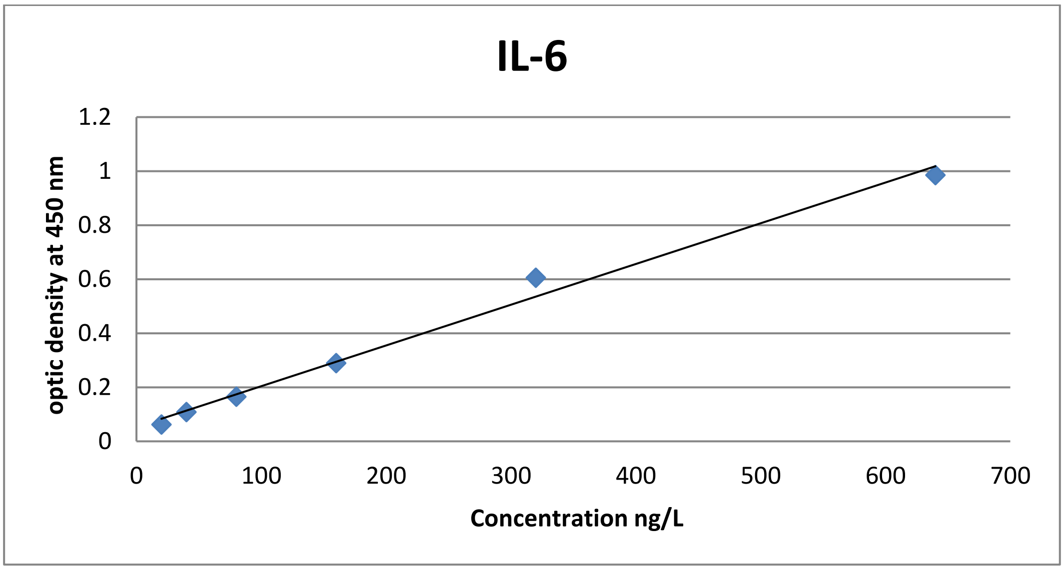

Figure 4:- Standard Curve of IL-6

2.7 Statistical analysis

The mean and standard deviation were used to describe the continuous variables. Both Paired samples t-test and independent samples t-test were used accordingly for the assessment of the mean differences between continuous variables. Pearson correlation coefficient was used to assess the presence of correlation between the study variables. A P-value equal to or less than 0.05 was considered significant.

3. Results

Concentration-dependent IL-6 response: Our results demonstrated a concentration-dependent increase in IL-6 production upon stimulation with bacterial endotoxin. Higher concentrations of LPS resulted in greater IL-6 secretion by PBMCs.

Time-dependent kinetics: The production of IL-6 showed a time-dependent response following LPS stimulation. IL-6 levels increased gradually over time, with a peak observed at a specific time point .Synergistic effects: We observed synergistic effects between LPS and other pro-inflammatory stimuli, such as toll-like receptor (TLR) ligands.

Co-stimulation with TLR ligands significantly potentiated IL-6 production compared to LPS stimulation alone. Cellular heterogeneity: Subpopulation analysis revealed variations in IL-6 response among different cell types within the PBMC population. Monocytes, in particular, showed higher IL-6 production compared to lymphocytes.

3.1 Detection Interleukin 6(IL-6) in experimental vitro by using ELISA method:

Table 1: Mean differences between the baseline and post-stimulation interleukin 6 (IL-6) as show in figure 4

IL-6 (ng/L)	Baseline	Post stimulation	P-value
Mean	208.189	283.827	<0.001
Standard deviation	85.236	117.565	

Table 2: Descriptive statistics for the mean difference (post-stimulation – baseline level) in the study parameter.

Variables	Mean difference	Standard Deviation
IL-6 (ng/L)	75.63	110.53

Figure 5: Histogram of the distribution of baseline and post-stimulation IL-6.

4. Discussion

Interleukin-6(IL-6) is known proinflammatory cytokine. It is also a stimulator of acute-phase proteins such as CRP. The acute phase response is a systemic reaction against inflammation, infection, or tissue injury. IL-6 plays a role in hematopoiesis Tanaka et al., 2014 [15].

Our results shows a significant difference (P-value < 0.001) in means of interleukin 6 (IL-6) between baseline and post-stimulation levels.

There was an increase in the level of IL-6 from 208.189 ng/L to 283.827 ng/L for post-stimulation, shows in table 1.

Our study agree with Thorsted et al., 2019 [16] also agree with Veiz et al., 2022 [17] may be the reason IL-6 can be elevated with inflammation, infection, autoimmune disorders, cardiovascular diseases, and some cancers. contributes to host defense through the stimulation of acute phase responses, hematopoiesis, and immune reactions Tanaka et al., 2014 [15].

disagreed with *Liu et al*., 2020 [18] that suggest reduce the size of infarction volume therefore, decreased pro-inflammatory cytokines.

Likewise Hua et al., 2021 [19], Saia et al., 2020 [20] agreement that findings indicate that the production of IL-6 in response to bacterial endotoxin is influenced by various factors. The concentration-dependent response suggests that the magnitude of IL-6 production can be modulated by adjusting the endotoxin concentration. This concentration-dependent effect might have implications for the severity of the inflammatory response in vivo and vitro.

Our results represented a description of the mean difference and standard deviation (between post-stimulation and baseline) for all the study variables. IL-6 show a positive mean difference which means an increase in post-stimulation levels more than the baseline levels Üçeyler et al., 2019 [21].

5. Conclusions

1. the correlation analysis was conducted to examine the relationships between the mean differences (post-stimulation - baseline level) of the five study parameter. The results are presented in Table 1.

2. our study sheds light on the factors driving IL-6 production in response to bacterial endotoxin stimulation. Targeting IL-6 signaling pathways may hold therapeutic potential for diseases characterized by dysregulated inflammation.

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Article submitted 10 Jun 2023.
Accepted at 21 July 2023.
Published at 30 September 2023.