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Differential Expression and Clinical Significance of Interleukin-6 in Iraqi COVID-19 Patients

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Abstract

Background: *Interleukin-6 (IL-6) acting a vital role in host defense against infectious diseases and tissue injury. The epidemic SARS-CoV-2 infects humans and causes upper respiratory illness from mild to severe disease. The proinflammatory cytokine IL-6 was reported as a predictable biomarker for cytokine storms associated with the severity and mortality in infected patients. This research article was aimed to measure the gene expression and soluble form of IL-6 in Iraqi infected patients.*

Materials and methods: *A case control study was conducted on 150 severely infected patients with SARS-CoV-2. The cases and samples were obtained from Baghdad Teaching Hospital, Baghdad, Iraq. In addition, 100 healthy subjects were enrolled in this study. The gene expression was standardized to the level of a housekeeping gene (GAPDH) and quantified by the ΔCt value and folding (2-ΔΔCt) method and serum IL-6 was measured using ELISA techniques.*

Results: *A significant elevated serum levels of IL-6 in Covid-19 patients compared to controls ($p<0.05$). The folding change of gene expression was dramatically raised in patients.*

Conclusion: *Overexpression of IL-6 may play potential therapeutic approaches for the management of cytokine storms, especially those associated with sepsis and multi-organ failure.*

Keywords: *Interleukin-6, IL-6; COVID-19, ELISA; RT-PCR.*

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1. Introduction

The COVID-19 pandemic is a novel Coronavirus termed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Most recorded patients with Covid-19 involved mild symptoms. However, up to 20% of identified cases of Covid-19 are complexed by severe pneumonia which might result in acute respiratory distress syndrome (ARDS) which causes acute hypoxemic respiratory failure (Chen et al., 2020). The proinflammatory cytokine IL-6 is released through infection or tissue injury that activates both innate and adaptive immune responses (Liu et al., 2020). Secreted by innate immune cells, for instance, macrophages, and other polymorphonuclear immune cells upon their recognition of damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) as one of the host defense approaches to eliminate infected cells or damaged tissue (Grifoni, 2020). Excessive production of IL-6 plays a central role in the development of chronic inflammatory diseases, such as rheumatoid arthritis (RA), and hyper-inflammation, including cytokine storms (Bhatraju, 2020), which are hyper-reactive immune responses that can arise in patients infected with pathogenic bacteria or viruses, including SARS-CoV-2 (Zhao, 2020). Throughout a cytokine storm, large amounts of varied active immune mediators, including proinflammatory cytokines, chemokines, growth factors and complements proteins are formed rapidly (Gao, 2020), which contributes to the development and progression of the associated diseases. Hyper-reactive immune responses can be repressed by treatment with a monoclonal antibody targeting the IL-6 or its receptor (A Zumla, 2020). The development of novel SARS-CoV2-associated pneumonia to a serious and life-threatening that related to cytokine storm. The serum concentrations of IL-6, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Sun et al., 2020), and other cytokines were reported being dramatically increased in patients with severe infections of viruses such as SARS-CoV-2 and H1N1 influenza virus (Pairo-Castineira et al., 2021). These inflammatory aspects can employee and stimulate immune cells in the lungs (Chen and Quach, 2021b) Numerous immune cells accumulate in the lungs and obstructive gas exchange between the alveoli and capillaries and affects the peripheral oxygen saturation (SpO2), and partial pressure of oxygen in arterial blood (PaO2), showing that IL-6 correlates with respiratory failure (Santa Cruz et al., 2021). Initiation of a cytokine storm consequences in the immune cells and proteins abolishing large numbers of healthy lung cells and impairing the normal function of the lungs. This damage seems on computed tomography (CT) images of the lungs as large white areas and can result in respiratory failure and death (Herold et al., 2020). This study was aimed to discover the gene expression of IL-6 and soluble levels in the blood and vital roles in developed severity and mortality in Iraqi populations.

2. Materials and Methods

2.1. Subjects

One hundred fifty of Iraqi patients infected with SARS-COV-2 were enrolled in this study, the samples were collected from Baghdad teaching hospital in Baghdad, Iraq, their age was ranged from 25 to 65 years and their age-matched to 100 apparently healthy individuals and their age ranged was 22 - 55 years. Based on laboratory tests and clinical examination for both patients and controls.

2.2. Samples collection and preparation

Ten ml of venous blood was collected, the aliquot was dispensed in gel tube for serum separation, and then serum was collected by centrifugation (3000 rpm for 10 min). The serum was frozen at -20°C until assessment for tests, and 5ml of the blood was subjected for the total RNA extraction tissue using Trizol (TRI Reagent®; ZYMO RESEARCH, USA).

2.3. Measuring of Interleukin-6

A quantitative sandwich ELISA kit for IL-6; SunLong Biotech Co., LTD; China.

2.4. Primers used in this study

The sequences of the primers used in the laboratory work were designed and manufactured and they were illustrated using the free site https://www.ncbi.nlm.nih.gov/tools/primer-blast/primertool. The forward primer for Homo sapiens IL-6 was 5' CCACCGGGAACGAAAGAGAA 3', reverse primer was 5' GAGAAGGCAACTGGACCGAA 3'. The housekeeping gene used in this study was Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH) the forward primer was 5'GACAGTCAGCCGCATCTTCT3', reverse primer was 5'GCGCCCAATACGACCAAATC3'. The primers were designed and used according to the manufacturer Takara Bio Inc., Shiga, Japan.

2.5. Preforming the Expression of IL-6

The RNA was isolated from blood samples using Direct-zol™ RNA MiniPrep, Zymo-Research/ USA. The Prime ScriptTM RT reagent Kit cDNA mixture reaction was used to convert the RNA to cDNA. The reaction was performed using a SaCycler-48 thermal cycler, Sacace, Italy. For quantitative PCR (qPCR), the reaction mixture was prepared using KAPA-SYBR® FAST-PCR Master Mix, KAPA, USA. the GAPDH housekeeping gene was used as the endogenous control. Lastly, the melting curve analysis was achieved the separation features of dsDNA during cycles with increasing denaturing temperature.

2.6. Statistical analysis

The software GRAPH PAD PRISM 8 was used to obtain mean and SE, $P<0.05$ considered as non-significant. The fold change was calculated by the equations, ΔCT = CT of target gene – CT of U gene, $\Delta\Delta CT$ = ΔCT of each sample - average control ΔC and the Fold change = $2\text{-}\Delta\Delta Ct$, respectively It was observed that the control value was considered as 1, the samples those are less than 1 are down-regaled and those the values more than 1 are up-regulated (Livak and Schmittgen, 2001).

3. Results

The overall mean age was 42.6 ± 11.7 years and 41.7 ± 13.46 years in the control group. Approximately the patients with severe symptoms had underlying diseases, including hypertension (28; 19%), diabetes (32; 22%), cardiovascular disease (26 17.3%), (22; 14.7%) hyperlipidemia, developed kidney failure (7; 4.66%) and there was no history of other diseases in the remaining patients (44, 29.33%). Clinical symptoms of the patients were mixed including, fever (68/150; 45.33%), dry cough (54/150; 36%) and dyspnoea (48/150; 32%), expectoration (26/95; 27.4%), nausea (14/95; 14.7%), myalgia (14/95; 14.7%), vomiting (11/95; 11.6%), diarrhea (9/95; 9.5%), dizziness (7/95; 7.4%), sore throat (5/95; 5.3%), headache (3/95; 3.2%) and abdominal pain (2/95; 2.1%). The mean length of the hospital stay was 16.8 ± 8.8 days. The survival rates were (109; 72.7%) and the mortality rates were (41; 27.3%) of the total cases. There were significant differences

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between patients and controls in the SPO2% level (54.243± 6.33 *vs* 94.33±2.45 %), p<0.05.

3.1. Serum level of IL-6

There were significant differences in serum level of IL-6 in patients (17.41± 5.935) Pg/ml and control (1.89± 0.81) Pg/ml, respectively (p<0.05) Figure (1).

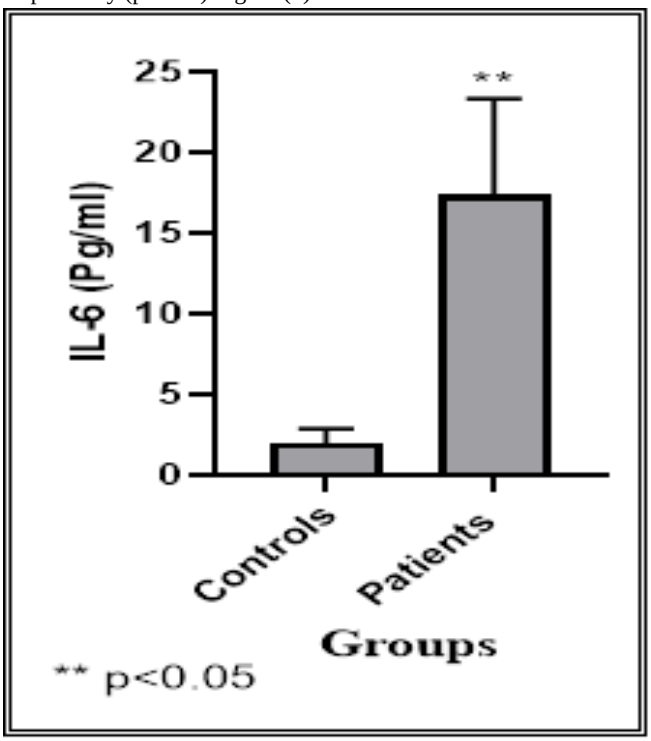
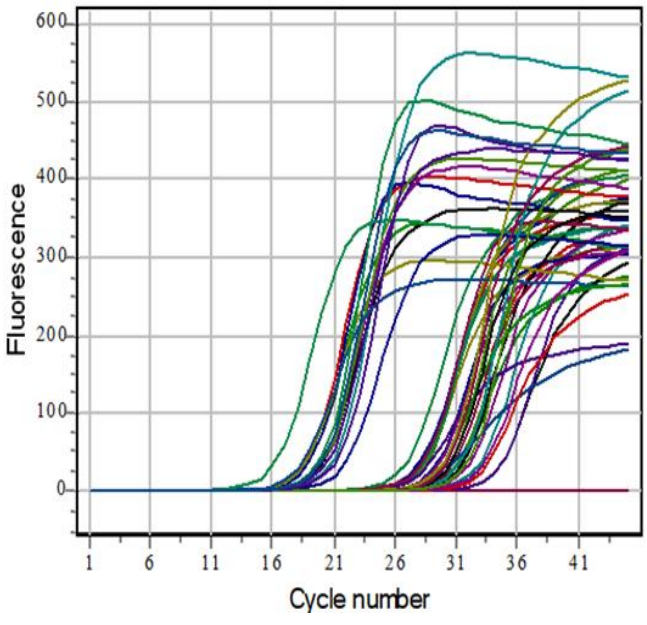


Figure 1. Serum level of IL-6 in Covid-19 patients and healthy.

3.2. Gene Expression of IL-6

Quantitative expression of IL-6 was confirmed qRT-PCR, in which the relative quantitation method was employed. The gene expression was normalized to the level of a housekeeping gene (GAPDH) and quantified by the folding (2−ΔΔCt) method. A representative RT-qPCR plot is given in figure (2).

A



B

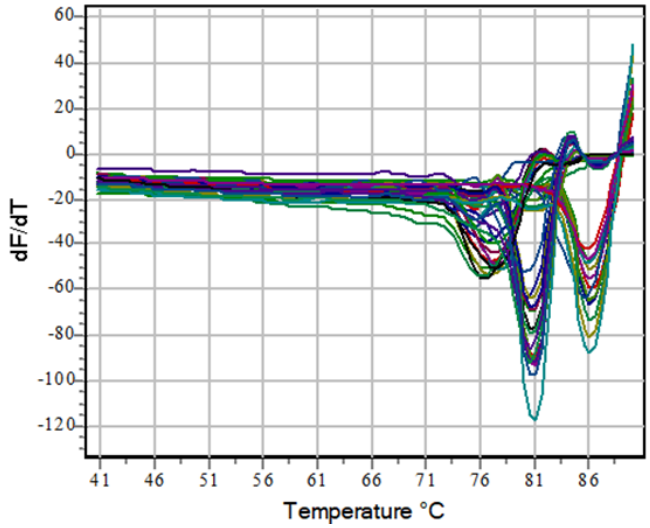


Figure (2); A; Results of RT-PCR IL-6 expression in patients and control normalized with GAPDH gene. B; melting curves followed amplification of target genes

The results showed that IL-6 was overexpressed in patients 59.764±8.342 in severe than in controls 1±0.000, p=0.001. (Table 1).

Table (1); Folding change expression of IL-6 in Covid-19 infected patients and control

IL-6	mean of ΔCt± SE	ΔΔCt	P value	Folding
Control	-3.5± 0.09	-1.26	0.001	**1.000±00.00**
Covid-19	-6.5± 0.7	3.8	0.001	**59.764±8.342****

3.3. ROC Analysis

ROC analysis recorded that IL-6 Sensitivity %: 83, Specificity %: 93, confidence interval: 0.92 to 0.98, Area: 0.95±0.01, cutoff value: 12.5, p.value: 0.000.

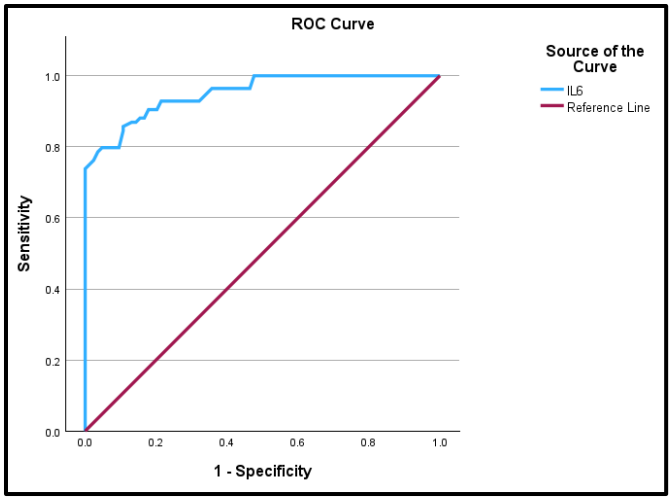


Figure 3-35: Infected individual ROC curve analysis (Wilson/Brown method) Sensitivity% and 1-Specificity %, of studied cytokines p<0.05.

4. Discussion

IL-6 is a multi-factorial cytokine with a variety of functions critical for managing immune responses and might consequently play an

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important role in the cytokine storm (Tharmarajah et al., 2021). Throughout the acute stage of inflammation, IL-6 mostly induces the proliferation, differentiation, and functional perfection of cells complicated in the initiation of immune responses. It was reported that the IL-6 has been used to diagnose transmittable diseases, including bacterial and viral infections (Tamayo-Velasco et al., 2021). Increased IL-6 is correspondingly a biomarker for the severity of other viral such as HPV, HBV, H1N1, and HCV infection (Diaz-Torne et al., 2018). Similarly, IL-6 might play an important role in the progress of novel coronavirus pneumonia. Soluble IL-6 have been thoroughly related to the clinical severity of COVID-19 (Yousif et al., 2021). Recent studies suggested that the IL-6 plays a significant role in lung injury and respiratory distress and the need for mechanical ventilation due to SARS-CoV-2 infection (Santa Cruz et al., 2021)'(Rose-John, 2021). The extreme excretion of pro-inflammatory cytokines, including TNF, IL-6, and IL-1β in the initial phase of the infection mains to increase the risk of high vascular permeability and multiple organ failure due to continuous productions of cytokines, and it ultimately principals to mortality (Leisman et al., 2020). The excessive production of cytokines in response to COVID-19 infection may stimulate the coagulation pathway and leads to pulmonary embolism and thrombosis (Chen and Quach, 2021a). Numerous studies published that the soluble IL-6 level in patients with COVID-19 rises 21, 2222, and predictable biomarker of inflammation (Hayden et al., 2017). It has been detected that chronic exposure to IL-6 represses the cytotoxic activity of natural killer (NK) cells, and down-regulating the expression of granzyme B and perforin in severe COVID-19 (Chen et al., 2019). These proceedings consequence in the failure of cytotoxic NK cells or T-lymphocytes to kill target cells through cell-mediated apoptosis mechanisms, thus triggering the survival time of target cells and enhancement of antigen stimulation. In general, it consequences in the extreme production of pro-inflammatory cytokines, leading to ARDS and MODS (Herold et al., n.d.). IL-6 might quickly stimulate pathogenic T-cells, which produce GM-CSF, IL-6, and other factors. GM-CSF additional stimulates CD16+ CD14+ inflammatory monocytes, constructing advanced amounts of IL-6 and creating an optimistic response and eventually damage to alveolar and pulmonary capillary endothelial cells (Mehta et al., 2020). Similar to our findings, were reported by worldwide publications which stated the dramatic elevation of soluble IL-6 and their vital role in the severity and progression of the respiratory failure 27' 2829 30. Our results recommended the principles of monitoring the IL-6 levels in severely infected patients with COVID-19 and proposed that could predict the progress of the life-threatening illness. The expression analysis specified that IL-6 was a respectable predictor of the therapeutic severity of COVID-19. Serum IL-6 concentrations ≥17.41 pg/mL were related to the progression to critical disease rank, representative of the necessary needs for an additional active interference to prevent supplementary worsening life-threatening situations [(30)]. This study presented that the gene expression of IL-6 was meaningfully linked to the severity of COVID-19 and that substantial increases in IL-6 could specify that the patient's underlining diseases worsening and becoming critical. IL-6 and other cytokines mediated storm levels should be measured directly after hospital admission of patients with COVID-19 and continuous monitoring subsequently. IL-6 has clinical bioactivity in assessing patient disorders and forecasting its deterioration, representative of the need for active therapeutic events.

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