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Gene expression of TLR-4 and TLR-3 in patients with SARS-CoV-2 in Basrah, south of Iraq

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ABSTRACT

Aims: TLR4 is a member of the family of receptors used for pattern recognition. They act as the first line of defense against infections because they are highly conserved receptors that identify pathogen-associated molecular patterns (PAMPs), including viral proteins and trigger the development of type I interferons and proinflammatory cytokines.
Methodology and results: The Study exhibited that the patients of the Iraqi population with SARS-CoV-2 have up-managed quality articulation for TLR4 and TLR3 contrasted with the control test. COVID-19 mortality is related to respiratory failure, cardiovascular breakdown and sepsis/multiorgan failure.
Conclusion, significance and impact of study: The role of TLR3 activation is proven to be advantageous against a wide variety of RNA viral infections, with TLR3 activation being more effective than TLR4 in SARS-COV-2.

Keywords: COVID-19, TLR3, TLR4

INTRODUCTION

Severe acute respiratory syndrome coronavirus type 2 SARS COV-2 has the same clinical features and genetic information as SARS and MERS, as they belong to the same family of beta-coronavirus (Pellegrino *et al.*, 2020). There is a similarity of 79.5% between SARS COV-2 and the SARS-COV-1 virus (Rouchka *et al.*, 2020). All seven human coronaviruses (HCOV) (OC43-NL63, E229, HKU1, MERS, SARS-COV and SARS-COV-2) have a zoonotic origin, such as mice, pangolin, bats and other pets (Gorbalenya *et al.*, 2020). SARS-COV-2 is considered the virus that causes COVID-19 disease (Gorbalenya *et al.*, 2020). Coronavirus was initially found in Iraq on the 24th of February by an Iranian understudy visiting the city of Najaf (Dawood and Dawoand 2021; Hammadi *et al.*, 2021). The rate of SARS-CoV-2 infections reached its highest level in September of the same year, with a weekly average of 4,500 cases. Then, the number of diseases gradually declined until reaching its lowest level during mid-January 2021, with a rate of 750 confirmed infections throughout Iraq (Al-Malkey and Al-Sammak, 2020; Hijaj *et al.*, 2020). The number of diseases continued until the onset of February 2021. Then, the condition curve began to increase at the beginning of August 2021 until the number of infections reached the highest record in the country when preparing this research, reaching approximately a weekly rate of 12 thousand confirmed diseases. This temporary decline between the first rise in injuries and the second rise was considered a first and second wave, according to what was stated in the report of the World Health Organization on Iraq (Habib *et al.*, 2020; WHO, 2021).

In disease development and clinical manifestation, COVID-19 is vital in the host immune response (Hammadi *et al.*, 2020). Toll-like receptors 1, 4 and 6 on the cell surface recognize structural components of the viral envelope, with the strongest affinity observed for TLR4, which is triggered by oxidized phospholipids generated during SARSCoV2 infection (Choudhury and Mukherjee, 2020; Choudhury *et al.*, 2021). TLRs7/8 in endosomes, on the other hand, recognize single-stranded positive-sense RNA (AlSaimary *et al.*, 2020b), whereas TLR3 senses the double-stranded RNA intermediate generated during viral replication (Zhao *et al.*, 2012; Lee *et al.*, 2020). TLR3 expression is more efficient than TLR4 activation in a mouse model and TLR3 activation is beneficial against a broad spectrum of RNA viral infections (Perales-Linares and Navas-Martin, 2013; Totura *et al.*, 2015; Mukherjee *et al.*, 2019). The strong binding affinity of SARS COV-2 non-structural protein 10 (nsp10) mRNA to TLR3 in a docking analysis implies that TLR3 downstream signaling may be induced (Choudhury *et al.*, 2021). TLR3 has also been shown to play a protective function in infections with COVID-19 viruses that are more closely related, such as SARS-CoV1 and

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the Middle East respiratory syndrome (MERS-CoV) in earlier research (Biswas and Khan, 2020).

SARS COV-2 most likely uses TLR4 to enter cells and increase angiotensin-converting enzyme 2 ACE2 expression. The virus could be utilizing TLR4 signaling to trigger the expression of ACE2 (or another receptor) through which it would enter the same or neighboring cells, according to the bio-computational analysis of the S spike to SARS-COV-2 glycoprotein (Aboudounya and Heads, 2021). LPS binds to MD-2 and TLR4 with the aid of CD14. This causes TLR4 to homodimerize, drawing MyD88 and Mal to the receptor complex. The necessary amino acids for TLR4 signaling and the relationships between TLR4 and MyD88/Mal have been the subject of several investigations (Mesquita *et al.*, 2014; Molteni *et al.*, 2016; Turner, 2016). These DAMPs cause fibro-inflammatory genes to be expressed at wound healing sites, resulting in maladaptive remodeling and fibrosis (Mckeown-Longo and Higgins, 2017). TLR4 is activated by viral PAMPs, which results in an innate immunological and inflammatory response. TLR4 recognizes LPS and viral proteins with the help of an accessory protein called MD2. MD2 binds to TLR4 inside the cell and is also required for TLR4 trafficking to the cell surface (Park and Lee, 2013). CD14 receptors mediate LPS attraction to the TLP4 sensor and are essential in cellular endocytosis (Zanoni *et al.*, 2011; Rajaiah *et al.*, 2015). ACE2 has low pulmonary expression and the spike protein has been proposed to have the most substantial protein-protein interaction with TLR4.

Upon reviewing and correlating the evidence for SARS-CoV-1 and SARS-CoV-2 having direct and indirect associations with TLR4. SARS-CoV-2 spike glycoprotein ties TLR4 and initiates TLR4 flagging. In addition, SARS-CoV-2 obliterates type II alveolar cells and blocks TLR4 in the lungs, subsequently advancing ARDS and irritation. SARS-CoV-2 myocarditis and infection of various organs are due to TLR4 abundance and excessive irritability in MERS patients. Subsequently, hyper irritation in Coronavirus patients. Consequently, TLR4 connects with the pathogenesis of SARS-CoV-2 and its over-activation causes delayed, inborn resistant reactions. Thus, TLR4 is a promising helpful objective in coronavirus patients (Aboudounya and Heads, 2021).

The twofold abandoned middle RNA shaped during viral replication is detected by TLR3 (Zhao *et al.*, 2012; Lee *et al.*, 2020; Choudhury *et al.*, 2021). Among them, TLR3 abundance is more successful than TLR4 in a murine model and the function of TLR3 actuation is gainful against an extensive variety of RNA infection diseases (Perales-Linares and Navas-Martin, 2013; Totura *et al.*, 2015; Mukherjee *et al.*, 2019). Strangely, the high restricting partiality of SARS-CoV-2 non-structure protein 10 (nsp10) mRNA to TLR3 in the docking study shows the potential for the enlistment of downstream TLR3 flagging (Choudhury *et al.*, 2021). Besides, the defensive function of TLR3 in diseases with all the more firmly related coronavirus infections like SARS-CoV-1, Center East respiratory disorder (MERS-CoV) and so forth has been recorded in past viruses (Biswas and Khan, 2020).

MATERIALS AND METHODS

Blood samples were collected from patients from December 2020 to September 2021 at Basrah Teaching Hospital, Basrah-Southern Iraq. It is 50 in hospital male patients, 25(50%). Females were 25(50%) suffering from a COVID-19 infection and distributed according to patient's status into 20(40%) and 20(40%) to moderate and severe symptomatic, respectively, in addition to ten samples (20%) as a control group. All patients with COVID-19 enrolled in this study were diagnosed according to the Iraqi national guidelines for the diagnosis and treatment of COVID-19, to the interim WHO guidelines. Common symptoms included dizziness, headache, shortness of breath, runny nose, sore throat, diarrhea and decreased appetite. The Ethics Committee approved the study, a competent committee in the Ministry of Health and the Ministry of Higher Education and Scientific Research in Iraq, and informed consent was obtained from the participating patients before collecting data and samples. Informed consent is waived for patients who were unable to obtain informed consent.

qPCR total RNA was extracted using a peripheral blood mononuclear cells lysis buffer (Promega, USA) and qRT-PCR reactions were performed using the master mix SYBR green Real-Time Detection kit (Promega, USA), which targeted gene expression of TLR4 and TLR3 by forward and reverse primers according to the manufacturer's protocol.

Briefly, PCR was run at an Applied Bio-RAD CFX96 Real-Time PCR instrument system in a volume of 25 µL containing 1 µL sample, 12.5 µL master mix, 1 µL from each forward and reverse primers, 9.5 µL nuclease-free water and 1 µL internal control. The PCR conditions were reverse transcription in two steps and loading cDNA protection in a thermo cycle device according to the program illustrated in Table 1.

Sequentially dilution gene-specific cDNA generated

Table 1: Thermo cycle for TLR3 and TLR4 gene expression using RT-PCR**.**

Steps	Temperature (°C)	Time	No. of cycles
Initial denaturation	95	120 sec	1
Denaturation	94	10 sec	40
Annealing	57	60 sec	
End cycle (storage)	4	15 min	1

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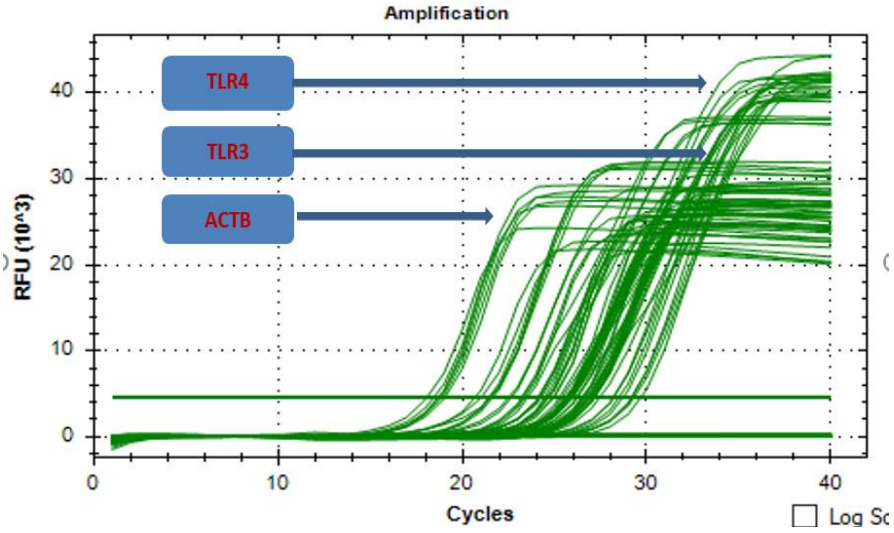

Figure 1: Amplification plots for TLR3, TLR4 and housekeeping gene on RT-qPCR using Syper Green.

Table 2: Sequences of conventional PCR primers for TLR3, TLR4 and ACTB.

Primer	Sequence 5' to 3'	RT-PCR chemistry	Source	Product size (bp)
TLR3	F: ATGCTTTCTCTTGGTTGGGC R: AGTTCCTAGTCAGCTGCAGG	SYBR Green	NM_003265.3	220
TLR4	F: GAGCCGCTGGTGTATCTTTG R: GTCCTCCCACTCCAGGTAAG	SYBR Green	NM_138557.3	169
ACTB	F: ACCAACTGGGACGACATGGA R: CCAGAGGCGTACAGGGATAG	SYBR Green	MN_001101	209

from TLR3, TLR4 and β-actin (ACTB) amplifications were used and listed in Table 1 and Table 2 to determine the copy number of PCR fragments. All primers were designed with Primer3 software. As a first step, the efficiency of the newly designed primers was validated by running them in conjunction with those in the previous research. Hence, two sets of target gene primers (TLR3 and TLR4) and one set of internal control gene primers (ACTB) were used for qPCR. Four replicates of each primer of the respective gene and three replicates of the internal control, primer negative control, and negative control (cDNA) were performed for each sample.

RESULTS

Results for estimating TLR3 and TLR4 expression levels were obtained from 50 patients. SARS-CoV-2 patients were classified into two main groups, severe and moderate. Genes of interest were quantified using qRT-PCR SYBR Green.

Gene expression of TLR3 and TLR4 pathway in SARS-CoV-2

For the TLR3 and TLR4 pathway genes, SYBR Green chemistry was used, and the dye binding was specific to the study genes, as the amplification plots for TLR3 and TLR4 genes are illustrated in Figure 1.

The expression of TLR3 and TLR4 pathways was investigated in SARS-CoV-2 patients according to the COVID-19 as follows:

Initially, the results of the qRT-PCR experiment showed that the TLR3 and TLR4 pathway genes were elevated compared to the healthy control in SARS-CoV-2 patients, whether these cases were severe or moderate subjects, males or females, Figure 2.

In severe cases, patients infected with SARS-CoV-2 were more likely to express both TLR3 and TLR4 genes than patients diagnosed with moderate symptoms. For the severity symptomatic group of patients, the means of expression for the TLR4 gene was (female 0.584 ± 0.01, male 0.747 ± 0.009) and the means of expression for the same gene for patients with moderate symptoms were (female 0.389 ± 0.03, male 0.664 ± 0.08). As for the gene expression of the TLR3 gene for severe and mild cases, it was (female 0.369 ± 0.01, male 0.592 ± 0.07) and (female 0.328 ± 0.01, male 0.414 ± 0.04), respectively, while TLR4 of healthy control was female 0.274 ± 0.02 and male 0.273 ± 0.02, respectively. TLR3 of healthy control was female 0.251 ± 0.01 and male 0.3 ± 0.004, respectively. Moreover, this shows that severe cases are also genetically predisposed to show this gene more than people who are considered moderate cases, as shown in Figure 2 and Figure 3.

To make the observation more accurate and because the differences are not significant between gene

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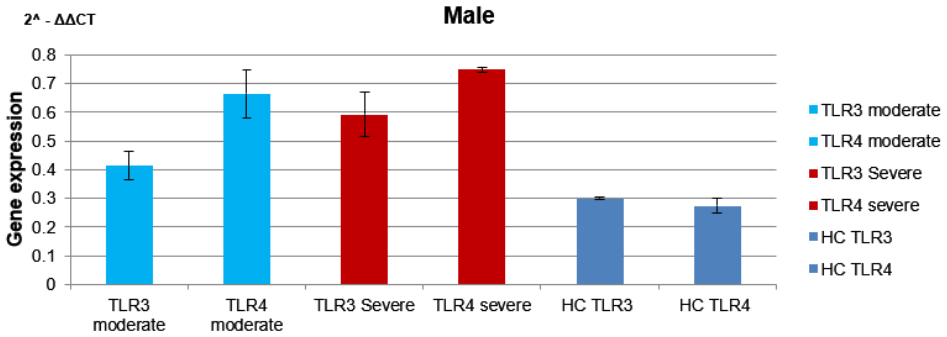

Figure 2: Gene expression levels of TLR3 and TLR4 in male patients infected with SARS-COV-2 severe and moderate symptomatic patients and healthy controls. The expression of TLR4 was higher in severe patients than in moderate symptomatic patients and HC compared to the two groups.

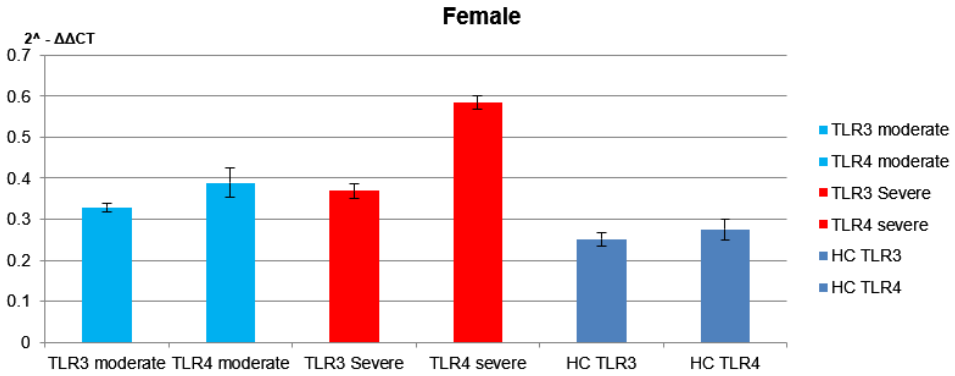

Figure 3: Gene expression levels of TLR3 and TLR4 in female patients infected with SARS-COV-2 severe and moderate symptomatic patients and healthy controls. The expression of TLR4 was highest in severe patients than in moderate symptomatic patients and HC compared to the two groups.

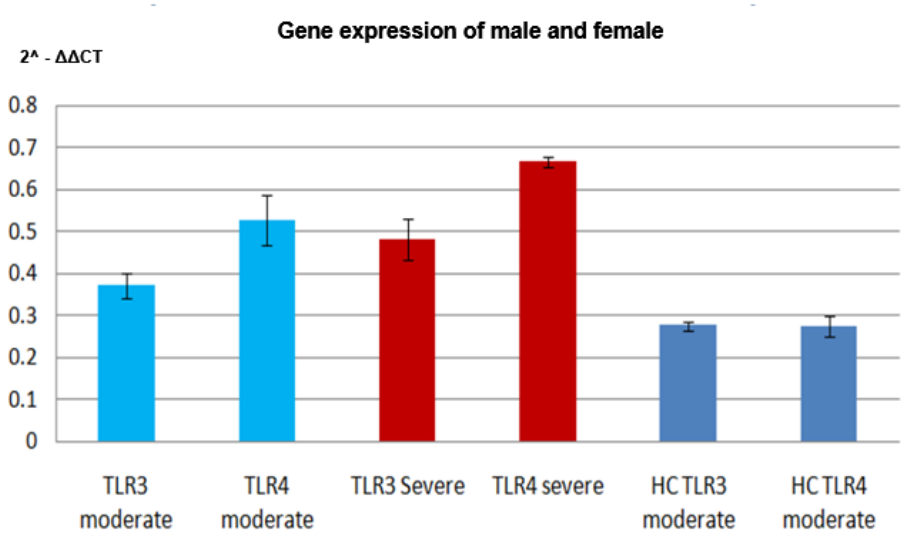

Figure 4: Gene expression levels of TLR3 and TLR4 in male and female populations infected with SARS-COV-2 severe and moderate symptomatic patients and healthy controls. The expression of TLR4 was highest in severe patients than in moderate symptomatic patients and HC compared to the two groups.

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expression between males and females, they have been combined in the following Figure 4.

This study investigates TLR3 and TLR4 gene expression in the innate immunity of COVID-19 patients. real-time transcription-polymerase chain reaction (RT-PCR) analysis was used to determine the gene expression in innate immune cells and their role in improving or deteriorating a person's health. The results show a high gene expression level in severe cases of TLR3 and TLR4 receptors, while the expression of the same toll-like receptors for moderate cases was lower. The gene expression of the healthy individual was the control.

DISCUSSION

Toll-like receptors 4 and toll-like receptors3 up-regulated TLR4 is most likely being used by SARS-COV-2 to enter cells and increase ACE2 expression. The virus could be utilizing TLR4 signaling to trigger the expression of ACE2 (or another receptor) through which it would enter the same or neighboring cells, according to the bio-computational analysis of the S spike to SARS-CoV-2 glycoprotein (Aboudounya and Heads, 2021). LPS, TLR4's usual ligand, activates it. DAMPs let out of lytic or necrotic cells, like HMGB1 and HSPs, or up-controlled at locales of tissue injury and persistent irritation, for example, on the other hand, joined fibronectin-additional space A (Fn-EDA) and other ECM-inferred DAMPs,

Such as low molecular weight hyaluronic (LMWHA) and sulfated proteoglycans can also activate it (Mesquita *et al.*, 2014; Molteni *et al.*, 2016; Turner, 2016). These DAMPs cause fibro-inflammatory genes to be expressed at wound healing sites, resulting in maladaptive remodeling and fibrosis (Mckeown-Longo and Higgins, 2017). TLR4 is activated by viral PAMPs, which results in an innate immunological and inflammatory response. TLR4 recognizes LPS and viral proteins with the help of an accessory protein called MD2. MD2 binds to TLR4 inside the cell and is also required for TLR4 trafficking to the cell surface (Park and Lee, 2013; AlSaimary *et al.*, 2020a). CD14 receptors mediate LPS attraction to the TLP4 sensor and are essential in cellular endocytosis (Zanoni *et al.*, 2011; Rajaiah *et al.*, 2015).

TLR3 is an interferon-inducing dsRNA sensor whose activation aids the defense against RNA viruses (Totura *et al.*, 2015; Mukherjee *et al.*, 2019). TLR3 signaling activates two immunological factors, NF-kB and interferon (IFN)-regulatory factor 3 (IRF3), in response to viral infection. TNF (tumor necrosis factor) is one of the cytokines produced due to activating immunological responses. On the other hand, increased inflammatory reactions can render a patient more vulnerable to pneumonia and autoimmune illnesses. In the absence of TLR3, a protective effect against deadly pneumonia has been demonstrated (Matsumoto *et al.*, 2011; Suresh *et al.*, 2019). TLR3, like other TLRs, appears to play a role in determining infection susceptibility via autophagy (Franco *et al.*, 2017). The autophagy pathway is critical during infection and for molecular functions such as cell maintenance and homeostasis (Johansen *et al.*, 2012). Autophagy is, in fact, one of the most critical cell defense systems against pathogens (Levine *et al.*, 2011). Different investigations on other COVID viruses, like the mouse hepatitis infection (MHV) and the contagious gastroenteritis infection (TGEV), have proposed that autophagy assumes a part (Prentice *et al.*, 2004; Guo *et al.*, 2016). It has likewise been recommended that it has a capability for SARS-CoV-2 contamination (Miao *et al.*, 2020; Carvalho-Schneider *et al.*, 2021). SARS-CoV-2, specifically, can smother autophagy, coming about in autophagosome development and viral leeway restraint, which, when joined with immunological brokenness and the enactment of various provocative cytokines, brings about a more severe type of coronavirus sickness (Jamwal *et al.*, 2020; Shojaei *et al.*, 2020).

Evidence binding to TLR4 or activating TLR4 via DAMP-related mechanisms with SARS-CoV-1 and SARS-CoV-2 proteins

Cytokine storms occur in severe cases of COVID-19 patients and cause many organ failures. TLR4 may cause this because of its association with the spike protein of SARS-CoV-2, through which it turns from a state of defense of the cell to a failure of the immune system and the virus uses it to enter the host cells. Choudhury and Mukherjee (2020) conducted an in-silico study. They discovered that, compared to other TLRs, the spike glycoprotein of SARS-COV-2 had the highest protein-protein interaction with the TLR4 receptor (Choudhury and Mukherjee, 2020).

Fu *et al.* (2020) presumed that the SARS-CoV-2 glycoprotein is more in liking with TLR4 than with ACE2; this is an urgent disclosure that should be exploited. Furthermore, when contrasted with other TLRs, TLR4 on the phone surface is bound to be associated with recognizing atomic examples from SARS-CoV-2 and setting off inflammatory reactions.

TLR4 articulation and downstream flagging systems give circuitous proof. *In vivo* examinations show that a particular ligand enacts both TLR4 downstream flagging pathways in SARS-CoV-1 disease: the exemplary MyD88-subordinate pathway and the option TRIF/Cable car subordinate pathway. This checks out because the host cells require both NF-B and IRF3 to mount a solid intrinsic safe reaction against the infection. NF-B sets off the record of proinflammatory cytokines and chemokines, while IRF3 communicates antiviral and mitigating interferons, which initiate the history of ISGs. In contrast with controls, Myd88−/− mice contaminated with SARS-CoV-1 had expanded mortality, weight reduction and viral burdens. Subsequently, MyD88 is essential for insurance against SARS-CoV-1 contamination, mainly as it is a crucial connector protein for various TLRs, not just the TLR-4 receptor (Sheahan *et al.*, 2008). TLR4 initiation instigated by moist. Another thought is that coronavirus causes irritation and fibrosis by delivering harm-related atomic examples (DAMPs) from lysed or passing on cells, which actuate TLR4 in the lungs and heart, causing

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aggravation and fibrosis. The presence of host DAMPs in the lungs of SARS-CoV-1 patients has been found, and they might assume a crucial part in intense lung injury (Imai *et al.*, 2008).

Besides, Andersson *et al.* (2020) proposed that TLR4 and the receptor for cutting-edge glycation end products (Fury) were both enacted by HMGB1 delivered as a Sodden or emitted by actuated safe cells, bringing about the creation of proinflammatory cytokines (Andersson *et al.*, 2020). In extreme/basically coronavirus patients, a Soggy TLR4 ligand is a reliable biomarker (Sohn *et al.*, 2020).

CONCLUSION

The study shows that the Iraqi population with SARS-CoV-2 patients has up-managed quality articulation for TLR4 and TLR3 contrasted with the control test. However, more often than not, the patients with serious side effects show higher up-directed quality articulation for TLR-4 and TLR3 in contrast with patients with moderate side effects.

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