

How to Cite:

Kadam, M. M., & Al-Abady, Z. N. (2022). Evaluating the expression of IDO and TNF- α and vitamin D levels in diabetic patients cured of MERS-CoV compared to the control groups. *International Journal of Health Sciences*, 6(S1), 10283–10292. <https://doi.org/10.53730/ijhs.v6nS1.7655>

Evaluating the expression of IDO and TNF- α and vitamin D levels in diabetic patients cured of MERS-CoV compared to the control groups

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Abstract---SARS-CoV-2, the new virus responsible for the current coronavirus 2019 (COVID-19) pandemic, has infected people around the world since the first case was reported in Wuhan, China. As more epidemiological data is collected on COVID-19 patients, factors that increase the severity of infection are identified and reported. It is well known that diabetes is one of the most frequently associated comorbidities with worse outcomes for COVID-19 patients, as it is considered a high-risk aggregate of COVID-19. In addition to low NAD availability and exacerbation of infection. These patients are predisposed to severe COVID-19 pathophysiology due to a metabolic imbalance. The current study looked at the expression of tumor necrosis factor (TNF- α) and IDO (the NAD-biosynthesizing enzyme), as well as serum vitamin D levels and triglycerides. This study included 100 participants: 25 patients with type 2 diabetes who recovered from COVID-19, 25 type 2 diabetic patients who were not infected with COVID-19, and 25 not-diabetic subjects who recovered from COVID-19 compared to 25 healthy control. TNF- α and IDO levels were measured with quantitative PCR, while vitamin D levels were measured with competitive fluorescent immunoassay technology. The findings revealed a decrease in the expression of TNF- α and IDO in addition to a decline in vitamin D levels. However triglyceride levels were elevated. These findings suggest that intracellular NAD contents might be associated with an inflammatory response marker (as TNF- α) and low vitamin D levels during COVID infection in diabetic patients.

Keywords---COVID-19, Diabetes mellitus, Vitamin D, TNF- α , IDO.

Introduction

Diabetes mellitus (DM) is described as an imbalance in sugar metabolism that results in an excessively high blood sugar level for a variety of reasons, including psychological, organic, excessive sugar intake, or genetic variables, and it develops as a result of an imbalance in sugar (1). It can be divided into four types: Type 1 diabetes mellitus (DM1) (due to autoimmune system-mediated destruction, which is usually the basis for absolute insulin deficiency), and type 2 diabetes (DM2) (due to a revolutionary deficiency of B-mobilized insulin secretion, often in the history of insulin resistance, and gestational diabetes mellitus (GDM), certain types of diabetes are recognized in the third trimester of pregnancy, and certain forms of diabetes are due to various causes, for example, monogenetic diabetes syndromes. (which includes neonatal diabetes and adult-onset diabetes in young adults [MODY] (2). Diabetes mellitus symptoms differ from person to person, but they are all influenced by the patient's health and diet (3). Polyphagia (increased hunger), polydipsia (increased thirst), and polyuria are all signs of diabetes mellitus (increased urination). Various organs, such as the heart (primarily myocardial infarction), kidneys (diabetic nephropathy), nerves (diabetic neuropathy), and eyes (diabetic retinopathy), frequently malfunction due to increased diabetes levels in the blood (4). Aside from the huge number of vulnerable diabetics, the condition DM increases the chance of developing SARSCoV-2-related symptoms or infection rates (5). First, diabetic patients are particularly vulnerable to infection pathogens, and both DM1 and DM2 patients are at risk of developing mucous membrane infection when exposed to causes. Second, if diabetic patients are exposed to SARS-CoV-2 and become infected, a severe form of COVID-19 is more likely, as diabetic people have a reduced rate of viral clearance and a higher affinity for the pathogen for cellular binding (6). Diabetes as a comorbidity for COVID-19 is a global concern, as diabetes has become a pandemic of the twenty-first century due to an increase in DM among the elderly and adolescents (7). There is less information on glucose metabolism and the development of acute diabetic complications in COVID-19 individuals (8). Infection of SARS-CoV-2 in patients with diabetes probably produces higher stress circumstances, with greater release of hyperglycemic hormones, leading to increased blood glucose levels and aberrant glucose variability (9). It was discovered that diabetic patients with COVID-19 had a higher rate of common comorbidities. Diabetic patients are more likely than non-diabetics to have a more serious infection, resulting in greater hospitalization and a higher chance of mortality (10).

The Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) virus, which first appeared in Wuhan, China (11), in December 2019, was the source of the COVID-19 outbreak. The causal agent was discovered as a new coronavirus and was given the name SARS-CoV-2 by the World Health Organization (WHO) (12). It may be asymptomatic or evident in three clinical phases: an initial upper respiratory tract infection, followed by a pneumonic phase in a few people, and a hyperinflammatory phase in an even smaller number of patients, which can be fatal (13). The fact that some risk factors were shown to be common in most worldwide investigations suggested that a comparable systemic aberration might be present in those at high risk, leading to severe illness or death. The nicotinamide adenine dinucleotide (NAD⁺)-indicates a possible common thread in

the pathogenesis of the hyperinflammatory response and higher mortality in this context(14). Multiple organs, including adipose tissue, skeletal muscle, the liver, and the hypothalamus, have lower intracellular NAD⁺ levels in type 2 diabetes mellitus. Low-grade inflammation is also a feature of the illness, which is linked to TNF-activation (8). Cells require electron transfer between NAD⁺ (the oxidized form) and its reduced form, NADH because it is required for glycolysis, the citric acid cycle, energy pathways, and NAD⁺ consumption enzymes such as ADP-ribose transferases, NADases such as CD38, sirtuins, and poly(ADP-ribose) polymerases (PARP). NAD⁺ can be synthesized from nicotinamide, nicotinic acid, or nicotinamide riboside, or acquired through the salvage pathway, and from tryptophan (through the *de novo* pathway), where the IDO enzyme represents the main regulatory enzyme in this pathway (15-17).

The current study aimed to determine whether the change in the expression of NAD-biosynthesis enzyme (IDO) and TNF- or vitamin D levels in T2DM patients, who had infected with COVID-19 in the last 6 months, might be associated with worsen of disease state to determine their potential diagnostic value.

Materials and Methods

Study Population

The study was conducted in Al-Diwaniyah Governorate in Iraq. Twenty-five diabetic subjects who had infected with COVID-19 for the last six months, were compared with twenty-five diabetic patients who did not infected with COVID-19, twenty-five non-diabetic subjects who had recovered from COVID-19, and twenty-five healthy subjects. All subjects were selected during the period from November 2021 to January 2022. The patients' ages ranged from 40 to 60 years, identical to the age included in the study. The patients are underwent diabetes treatment properties.

Methods

Blood sample (5 ml) were withdrawn from 100 people, and the samples were divided into three sections: the first section was one ml of blood in a K2EDTA tube for gene expression; the second section was kept in a gel tube. The serum was centrifuged at 3600 rpm for 10-15 minutes at 4 °C to separate the serum, which was stored at -20° C for vitamin D level measurement using competitive fluorescent immunoassay technology, and the remaining section was kept in a sodium citrate tube to get plasma. To measure triglycerides, assayed with enzymatic methods on neutralized perchloric extracts of plasma.

Blood RNA was extracted using a TRIzol™ RNA extraction kit and reverse-transcribed using the ProtoScript® First Strand cDNA Synthesis Kit and the Luna Universal qPCR Master Mix kit (NEB, UK). The cDNA that resulted was combined with TNF-α or IDO -specific forward and reverse universal primers, as well as the Bright Green cDNA master mix. GAPDH served as the endogenous control. The comparative threshold cycle (Ct) and ($2^{-\Delta\Delta Ct}$) were used to calculate the relative levels of TNF-α or IDO.

Statistical Analysis

Data were summarised, analyzed, and presented using GraphPad Prism 9.2.0 and Microsoft Office Excel 2013. Numeric data were expressed as mean $\pm$ standard error mean. One-way ANOVA test and unpaired t-test were used to compare the mean values among the different groups in the case of normally distributed variables. *p-value* was considered significant at *p-value* $\leq$ 0.05.

Results

The relationship between NAD⁺ and TNF- α is now well established in a variety of cell types. It has been proposed that NAD⁺ regulates TNF- α production in a sirtuin-dependent manner (18). As well as, TNF-, on the other hand, has been shown to modulate the expression of several enzymes involved in NAD⁺ homoeostasis (19)

As a result, the decrease in NAD-biosynthesized enzyme expression levels of IDO is shown in all studied groups diabetic patients previously infected with COVID 19 (1.046105 ± 1.121538) compared to the diabetic patients (0.922449 ± 0.860329) and non-diabetic subjects previously infected with COVID-19 (0.856235 ± 0.698368) compared to the control group (Fig. 1).

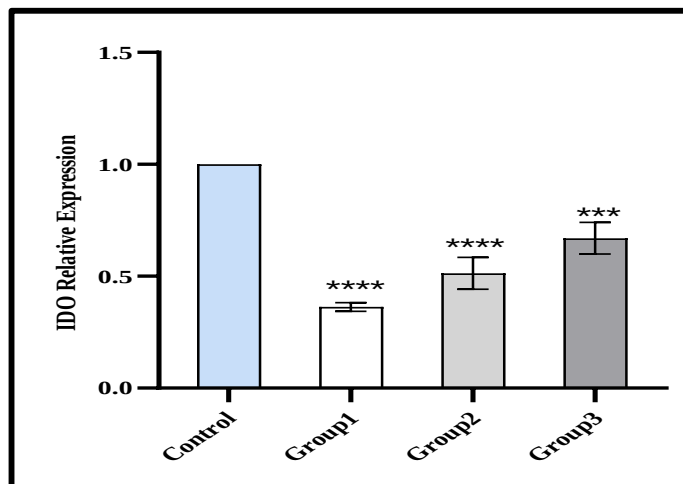


Figure 1: IDO levels in serum patients with, Group1: (COVID-19), Group2: (Diabetes), Group3: (Diabetes+COVID-19), and control group the significant difference (*p-value* $\leq$ 0.0001). Data are expressed as means $\pm$ SEM. indicates * significant differences compared to the control, $P \leq 0.05$.

Interestingly, there was a similar decrease in the expression levels of the pro-inflammatory cytokine (TNF-) as it is shown in all studied groups, it was low in diabetic patients previously infected with COVID 19 (0.179474 ± 0.30576) compared to the diabetic patients (0.263654 ± 0.286643) and non-diabetic subjects previously infected with COVID-19 (0.17031 ± 0.159676) compared to the control group (Fig.2)

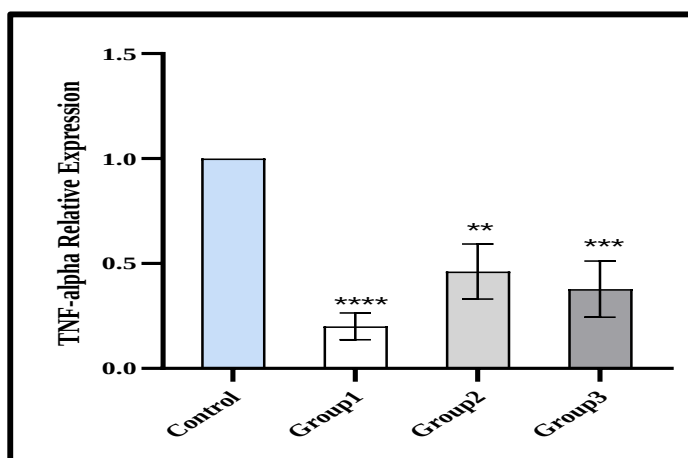


Figure 2: TNF- α levels in serum patients with, Group1: (COVID-19), Group2: (Diabetes), Group3: (Diabetes+COVID-19), and control group the significant difference (p -value < 0.0001). Data are expressed as means $\pm$ SEM. indicates * significant differences compared to the control, $P \leq 0.05$.

Table 1
Primers used for qPCR experiments

Genes	Primers	Size	GC %	Product (bp)
GAPDH	For: <u>CCCACTCCTCCACCTTTGAC</u>	20	60	100
	Rev: <u>CTGTTGCTGTAGCCAAATTCGT</u>	22	45	
IDO	For: GCCTGCGGGAAGCTTATG	18	61	100
	Rev: TGGCTTGCAGGAATCAGGAT	20	50	
TNF- α	For: CTCTTCTGCCTGCTGCACCTTG	22	55	135
	Rev: ATGGGCTACAGGCTTGTCATC	22	55	

It is worth noting that the levels of D vitamin was also changed in all groups. The results show low levels of D vitamin in all studied groups: diabetic patients previously infected with COVID-19 (22 ± 1.66 ng/ml), diabetic patients (26 ± 1.76 ng/ml), and non-diabetic subjects previously infected with COVID-19 (29.88 ± 2.23 ng/ml) compared to the control group (38.5 ± 3.96 ng/ml) (Fig.3).

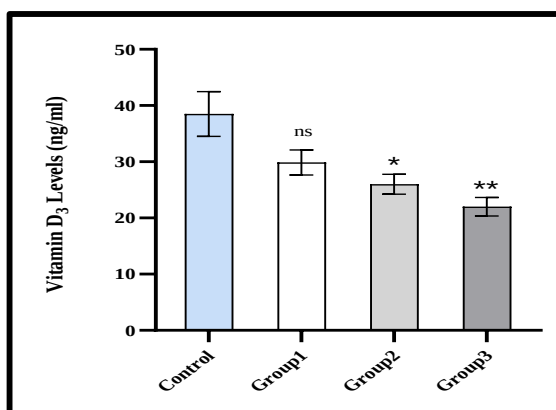


Figure 3: vitamin D₃ levels in serum patients with, Group1: vitamin D₃ and (COVID-19), Group2: (Diabetes), Group3: (Diabetes+COVID-19), and control group the significant difference (p -value = 0.0008). Data are expressed as means $\pm$ SEM. indicates * significant ns, not significant differences compared to the control, $P \leq 0.05$

The last parameter has been investigated in diabetic patients and all other groups is triglycerides levels. Triglycerides have been shown to be increased in patients with type 2 diabetes mellitus (178.88 ± 10.14 mg/dL) (patients previously infected with COVID 19) compared to all other groups (172.75 ± 15.61 mg/dL) and (154.88 ± 12.53 mg/dL) respectively and the control (127.75 ± 13.45 mg/dL) (Fig.4).

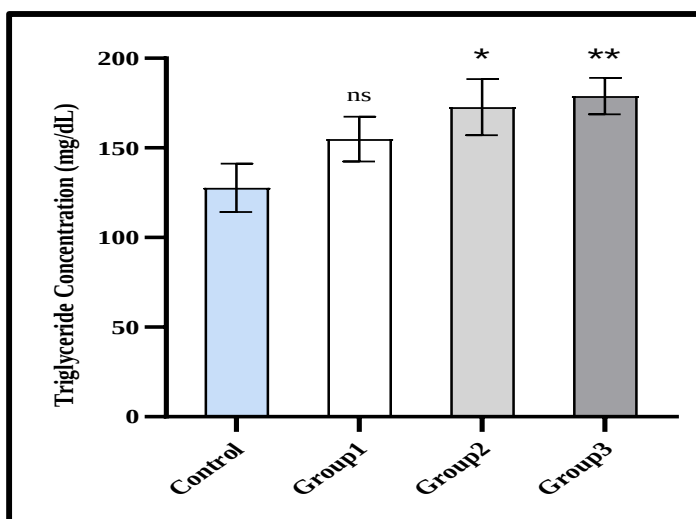


Figure 4: Triglyceride levels in serum patients with, Group1: (COVID-19), Group2: (Diabetes), Group3: (Diabetes+COVID-19), and control group the significant difference (p -value = 0.0439). Data are expressed as means $\pm$ SEM. indicates * significant ns not significant differences compared to the control, $P \leq 0.05$.

Discussion

Nicotinamide adenine dinucleotide (NAD⁺) is a multifunctional chemical molecule that regulates a variety of biological processes and serves as a substrate for a variety of species, it is a necessary coenzyme in many redox activities that drive energy metabolism (20). The most important enzyme involved in NAD biosynthesis is IDO (indoleamine 2, 3-dioxygenase), it catalyzes the first step in tryptophan catabolism via the kynurenine degradation pathway(21). It is thought to play a role in a variety of pathophysiological processes, including antimicrobial and antitumor defense, neuropathology, immune-regulation, antioxidant activity, and autoimmunity suppression (22). Low IDO expression in the present study suggested low intracellular NAD levels and might also suggest the role of other NAD-biosynthesis pathway to regulate NAD levels such as Salvage pathway rather than *de novo* pathway. Different cytokines might also activate IDO and increase its expression such as TNF, IL-1, and IFN (23). The reduction levels of TNF might be a normal mechanism to explain the decrease in IDO levels in our study. This reduction in TNF- α might be associated with the levels of NAD-dependent enzymes such as sirtuins or intracellular NAD levels and their important roles in regulating TNF- α protein synthesis (18,24). In COVID-19 patients, inflammatory cytokines such as TNF- α and interleukin-6 (IL-6) are elevated, particularly in the severe group (25, 26). However our findings show that diabetic and non-diabetic patients that previously were infected or not with COVID-19 have a lower TNF- α gene expression which may aid in developing effective COVID-19 treatment strategies.

The other interesting biochemical marker to study is Vitamin D, this vitamin has several anti-infective effects, including increasing T-lymphocyte chemotaxis and eliminating respiratory pathogens by inducing apoptosis and autophagy in the affected epithelium (27). Low T-lymphocyte counts have been found in COVID-19 patients with severe symptoms (28). Importantly, vitamin D administration has shown to increase T-lymphocyte levels (29), these findings confirmed that vitamin D may be useful in the treatment of COVID-19. According to our results, low vitamin D levels are associated with an increased risk of COVID-19 infection. COVID-19 infected people had lower vitamin D levels than non-infected people, these findings are similar to the study of Nanyang Liua et al (30). The relationship between low vitamin D levels and metabolic, autoimmune, and infectious diseases has received a lot of attention. In some studies, low vitamin D levels have been linked to an increased risk of respiratory infections (31). Low levels of serum 25-hydroxyvitamin D (25(OH)D) were linked to a higher prevalence of community-acquired pneumonia and the severity of the disease.(32). Vitamin D deficiency has been also reported in people with Type 2 diabetes mellitus (33), which is all consistent with the current results.

Finally, investigating of triglycerides levels might help to predict its role in the developing of several disease such as CHD, particularly in patients with type 2 diabetes (34), many studies confirmed that hypertriglyceridemia is closely related to insulin (35). Currently it was also mentioned that corona patients have higher triglycerides than the control group (36). In the present study high triglyceride levels might also be involved in the developing of diabetes. In conclusions, the change in IDO expression and intracellular NAD levels in addition to the change

in TNF- α expression, and low vitamin D3 levels with high triglycerides might all be involved with the development of type 2 diabetes mellitus.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

1. Authors Contribution

Muna Mansoor Kadhim, and Dr. Zainab N Al-Abady were contributed to design the research, to the analysis of the results and to the writing of the manuscript. The authors approved the final version for submission

2. Authors Contribution

Muna Mansoor Kadhim, and Dr. Zainab N Al-Abady were involved in the design of the study, the analysis of the findings, and the drafting of the report. The writers gave their approval for the final version to be submitted.

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