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Folding expression of IL-32, IL-33 and TNF-α in Patient's Co-infected with Hepatitis C Virus and Toxoplasmosis

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TOXOPLASMOSIS remains a common parasitic zoonosis with an international spread. Many warm-blooded animals, including sheep and humans, are prone to infection. Hepatitis C virus (HCV) and toxoplasmosis co-occurrence is a rare but potentially serious disease. The microbe *Toxoplasma gondii* is the source of the parasitic disease toxoplasmosis, whereas HCV is a viral infection known for its ability to damage the liver. To assess the Co-occurrence of hepatitis C virus (HCV) and toxoplasmosis. A Total of 100 blood samples were collected from HCV and toxoplasmosis co-infected patients (52 males and 48 females) whose ages ranged between 5 and 65 years, and 50 healthy individuals (25 males and 25 females) whose ages ranged between 5 50 - years as a control group. Folding expression detection and estimation of IL-32, IL-33, and TNF-α levels for patient samples and the control group. When the control group›s mean levels of IL-32, IL-33, and TNF-α were compared to those of the co-infection patients 3.919±0.226, 4.775±0.249, and 4.655±0.271, a very significant increase in these levels was seen 10.110±0.596, 24.914±2.308, and 12.356±1.369.The results also showed highly significant differences ($p<0.001$) in folding expression between IL-32, IL-33 and TNF-α in the patients group (7.598±1.533, 29.089±5.785 and 10.744±2.289) respectively when compared with their folding expressions in the control group (1.324±0.5, 1.214±0.317 and 1.544±0.59) respectively. Patients with co-infection (HCV and toxoplasmosis) had considerably higher levels of IL-32, IL-33, and TNF-α as well as their folding expressions.

Keywords: HCV, Toxoplasmosis, IL-32, IL-33, TNF-α, Folding expression**.**

Introduction

One of the most important parasites that affect public health is *Toxoplasma gondii*, which interacts with warm-blooded animals and humans as an intermediate host. Often infected are humans and other warm-blooded animals, especially sheep [1]. The association between HCV infection and acute and chronic hepatitis, as well as liver cancer, has been firmly established [2]. The co-occurrence of hepetitis C virus (HCV) and toxoplasmosis is an uncommon yet potentially significant condition. Although the bacteria Toxoplasma gondii is the source of the parasitic illness toxoplasmosis, the human coronavirus (HCV) is well recognized for its capacity to harm the liver. The consequences and symptoms of two infections happening simultaneously may be more severe than those of an isolated infection [3]. Fever is the main indicator of co-infection and is often followed by fatigue, muscular stiffness, and joint discomfort. Jaundice, an upset stomach, nausea, and vomiting are other symptoms. Rarely, the infection may

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cause damage to the liver and increase the risk of cirrhosis and liver failure [4]. A combination of imaging methods, blood tests, and biopsies is often used to diagnose co-infections. In treatment, antiviral and antiparasitic drugs are often employed. In certain cases, surgery may be required to remove the injured tissue [5]. The diagnostic technique involves analyzing signs and symptoms to determine whether a certain illness or condition exists. Molecular and serologic testing is the main diagnostic approach used for HCV. These tests are used to identify and investigate HCV antibodies and genetic material [6]. Various methods are used to diagnose toxoplasmosis, but serological tests are most commonly used. These tests are used to establish the diagnosis due to the resemblance of clinical symptoms in immunocompetent individuals to other infections or even malignancies. It is crucial to maintain a high level of suspicion in order to determine the correct diagnosis [7].

Cytokines are crucial for maintaining a balanced immune system, but when their regulation goes awry, it can contribute to different diseases. Excessive production of pro-inflammatory cytokines, referred to as a cytokine storm, can occur in conditions like sepsis, autoimmune diseases, and specific viral infections, this exaggerated immune response can cause harm to tissues and lead to organ dysfunction. Conversely, deficiencies in certain cytokines or impaired responsiveness to cytokine signaling can result in immunodeficiency disorders, where the immune system is unable to effectively fight against infections [8, 9].

When compared to healthy controls, individuals with viral hepatitis had different amounts of zinc, copper, iron, cadmium, and lead. [10]. Studies have shown that individuals infected with the other widespread zoonotic coronaviruses frequently have liver damage. [11]. Among patients with β-thalassemia, the frequency of HCV was 1.6% for HCV viremia with genotype 3a and 17.6% for anti-HCV antibodies. These findings show that β-thalassemia patients have a higher frequency of HCV infection than the general population, suggesting that HCV infection is still present in the thalassemia community in south Iran. [12]. Therefore, this study aimed to discover the co-occurrence of hepatitis C virus (HCV) and toxoplasmosis.

Material and Methods

Ethical Considerations

Ethical review board of Middle Technical University, College of Health and Medical Technology Guidelines Ethical approval number 3/2201 ethically cleared the study. The study is reported by ARRIVE guidelines.

In this case-control study, the study was conducted at the Medical City, Gastroenterology & Hepatology centre. Ibn Al-Balady Children & Maternity Hospital and Balad general hospital-Iraq, during the period from May to July 2023. 100 patients who also had co-infection with the Hepatitis C virus (HCV) and 50 healthy people served as the control group. Venous blood samples were taken from both groups.

Information sheet was prepared and designed according to a questionnaire, which covers all information. After clotting, the blood samples were put in plane tubes and centrifuged for 15 minutes at 3000 rpm to extract serum, which is kept at -20 C until needed.

The levels of IL33, IL32, and TNF cytokines were estimated for both the patients and healthy control group by using the Q- PCR was used for folding expression detection of IL33, IL32, TNF cytokines for the patient's samples and the control groups described in the steps below.

RNA Purification

Following the TRIzolTM Reagent procedure, RNA was extracted from the sample using the following steps:

Blood sample

After adding 0.5 mL of TRIzol™ Reagent and 0.4 mL of blood to each tube, the lysate was homogenised by repeatedly pipetting up and down.

For three phase's separations

The lysate in each tube was mixed with 0.2 mL of chloroform before the tube cap was fastened. After being incubated for two to three minutes, the mixtures were centrifuged for ten minutes at 12,000 rpm to separate them into a lower organic phase, interphase, and a colourless upper aqueous phase. A fresh tube was filled with the RNA-containing aqueous phase.

RNA precipitation

After combining the aqueous phase with 0.5 mL of isopropanol, the mixture was incubated for ten minutes and centrifuged at 12,000 rpm for ten minutes. At the bottom of the tube, a white pellet that resembled gel was the product of total RNA precipitation. After that, the supernatant was discarded.

RNA washing

After adding 0.5 mL of 70% ethanol to each tube and quickly vortexing them, the tubes were centrifuged at 10,000 rpm for five minutes. The pellet was then air-dried and inhaled by ethanol.

RNA solubility

Pellet was rehydrated in 20–50 µl of Nuclease Free Water and incubated for 10–15 minutes at 55–60°C in a water bath or heat block.

Determine RNA yield

A-Fluorescence Method

The concentration of extracted RNA was evaluated using a Quantus Fluorometer to evaluate the quality of samples for use in further procedures. A solution of 200µl diluted Quanti Flour Dye and 1 µl RNA were mixed. Values for RNA concentration were discovered during a 5-minute incubation time in a dark, room temperature setting.

Primer preparation

These primers were given in lyophilized form by The Macrogen Company. Lyophilized primers were dissolved in nuclease-free water to produce a stock solution with a final concentration of 100 pmol/µl. 90µl of nuclease-free water and 10µl of primer stock solution, which was stored at -20 C in the freezer, were combined to form a practical primer solution with a concentration of 10 pmol/µl (**Tables 1 - 5**)

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Analysis Gene Expression using Livak Method

Folding =2-ΔΔCT - ΔCT =CT Gene - CT House Keeping gene - ΔΔCT =ΔCT Treated or Control - ΔCT Control

TABLE 1. Primers used in this study

Primer Name	Concentration (pmol/µl)	Vol. of nuclease free water (µl)
β-Globin-F	100	300
β-Globin-R	100	300
IL33_exp-F	100	300
IL33_exp-R	100	300
IL32_exp-F	100	300
IL32_exp-R	100	300
IFNG_exp-F	100	300
IFNG_exp-R	100	300

TABLE 2. Primers types and program

Primer Name	Sequence 5`-3`	Annealing Temp. (˚C)	Product size (bp)
rs7044343-F	TGTAAAACGACGGCCAGTTGTCTCACCAGAGGGATTT	60	996
rs7044343-R	CAGGAAACAGCTATGACCATCAACACCGTCACCTTAC		
rs12934561-F	TGTAAAACGACGGCCAGTCCTCCAAATCTCGGGTTTAAG		1025
rs12934561-R	CAGGAAACAGCTATGACGCAAAGGTGGTGTCAGTATC		
rs2430561-F	TGTAAAACGACGGCCAGTCGTTGCTCACT GGGATTT	55	1029
rs2430561-R	CAGGAAACAGCTATGACCATGTCTTCCTT GATGGTCTC		

TABLE 3. PCR component calculation

No. of reaction	20	Annealing temperature of primers	run	57, 6,63, 65
Reaction volume /run	10	No. of primers	µl	4

TABLE 4. PCR Master Mix

Master mix components	Unit	Stock	Unit	Final	Volume
		Sample			
qPCR Master Mix	X	2	X	1	5
RT mix	x	50	X	1	0.25
$MgCl_2$					0.25
Forward primer	µM	10	µM	0.5	0. 5
Reverse primer	µM	10	µM	0.5	0. 5
Nuclease Free Water					2.5
RNA	ng/µl		ng/µl		1
Total volume					10
Aliquot per single rxn	9µl of Master mix per tube and add 1µl of Template				

TABLE 5. Real Time PCR program

Steps	m: s	°C	Cycle
RT. Enzyme Activation	15:00	37	1
Initial Denaturation	05:00	95	
Denaturation	00:20	95	
Annealing	00:20	58,60,63 or 65	40
Extension	00:20	72	

Unsuitable or invalid samples, non-irrelevant samples, and co-infection with other viruses, such as the hepatitis B virus (HBV), were among the circumstances that precluded the study›s participation. Individuals with chronic illnesses including haemophilia, thalassemia, and renal failure, as well as samples with missing or insufficient data, may have viral hepatitis C.

Statistical analysis

Version 20.0 of the SPSS software programmed was used for data analysis. While qualitative data were given as a number and percentage, the mean and standard error were supplied for the quantitative data. The Student's t test and Pearson correlation coefficient were employed when appropriate, and a p value of 0.01 was used to assess the significance of the findings.

Results

The concentrations of IL-32, IL-33, and TNF-α were higher in the co-infected individuals compared to the healthy controls. The mean and standard deviation of IL-32 concentration in the control group was 3.919±0.226, whereas in the patient group it was 10.110±0.596, $p<0.001$, indicating a highly significant difference. The concentration of TNF-α was also significantly higher in the co-infected individuals compared to the control group.The mean and standard deviation of TNF-α in the control group was 4.655±0.271, whereas in the patient group, it was 12.356±1.369, demonstrating a significantly large difference ($p<0.001$). On the other hand, the mean and standard deviation of the IL-33 levels in the co-infected patients and the control group differed significantly ($p<0.001$), with the mean and SD

of (4.775±0.249) for the former and the latter, respectively (**Table 6**).

The control group's mean and standard deviation of IL-32 folding expression was 1.324±0.5, but the co-infection patient group's was 7.598±1.533, A highly significant difference ($p<0.001$) is seen in **Table (7).**

The mean and standard deviation of IL-33 folding expression were significantly different in the co-infection patient group (29.089±5.785) compared to the control group (1.214±0.317) according to the findings of the IL-33 folding expression analysis (**Table 8**).

The TNF-α folding expression results indicated that the co-infection patient group had a very significant difference in mean and SD of TNF-α folding expression (10.744±2.289) compared to the control group's (1.544±0.59). **Table (9)** illustrates this difference.

Discussion

Given that circulating levels of IL-32α, the isoform of IL-32 that is most common in patients with chronic HCV, have been demonstrated [13,14], and that peripheral IL-32α levels are connected with hepatic fibrosis scores in patients with chronic HCV [15], IL-32 may play a role in chronic HCV. There is proof that IL-32 contributes to the development of long-term HCV infection. [16]. Moreover, rheumatoid arthritis synovial tissues, serum from psoriasis patients, and inflammatory tissues linked to inflammatory bowel disease all show substantial co-localization of IL-32 with pro-inflammatory cytokines (TNF, IL-1β, and IL-6). Taken as a whole, the data unambiguously show that IL-32 induces inflammation in the specific regions of injured tissues. [17].

Depending on the kind of infectious agents, the tissue affected, the acute or chronic infection stage, and the host immunological microenvironments, L-33 plays a variety of vital and varied functions in infectious disorders [18]. The IL-33/ST2 axis has been shown to have protective functions in the liver during chronic viral infection. These roles include stimulating type 2 innate lymphoid cells (ILC2), suppressing the inflammatory cytokine TNF-α, boosting CD8+ T-cell responses, and shielding the liver after acute adenovirus infection [19].

TABLE 6. Distribution of IL-32, IL-33 & TNF- -α among the study groups (patients and controls)

Parameter	Groups	Mean ± S.D	P value
IL-32	**Control**	3.919±0.226	p<0.001 (HS)
	Co-infection patients	10.110±0.596	
IL-33	**Control**	4.775±0.249	p<0.001 (HS)
	Co-infection patients	24.914±2.308	
TNF-α	**Control**	4.655±0.271	<0.001 (HS)
	Co-infection patients	12.356±1.369	

TABLE 7. IL-32 folding expression among the control and co-infection groups

Parameter	Groups	Folding expression (Mean ±S.E)	P value
IL-32	**Control**	1.324±0.5	P = 0.001 (HS)
	Co-infection patients	7.598±1.533	

TABLE 8. IL-3 folding expression among the control and co-infection groups

Parameter	Groups	Folding expression (Mean ±S.E)	P value
IL-33	**Control**	1.214±0.317	p<0.0001
	Co-infection patients	29.089±5.785	

TABLE 9. TNF-α folding expression among the control and co-infection groups

Parameter	Groups	Folding expression (Mean ±S.E)	P value
TNF-α	Control	1.544±0.59	0.001**
	Co-infection patients	10.744±2.289	

Th2 cell differentiation and mast cell activation are biologically induced by IL-33, which results in Th2 cytokine production, Th2 response, and Th2 inflammation in the lungs and mucous membranes [20].

Because interleukins control the flow of information between distinct types of leukocytes throughout different phases of the immunological or inflammatory response, IL-33 raises the quantity of IgG in the blood of toxoplasmosis patients. This is ensured by a rise in IgG, IgA, and IgM; this return may suggest that cytokine production in toxoplasmosis patients may be influenced by autoimmune diseases such as toxoplasmosis. [21].

Numerous cell types, including cytotoxic T cells and monocytes/macrophages, generate TNFa, a cytokine with a range of functions in inflammation and immunity. It has been demonstrated by us and other researchers that following HCV infection, cultured liver cells produce TNFa. Here, we demonstrate how TNFa mediates HCV antiviral action [22].

In this investigation, individuals infected with both HCV and toxoplasmosis had considerably higher folding expression levels of IL-32, IL-33, and TNF-α.

It was discovered that the HCV core protein stimulates the production of IL-32, which has been shown to be crucial for the development of hepatic inflammation during HCV infection [23].

To maintain a replicative niche and produce a prolonged infection, T. gondii modifies the host's immune system. The regulation of host gene transcription results in an imbalance in inflammatory signalling pathways, which in turn triggers this process. This imbalance leads to cell death as well as the release of immunoregulatory cytokines, leukocyte migration, and alteration of their adherence to the endothelium [24]. Interleukin 32 (IL32), an intracellular pluripotent cytokine, is implicated with both inflammation and tumorigenesis. It is produced by monocytes, T-lymphocytes, epithelial cells, and natural killer cells. You may find the IL32 gene on chromosome 16p13.3. It comprises six splice variants (α, β, γ, δ, ε, and ξ) and eight exons. Although its precise role is still unknown, IL32 has been linked to the onset of several inflammatory illnesses, including cancer [25].

Subsequent analysis showed that, in comparison to individuals who do not express the HCV core, the genetic variation of this SNP is associated with a greater risk HCV core protein ($P < 0.01$ and P 0.001, respectively).The production of IL-32 was considerably decreased ($P < 0.001$) when PI3K was suppressed by KEGG and LY294002, a particular signalling pathway inhibitor.To sum up, the hepatic cells' PI3K pathway triggers the HCV core protein to increase the synthesis of IL-32, a crucial element in the emergence of HCV-associated severe hepatitis [26].

Interleukin-33, a new member of the IL-1 class of cytokines, is mostly generated by stromal cells, which include endothelial and epithelial cells. Pro-inflammatory stimuli cause an increase in its production [27]. It performs the dual roles of a conventional cytokine and a nuclear factor that controls transcription of genes [28].

IL-1 receptor accessory protein (IL-1RAcP) and ST2 (also known as IL1RL1) facilitate the binding of IL-33 to a heterodimeric receptor complex as an external signal [29]. Interleukin 33 induces a type 2 immune response by acting on CD4+Th2 cells, mast cells, macrophages, dendritic cells, and group 2 innate lymphoid cells (ILC2). Additionally, the cytokine can activate Treg ST2 cells, indicating that it can suppress inflammation [30]. IL-33 controls transcription, which is another way it functions inside cells. Several scientists attempted to identify the genes that IL-33 targets, including RELA and IL-6. (p65 NF-kB) [31]. IL-33 serves a variety of purposes. In actuality, IL-33 functions to model and repair tissue in the nucleus; nevertheless, extracellular secretion of IL-33 causes pro-inflammatory consequences. In these situations,

subtle epigenetic interactions involving immune genes and cascade pathways may be able to modestly modify immune activation [32]. DNA methylation and post-translational alterations to histone tails (acetylation and methylation) are examples of chromatin epigenetic modifications that can affect the expression of genes. As a result, transcriptional factors have either increased or decreased access to gene promoters and enhancers that can control inflammation [33].

Following infection with *T. gondii*, proinflammatory cytokines such as IL1, IL6, IL12, and TNF-α, together with the anti-inflammatory cytokine IL10, have been shown to play a role in the development of immunological responses [34].

Only T cells and a small number of other cell types express TNFR2, despite the fact that most other cell types express TNF receptor (TNFR) 1.When Thirteen TNFR1 recruits the TNFR1-associated death domain protein, signalling pathways for nuclear factor-kB, c-Jun N-terminal kinase, p38, and cell death are initiated. The sum of several downstream signals determines the transduction consequences of TNFR1 signal [35].

The majority of current research focuses on the relationship between the outcome of HCV infection and polymorphisms in the TNF-α promoter region at positions -308, -238. However, inconsistent findings from previous studies exist. To improve statistical power and reach a more certain conclusion on the link between the polymorphisms at TNF-α-308, -238 and the susceptibility and outcome of HCV infection, we performed a meta-analysis on TNF-α polymorphism and HCV infection. This will support the development of control and preventive measures by giving future research a clear direction and a strong foundation. The precision of the findings may be impacted by the intricacy of HCV infection and the calibre of the tiny sample used in the study [36].

Conflicts of interest

"The study's authors attest that there are no known conflicts of interest related to it.".

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Author`s contributions

"Amjad Tareq Hameed, Amani M.Jasim, Nazar Sihyaa Mohammed and Ashraf Mohamed Barakat sharing in Conceptualization, Methodology, Data curation, Writing- Original draft preparation, Supervision, Writing- Reviewing and Editing".

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التعبير القابل للطي للإنترلوكينز - IL-32 و IL-33 و TNF-α في المرضى المصابين بفيروس التهاب الكبد C وداء المقوسات

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يظل داء المقوسات مرضًا طفيليًا شائعًا حيوانيًا انتقاليا او مشتركا منتشرًا على المستوى الدولي. العديد من تكون الحيوانات ذوات الدم الحار، بما في ذلك الأغنام والبشر والانسان، معرضة للإصابة. إن الإصابة بفيروس التهاب الكبد الوبائي سي (HCV) وداء المقوسات هو مرض نادر ولكنه قد يكون خطيرا يعد التواجد المشترك لفيروس التهاب الكبد) (HCV-C) التهاب الكبد الوبائي سي) وداء المقوسات حالة غير شائعة ولكنها قد تكون خطيرة. داء المقوسات هو عدوى طفيلية تسببها الكائنات الحية الدقيقة التوكسوبلازما جوندي ، في حين أن فيروس التهاب الكبد الوبائي (HCV) هو عدوى فيروسية معروفة بقدرتها على إتلاف الكبد. لتقييم التواجد المشترك لفيروس التهاب الكبد HCV) C) وداء المقوسات. المواد والطرق: تم جمع ما مجموعه مائة عينة دم من (١٠٠) مريض مصاب بفيروس التهاب الكبد سي وداء المقوسات (٥٢ ذكراً و٤٨ أنثى) والذين تراوحت أعمارهم بين (٥ و٦٥) سنة، و(٥٠) فرداً سليماً (٢٥ ذكراً). و٢٥ أنثى) تراوحت أعمارهن بين (٥ – ٥٠) سنة كمجموعة ضابطة سيطرة. تقدير تم تقدير قيم IL-32 وIL-33 وTNF-α واكتشاف التعبير القابل للطي لعينات المريض المرضى والمجموعة الضابطة السيطرة. النتائج والاستنتاج: كشفت النتائج عن زيادة معنوية للغاية اختلافات معنوية عالية في متوسط مستويات IL-32 وIL-33 و TNF-α لدى مرضى العدوى المشتركة (١٠,١١٠ ± ٠,٥٩٦، ٢٤,٩١٤ ± ٢,٣٠٨ و١٢,٣٥٦ ± ١,٣٦٩) مقارنة مع المجموعة الضابطة السيطرة (٣,٩١٩ ± ٠,٢٢٦، ٤,٧٧٥ ± ٠,٢٤٩ و٤,٦٥٥ ± ٠,٢٧١). أظهرت النتائج أيضًا اختلافات كبيرة جدًا (P <0.001) في تعبير الطي بين IL-32 وIL-33 وTNF-α في مجموعة المريض المرضى (٧,٥٩٨±١,٥٣٣، ٢٩,٠٨٩±٥,٧٨٥ و١٠,٧٤٤±٢,٢٨٩) على التوالي عند مقارنتها مع مجموعة المرضى السيطرة. تعبيرات قابلة حيث كانت التعبيرات القابلة للطي في المجموعة الضابطة السيطرة (١,٣٢٤ ± ٠,٠٥، ١,٢١٤ ± ٠,٣١٧ و١,٥٤٤ ± ٠,٥٩) على التوالي. الاستنتاج: يمكن الاستنتاج أن مستويات IL-32 وIL-33 وTNF-α وتعبيراتها القابلة للطي تزداد بشكل ملحوظ في المرضى الذين لديهم عدوى مصاحبة مع مرض التكسوبلازما (فيروس التهاب الكبد الوبائي وداء المقوسات)

الكلمات الدالة: فيروس التهاب الكبد C، داء المقوسات، الإنترلوكينز , تعبير القابل الطي.