الآيـــــة

بسم الله الرحمن الرحيم

قال تعالى: {وَوَصَّيْنَا الْإِنسَانَ بِوَالِدَيْهِ إِحْسَانًا ۖ حَمَلَتْهُ أُمُّهُ كُرْهًا وَوَضَعَتْهُ كُرْهًا ۖ وَحَمْلُهُ وَفِصَالُهُ ثَلَاثُونَ شَهْرًا ۚ حَتَّىٰ إِذَا بَلَغَ أَشُدَّهُ وَبَلَغَ أَرْبَعِينَ سَنَةً قَالَ رَبِّ أَوْزِعْنِي أَنْ أَشْكُرَ نِعْمَتَكَ الَّتِي أَنْعَمْتَ عَلَيَّ وَعَلَىٰ وَالِدَيَّ وَأَنْ أَعْمَلَ صَالِحًا تَرْضَاهُ وَأَصْلِحْ لِي فِي ذُرِّيَّتِي ۖ إِنِّي تُبْتُ إِلَيْكَ وَإِنِّي مِنَ الْمُسْلِمِينَ} .

صدق الله العظيم

(الأحقاف 15)

DEDICATION

To my Mothers...

The love of our live...

To my Fathers...

The candles lightening our road ...

To my Teachers...

And to everyone that support and encourage us during our education and through our life...

ACKNOWLEDGMENTS

Piously my gratitude and prayers to **ALMIGHTY ALLAH** for the mercy that followed me during the long way of this research and granted me ability to accomplish this work . I would like to express my special thanks and my sincere gratefulness to **Dr Hind Haider Ahmed** for her immense effort not only to accomplish this work but also to inculcate the researcher's soul on me . Special thank to my colleagues **Reem ZainAlabidin and Wafaa Alabed Talha** for their efforts and boundless support . Then I would like to thank my parents and friends who have helped me with their valuable suggestions and guidance has been helpful in various phases of the completion of the project

ABASTRACT

One of the most important factor playing in hepatitis B pathogenesis is cytokine release and one of the cytokine with antiviral characteristic is interferon-γ (IFN-γ) . The aim of the present study was to estimate (IFN-γ) levels among Sudanese patients infected with hepatitis B virus compared to healthy control group in Khartoum state during period from March to December 2019.

Three milliliter of Venous blood sample were collected in EDTA container from each subjects and separated using centrifuge to obtain plasma. (IFN-γ) concentration was measured by Enzyme Linked Immunosorbent Assay (ELISA) in Ibn Sina University laboratory. The data was analyzed using statistical package of social science programme (Version 20) .students T test and one way ANOVA test were used to compare between means. *P.value* significant when ≤0.05.

Sixty subjects, 30 case and 30 healthy control with age and sex matched were selected for this study their age varies from 12-50 years (28.63± 7.91 years) .

The result of this study showed that the mean level of (IFN-γ) was statistical significantly decreased in hepatitis B virus patients (5.73±1.67pg/ml) when compared to control group (11.35±6.33pg/ml) with *p.value* 0.00.

The mean level was higher in females (6.18±1.87pg/ml) than in males (5.27±1.35pg/ml) , which is statistically insignificant *p.value* 0.138.

Also there was no statistically difference between (IFN-γ) level in acute and chronic hepatitis B , mean were (6.13±1.9 and 5.32±1.26 respectively) patients *P. value* 0.193.

Regarded to treatment showed that insignificantly decreased in (IFN-γ) level between patients using treatment compared to patients do not using treatment with mean (5.36±1.21 and 6.28±2.13 respectively) *p. value* 0.195.

According to jaundice : there was no significant difference in (IFN-γ) level between patients with symptoms of jaundice (5.74±0.82 pg/ml) and patients have no symptoms of jaundice (5.72±1.78pg/ml) *p. value* 0.982.

The mean of (IFN-γ) in patients who didn't receive blood transfusion (5.86±1.61pg/ml) was higher than patients who received blood transfusion (3.86±1.91pg/ml) mean with insignificant statistical correlation with *p.value* 0.104

One way ANOVA test showed significant association between age group and IFN-γ *P.value* 0.00 (Table 4-4) .There was insignificant correlation between IFN-γ and age in hepatitis B virus group (*P. Value* 0.590, r=0.060) .

This study concluded that (IFN-γ) concentration was lower in Hepatiti B patient in comparison to healthy individuals .Its recommended to study gene polymorphism that may be associated with lower level of (IFN-γ) among Hepatitis B patient individuals .

مستخلص البحث

احد اهم العوامل التي تلعب دورا في التهاب الكبد الوبائي الفيروسي "ب" هي افراز السايتوكينات واحد هذه السايتوكينات والذي له خاصية مضادة للفيروسات هو "الانترفيرون قاما" . الهدف من هذه الدراسة كان تقييم مستوى الانترفيرون قاما لدى المرضى السودانيين المصابين بالتهاب الكبد" ب" الفيروسي مقارنة مع الأفراد الطبيعيين بولاية الخرطوم في الفترة من مارس حتى ديسمبر2019.

سحبت 3 مللتر عينة دم وريدية من كل مشارك في أنبوبة تحتوي على EDTA و فصلت باستخدام الطرد المركزي و حصل على البلازما. قيس تركيز الانترفيرون قاما عن طريق فحص الممتز المناعي المرتبط بالإنزيم . في معمل جامعة ابن سينا . حللت البيانات باستخدام الحزمة الإحصائية للمجتمع (نسخة 20). استخدم أنوفا (ANOVA) و اختبار T لمقارنة الأوساط و كانت القيمة المطلقة متوافقة عند اقل من 0.05 .000.

تم اختيار ستين شخصا و 30 حالة و 30 سيطرة صحية مع العمر والجنس المتطابقة عشوائيا لهذه الدراسة تتراوح أعمارهم بين 12-50 سنة (7.91 ±28.63 سنة).

أظهرت نتائج هذه الدراسة أن المستوى المتوسط لـ IFN-γ كان إحصائيا انخفض بشكل ملحوظ في مرضى فيروس التهاب الكبد B (1.67pg ± 5.73 / مل) بالمقارنة مع مجموعة التحكم (11.35 ± 6.33 pg / ml) مع قيمة احصائية 0.00.

كان المستوى المتوسط أعلى عند الإناث (6.18 ± 1.87 pg / ml) منه في الذكور (5.27 ± 1.35 pg / ml) ، وهو غير ذي قيمة إحصائية 0.138.

أيضا لم يكن هناك فرق إحصائي بين (IFN-level) المستوى في مرضى التهاب الكبد B الحاد والمزمن (1.9±6.13 و 1.26±5.32 على التوالي) مع قيمة احصائية 0.193.

فيما يتعلق بالعلاج ، أظهرت انخفاض غير ملحوظ في مستوى IFN-γ بين المرضى الذين يستخدمون العلاج مقارنة بالمرضى الذين لا يستخدمون العلاج (2.13±6.28 و 1.21±5.36 على التوالي)مع قيمة احصائية 0.195.

وفقًا لليرقان: لم يكن هناك اختلاف كبير في مستوى IFN-γ بين المرضى الذين يعانون من أعراض اليرقان(0.82±5.74 pg/ml) والذين ليس لديهم أعراض لليرقان (1.78±5.72pg/ml) مع قيمة احصائية 0.982.

كان متوسط IFN-γ في المرضى الذين لم يتلقوا نقل الدم (1.61±5.86pg/ml) أعلى من المرضى الذين تلقوا نقل الدم (1.91±3.86pg/ml) مع عدم وجود علاقة إحصائية (0.104).

أظهر اختبار ANOVA بطريقة واحدة وجود علاقة معنوية بين الفئة العمرية ومعدل IFN-γ (القيمة الاحتمالية 0.00) وكان هناك ترابط ضئيل بين مستوى IFN-γ والعمر في مجموعة مرضى التهاب الكبد الوبائي(r=0. 060 , وقيمة احتمالية 0.590.

وخلصت هذه الدراسة إلى أن تركيز الانترفيرون قاما يقل في مرضى الكبد الوبائي بالمقارنة مع الافراد الطبيعين ، من الموصى به القيام بدراسة تعدد الاشكال الجينية التي قد تترافق مع انخفاض مستوى الانترفيرون قاما لدى الاشخاص المصابين بمرض الكبد الوبائي .

List of Contents

List of Table

List of Figure

List of Abbreviations

ALT	Alanine transaminase
APCs	Antigen presenting cell
cccDNA	Convently closed circular Deoxyribo nucleic acid
CD4	Cluster differentiation 4
CD8	Cluster differentiation 8
CEIA	Chemiluminesence immunoassay based enzyme reactivity
CTL	Cytotoxic T lymphocyte
DNA	Deoxyribo nucleic acid
EIA	Impact assessment environmental
ELISA	Enzyme Linked Immunosorbant Assay
ER	Endoplasmic reticulum
HBcAg	Hepatitis core antigen
HBeAg	Hepatitis envelope antigen
HBsAg	Hepatitis surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human immunodefficiency virus
IFN-α	Interferon alpha
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IL	Interleukin
Kb	kilobase

MHC	Major histocompatibility complex
NKC	Natural killer cell
PCR	Polymerase chain reaction
Pg RNA	Pregenomic Ribo nucleic acid
RC-DNA	Relaxed circular DNA
RNA	Ribo nucleic acid
RT	Reverse transcriptase
SGPT	Serum glutamic-pyruvic transaminase
SNP	Single nucleotide polymorphism
SPSS	Statistical Package of Social Sciences
Th1	T helper 1
Th2	T helper 2
TNF	Tumer necrosis factor
TMB	Tetramethylbenzidine

CHAPTER ONE

INTRODUCTION

1.Introduction

Hepatitis B virus (HBV), a DNA virus of the family Hepadnaviridae is the causative agent of hepatitis B infection. Hepatitis B is one of the most common infectious diseases in the world and a major health problem. According to the most recent World Health Organization estimate (WHO), 2 billion people worldwide have serologic evidence of past or present HBV infection, and 350 million are chronically infected and at risk for HBV-related liver disease. (Amniu and MD,2014)

Infection with hepatitis B virus (HBV) may lead to acute or chronic hepatitis. HBV infections were previously much more frequent but there are still 240 million chronic HBV carriers today and ca. 620,000 die per year from the late sequelae liver cirrhosis or hepatocellular carcinoma. Hepatitis B virus (HBV) has infected more than 2000 million persons alive today and 350 million persons are chronically infected carriers of the virus, at high risk of death from active hepatitis, cirrhosis and primary hepatocellular cancer. Each year approximately 1 million people die from the acute and chronic sequelae of HBV infection, making it one of the major causes of morbidity and mortality in man (WHO,2017).

Hepatitis B virus is a blood borne and sexually transmitted pathogen that is spread through contaminated blood or other body fluids (saliva, sweat, semen, vaginal secretions, breast milk, urine, and feaces) (Chang,2007)

There are approximately 400 million HBV carriers in the world complications such as fulminant hepatic failure, cirrhosis and hepatocellular carcinoma develop annually in 250 000 of them. While the majority of adult infections remit, chronicity develops in 5−10% of cases .Adaptive immune response, which develops against HBV (a hepatotropic non-cytopathic virus) plays a key role in infection control. After the virus is taken into the livepr cells infection ensues and host immune response is initiated which result in cytokines secretion and designation of immune response according to them (Ozguler et al., 2014).

Interferon originally named after their interfering effect on viral replication . Depending on the cellular receptor, three types of IFNs have been identified: types I, II, and III (IFN-α, IFN-βand IFN-γ). (IFN-γ) is a pleiotropic cytokine that modulates both innate and adaptive immune networks; it is the most potent activator of macrophages and a signature cytokine of activated T lymphocytes. Interferon-gamma (IFN-γ)was first discovered as a soluble macromolecule with antiviral activity Though (IFN-γ) is now appreciated to have a multitude of roles in immune modulation and broad-spectrum pathogen defense, it was originally discovered, and named, as a secretory factor that interferes with viral replication. (Kang *et al.*,2018).

IFN-gamma (IFN-γ) is a key players in driving cellular immunity it can exhibit its immunomodulatory effect by antigen processing and presentation, increasing leucocyte trafficking , inducing an anti -viral state ,boosting the anti – microbial function and effecting cellular proliferation and apoptosis . the main producer of (IFN-γ) include T-cell , natural killer cell (NK cell) and antigen presenting cells (APCs) has been implicated in early host defense and autocrine regulation .Tcells are the major source of (IFN-γ)during adaptive iummunity (Kak *et al.*,2018)

1.2 Rationale

Hepatitis B virus is considered as one of the most common viruses which can transmitted percutaneously, sexually, and perinatally, affects 1.25million persons in the United States and 350 to 400 million persons worldwide. HBV infection accounts annually for 4000 to 5500 deaths in the United States and1 million deaths worldwide from cirrhosis, liver failure, and hepatocellular carcinoma. (Dienstag, 2015).

Hepatitis B virus is 50 to 100 times more infectious than HIV and 10 times more infectious than hepatitis C virus (HCV) with many carriers not realizing they are infected with the virus. It is an important cause of liver diseases such that chronic infection with HBV is a common cause of death associated with liver failure, cirrhosis and liver cancer (Aminu and MD,2014). Because of the asymptomatic nature of chronic hepatitis B, most people infected with HBV are not aware that they have been infected until they have symptoms of cirrhosis or a type of liver cancer ,hepatocellular carcinoma (Colvin and Mitchell,2010).

Interferon-gamma (IFN-γ) is among the principal mediators inducing a host resistance against HBV and the clearance of this virus from hepatocytes , IFN-γ as a pro-inflammatory Th1 cytokine has a vital role in the antiviral activity, and genetic variation of individuals affects immunity in response to a pathogen (Naghizadeh *et al*.,2018) Results of several studies suggest possible role of interferon gamma (IFN-γ) in the modulation of immune persistent infection progression of the infection from acute into long term established ,therefore the aim of this study was to estimate the interferon gamma (IFN-γ) levels among hepatitis B infected patients .

1.3 Objectives:

1.3.1 General Objectives

Estimation of Interferon Gamma (IFN-γ) Level among Sudanese Hepatitis B Patients in Khartoum State.

1.3.2 Specific Objectives

1- To estimate plasma Interferon gamma (IFN-γ) levels among hepatitis B infected patient using ELISA technique. .

2- To compare plasma Interferon gamma (IFN-γ) levels in hepatitis B patients and healthy controls using ELISA technique e .

3- To correlate Interferon gamma (IFN-γ) level with possible risk factors (age ,gender , treatment , blood transfusion , jaundice and duration of disease).

CHAPTER TWO

LITERATURE REVIEW

2.1.Background

Hepatitis B Virus (HBV) infection is a global health problem: an estimated two billion people (one-third of the global population) have been infected with HBV at some point in their life; of these, more than 350 million suffer from chronic HBV infection, resulting in over 600,000 deaths each year, mainly from cirrhosis or liver cancer (Jayalakchmi *et al*.,2013).

Hepatitis refer to an inflammation of the liver cell and damage to the liver ,the liver function include detoxifying the blood ,storing vitamin and producing hormone ,hepatitis disrupt these process (Felman *et al.*,2017) .

HBV infection was first identified in 1965 when Blumberg and co-workers found the hepatitis B surface antigen (HBsAg), originally termed as Australia antigen. Enhanced viral replication leading to a vigorous and extensive immune response may lead to massive liver injury resulting spontaneously into fulminant hepatic failure. The seriousness of disease incidence is mainly related to various host factors (age, gender, duration of infection, immune response and viral factors as viral load, genotype, quasispecies) (Jayalakchmi *et al.*,2013).

viral hepatitis is caused by infection with five distinctly different human hepatitis viruses A, B, C, D and E, which cannot be distinguished from one another without serologic testing, other type of hepatitis can caused by over consumption of alcohol or an autoimmune condition (Felman *et al.*,2017).

2.2 Hepatitis B Virus biology

Human hepatitis B virus (HBV) is the prototype of a family of small DNA viruses that productively infect hepatocytes and, for the most part, non-cytopathic. Although *hepadnaviruses* have a strong preference for infecting liver cells, but small amounts of hepadnaviral DNA can be found in the bile duct epithelium of the liver, rare exocrine cells and alpha and beta islets of the pancreas, proximal tubular epithelium of the kidney, and possibly a subset of splenic cells. Infection of the liver may be either transient (<6 months) or chronic and life long, depending on the ability of the host immune response to clear the infection. Chronic infections can cause immune mediated liver damage progressing to cirrhosis and hepatocellular carcinoma (HCC). The mechanisms of carcinogenesis are unclear. (Seeger and Mason ,2016).

The incubation period for acute hepatitis B ranges from 45 to 180 days (average 120 days). The clinical manifestations of acute HBV infection are age dependent. Infants, young children (younger than 10 years of age), and immunosuppressed adults with newly acquired HBV infection are usually asymptomatic. older children and adults are symptomatic in 30%–50% of infections. When present, clinical symptoms and signs might include anorexia, malaise, nausea, vomiting, abdominal pain, jaundice, dark urine, and clay-colored or light stools. Occasionally, extrahepatic manifestations occur and include skin rashes, arthralgia, and arthritis. Among adults with normal immune status, most (94%–98%) recover completely from newly acquired HBV infections, eliminating the virus from the blood and producing neutralizing antibodies that confer immunity from future infection. In infants, young children, and immunosuppressed persons, most newly acquired HBV infections result in chronic infection.Infants are at greatest risk, with a 90% chance of developing chronic infection if infected at birth. Although the consequences of acute hepatitis B can be severe, most of the serious sequelae occur in persons in whom chronic infection develops. Chronic liver disease develops in two-thirds of these persons, and approximately 15%–25% die prematurely from cirrhosis or liver cancer. Persons with chronic HBV infection are often detected in screening programs, such as those for blood donors, pregnant women, and refugees.Persons with chronic HBV infection are a major reservoir for transmission of HBV infections. Any person testing positive for hepatitis B surface antigen (HBsAg) is potentially infectious to both household and sexual contacts. (Winston..et al,2014).

2.3 Transmission

Although the highest concentrations of virus are found in blood, other body fluids, such as semen and saliva, have also been demonstrated to contain HBV. it is predominantly a blood and sexually transmitted infection and is transmitted by percutaneous and mucosal exposure to infectious body fluids (Winston *et al.*,2014).

HBV is transmitted through exposure to infected blood and body fluids (particularly semen and vaginal secretions). HBV survives for prolonged periods outside the body . Although HBV has been detected in saliva, tears, breast milk, sweat, and urine, there minimal evidence of transmission through exposure to these fluids where no blood is present, and breastfeeding has not been shown to increase risk of infection Most infections worldwide are acquired through perinatal transmission at birth, through horizontal transmission to/between young children, through sexual contact, and through injecting drug use (McLachlan and Cowie,2015)

Heterosexual-and especially homosexual-activity is an important rout of transmission , HBsAg prevalence estimate range from<1% to 4% in men who have sex with men in 3 of the 34 countries (Hahne *et al* ., 2013). Prisoner are at high risk of HBV infection due to social and environmental risk factors , in addition to specific features of the prison environment (i.e. restricted space and overcrowding) . In prison , prevention programmes for vaccination against HBV could be both a challenge and an opportunity; many people who do not otherwise have access to health care can be reached and followed for a certain period , although the environment itself presents an increase risk for HBV transmission (Stasi *et al* .,2016).

The hepatitis B virus can survive outside the body for at least 7 days. during this time, the virus can still cause infection if it enters the body of a person who is not protected by the vaccine. The incubation period of the hepatitis B virus is 75 days on average, but can vary from 30 to 180 days. The virus may be detected within 30 to 60 days after infection and can persist and develop into chronic hepatitis B (WHO,2019).

2.4 Classification and Structure

The hepatitis B virus (HBV) is a small DNA virus with unusual features similar to retroviruses.

It is a prototype virus of the *Hepadnaviridae* family. Related viruses are found in woodchucks, ground squirrels, tree squirrels, Peking ducks, and herons. Based on sequence comparison, HBV is classified into eight genotypes, A to H. Each genotype has a distinct geographic distribution. Three types of viral particles are visualized in infectious serum by electron microscopy. Two of the viral particles are smaller spherical structures with a diameter of 20 nm and filaments of variable lengths with a width of 22 nm . The spheres and filaments are composed of hepatitis B surface antigen (HBsAg) and host-derived lipids without viral nucleic acids and are therefore noninfectious. The infectious HBV virion (Dane particle) has a spherical, double-shelled structure 42 nm in diameter, consisting of a lipid envelope containing HBsAg that surrounds an inner nucleocapsid composed of hepatitis B core antigen (HBcAg) complexed with virally encoded polymerase and the viral DNA genome. The genome of HBV is a partially double-stranded circular DNA of about 3.2 kilobase (kb) pairs. (Liang,2010). The viral genome consists of a partially double-stranded, relaxed-circular DNA (RC-DNA), comprising a complete coding strand (negative strand) and an incomplete non-coding strand (positive strand), which replicates by reverse transcription *via* an RNA intermediate. Due to reduced fidelity of the reverse

transcription process, this pregenomic RNA (pgRNA) is prone to mutation (Jayalakchmi *et al*.,2013).

2.5 Antigenic properties

The HBV genome encodes four overlapping reading frames that are translated to make the viral core protein (HBcAg), the surface proteins (HBsAg) , a reverse transcriptase (RT), and the hepatitis B "x" antigen (HBxAg) (Jayalakchmi *et al*.,2013).

.Hepatitis B virus surface antigen (HBsAg) it has been used as the hallmark for the diagnosis of HBV infection . HBsAg is the prototype serological marker of HBV infection that characteristically appears after 1 to 10 weeks of an acute exposure to HBV but before the onset of visible symptoms .in addition to playing a major role in cell membrane attachment to initiate the infection process by binding to the hepatocyte plasma membrane , Persistence of HBsAg for more than 6 months indicates chronic infection . The immune response enhancing ability of HBsAg is not clear yet it is understood that large amounts of HBsAg may induce T cell anergy, leading to decreased antibody-mediated neutralization of HBV and generalized hyporesponsiveness towards pathogens (Jayalakchmi *et al.*,2013).

The c antigen (HBcAg) is present on the surface of core particles, HBcAg and core particles are not present in the blood in a free form , but are found only as internal components of virus particles. The core antigen shares it sequences with the e antigen (HBeAg), identified as soluble antigen but no cross reactivity between the two proteins are observed (Robinson, 1999).

2.6 Replication

The life cycle of the HBV is complex. Hepatitis B is one of the few known non-retroviral viruses which used reverse transcription as a part of its replication process. The virus gain entry into the cell by binding to an unknown receptor on the surface of the hepatocytes and enter it by endocytosis. Because virus multiplies via RNA made by a host enzyme, the viral genomic DNA has to be transformed to the cell nucleus by host protein called chaperones. The partially double stranded viral DNA is then made fully double stranded and transform in covalently closed circular DNA (cccDNA) that serves as template, for transcription of four viral mRNAs. (Levinson, 2006).

These transcripts are polyadenylated and transported to the cytoplasm, where they are translated into the viral nucleocapsid and pre-core antigen (C, pre-C), polymerase (P), envelope L (large), M (medium), S (small) and transcriptional transactivating proteins (X) .The envelope proteins

insert themselves as integral membrane proteins into the lipid membrane of the endoplasmic reticulum (ER), the 3.5 kb species ,spanning the entire genome and termed pre-genomic RNA (pgRNA), is packaged together with HBV polymerase and a protein kinase into core particles where it serves as a template of reverse transcription of negative strand DNA (Ganem and Schneider, 2001). These four viral transcripts undergo additional processing and go on to form progeny virions which are released from the cell or returned to the nucleus and recycled to produce even more copies. The long mRNA is then transported back to the cytoplasm where the virion p protein synthesized DNA via its reverse transcriptase activity (Levinson, 2006).

2.7 Immunopathology

Most people develop acute hepatitis B virus (HBV)-related hepatitis that is controlled by both humoral and cellular immune responses following acute infection. However, a number of individuals in HBV-endemic areas fail to resolve the infection and consequently become chronic carriers (Sandhu et al .,2017).

Kupffer cells, tissue resident macrophages within the sinusoidal compartment of liver, act as first line of defense against pathogens invading the liver (Sandhu et al .,2017) . After entering the blood, the virus infects hepatocytes, and viral antigens are displayed on the surface of the cells. Cytotoxic T cells mediate an immune attack against the viral antigens, and inflammation and necrosis occur. Immune attack against viral antigens on infected hepatocytes is mediated by cytotoxic T cells. The pathogenesis of hepatitis B is probably the result of this cell-mediated immune injury, because HBV itself does not cause a cytopathic effect. Antigen−antibody complexes cause some of the early symptoms (e.g., arthralgias, arthritis, and urticaria) and some of the complications in chronic hepatitis (e.g., glomerulonephritis, cryoglobulinemia, and vasculitis) (Levinson,2014).

Innate immunity generally plays a role immediately after infection to limit the spread of the pathogen and initiate efficient development of an adaptive immune response. Innate host responses during the early phases of viral infections are mainly characterized by the production of type 1 interferon (IFN)- α/β cytokines and the activation of natural killer (NK) cells (Bertoletti and Gehring ,2006). HBV is called a “stealth” virus as it effectively evades the innate immune response, which leads to undetected infection for months (Sandhu *et al* .,2017)..

Thus, most viral DNA (at least 90% in the chimpanzee model) is cleared without the destruction of liver cells by non-cytolytic mechanisms likely triggered by IFN- γ and TNF- α primarily

produced by non-T cells. Indeed, macrophages, NK cells and NK-T cells represent the main cell populations in the infected liver before the onset of liver disease (Ferrari *et al.*,2003). NK cells have been suggested to have a role in acute HBV although there is conflicting evidence about their contribution depending on the stage of pathogenesis of the patients (Schuch *et al*.,2014).

In acute resolving infections, the response of the innate and adaptive immune system to HBV is efficient and timely. Viral clearance involves the induction of a robust adaptive T cell reaction inducing both a cytolytic dependent and independent antiviral effect via the expression of antiviral cytokines, as well as the induction of B cells producing neutralizing antibodies preventing the spread of the virus , When the acute infection becomes chronic, there is a progressive impairment in HBV specific T cell function (EASL ,2017). Cell-mediated immunity is critical for clearance of HBV infection from hepatocytes. CD8+ and CD4+ T cells have been shown to be indispensable for resolution of HBV infection in infected chimpanzees .Interestingly, the mechanism for this clearance was non-cytolytic for hepatocytes and occurred through interferon-α and TNF-α provided by CD8+ and CD4+ cells when infected with high viral inoculum(Sandhu *et al* .,2017)

Chronic HBV infection is characterized by the persistence of HBsAg, HBeAg, and HBV DNA for more than 6 months (Gerlich,2013) Chronic HBV carriers can remain asymptomatic but a small percentage of patients can undergo viral re-activation and develop hepatocellular carcinoma (HCC) (Dienstag ,2008).

The clearance of infected virus during acute and probably also in chronic HBV infection is due to the body's adaptive immune response both CD4 T cells (T helper cells) and CD8 T (cytotoxic T-lymphocyte [CTL]) cells is activated , which usually prompts the death of infected hepatocytes leading to hepatic injury and damage. Serum glutamic-pyruvic transaminase (SGPT), known also as alanine transaminase (ALT), is one of the markers for diagnosing liver damage. Dead hepatocytes release ALT, increasing its level in the blood(Lu ,2011).

2.8 Hepatitis B infection

The hepatitis B virus (HBV) causes acute and chronic liver disease and is endemic in many areas of the world. The virus is transmitted through contact with blood or other body fluids from an infected person . Every year, there are over 4 million acute clinical cases of HBV and 250 million are chronically infected and are at risk of developing HBV-related liver disease 15–40%

of chronically infected patients will develop cirrhosis, progressing to liver failure and/or HCC during their lifetime (Feld and Janssen ,2015).

2.8.1 Acute Hepatitis B virus infection

About two-thirds of patients with acute HBV infection have a mild, asymptomatic and subclinical illness that usually goes undetected. Approximately one-third of adults with acute HBV infection develop clinical symptoms and signs of hepatitis, which range fom mild constitutional symptoms of fatigue and nausea, to more marked symptoms and jaundice, and rarely to acute liver failure. The clinical incubation period of acute hepatitis B averages 2–3 months and can range from 1–6 months after exposure, the length of the incubation period correlating, to some extent, with the level of virus exposure. The incubation period is followed by a short preicteric or prodromal period of constitutional symptoms such as fever, fatigue, anorexia, nausea, and body aches. During this phase, serum ALT levels increase of 3- 10-fold to to striking increase of >100- fold and high levels of HBsAg and HBV DNA are detectable. The preicteric phase lasts a few days to as long as a week and is followed by onset of jaundice or dark urine (Liang ,2010). The icteric phase of acute viral hepatitis begins usually within 10 days of the initial symptoms with the appearance of dark urine followed by pale stools and yellowish discoloration of the mucous membranes ,conjunctivae, sclerae, and skin. Jaundice becomes apparent clinically when the total bilirubin level exceeds 20 to 40 mg/l. It is accompanied by hepatomegaly and splenomegaly. About 4-12 weeks thereafter, the jaundice disappears and the illness resolves with the development of natural, protective antibodies (anti-HBs), in about 95% of adults(WHO ,2002).

In the acute stage there are signs of inflammation in the portal tracts; the infiltrate is mainly lymphocytic. In the liver parenchyma, infected hepatocytes show ballooning and form acidophilic (Councilman) bodies as they die (Aryal ,2019).

The diagnosis of acute hepatitis B is based on the detection of HBsAg and anti-HBc (immunoglobulin M). During the initial phase of infection, markers of HBV replication—HBeAg and HBV DNA—are also present. Recovery is accompanied by the disappearance of detectable HBV DNA, HBeAg seroconversion to anti-HBe, and subsequent clearance of HBsAg with seroconversion to anti-HBs and appearance of anti-HBc (IgG) (Feld and Janssen ,2015).

2.8.2. Chronic hepatitis B virus infection

In chronic hepatitis, damage extends out from the portal tracts, giving a piecemeal necrosis appearance. Some lobular inflammation is also seen. As the disease progresses, fibrosis and, eventually, cirrhosis develops. Chronic liver damage results from continuing, immune-mediated destruction of hepatocytes expressing viral antigens. In addition, autoimmune reactions may contribute to the damage as immune responses are induced to various liver-specific antigens(Aryal , 2019).

The diagnosis of chronic infection is based on the persistence of HBsAg for more than 6 months. Patients with chronic HBV infection are commonly diagnosed by laboratory means but not by clinical presentations. Past HBV infection is defined by the coexistence of anti-HBs and IgG anti-HBc (song and kim ,2016).

CHB is a dynamic disease that fluctuates over time, likely relating to interactions between the virus and the host immune system. The following five—not necessarily sequential—phases can be identified in chronic HBV infection.

2.8.2.1. Immune-tolerant phase

Characterized by high levels of serum HBV DNA, HBeAg positivity, normal alanine aminotransferase (ALT) levels, and absent liver necroinflammation. — Disease progression is minimal in patients who remain in this phase (Feld and Janssen ,2015). .

2.8.2.2. Immune-reactive phase(HBeAg-positive CHB)

The immune system becomes more active and the infected hepatocytes are attacked. Characterized by highly fluctuating, but progressively decreasing, HBV-DNA levels, elevated ALT, and hepatic necroinflammation (HBeAg-positive CHB). A prolonged immune-active phase with multiple ALT flares may result in progressive liver fibrosis, leading to cirrhosis (Feld and Janssen ,2015)..

2.8.2.3. Immune-control phase

(and inactive carrier state): Transition into this phase as an outcome of the immune-active phase is marked by seroconversion from HBeAg to anti-HBe positivity. Characterized by low (< 2000 IU/mL) or undetectable serum HBV DNA, normal ALT levels, and disappearance of liver necroinflammation (inactive carrier state) (Feld and Janssen ,2015).

2.8.2.4. Reactivation phase (HBeAg-negative CHB)

Despite HBe seroconversion, reactivation of HBV replication may occur due to the selection of HBeAg-defective HBV mutants. Characterized by positive anti-HBe antibody levels, fluctuating HBV DNA and ALT levels, and a high risk of progression to severe hepatic fibrosis (HBeAg-negative CHB) (Feld and Janssen ,2015).

2.8.2.5. HBsAg-negative phase

After HBsAg loss, low-level HBV replication HBV DNA in the liver and rarely in the serum (Feld and Janssen ,2015).

HBcAb is present in those with acute infection and chronic infection, as well as in those who have recovered from acute infection Therefore, it cannot be used to distinguish between acute and chronic infection (Levinson ,2014).

2.9 Occult hepatitis B infection :

Occult hepatitis B is defined by the presence of hepatitis B virus DNA in serum or liver in the absence of HBs Ag. Serum HBV level usually less than 104 copies/ml . Although occult HBV infection has identified in patient with chronic liver disease two decades ago. Occult HBV infection has been found in patient with hepatocellular carcinoma (HCC), past HBV infection, or chronic hepatitis C virus, and individual without hepatitis B serological markers, the frequency of the diagnosis depends on the relative sensitivity of HBV DNA assay and the prevalence of HBV infection in the population. Collectively around 30% to 35% of HBs Ag negative. Subject with chronic hepatitis with or without HCC have positive serum HBV DNA (range from 5% to 55%) (Mumtaz *et al.*, 2011).

2.10 Laboratory diagnosis of hepatitis B virus infection

The diagnosis of HBV infection and its associated disease is based on a constellation of clinical, biochemical, histological, and serologic findings. The laboratory can test for a wide range of HBV antigens and antibodies Focuses on the detection of the hepatitis B surface antigen HBsAg, using immnoassays based on enzyme reactivity (EIA) or chemiluminesence (CLIA) and ELISA. HBV DNA can be quantified in serum or plasma using real time polymerase chain reaction (PCR) assays (Aryal ,2019).

Acute HBV infection is characterized by the presence of HBsAg and immunoglobulin M (IgM) antibody to the core antigen, HBcAg. During the initial phase of infection, patients are also seropositive for hepatitis B e antigen (HBeAg). HBeAg is usually a marker of high levels of

replication of the virus. The presence of HBeAg indicates that the blood and body fluids of the infected individual are highly contagious (Aryal ,2019).

Chronic infection is characterized by the persistence of HBsAg for at least 6 months (with or without concurrent HBeAg). Persistence of HBsAg is the principal marker of risk for developing chronic liver disease and liver cancer (hepatocellular carcinoma) later in life (Aryal ,2019).

HBV DNA is a directed measurement of the viral load , which reveals the replication activity of virus . It is detectable at the early stage of infection (1 month after HBV infection) and increases up to peak level (more than 108 copies/ml) approximately 3 months after the exposure to HBV and then gradually diminishes in chronic infection Or disappears at the recovery from HBV infection . (Eun and Young , 2016). As the prevalence of serologically negative HBV infection (HBeAg-negative CHB and occult HBV infection) has increased , HBV-DNA detection has obtained more a wareness in clinical medicine (Datta *et al* ., 2014). There are two principles of techniques to identify and quantify HBV DNA: singal amplification such as hybrid capture and branced DNA technology ; target amplification such as polymerase chain reaction (PCR) (Caliendo *et al* ., 2011). Real-time PCR can detect wide dynamic range of viral load (lower range , 10-15 IU/mL; upper range, 107-108 IU/mL). For this reason , it has come to be the standard method to detect and quantify HBV DNA in clinical setting . Furthermore , it can be fully automated and dose not generate carry-over contamination (Eun and Young 2016).

2.11 Cytokines

Cytokines are a cell-signaling group of low molecular weight extracellular polypeptides/ glycoproteins synthesized by different immune cells, mainly, by T cells, neutrophils and macrophages, which are responsible to promote and regulate immune response (i.e. activity, differentiation, proliferation and production of cells and other cytokines). These polypeptides act on signaling molecules and cells, stimulating them toward sites of inflammation, infections, traumas, acting on primary lymphocyte growth factors and other biological functions. Cytokines may act in the site where they are produced (autocrine action), in nearby cells (paracrine action) or in distant cells (endocrine action). In this sense, they are important in the development and regulation of immune system cells. Different types of cytokines had been discovered, including chemokines, interferons (IFN), interleukins (IL), lymphokines and tumor necrosis factor (TNF) (Ferreira *et al*,2018).

2.12 Interferon gamma (IFN-γ)

Interferons were originally named after their interfering effect on viral replication . Depending on the cellular receptor, three types of IFNs have been identified: types I, II, and III . Virtually all cells have the receptor for prototypical type I IFNs (e.g., IFN-α, IFN-β) and can produce them upon detection of viral or microbial invasion. Type II IFN receptors are also ubiquitously expressed, though only the aforementioned limited subsets of immune cells produce (IFN-γ).type III IFN receptors are only expressed in certain types of cells, such as mucosal epithelial cells, but most cells can produce type III IFNs (e.g., IFN-lambda) (Kang *et al.*,2018).

IFN-γ is produced predominantly by natural killer (NK) and natural killer T (NKT) cells as part of the innate immune response, and by Th1 CD4 and CD8 cytotoxic T lymphocyte (CTL) effector T cells once antigen-specific immunity develops (Schoenborn and Wilson ,2007)

IFN-γ has since been characterized as a homo-dimeric glycoprotein with pleiotropic immunologic functions IFN-γ can promote macrophage activation, mediate antiviral and antibacterial immunity, enhance antigen presentation, orchestrate activation of the innate immune system, coordinate lymphocyte-endothelium interaction, regulate Th1/Th2 balance, and control cellular proliferation and apoptosis (Tau and Rothman ,2014). Interferon-gamma (IFN-gamma) is crucial for immunity against intracellular pathogens and for tumor control. However, aberrant IFN-gamma expression has been associated with a number of autoinflammatory and autoimmune diseases (Schoenborn and Wilson ,2007).

Macrophages represent a class of APCs which are versatile sentinels of the immune system. A pathogen is most likely to encounter a macrophage soon after its entry into the host and IFN-γ -stimulated macrophages show enhanced anti-microbial activity. Thus, IFN-γ activates macrophages and makes them better able to mount an effective immune response, such as enhanced antigen processing and presentation through upregulation of class II MHC, increased ROS and NOS production, induction of autophagy for clearance of intracellular pathogens and increased secretion of pro-inflammatory cytokines. Additionally, it can activate NK cells, increase their tumoricidal activity and also regulate antibody production to modulate B cell responses. Growth and maturation of other cell types and leukocyte migration are also facilitated by IFN-γ. The heightened immune activation ultimately leads to effective clearance of pathogens through enhanced phagocytosis, pro-inflammatory responses and lymphocyte recruitment (Kak *et al.*,2018) . NK cells are lymphocytes of innate immune system, which provide defense against

viral infections by secreting cytokines (mainly IFN-γ) and killing infected cells (Ferreira *et al*,2018).

Antiviral mechanisms of IFN-γ have not been well characterized. This is partly due to its overlapping antiviral effect with type I IFNs . IFNs activate an overlapping set of genes via similar Janus kinase/signal transducer and activator of transcription (JAK/STAT) protein signal transduction pathways, and thus antiviral activities of IFNs are somewhat redundant (Kang *et al*.,2018). the protective effects of IFN- γ have been harnessed for therapeutic purposes as well. This further reiterates its protective role in the host system (Kak *et al.*,2018).

2.13 Genetic Polymorphism of IFN-γ Gene

Interferon-γ (IFN-γ), or type II interferon, is a cytokine that is critical for innate and for innate and adaptive immunity against viral, some bacterial and protozoa infections . IFN-γ gene is located on chromosome 12 at position 12q14, and the IFN-γ monomer consists of a core of six α-helices and an extended unfolded sequence in the C-terminal region (Al-Saffar and Ad'hiah ,2018). The human IFN-γ gene, which consists of four axons and three introns, is located at chromosome 12q24.1 . Functional studies have demonstrated that the single nucleotide polymorphism (SNP) within the first intron of the IFN-γ gene (known as IFN + 874A/T), which has been confirmed as a possible binding site for nuclear factor kappa B, can increase or decrease the overall expression and secretion of IFN- and ultimately determine the outcome of the infection (Ghasemian and hahbazi1 ,2016).

As previously reported, genetic variations in IFN-γ may be related to virus-related diseases, such as cervical cancer caused by human papillomavirus infection and leprosy caused by Mycobacterium leprae . The IFN-γ gene polymorphisms are also widely studied in HBV infection and HBV (sun *et al* .,2015) .

The SNPs in the IFN-γ gene region can influence the IFN-γ production, which may increase the risk of viral infection .The IFN-γ +874 T/A (rs2430561) in the first intron of the IFN-γ gene, in which the TT genotype produces a high level of IFN-γ, helps the host's defense against viral infection. Conversely, the genotypes AA and AT cause low IFN-γ production, which may increase the risk of viral infection (sun *et al* .,2015) .

2.14 IFN-γ and Hepatitis B virus

Hepatitis B virus engages different immune components over time as it progresses through its pathogenesis. It is primarily a concerted action of interferon-γ (IFN-γ) and cytolytic CD8+ T cells that target infected hepatocytes during acute phase of infection (Sandhu et al .,2017).

IFN-γ is a pro-inflammatory Th1 cytokine with a fundamental role in downregulating the gene expression of HBV in infected hepatocytes by disrupting the stability of viral RNA and abrogating HBV nucleocapsid particles (Sun *et al*.,2015).

T lymphocytes and natural killer cells are the only cells that are responsible for secreting IFN-γ. These cells release IFN-γ once activated by antigens, alloantigens, or mitogens (Liu *et al*.,2006). IFN-γ induces antiviral responses by the enhanced expression of major histocompatibility complex (MHC) antigens on hepatocytes (Nieters *et al*., 2005). This cytokine also seems to be involved in the clearance of HBV infection as it suppresses the replication of HBV-infected cells and adjusts the antiviral capacity of cytotoxic lymphocytes to, directly and indirectly, diminish the viral load (Korachi *et al*.,2013).

An intronic single nucleotide polymorphism at *IFN-γ* (+874 T/A) may affect the production of the cytokine and biologically change the function of regulatory elements by modifying their affinities to transcription factors . A positive association of this T to A transition polymorphism with human diseases has been consistently reported (sun *et al*., 2015).a resent studies suggested that IFN-γ +874 T haplotype is potentially protective against persistent HBV infection and IFN-γ+ 874 AA genotype showed to be associated with an increased risk of chronic HBV infection (Naghizadeh *et al*.,2018) Therefore, the low IFN-γ expression may reduce the host immune response to HBV, and these individuals are more prone to the infection. A study conducted by Sun and his colleagues (2015).

2.15 Previous studies

In Hospital Santa Casa de Misericórdia in al- Brazil demachki *et al* (2013) evaluate serum level of IFN-γ in chronic hepatitis B patient and healthy control The mean serum level of IFN-γ in the patients with chronic hepatitis B was significantly lower ($p < 0.0001$) than that of the control Group **(D**emachki *et al* 2013).

In Tran Hung Dao Hospital in Vietnam Song and his colleagues (2003) measured serum level in Vietnams patients, Samples collected from a total of 154 HBV-infected patients with well-characterised clinical profiles and 56 healthy controls were assessed. The result showed that

Serum IFN-γ levels were uniformly low and showed no association with clinical presentation (song et al,2003).

Al-Saffar and Ad'hiah investigated serum level and gene polymorphism of Interferon gamma (IFN-γ) were in 38 Iraqi Arab hepatitis B patients as well as 24 ethnicity, age and gender-matched controls. decreased level was observed in HBV patients , but the difference was not significant (Al-Saffar and Ad'hiah,2018).

Rizvi and his colleagues evaluated serum IFN-γ in acute and chronic HB and show that significantly elevated of IFN-γ level in patient with AHB and CHB compare to control (*P value* <0.05) Decreased level of (IFN-γ) in CHB have also been reported .Resent studies have reported rather very low in different clinical group or elevated levels in those with chronic HBV (Rizvi *et al*.,2012).

Hosseini Khorami and his colleges enrolled Sixty patients with chronic hepatitis B and 60 age and gender-matched healthy controls in there study . the result showed that The concentration of IFN-γ was significantly higher in patients compared with the healthy controls ($P < 0.05$) (Khorami *et al*.,2018).

Ben-Ari and his colleagues study population consisted of 77 patients with chronic HBV infection The genetic profile of five cytokines was analyzed by polymerase chain reaction. A highly statistically significant difference in the distribution of the IFN-γ gene polymorphism (at position+ 879) was observed between patients with chronic HBV infection and controls. The majority of the patients (65.2%) exhibited the potential to produce low levels of IFN-γ (A/A genotype) compared with 37.5% of the control group ($p= 0.003$) (Ben-Ari *et al*.,2003).

In India Srivastava and his colleagues estimate the level of IFN-γ in total of 232 patients along with 76 healthy controls . Allele-specific primers for IFN-γ were used ,The study indicated that low IFN-γ expression and Significantly, AA homozygous genotype was dominant in chronic hepatitis B cases in IFN-γ+874 and is associated with increased risk of persistent infection (Srivastava *et al*.,2014).

2.16 Treatment

No antiviral therapy is typically used in acute hepatitis B. For chronic hepatitis B, entecavir (Baraclude) or tenofovir (Viread) are the drugs of choice. They are nucleoside analogues that inhibit the reverse transcriptase of HBV. Interferon in the form of peginterferon alfa-2a (Pegasys) is also used. Other nucleoside analogues such as lamivudine (Epivir-HBV), adefovir

(Hepsera), and telbivudine (Tyzeka) are used less frequently. A combination of tenofovir and emtricitabine (Emtriva) is also used. These drugs reduce hepatic inflammation and lower the viral load of HBV in patients with chronic active hepatitis. Neither interferon nor the nucleoside analogues cure the HBV infection. In most patients when the drug is stopped, HBV replication resumes (Levinson, 2014).

2.17 Prevention

Broadly there are two approaches to the prevention of infection with HBV modification of risk behavior and immunization. Measures for the former include avoiding unprotected sexual contact by the use of condoms and reducing needle-sharing among injecting drug users through needle exchange schemes implementation of sensible infection control policies can reduce the risks considerably to healthcare workers and patients .it is essential that blood for transfusion and organ donors for transplantation are screened (Greenwood *et al.*, 2012).

Asafe and effective vaccine that offers a 98-100% protection against hepatitis B is available preventing hepatitis B infection averts the development of complications including the development of chronic disease and liver cancer. (WHO,2019).

CHAPTER THREE
MATERIALS AND METHODS

3. Materials and methods

3.1 Study design

This was analytical case control study .

3.2 Study area

The study was conducted in Saba Medical center in Khartoum state .

3.3 Study duration

This study was conducted during the period from May to December (2019).

3.4 Study population

Study population include 60 individual age and sex matched ,(30) infected hepatitis B patients as case group , and (30) healthy individual as control group .

3.5 Inclusion criteria

Known cases of HBV infection acute or chronic positive ELISA patients and control group was included age and sex matched apparently health subjects.

3.6 Exclusion criteria

Hepatitis B patients with other disease such as autoimmune diseases, infectious disease, allergy, hypersensitivities, cancer, heart failure, Parkinson disease and diabetic.

3.7 Sample size

Total of sixty (n=60) individuals were included in this study , (30) were from hepatitis B infected patient and (30) from healthy volunteers .

3.8 Sample technique

This study based on non-probability convenience sampling technique .

3.9 Ethical consideration

Approval of carry out of study was obtained from Sudan university of since and technology ,College of graduate studies and Medical Microbiology Department , permission of Saba manger and patient was taken before beginning .

3.10 Data collection

Data collected with self-administered questionnaire .

3.11 Specimen collection

3 ml of venous blood were collected from patients and control in EDTA container. Then the samples were centrifuged and plasma separated in cryovial tube and stored at -20ºC until analysis. plasma levels of (IFN-γ) will be measured using ELISA (Biolegend's ELISA MAXTM).

3.12 Enzyme Linked Immunosorbent Assay(ELISA) technique for Estimation of (IFN-γ)

3.12.1 ELISA principle

Biolegend's ELISA MAXTM Deluxe Set is a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). A human (IFN-γ)specific monoclonal antibody is irst coated on a 96-well plate. Standards and samples are added to the wells , and (IFN-γ)binds to the immobilized capture antibody . Next , a biotinylated anti-human (IFN-γ)detection antibody is added , producing an antibody-antigen-antibody "sandwich". Avidin-horseradish preoxidase is subsequently added , followed by TMB Sustrate Solution , producing a blue color in proportion to the concentration of (IFN-γ)present in sample . Finally , the stop Solution changes the reaction color from blue to yellow , and the microwell absorbance is read at 450 nm with a microplate reader (biolegend , USA , 2016).

3.12.2 ELISA Procedure

100 µL of diluted capture antibody solution was added to each well , seal the plate and incubate overnight between 20 and 80 C .Then plate washed 4 times with at least 300µL of wash buffer per well and blot any residual buffer by firmly tapping the plate upside down on clean absorbent paper . To block the plate by adding 200 µL 1X Assay diluents A to each well , seal the plate and incubate at room temperature for 1 hour with shaking at approximately 500 rpm (with a 0.3 cm circular orbit). All subsequent incubation with shaking should be performed similarly. Plate washed 4 times ; add 100 µL Diluted stanndareds and samples to the appropriate wells . seal the plate and incubate at room temperature for 2 hours with shaking . then wash plate 4 times ; add 100µL diluted detection antibody solution to each well , seal the plate and incubate at room temperature for 1 hour with shaking . wash plate 4 times ; add 100 25 µL diluted Avidin-HRP solution to each well , seal the plate and incubate at room temperature for 30 minutes with shaking . wash plate 5 times ; soaking for 30 seconds to 1 minute per wash . add 100µL of freshly mixed TMB substrate solution to each well and incubate in the dark for 30 minutes. Add

100µL stop solution to each well . read absorbance at 450 nm and 570 nm within 15 minutes . The absorbance 570 nmm can be substracted from the absorbance at 450 nm .

3.12.3 ELISA Washer principle

First the wash solution is pump from the wash bottle , the solution is dispense to the cuvette by short pins , and then the wash liquid is aspirate from the cuvette by long pins , at the end the waste liquid was pumped into the waste bottle by the vacuum pupp.(www.diasource.be 2019).

3.12.4 ELISA reader principle

White light produced by the lamps is focused into a beam by the lens and passes through the sample. Part of the light is absorbed by the sample and the remaining light is transmitted . It is filtered by interference filters and focused onto the photodiodes . The photodiode converts the received light into an electrical signal which is transformed into a digital form , from which the microprocessor calculates the absorbance , taking in account of the blank and dichromatic selection (www.diasource.be 2019).

3.13 Statistical analysis

Statistical analysis was perform using Statistical Package for Social Science (SPSS) Version 20 .The data display as {mean ±SD}, frequencies, person correlation, T test and P.value $\leq$ 0.05.

CHAPTER FOUR

RESULTS

Sixty volunteers with age varies from 12-50 years were enrolled in this study,30 subjects are hepatitis B virus patients with mean of age 28.63± 7.91 years,15 of them (50%) were males and 15 (50 %) are females. Other 30 subjects are apparently healthy control group with mean of age 31.10± 9.21 years, 15 (50 %) of them were males and 15 (50 %) were females (Table 4-1).

Mean level of IFN-γ was significantly decreased in hepatitis B virus patient (5.73±1.67pg/ml) when compared to control group (11.35±6.33pg/ml) with *p.value* 0.00 (Table 4-2) .

Comparisons between IFN-γ levels in hepatitis B patients with sex , IFN-γ mean level was lower in males (5.27±1.35pg/ml) than females (6.18±1.87pg/ml), which is statistically insignificant *p.value* 0.138 (Table 4-3).

According to duration of disease high plasma level of IFN-γ in patients with acute hepatitis B compared to chronic hepatitis B patients ,mean were 6.13±1.9 and 5.32±1.26respectively, this difference was statistically insignificant *P. value* 0.193 (Table 4-3).

Regarded to treatment the result showed that insignificantly increased between patients using treatment compared to patient not use treatment (5.36±1.21 and 6.28±2.13respectively) *p.value* 0.195 (Table 4-3).

There was no significant difference in IFN-γ level between patients with symptoms of jaundice (5.74±0.82 pg/ml) and patients have no symptoms of jaundice (5.72±1.78pg/ml) *p. value* 0.982 (Table 4-3).

In blood transfusion IFN-γ level was decreased in patients received blood transfusion (3.86±1.91pg/ml) when compared to patients didn't receive blood transfusion (5.86±1.61pg/ml) and this difference was statistically insignificant *p.value* 0.104.(Table 4-3).

One way ANOVA test showed significant association between age group and IFN-γ *P.value* 0.00 (Table 4-4) .There was insignificant correlation between IFN-γ and age in hepatitis B virus group (*P. Value* 0.590, r=0.060) (Figures 4-2).

Table (4-1): Gender distribution among study groups

Gender	Case	Control
Male	15 **(**50%)	15 (50%)
Female	15 (50%)	15 (50%)
Total	30 (100%)	30 (100%)

Tables (4-2): Association Of (IFN-γ) Level between Hepatitis B Patients Group and Healthy Control Group.

Parameter	Hepatitis B virus patients (Mean ± SD)	Healthy control group (Mean ± SD)	*P.Value*
(IFN-γ)	5.73±1.67	11.35± 6.33	0.00

*$p.value \leq 0.05$ = significant

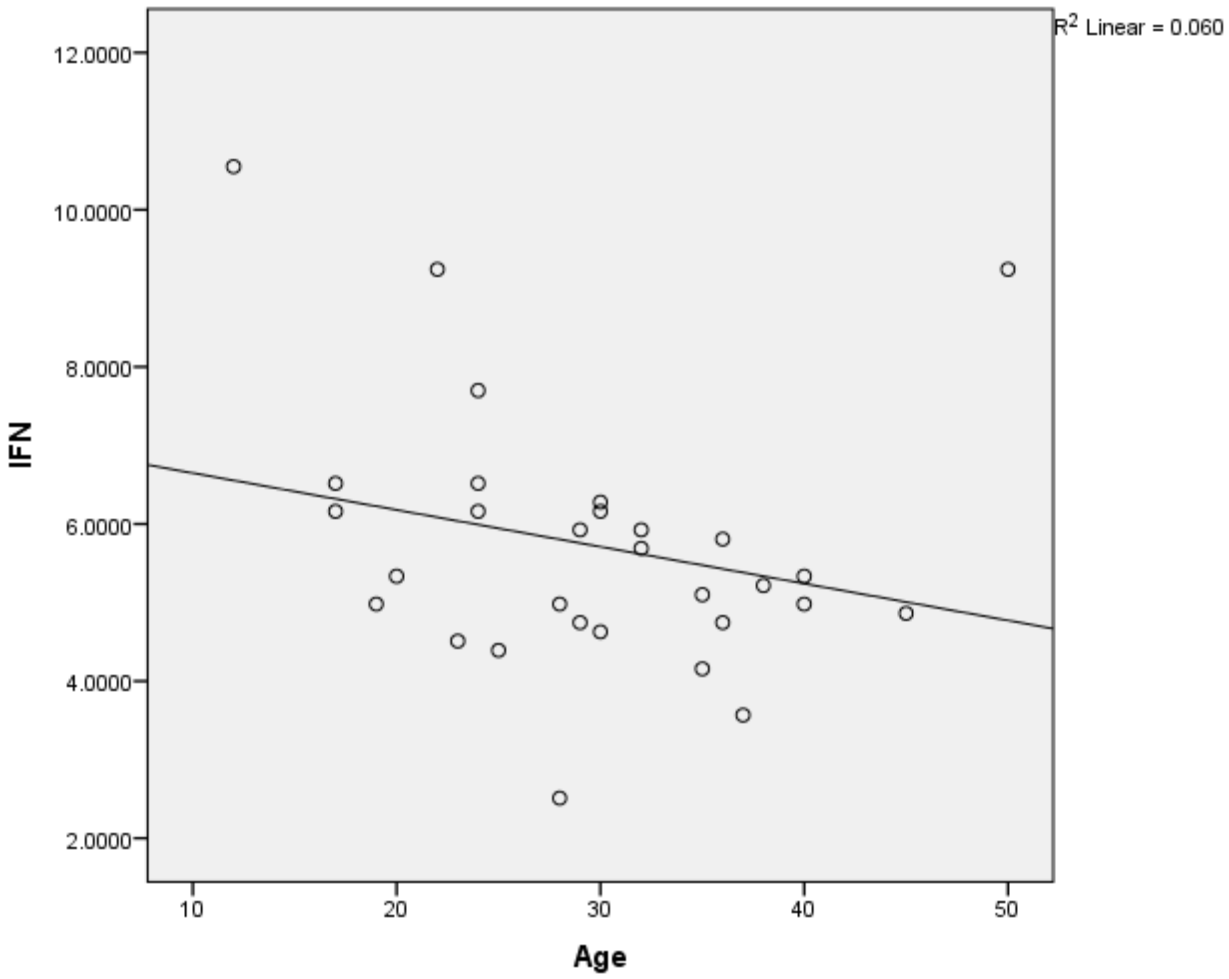
Table (4-3): Association Of (IFN-γ) Level and Different Study Variables in Hepatitis B Virus Patients

Variable		Mean ± SD	*P*.value
Sex	Male	5.27±1.35	0.138
	Female	6.18±1.87	
Duration	Acute	6.13±1.9	0.193
	Chronic	5.32±1.26	
Treatment	Yes	5.36±1.21	0.195
	No	6.28±2.13	
Blood transfusion	Yes	3.86±1.91	0.104
	No	5.86±1.61	
Jaundice	Yes	5.74±0.82	0.982
	No	5.72±1.78	

**p.value* ≤ 0.05 = significant

Table (4-4) : Association between (IFN-γ) and Different Age Group

Age groups	**Mean ± SD**	***P*.value**
<15	10.5± 9.5	0.00
15-45	5.4± 1.2	
>45	9.2± 1.6	



P. Value 0.590

R=0.060

Figure (4-1): Correlation between (IFN-γ) level and age of hepatitis B patients

CHAPTER FIVE

DISCUSSION , CONCULUSION AND RECOMMENDATIONS

5.1. Discussion

Interferon gamma (IFN-γ) is a key regulatory cytokine, which plays an important role in antiviral defense by an infected host. IFN-γ can exert anti proliferative activity and thought to play major role in combating chronic hepatitis B, liver cirrhosis , and hepatocellular carcinoma (sun *et al*.,2015).

In the present study plasma level of IFN-γ (pg/ml) is significantly lower in hepatitis B virus patients compared to healthy control group *(p value* 0.00) , This finding was supported by Song *et al* in vietnams patients showed that IFN-γ levels were uniformly low in case compare to control group (song *et al*.,2003) and also decreased level of IFN-γ was observed in Iraqi Arab hepatitis B patients when they are investigate against healthy subject (Al-Saffar and Ad'hiah,2018). Low level of (IFN-γ) in CHB have been reported , Resent studies have reported rather very low in different clinical group or elevated levels in those with chronic HBV (Rizvi *et al*.,2012).

According to previous studies which have revealed the association of A and T alleles with low and high IFN- production respectively (Ghasemian and Shahbazi,2016) .The resent studies suggest that The TT genotype is associated with increased IFN production when the immune system responds to stimuli . In contrast, the AA and TA genotypes are linked to low IFN production so many studies have mainly focused on the IFN-γ +874T/A polymorphism (sun *et al*.,2015).

In addition this study supported by Naghizadeh *et al* ,(2018) his results exhibited a statistically significant difference between patients and control individuals (*p*-value<0.001) the obtained results showed that HBV infected individuals with T allele have less risk of progressing to chronic HBV infection. It also suggests that the homozygous carriers of the A allele are more vulnerable to chronic HBV infection (Naghizadeh *et al* , 2018).

Our study agree with study conducted in al- Brazil by Demachki and his colleges (2013) who evaluated serum level of IFN-γ in chronic hepatitis B and healthy control and significantly lower level of IFN-γ is reported ($p < 0.0001$) **(D**emachki *et al* 2013) and disagree with other study enrolled by Khorami and his colleagues reported that significantly higher level of IFN-γ in patients when compared with healthy controls ($P < 0.05$) .

On the other hand, a study from Iran in 2016 showed no association between this polymorphism and HBV clearance in a population from Golestan Province (Ghasemian , Shahbazi .,2018) .

The lower in the results in pur study might be due to gene polymorphism which confiermed by many pervious study or may attributed to the ethnic background of the studied populations and sample size .

5.2. Conclusions:

This study concluded that:

The plasma IFN-γ level was significantly lower in in hepatitis B patients group when compared with healthy subjects group .

There was significant association between IFN-γ level and age group .

There was no association between IFN-γ level and gender ,duration of disease ,treatment ,blood transfusion and jaundice .

5.3. Recommendations

Regular measurement of IFN-γ level in Sudanese hepatitis B patients .

Large sample size (chronic carriers , patients on antiviral therapy , patients has not starting antiviral treatment yet , patients with liver cirrhosis and hepatocellular carcinoma) is likely to enhance our understanding of IFN-γ roles and biological activities .

Taking this work into the next levels the genomic levels and estimating the effects of different mutation and gene polymorphisms using a number of molecular and bioinformatics tools will expanse our knowledge about the role of IFN-γ in the pathogenesis of hepatitis B infection among Sudanese patients and so many other diseases .

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Appendices

Appendix (1)

Sudan University of Sciences and Technology

College of Post Graduate Studies

Estimation of Interferon Gamma (IFN-γ) Level among Sudanese Hepatitis B Patients in Khartoum State

Date / 2019

ID Number:……..

Age:……Years

Gender:

Male ( ) Female ( )

Duration of disease:

Less than six months ( ) More than six months ( )

Treatment:

Yes ( ) No ( )

Blood transfusion:

Yes ( ) No ( )

Jaundice:

Yes ( ) No ( )

Any chronic disease:

Yes ( ) No ( )

Result of (IFN-γ) –level =

Appendix (2)

Color plate (1): ELISA Kits

Appendix (3)

Color plate (2): ELISA Washer

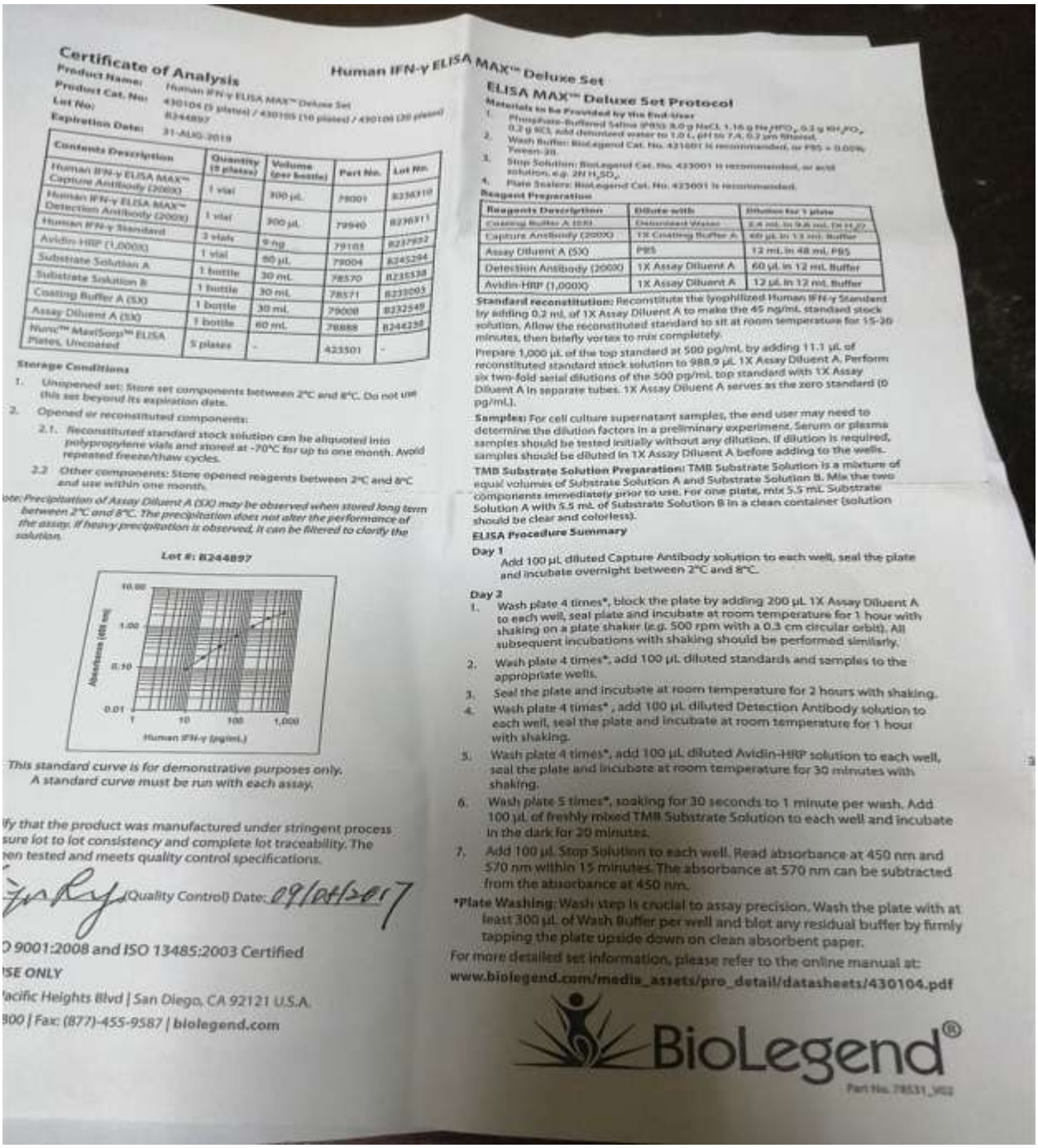
Appendix (4)

Color plat (3): ELISA Reader

Appendix (5)

Color plate (4) : ELISA micro plate

Appendix (6)



Certificate of Analysis

Human IFN-γ ELISA MAX™ Deluxe Set

Product Name: Human IFN-γ ELISA MAX™ Deluxe Set
Product Cat. No: 430104 (5 plates) / 430105 (10 plates) / 430106 (20 plates)
Lot No: B244897
Expiration Date: 31-AUG-2019

Contents Description	Quantity (5 plates)	Volume (per bottle)	Part No.	Lot No.
Human IFN-γ ELISA MAX™ Capture Antibody (200X)	1 vial	300 µL	79001	B236310
Human IFN-γ ELISA MAX™ Detection Antibody (200X)	1 vial	300 µL	79940	B236311
Human IFN-γ Standard	2 vials	9 ng	79103	B237932
Avidin-HRP (1,000X)	1 vial	60 µL	79004	B245294
Substrate Solution A	1 bottle	30 mL	78570	B235538
Substrate Solution B	1 bottle	30 mL	78571	B235005
Coating Buffer A (5X)	1 bottle	30 mL	79008	B232549
Assay Diluent A (5X)	1 bottle	60 mL	78888	B244238
Nunc™ MaxiSorp™ ELISA Plates, Uncoated	5 plates	-	423501	-

Storage Conditions

1. Unopened set: Store set components between 2°C and 8°C. Do not use this set beyond its expiration date.
2. Opened or reconstituted components:
   - 2.1. Reconstituted standard stock solution can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze/thaw cycles.
   - 2.2. Other components: Store opened reagents between 2°C and 8°C and use within one month.

Note: Precipitation of Assay Diluent A (5X) may be observed when stored long term between 2°C and 8°C. The precipitation does not alter the performance of the assay. If heavy precipitation is observed, it can be filtered to clarify the solution.

Lot #: B244897

This standard curve is for demonstrative purposes only. A standard curve must be run with each assay.

...fy that the product was manufactured under stringent process ...sure lot to lot consistency and complete lot traceability. The ...en tested and meets quality control specifications.

...(Quality Control) Date: 09/08/2017

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ELISA MAX™ Deluxe Set Protocol

Materials to be Provided by the End User

1. Phosphate-Buffered Saline (PBS): 8.0 g NaCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, add deionized water to 1.0 L, pH to 7.4, 0.2 µm filtered.
2. Wash Buffer: BioLegend Cat. No. 421601 is recommended, or PBS + 0.05% Tween-20.
3. Stop Solution: BioLegend Cat. No. 423001 is recommended, or acid solution, e.g. 2N H_2SO_4.
4. Plate Sealers: BioLegend Cat. No. 423001 is recommended.

Reagent Preparation

Reagents Description	Dilute with	Dilution for 1 plate
Coating Buffer A (5X)	Deionized Water	2.4 mL in 9.6 mL DI H_2O
Capture Antibody (200X)	1X Coating Buffer A	60 µL in 11 mL Buffer
Assay Diluent A (5X)	PBS	12 mL in 48 mL PBS
Detection Antibody (200X)	1X Assay Diluent A	60 µL in 12 mL Buffer
Avidin-HRP (1,000X)	1X Assay Diluent A	12 µL in 12 mL Buffer

Standard reconstitution: Reconstitute the lyophilized Human IFN-γ Standard by adding 0.2 mL of 1X Assay Diluent A to make the 45 ng/mL standard stock solution. Allow the reconstituted standard to sit at room temperature for 15-30 minutes, then briefly vortex to mix completely.

Prepare 1,000 µL of the top standard at 500 pg/mL by adding 11.1 µL of reconstituted standard stock solution to 988.9 µL 1X Assay Diluent A. Perform six two-fold serial dilutions of the 500 pg/mL top standard with 1X Assay Diluent A in separate tubes. 1X Assay Diluent A serves as the zero standard (0 pg/mL).

Samples: For cell culture supernatant samples, the end user may need to determine the dilution factors in a preliminary experiment. Serum or plasma samples should be tested initially without any dilution. If dilution is required, samples should be diluted in 1X Assay Diluent A before adding to the wells.

TMB Substrate Solution Preparation: TMB Substrate Solution is a mixture of equal volumes of Substrate Solution A and Substrate Solution B. Mix the two components immediately prior to use. For one plate, mix 5.5 mL Substrate Solution A with 5.5 mL of Substrate Solution B in a clean container (solution should be clear and colorless).

ELISA Procedure Summary

Day 1

Add 100 µL diluted Capture Antibody solution to each well, seal the plate and incubate overnight between 2°C and 8°C.

Day 2

1. Wash plate 4 times*, block the plate by adding 200 µL 1X Assay Diluent A to each well, seal plate and incubate at room temperature for 1 hour with shaking on a plate shaker (e.g. 500 rpm with a 0.3 cm circular orbit). All subsequent incubations with shaking should be performed similarly.
2. Wash plate 4 times*, add 100 µL diluted standards and samples to the appropriate wells.
3. Seal the plate and incubate at room temperature for 2 hours with shaking.
4. Wash plate 4 times*, add 100 µL diluted Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour with shaking.
5. Wash plate 4 times*, add 100 µL diluted Avidin-HRP solution to each well, seal the plate and incubate at room temperature for 30 minutes with shaking.
6. Wash plate 5 times*, soaking for 30 seconds to 1 minute per wash. Add 100 µL of freshly mixed TMB Substrate Solution to each well and incubate in the dark for 20 minutes.
7. Add 100 µL Stop Solution to each well. Read absorbance at 450 nm and 570 nm within 15 minutes. The absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

***Plate Washing:** Wash step is crucial to assay precision. Wash the plate with at least 300 µL of Wash Buffer per well and blot any residual buffer by firmly tapping the plate upside down on clean absorbent paper.

For more detailed set information, please refer to the online manual at:
www.biolegend.com/media_assets/pro_detail/datasheets/430104.pdf

BioLegend®

Part No. 78531_V02

Color plate (5) :Leaflet of (IFN-γ) -Kits