Sudan University of Sciences and Technology

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Evaluation of Plasma Interleukin-8 Levels among Sudanese Hepatitis B Patients in Khartoum

تقييم مستويات البلازما للمادة الخلوية 8 وسط السودانين المصابين بالتهاب الكبد ب الوبائي في الخرطوم

A dissertation Submitted in Partial Fulfillment of the Requirements for the Award MS.c Degree in Medical Laboratory Science (Microbiology)

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الآية

قال تعالى:

(أَوَلَمْ يَتَفَكَّرُوا فِي أَنْفُسِهِمْ مَا خَلَقَ اللَّهُ السَّمَاوَاتِ وَالْأَرْضَ وَمَا بَيْنَهُمَا إِلَّا بِالْحَقِّ وَأَجَلٍ مُسَمًّى وَإِنَّ كَثِيراً مِنَ النَّاسِ بِلِقَاءِ رَبِّهِم لَكَافِرُون).

صدق الله العظيم

(سورة الروم الأية 8)

Dedication

To my beloved parents who made me what I'm today

To my respectful brothers, sister and friends

To all those wonderful persons

I am trying to say thank you

Acknowledgments

First of all, thanks to ALMIGHTY ALLAH for giving me patience and strength to complete this study.

I would like to express my sincere gratefulness and respect to Dr. Kawthar Abdelgaleil Mohammed Salih, for her valuable guidance, kind supervision and great help.

Thanks extended to volunteers participated in this study and Saba medical center staff.

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Abstract

One of the most important factors playing role in hepatitis B pathogenesis is cytokine release and the most effective cytokines that with proinflammatory characteristic is interleukin-8 (IL-8).

This is a case-control study was aimed to evaluate the IL-8 level in Sudanese hepatitis B cases and in control subjects in Khartoum state during the period from March to September (2019).

Sixty subjects, selected randomly in this study, with age varies from 12-50 years, 30 subjects were hepatitis B patients (17 male and 13 female) as case group and 30 subjects (17 male and 13 female) were age and sex matched healthy control group. Venous blood sample (3ml) was collected in EDTA container from each subject. IL-8 concentration was measured using Enzyme Linked Immunosorbent Assay (ELISA) in Ibn Sina University laboratory. The data was analyzed using Statistical Package 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.

Means of IL-8 were 43.11 ±30.01, 28.8±22.8 in the case group and control group respectively. IL-8 level was significantly elevated in hepatitis B patients than the and control group (*P. values* 0.042). Also there was no statistical correlation between IL-8 and age in case and control groups (*P. value* 0.524 and 0.994 respectively). The difference in mean of IL-8 between males and females of the case group and control group was not statistically significant (*P. value* 0.334 and 0.341 respectively).

The difference in mean level of IL-8 in case group for selected variables(Duration, Treatment, Blood transfusion and jaundice) was no statistically significant (*P. values* 0.627, 0.982, 0.486 and0.339 respectively).

The study concluded that in the studied population, IL-8 concentration may be useful as prognostic marker for hepatitis B infection.

مستخلص البحث

احد اهم العوامل التي تلعب دوراً في إمراضية إلتهاب الكبد ب الفيروسي هي إفرازالمواد الخلوية واكثر المواد الخلوية فعالية والذي له خاصية محفزه للالتهاب هو المادة الخلوية 8.

هذه دراسة حالة ضابطه هدفت الي تقييم مستوى المادة الخلوية 8 لدى المرضى السودانيين المصابين بالتهاب الكبد ب الفيروسي مقارنة مع الافراد الطبيعيين بولاية الخرطوم في الفترة من مارس حتى اكتوبر 2019.

اختير ستين فردا عشوائيا لهذه الدراسة و كانت اعمارهم تتراوح من 12–50, 30 مريضا من مرضى التهاب الكبد ب الفيروسي(17 رجل و 13 امرأة) و 30 من الافراد الطبيعيين (17 رجل و 13 امرأة) متوافقين في الجنس. سحبت 3 مل عينة وريدية من كل مشارك في انبوبة تحتوي على مناعة التجلط EDTA. قيس تركيز المادة الخلوية 8 عن طريق فحص الممتز المناعي المرتبط بالانزيم في معمل جامعة ابن سينا. حللت البيانات باستخدام الحزمة الاحصائية للمجتمع (نسخة 20) استخدم أنوفا (ANOVA) و اختبار T لمقارنة الاوساط وكانت القيمة المطلقة متوافقة عند اقل من 0.05.

كان الوسط الحساب للمادة الخلوية 8 43.11 ±30.01, 28.8±22.8 في المرضى بالتهاب الكبد ب الفيروسي وفي الأفراد الطبيعيين بالترتيب. هنالك ارتفاع ذو دلالة احصائية في معدل المادة الخلوية 8 في المرضى بالتهاب الكبد ب الفيروسي والأفراد الطبيعيين (كانت القيم الاحتمالية 0.042). ليس هنالك علاقة بين معدل المادة الخلوية 8 و العمر في المرضى بالتهاب الكبد ب الفيروسي وفي الأفراد الطبيعيين (القيم الاحتمالية0.524 في المرضى بالتهاب الكبد ب الفيروسي و0.994 في الأفراد الطبيعيين).

لاتوجد دلالة وصفية حسابية للمادة الخلوية 8 بين الرجال والنساء في المرضى بالتهاب ب الكبد الفيروسي وفي الأفراد الطبيعيين (القيم الاحتمالية0.334 في المرضى بالتهاب الكبد ب الفيروسي و 0.341 في الأفراد الطبيعيين).

لاتوجد دلالة وصفية حسابية للمادة الخلوية 8 في المرضى بالتهاب الكبد الفيروسي ب و المتغيرات المختارة (فترة المرض, العلاج, نقل الدم و اعراض اليرقان) و كانت (القيم الاحتمالية 0.627, 0.982, 0.486و 0.339 بالترتيب).

لقد توصلت الدراسة الى ان تركيز المادة الخلوية 8 يمكن ان يستخدم كعلامة لتكهن بالتهاب الكبد ب الفيروسي.

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HAV	hepatitis A virus
HBV	hepatitis B virus
HCV	hepatitis C virus
HDV	hepatitis D virus
HEV	Hepatitis E Virus
Th	T helper
CD4	Cluster of Differentiation 4
IL-8	Interleukin-8
CXC	Cysteine X Cysteine
IFN-α	Interferon alfa
ELISA	Enzyme Linked Immune Sorbent Assay
HIV	Human Immunodeficiency Virus
DNA	Deoxyribonucleic Acid
HBIG	Hepatitis B Immune Globulin
HBsAg	Hepatitis B Surface Antigen
HBcAg	Hepatitis B core Antigen
RNA	Ribonucleic Acid
mRNA	Messenger Ribonucleic acid
AHB	Acute Hepatitis B
IFN-γ	Interferon gamma
NK	Natural killer
CTL	Cytotoxic T Lymphocyte

CD8	Cluster of Differentiation 8
CHB	Chronic Hepatitis B
MDSCs	Myeloid-derived Suppressor Cells
PD-1	Programmed cell Death protein 1
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
ALT	Alanine aminotransferase
PCR	Polymerase Chain Reaction
Treg cells	regulatory T cells
IL-2	Interleukin-2
TNF-β	Tumor Necrosis Factor- beta
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-13	Interleukin-13
IL-17	Interleukin-17
TGF-β	Transforming Growth Factor – beta
IL-35	Interleukin-35
CXCR1	Cysteine X Cysteine Receptor 1
CXCR2	Cysteine X Cysteine Receptor 2
NCF	Neutrophil Chemotactic Factor
P. value	Probability value
SPSS	Statistical Package for Social Science

CHAPTER ONE

INTRODUCTION

Chapter One
Introduction

1.1. Introduction:

Hepatitis (inflammation of the liver) can be caused by a variety of organisms and toxins. For example, there are many viral diseases that involve some degree of liver damage as a secondary effect (such as infectious mononucleosis caused by the Epstein-Barr virus). However, the viruses referred to as "hepatitis viruses'' are those whose pathogenesis specifically involves replication in and destruction of hepatocytes (Harvey *et al*., 2013). Five medically important viruses are commonly described as "hepatitis viruses" because their main site of infection is the liver. These five are hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV, delta virus), and hepatitis E virus (HEV) (Levinson, 2014). Hepatitis B virus (HBV) infection is a severe public health burden, and approximately one third of the world population has serological evidence of resolved or ongoing infection, It infection causes a broad spectrum of liver diseases ranging from acute to chronic HBV infection with no biochemical evidence of liver injury to progressive chronic HBV infection, which may advance to liver cirrhosis, liver failure, and hepatocellular carcinoma (Yu *et al*., 2016).

The three main modes of transmission are via blood, during sexual intercourse and perinatally from mother to newborn. No antiviral therapy is typically used in acute hepatitis B. for chronic hepatitis B, peginterferon alfa-2a(pegasys) or peginterferon alfa-2b(peg-Intron) and/ or a nucleoside analogues. The prevention involves the use of either the vaccine or hyperimmune globulin or both (Levinson, 2014). The outcome of HBV infection largely depends on the host–virus interaction, mediated

by the adaptive immune response. The virus-specific T cell response is one of the key factors in the pathogenesis of HBV infection. Viral variants may influence the course and outcome of the disease. The effect of host factors on the progression of disease is poorly understood. Only very rarely (when there is profound immune suppression) does the hepatitis B virus probably become directly cytopathic (Feld and Janssen, 2015).

Many cytokines involved in the host's innate and adaptive immune responses have been suggested to contribute to effective antiviral immunity and outcomes of HBV infection. HBV specific cytotoxic T lymphocytes and CD4 T helper (Th) lymphocytes are 2 major components of the HBV-specific immune response. CD4 Th cells are a group of lymphocytes that produce cytokines regulating strength and duration of immunity and inflammation (Yu *et al*., 2016). Cytokines are soluble proteins that mediate immune and inflammatory reactions and are responsible for communications between leukocytes and between leukocytes and other cells. (Abbas *et al*., 2016). Chemokines are a family of cytokines that cause the directed migration of leukocytes along a concentration gradient, resulting in the accumulation of the migrating cells at the source of chemokine production. IL-8, also known as CXCL8, is a pro-inflammatory CXC chemokine that was discovered for its role in promoting chemotaxis and degranulation of neutrophils (David *et al*., 2016). Interleukin-8 (IL-8) is a pro-inflammatory member of the CXC chemokine family that produced by many types of cells such as macrophages, dendritic cell and vascular endothelial cell (Owen *et al*., 2013). Interleukin (IL)-8 is a pro-inflammatory cytokine that has a direct effect on immune cells, including polymorphonuclear cells. Keratinocytes are a rich source of IL-8. However, there is little knowledge on the role of IL-8 in clinical wound healing and the direct biological effect of IL-8 on keratinocyte (Jiang *et al*., 2012). The imbalance of T-

helper lymphocyte cytokine production plays an important role in pathogenesis of chronic hepatitis B virus (HBV) infection During viral infection various cytokines are in both viral clearance and tissue damage (khorami *et al*., 2018).

1.2. Rationale:

Hepatitis B virus infection is the most common chronic viral infection worldwide and is notable for being one of the diseases which has seen an increase in morbidity and mortality over the last 20 years (Koffas *et al*., 2019). Study results indicate that HBV activates IL-8 gene expression by targeting the epigenetic regulation of the IL-8 promoter and that IL-8 may contribute to reduce HBV sensitivity to IFN-α and play an important role in the immunopathogenesis of HBV infection (Pollicino *et al*., 2013). Recently, it has been revealed that the HBV induces expression of the proinflammatory chemokine IL-8 to partially inhibit the antiviral actions of IFN-α in vitro. IL-8 has been used as a marker for liver damage for diagnosis of various types hepatitis virus infection (Yang *et al*., 2014). This study was done to evaluate the utility of using IL-8 as biomarker for monitoring the progression of hepatitis B infection.

There are no previous studies about IL-8 levels in Sudanese hepatitis B patients.

1.3. Objectives:

1.3.1. General objective:

-To evaluate plasma level of IL-8 among Sudanese Hepatitis B Patients in Khartoum.

1.3.2. Specific objectives:

- To measure plasma level of IL-8 in hepatitis B patients and in healthy using Enzyme linked immune sorbent assay.

-To compare between the results of IL8 in hepatitis B patients and normal controls.

-To correlate IL-8 level with possible risk factors e.g age, gender, duration of disease, blood transfusion and jaundice.

CHAPTER TWO

LITERATURE REVIEW

Chapter Two

Literature review

2.1. Background:

Viral hepatitis is a global health problem, the current scale of which is second only to that posed by HIV. This problem is associated particularly with chronic infections by hepatitis B and C viruses, which give rise to a huge burden of late onset liver disease (Dimmock *et al*., 2016).

Many viruses cause hepatitis, of these, five medically important viruses are commonly described as "hepatitis viruses" because their main site of infection is the liver. These five are hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV, delta virus), and hepatitis E virus (HEV) Other viruses, such as Epstein–Barr virus (the cause of infectious mononucleosis), cytomegalovirus, and yellow fever virus, infect the liver but also infect other sites in the body and therefore are not exclusively hepatitis viruses (Levinson, 2014).

The family Hepadnaviridae (hepatotropic DNA viruses) consists of hepatitis-causing viruses with DNA genomes. Each hepadnavirus has a narrow host range in which it produces both acute and chronic, persistent infections, but HBV is the only member of this family that infects humans (*Harvey et al*., 2013).

Hepatitis B (HBV) infection is one of the most frequent viral infections and major global public health problems. In 2011, according to the World Health Organization, over 2 billion persons had been infected with the hepatitis B virus, one sixth of whom are chronically infected (Sodsai *et al*., 2013).

Infection may associate with a large spectrum of clinical forms, ranging from very mild and asymptomatic clinical pictures to the most severe liver diseases, including fulminant hepatitis, cirrhosis, and hepatocellularcarcinoma. Chronic HBV infection is a dynamic condition in which the Interaction between virus and the host immune response influences the outcome of the disease (Pollicino *et al*., 2013).

Mild HBV infections are treated by managing symptoms, whereas chronic infection may be treated with interferon, which stops virus multiplication. Passive immunization with hepatitis B immune globulin (HBIG) is administered to those who may have been exposed to the virus through needle punctures or other contact with blood. Extreme care when handling needles, broken blood vials, and splashed blood or serum is essential to reduce the risk of occupational exposure to the virus. Vaccination is available and is recommended for persons under 18 years of age and for adults with potential medical or occupational exposure to blood and blood products (Talaro and Chess, 2015).

2.2. Virology:

The hepatitis B virus (HBV) is a small DNA virus of the Hepadnaviridae family. 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 (Liang *et al*., 2009).

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. The viral polymerase is covalently attached to the 5′ end of the minus strand (Liang *et al*., 2009)

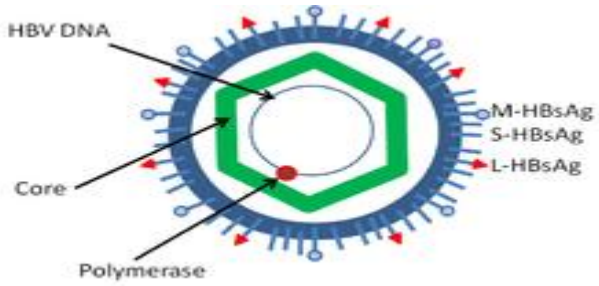

Figure (1): The structure of Hepatitis B virus

HBsAg - Hepatitis B surface antigen (HBsAg) was the first hepatitis B virus protein to be discovered.It is concist from small (S), medium (M) and large (L) protein (https://en.wikipedia.org/wiki/Hepatitis_B_virus).

2.3. Replicative Cycle:

The first step in the HBV life cycle is its attachment to the hepatocyte through the interaction of its envelope proteins with the host cell receptors. Then it penetrates in the hepatocyte, uncoating, and the viral genome, organized as relaxed circular partially double stranded DNA, is sent to the nucleus and converted into covalently closed circular DNA (ccc DNA). The cccDNA acts as template for transcription of four co-terminal mRNAs: 3.5 kb pre-core (pre-C) and progenomic RNA (pgRNA), 2.4 kb large surface mRNA, 2.1 kb middle and small surface mRNA and 0,7 kb X

mRNA. pgRNA serves as template for the reverse transcriptase and, after being transported to the cytoplasm, encodes viral capside protein and viral polymerase, thus playing an important role in viral genome amplification and replication. The latter is transcripted into viral RNA gene products: HBV surface protein, structural core protein, non-structural core protein (secreted HBeAg), X protein and viral polymerase. After this step the viral assembly occurs (encapsidation by the core protein to form the viral nucleocapsid) (Caligiuri *et al*., 2016).

Hepadnaviruses are the only viruses that produce genome DNA by reverse transcription with mRNA as the template. (Note that this type of RNA-dependent DNA synthesis is similar to but different from the process in retroviruses, in which the genome RNA is transcribed into a DNA intermediate.) Some of the progeny DNA integrates into the host cell genome, and this seems likely to be the DNA that maintains the carrier state. Progeny HBV with its HBsAg-containing envelope is released from the cell by budding through the cell membrane (Levinson, 2014).

2.4. Immunopathology:

HBV is a stealth virus that barely induces innate immunity in the early phase of infection. In AHB, innate immune responses could be established, followed by strong and robust adaptive immune responses, which both contribute to viral clearance. The noncytopathic pathway mediated by IFN-γ secreted by NK cells, NKT cells, and T cells contribute to the early control of HBV infection before emergence of the CTL response (Tseng and Huang, 2017).

CD4+ T cells facilitate the production of neutralizing antibodies and the induction of CTL responses, whereas CD8+ T cells contribute to the production of antiviral cytokines (eg, IFN-γ) and perform cytotoxic activities to eliminate virus-infected hepatocytes, which also induce immunopathology. In patients with CHB, both

innate and adaptive immune responses are weak. Suppressive cell populations and molecules (eg, NK cells, granulocytic MDSCs, Kupffer cells, PD-1, and CTLA-4) are key components and contribute to the chronicity of HBV infection (Tseng and Huang, 2017).

2.5. Epidemiology and Transmission:

Globally, liver disease caused by HBV is an enormous problem with an estimated 400 million people who are carriers of the virus. It is estimated that HBV well infect more than 2 billion people alive today at some point in their lives. About 80% of all chronic carriers live in Asia and the Western Pacific Rim region, where the prevalence of chronic hepatitis B is more than10% (kumar *et al*., 2013).

In United States there are approximately 185,000 new infections per year. HBV is found in the blood during the last stages of a prolonged incubation period (4 to 26 weeks) and during active episodes of acute and chronic hepatitis. It is also is present in all physiologic and pathologic body fluids, with the exception of stool. HBV is a hardy virus and can withstand extremes of temperature and humidity. Thus, whereas blood and body fluids are the primary vehicles of transmission, virus also may be spread by contact with body secretions such as semen, saliva, sweat, tears, breast milk and pathologic effusions. In endemic regions, vertical transmission from mother to child during birth constitutes the main mode of transmission. In areas of low prevalence, horizontal transmission via transfusion, blood products, dialysis, needle stick accidents among health care workers, sharing of needles in intravenous drug use, and sexual transmission constitute the primary mechanisms for HBV infection (kumar *et al*., 2013).

2.6. Hepatitis B Carriers:

Carriers are of two types the first is Super carriers which have HBeAg, high titters of HBsAg and DNA polymerase in their blood. HBV may also be demonstrable in their blood. Very minute amount of serum or blood from such carriers can transmit the infection. About a quarter of the carriers in India are HBeAg positive and second Simple carriers These are more common types of carriers who have low titer of HBsAg in blood, with negative HBeAg, HBV and DNA polymerase. They transmit the infection only when large volumes of blood are transferred as in blood transfusion. Many super carriers in time become simple carriers (Kumar, 2016).

2.7. Clinical Syndromes:

Hepatitis B virus is one of the most important causes of acute and chronic hepatitis. The clinical manifestations vary from subclinical hepatitis to symptomatic and icteric hepatitis. The incubation period varies from 6 weeks to 6 months. The clinical manifestations of HBV infection depend on age of infection, immune status of the host, and the level of HBV (Parija, 2012).

Acute Hepatitis B the incubation period of AHB varies from 1 to 4 mo postinfection. Clinical presentation varies from asymptomatic infection in two-thirds of patients to icteric hepatitis and, rarely, fulminant liver failure. A serum-sickness-like illness, characterized by fever, arthralgias, and rash, may occur in the prodromal period, followed by constitutional symptoms, anorexia, nausea, jaundice, and right upper quadrant discomfort. Clinical symptoms coincide with biochemical abnormalities. The biochemical diagnosis of acute hepatitis is characterized by elevations in the concentration of serum alanine aminotransferase (ALT) and bilirubin. The symptoms and jaundice generally disappear after 1– 3

months, although fatigue may persist for months in some patients, even after normalization of liver function tests (Burns and Thompson, 2014).

Chronic HBV infection the risk of chronicity in acute HBV infection is related to age at primary infection. Adults who become chronically infected during childhood have a 15–25% lifetime risk of dying from HBV -related cirrhosis or liver cancer. 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. (Feld and Janssen, 2015).

Immune-tolerant phase: Characterized by high levels of serum HBV DNA, HBe Ag positivity, normal alanine aminotransferase (ALT) levels, and absent liver necroinflammation. Disease progression is minimal in patients who remain in this phase Patients are highly contagious in this phase. Immune-reactive phase (HBeAg-positive CHB): Patients enter this phase after a variable time, linked to the age when HBV infection occurred.The immune system becomes more active and the infected hepatocyte are attacked. Characterized by highly fluctuating, but progressively decreasing, HBV-DNA levels, elevated ALTand hepatic necroinflammation (HBeAg-positive CHB). A prolonged immune-active phase with multiple ALT flares may result in progressive liver fibrosis, leading to cirrhosis. Immune-control phase (and inactive carrier state): Transition into this phase as an outcome of the immune-active phase is marked by seroconversion from HBe Ag 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).

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). Periodic ALT flares with intervening normalizationmay make it difficult to distinguish between HBeAg-negative CHB and inactive disease, and thus continued follow-up is required before patients with normal ALT and low HBV DNA levels are designated as inactive carriers. HBsAg-negative phase: After HBsAg loss, low-level HBV replication may persist, with detectable HBV DNA in the liver and rarely in the serum In patients with "occult" HBV infection, persistence of effective HBV immunological control has been demonstrated Significant immunosuppression may lead to HBV reactivation, with reappearance of HBsAg, known as "reverse seroconversion (Feld and Janssen, 2015).

2.8. Laboratory diagnosis:

The initial diagnosis of hepatitis can be made on the basis of the clinical symptoms and the presence of liver enzymes in the blood. However, the serology of HBV infection describes the course and the nature of the disease. Acute and chronic HBV infections can be distinguished by the presence of HBsAg and HBeAg in the serum and the pattern of antibodies to the individual HBV antigens. HBsAg and HBeAg are secreted into the blood during viral replication. The detection of HBeAg is the best correlate to the presence of infectious virus. A chronic infection can be distinguished by the continued finding of HBeAg, HBsAg, or both, and a lack of detectable antibody to these antigens(Murray *et al*., 2013).

Antibody to HBsAg indicates resolution of infection or vaccination. Antibody to HBcAg indicates current or prior infection by HBV and IgM anti-HBc is the best way to diagnose a recent acute infection, especially during the period when neither HBsAg nor anti-HBs can be detected (the window). Detection of antibodies to HBeAg and HBsAg is obscured during infection because the antibody is complexed with antigen in the serum. The amount of virus in blood can be determined by quantitative genome assays using polymerase chain reaction (PCR) and related techniques. Knowing the virus load can help in following the course of chronic HBV infection and antiviral drug efficacy (Murray *et al*., 2013).

2.9. Treatment and Prevention:

There is no specific treatment recommended for acute hepatitis B. A high-calorie diet is desirable. Treatment should be considered for patients with rapid deterioration of liver function, cirrhosis or complications such as ascites, hepatic encephalopathy, or hemorrhage as well as those who are immunosuppressed. For chronic hepatitis B diseases, pegylated or regular interferon-α provides benefit in some patients. Lamivudine (3TC), a potent inhibitor of HIV reverse transcriptase, and other nucleoside analogs (entecavir, telbivudine) as well as certain nucleotide analogs (adfovir) are active against hepatitis B. These antivirals inhibit viral replication and may reduce viral load but do not cure HBV infection (Ryan *et al*., 2014).

The original vaccine was produced in 1981 and consisted of purified HBsAg, prepared from the plasma of carriers, which was chemically treated to kill any contaminating viruses. The current vaccine is genetically engineered HBsAg produced in yeast or mammalian cells. Three injections of vaccine over a 6-month period give good protection in over 90% of healthy adults. After accidental

exposure to infection, hepatitis B immunoglobulin (HBIG) can be used to provide immediate passive protection. This is prepared from the serum of individuals with high titres of HB surface antibody. It may also be used together with hepatitis B vaccine to prevent transmission to children of HBV-carrier mothers (Goering *et al*., 2013).

2.10. Cytokines:

Cytokines are small, non-structural proteins with low molecular weights which have a complex regulatory influence on inflammation and immunity. It has long been considered that development of immune and inflammatory response involves hematopoietic cells, lymphoid cell, and various pro-inflammatory and anti-inflammatory cells, and cytokines mediate the complex interactions of these cells. Cytokines are the intercellular messengers in the immune system where they integrate function of several cell types in various body compartments into a coherent immune response (Gulati *et al*., 2016). Cytokines are soluble proteins that mediate immune and inflammatory reactions and are responsible for communications between leukocytes and between leukocytes and other cells. Most of the molecularly defined cytokines are called interleukins, by convention, implying that these molecules are produced by leukocytes and act on leukocytes (Abbas *et al*., 2016).

Cytokines exhibit the attributes of pleiotropy, redundancy, synergism, antagonism, and cascade induction, which permit them to regulate cellular activity in a coordinated, interactive way. A cytokine that induces different biological effects depending on the nature of the target cells is said to have a pleiotropic action, whereas two or more cytokines that mediate similar functions are said to be redundant. Cytokine synergy occurs when the combined effect of two cytokines on

cellular activity is greater than the additive effects of the individual cytokines. In some cases, the effects of one cytokine inhibit or antagonize the effects of another. Cascade induction occurs when the action of one cytokine on a target cell induces that cell to produce one or more additional cytokines (Owen *et al*., 2013).

2.10.1. CD4+T cell and their cytokines:

CD4+ T helper (Th) cells are critical for proper immune cell homeostasis and host defense, but are also major contributors to pathology of autoimmune and inflammatory diseases. This includes Th1, Th2, Th17, Th22, Th9, and Treg cells which are characterized by specific cytokine profiles (Raphael *et al*., 2015). The Th1 subset secretes IL-2, IFN-γ, and Lymphotoxin-α (TNF-β), and is responsible for many classic cell-mediated functions, including activation of cytotoxic T lymphocytes and macrophages. The Th2 subset secretes IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, and regulates B-cell activity and differentiation (Owen *et al*., 2013). Networks of cytokines and transcription factors are critical for determining CD4+ T-cell fates and effector cytokine production (Zhu and Paul, 2010). Th1 cells are involved with the elimination of intracellular pathogens and are associated with organ-specific autoimmunity. Th2 cells mount immune response to extracellular parasites, including helminthes, and play major role in induction and persistence of asthma as well as other allergic diseases. (Luckheeram *et al*., 2012). Treg cells is a major T cell subset with immunosuppressive function, which mainly produce immunosuppressive cytokines such as TGF-β, IL-10 and IL-35 and inhibit the activation of Th1 or Th2 cells (Li *et al*., 2016). Th17 cells are characterized by production of IL-17 and may have evolved for host protection against microbes that Th1 or Th2 immunity are not well suited for, such as extracellular bacteria and some fungi (Tesmer *et al*., 2008).

2.10.2. Chemokines:

Chemokines are a family of cytokines that cause the directed migration of leukocytes along a concentration gradient, resulting in the accumulation of the migrating cells at the source of chemokine production (David *et al*., 2016). There are two major chemokine sub-families based upon cysteine residues position: CXC and CC. As a general rule, members of the CXC chemokines are chemotactic for neutrophils, and CC chemokines are chemotactic for monocytes and sub-set of lymphocytes, although there are some exceptions (Palomino and Marti, 2015).

2.10.3. Interleukin-8 (IL-8, CXCL8):

The IL-8 chemokine is an important mediator of the innate immunity with well-defined immunomodulatory effects on T-cell function and inflammatory response (Pollicino *et al*., 2013). IL-8 is an 8.4 kDa nonglycosylated protein produced by processing of a precursor protein of 99 amino acids belonging to the CXC subfamily of chemokines which is characterised by two essential cysteine residues, separated by a third intervening amino acid (Qazi *et al*., 2011). IL-8 is a pro-inflammatory chemokine produced by various cell types to recruit leukocytes to sites of infection or tissue injury. Acquisition of IL-8 and/or its receptors CXCR1 and CXCR2 are known to be a relatively common occurrence during tumor progression (David *et al*., 2016). IL-8 was originally identified for its role in chemoattraction of neutrophils, for which it was named neutrophil chemotactic factor (NCF) and neutrophil activating protein (Turner *et al*., 2014).

2.10.4. Pro-inflammatory effects of IL-8:

IL-8 is an oxidative stress-responsive proinflammatory chemokine, released from epithelial cells following particle-induced oxidative stress leading to neutrophil

influx and inflammation. Proinflammatory stimuli are considered to be a major regulator of IL-8 levels in response to injury. IL-8 is involved in many of the wound healing processes. It not only serves as a chemotactic factor for leukocytes and fibroblasts but also stimulates fibroblast differentiation into myofibroblasts and promotes angiogenesis (Qazi *et al*., 2011).

2.11. Relation of IL-8 with HBV infection:

NK cells may contribute to liver inflammation by TRAIL-mediated death of hepatocytes and demonstrate that this non-antigen–specific mechanism can be switched on by cytokines (IL-8) produced during active HBV infection (Dunn *et al*., 2007).

2.12. Previous Studies:

In Messina,Italy Pollicino and his colleagues estimate the serum level of IL-8 in 26 CHB patients and in 22 healthy volunteers. The result revealed that high levels of serum interleukin-8 have been detected in chronic hepatitis B patients during episodes of hepatitis flares (*P. value* 0.001) (Pollicino *et al*., 2013).

In Wuhan, China Yu and his colleagues estimate the serum levels of IL-8 in 20 CHB patients and in 20 healthy volunteers. IL-8 levels in patients with chronic hepatitis B were significantly higher than in healthy controls, and large fluctuations in IL-8 concentrations in patients' sera associated with hepatic flares were observed (*P. value* less than 0.05) (Yu *et al*., 2011).

In 2017, Iran Bahramabadi and his colleagues measured serum level of IL-8 in 60 CHB patients and in 60 healthy controls. In patients with CHB compared with healthy controls, serum levels of IL-8 were significantly increased (*P. value* less than 0.001) (Bahramabadi *et al*., 2017).

In Aligarh Rizvi and his colleagues estimate the serum levels of IL-8 in 840 patients with HBV and 30 healthy controls. The result revealed that IL-8 levels were significantly higher than in the healthy controls (*P. value* less than 0.05) (Rizvi *et al*., 2013).

In China Deng and his colleagues estimate the serum levels of IL-8 in 235 patients with HBV infection and the result showed that circulating IL-8 levels were increased in patients with liver fibrosis in chronic HBV-infected patients (*P. value* 0.027) (Deng *et al*., 2015).

In Seoul Korea Shin and his colleagues estimate the serum levels of IL-8 in 16 patients with acute HBV infection and 14 healthy controls. The result showed that the IL-8 were significantly elevated in acute hepatitis B virus infection (Shin *et al*., 2016).

Hellstrom and Sylvan estimate the serum levels of IL-8 in 56bpatients with HBV infection and 46 healthy controls. The IL-8 levels in serum have been found to be significantly higher in patients with acute and chronic hepatitis B virus infection (*P. value* less than 0.001) (Hellstrom and Sylvan, 2012).

Shata and his colleagues estimate the serum levels of IL-8 in 30 patients with HBV infection and 20 healthy control the result showed that IL-8 levels were increased in patients (*P. value* less than 0.05) (Shata *et al*., 2019).

CHAPTER THREE

MATERIALS AND METHODS

Chapter Three

Materials and Methods

3.1. Study design:

This study is analytical prospective and case control study.

3.2. Study area:

The study conducted at Saba Medical Center in Khartoum State.

3.3. Study duration:

The study conducted during the period from March to September (2019).

3.4. Study population:

Sixty Sudanese individuals of age between 12-50 years divided as 30 hepatitis B patients and 30 apparently health subjects as control.

3.5. Inclusion criteria:

Case definitions for patients infected with hepatitis B virus and Control group will include age and sex matched apparently health subjects.

3.6. Exclusion criteria:

All hepatitis B patients with other disease that affect cytokines levels such as autoimmune diseases, infectious disease, allergy, hypersensitivities, cancer, heart failure, Parkinson disease and diabetes disease.

3.7. Sample size:

A total of 60 subjects were enrolled in this study. 30 samples were collected from hepatitis B patients and 30 samples were collected from healthy volunteer.

3.8. Data collection:

Samples were collected randomly. Structured questionnaire used to both collect demographic and clinical data.

3.9. Sampling Techniques:

Three 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 IL-8 was measured using ELISA (Biolegend's ELISA MAXTM).

3.10. Principles and procedures:

3.10.1. Principle of the ELISA:

BioLegend's ELISA MAX™ Deluxe Set is a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). A human IL-8 specific monoclonal antibody is first coated on a 96-well plate. Standards and samples are added to the wells, and IL-8 binds to the immobilized capture antibody. Next, a biotinylated anti-human IL-8 detection antibody is added, producing an antibody-antigen-antibody "sandwich". Avidin-horseradish peroxidase is subsequently added, followed by TMB Substrate Solution, producing a blue color in proportion to the concentration of IL-8 present in the 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 (www.biolegend.com).

3.10.2 ELISA procedure:

Frist 100 µL of diluted Capture Antibody solution was added to all wells of a 96-well plate; plate sealed and incubated overnight (16-18 hrs) between 2°C and 8°C.Then plate washed 4 times with at least 300 µL Wash Buffer per well and residual buffer was blotted by firmly tapping plate upside down on absorbent paper. To block non-specific binding and reduce background, 200 µL 1X Assay Diluent was added per well, plate sealed and incubated at RT for 1 hour with shaking at 200 rpm on a plate shaker.Plate washed 4 times with Wash Buffer. 50 µL of Matrix Diluent was added to the standard wells. 50 µL of Assay Diluent A was added to the sample wells. 50 µL/well of standards were added to the standard wells. 50 µL/well of serum samples were added to the sample wells. Plate sealed and incubated at RT for 2 hours with shaking. Plate washed 4 times with Wash Buffer. 100 µL of diluted Detection Antibody solution was added to each well, plate sealed and incubated at RT for 1 hour with shaking. Plate washed 4 times with Wash Buffer.100 µL of diluted Avidin-HRP solution was added to each well, plate sealed and incubated at RT for 30 minutes with shaking. Plate washed 5 times with Wash Buffer. For the final wash, wells were soaked in Wash Buffer for 30 seconds to 1 minute for each wash. To minimize background.100 µL of TMB Substrate Solution C was added and incubated in the dark for 15 minutes. Positive wells were turned blue in color. Reaction was stopped by addition of 100 µL of Stop Solution to each well. Positive wells were turned from blue to yellow. Absorbance was read at 450 nm within 30 minute. For results calculation the standard curve was plotted on log-log axis graph paper with analyte concentration on the x-axis and absorbance on the y-axis. A best fit line was drawn through the standard points. To determine the unknown analyte concentrations in the samples, the absorbance value of the unknown on the y-axis was found and a horizontal line

was drawn to the standard curve. At the point of intersection, vertical line was drawn to the x-axis and the corresponding analyte concentration was read.

3.10.3. 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 the long pins, at the end the waste liquid was pumped into the waste bottle by the vacuum pump (www.diasource.be).

3.10.4. 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)

3.11. Ethical Consideration:

This study approved by local Ethics and Scientific Committee of Ministry of Health Khartoum State, permission of Saba manger were taken before beginning and written informed consent was taken from every volunteers.

3.12. Data Analysis:

Statistical analysis was performed using Statistical Package for Social Science {SPSS} Version 20 .The data display as {mean ±SD}, frequency, person correlation, T test, one way ANOVA test and *P. value* ≤ 0.05.

CHAPTER FOUR

RESULTS

Chapter Four

Results

4. Results:

Sixty volunteers of age between 12-50 year were enrolled in this study 30 were hepatitis B patients with mean of age 30.23±10.12, 17(56.7%) were male and 13(43.3%) were female. 30 were apparently healthy subjects with mean of age 31.03 ± 9.11, 17(56.7%) were male and 13 (43.3 %) were female (Table 3-1).

The mean level of IL-8 (pg/ml) were 43.11 ±30.01, 28.8±22.8 in the case group and control group respectively. Statistically there was significant correlation in IL-8 level between case and control *P. values* was 0.042 (Table 3-2).

Mean level of IL-8 in males and females of the case group were 37.9±20.6 and 49.8±10.8 respectively, *P. value* was 0.334 and mean of IL-8 in control group were 24.8±10.1 in males and 34.1±32.8 in females, *P. value* was 0.341 (Figure 2). There was no statistical correlation between IL-8 level and gender in both case and control *P. value* 0.334 and 0.341 respectively. There was no statistical correlation between IL-8 level and age in both case (Figure 4) and control *P. value* 0.524 and 0.994 respectively.

The age grouped in three age groups and age group 2 (15-45year) high frequent 27(90%), follow by age group 3 (more than 45) 2(6.7%) and age group 1 (less than 15) was lowest 1(3.3) in case compared to control age group 2 high frequent 28(93.3%) follow by age group 3 was low 2(6.7%) and age group 1 was zero frequent. One way ANOVA test showed insignificant comparison between age group and IL-8 in both case(Figure 3) and control *P. value* 0.356 and 0.910 respectively.

Mean level of IL-8 in case taking treatment or not take was 43.2±33.7and 42.9±23.6 respectively, with no statistical significant *P. values* 0.982. Also mean IL-8 level in blood transfusion patients and not blood transfusion 57.7±31.7 and 42.1±30.2 respectively, *P. values* 0.486. The mean IL-8 level in patients have jaundice and not 53.8±41.4 and 40.4±26.9 respectively, *P. values* 0.339. The mean IL-8 level in patients less than six month (acute) and more than six month (chronic) 40.4±20.9 and 45.8±37.6 respectively, *P. values* 0.627. There was no significant correlation between IL-8 level and treatment, blood transfusion, jaundice and duration of disease (Table 3-3).

Table (3-1): Mean ± SD of age and gender distribution among the study groups:

Study group	**Mean ± SD of age**	**Male**	**Female**	**Total**
Case group	30.23±10.12	17 (56.7%)	13 (43.3%)	30 (100%)
Control group	31.03 ± 9.11	17 (56.7%)	13 (43.3%)	30 (100%)
Total		30	30	60 (100%)

Table (3-2): Comparison of IL-8 level between case group and control group:

Subject	case group (N = 30)	control group (N = 30)	*P. value*
Mean ±SD of IL-8	43.1 ±30.0	28.8±22.8	0.042

Table (3-3): Comparison of IL-8 level with selected variables in case group:

Variable	Mean of IL-8±STD	*P. value*
Duration		
Acute	40.4±20.9	
Chronic	45.8±37.6	0.627
Treatment		
Yes	43.2±33.7	
No	42.9±23.6	0.982
Blood transfusion		
Yes	57.7±31.7	
No	42.1±30.2	0.486
Jaundice		
Yes	53.8±41.4	0.339
No	40.4±26.9	

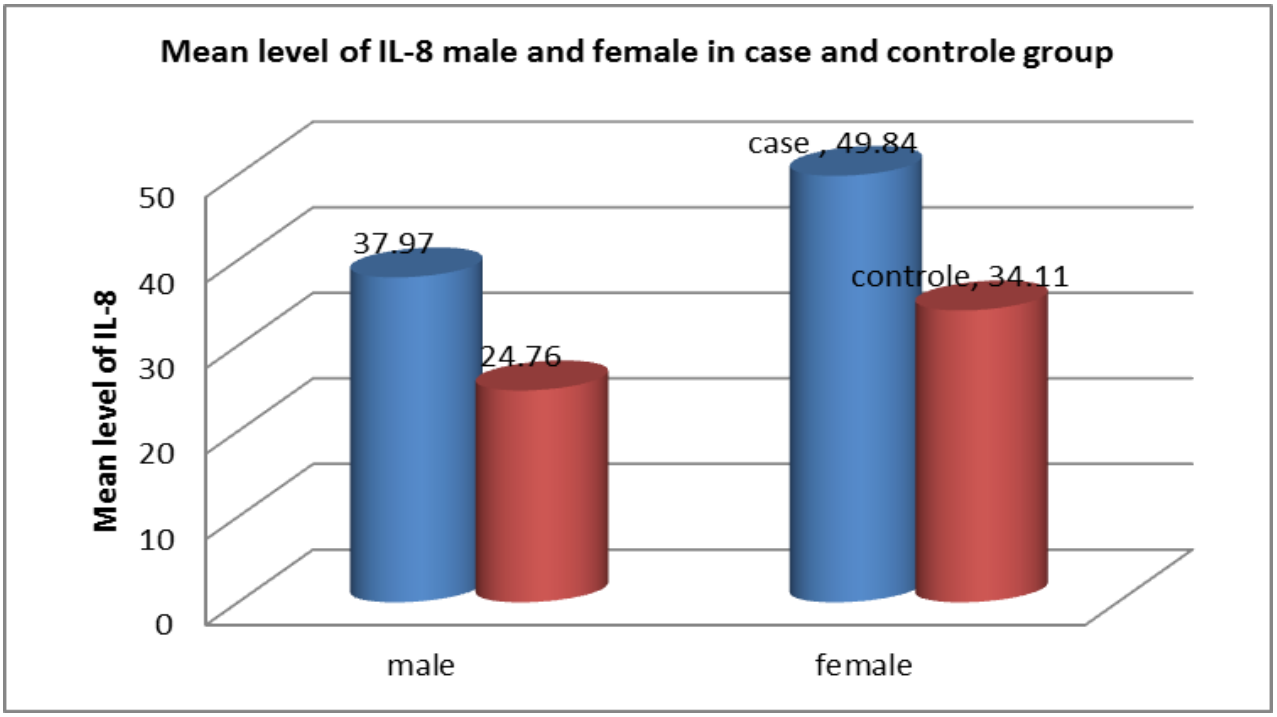

Figure (2): Comparison mean level of IL-8 between males and females in case and control group.

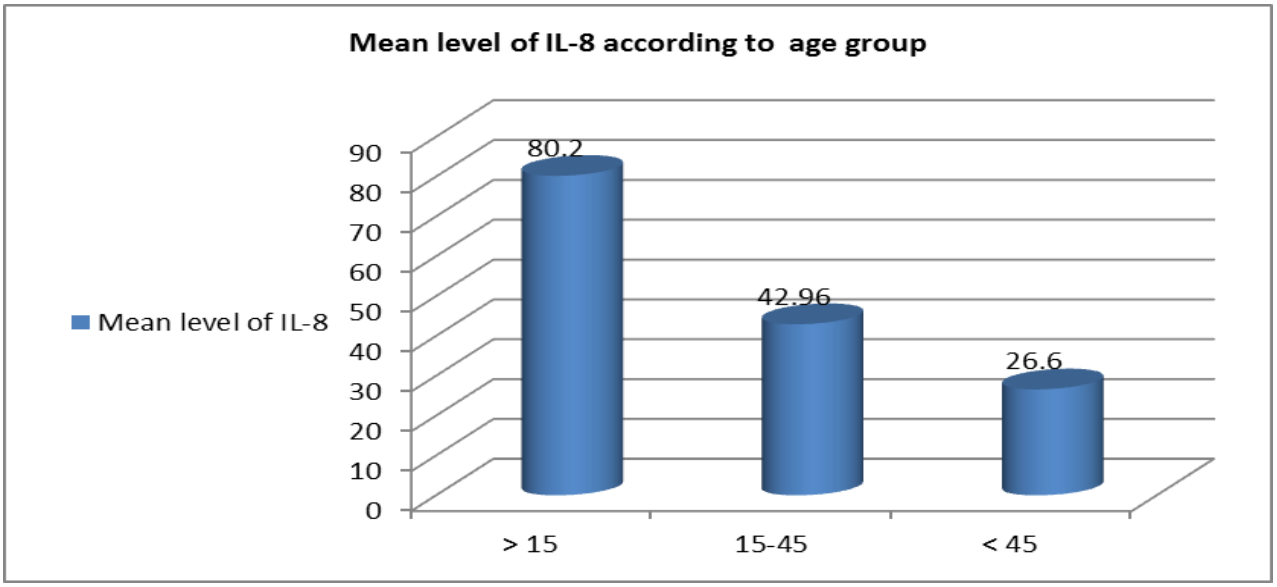

Figure (3): Mean level of IL-8 according to age group in case volunteer (*P. value* 0.356)

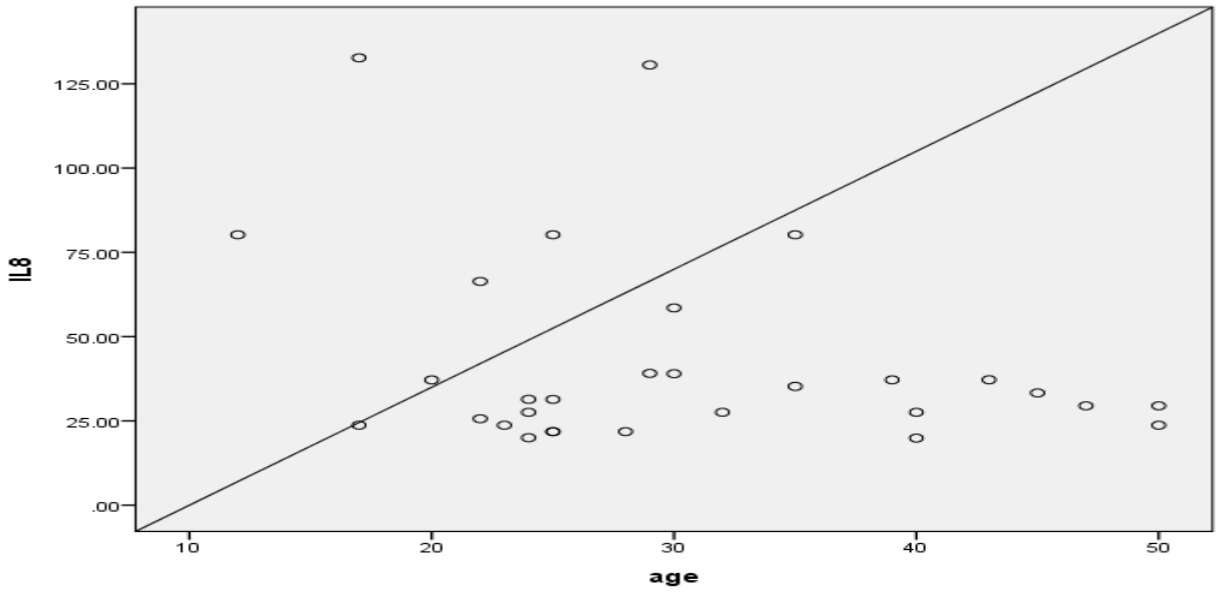

Figure (4): correlation between IL-8 level and age of cases. There was no correlation between IL-8 level and age of cases *P. value* 0.52.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

Chapter Five

Discussion, Conclusions and Recommendations

5.1. Discussion:

Interleukin-8 (IL-8) is an important mediator of inflammation and the immune response in human disease. IL-8 plays a key role in the body's defense mechanism by regulating neutrophil activity, but prolonged presence of inflammation induced IL-8 in circulation may cause variable degrees of tissue damage (Dong and Zheng , 2015).

In the present study plasma level of IL-8 was measured in 30 hepatitis B patients and in 30 apparently health control. The result relieve that the mean level of IL-8 in case group was higher than control and the difference was significant (*P.value* 0.042). This result was supported by Shin *et al* who demonstrated that significantly elevated IL-8 level in case than control *P.value* less than 0.05 (Shin *et al*., 2016). The mean level of IL-8 in the present study was agree with Xu *et al* result which show that IL-8 level was significantly increase in case than control *P.value* less than 0.05 (Xu *et al*., 2016). Another study conducted by Qian *et al* in china found that the mean level of IL-8 was significantly higher in hepatitis B patients than normal health individual (*P.value* < 0.05) (Qian *et al*.,2017). Similar result found by Bahramabadi *et al* which measured IL-8 in patients with CHB compared with healthy controls, serum levels of IL-8 were significantly increased (*P. value* less than 0.001) (Bahramabadi et al., 2017). Shata and his colleagues estimate the serum levels of IL-8 in 30 patients with HBV infection and 20 healthy control the result showed that IL-8 levels were increased in patients (*P. value* less than 0.05) (Shata et al., 2019) which supported my study.

In the present study there is no correlation between the mean level of IL-8 and age in both case and control *P.value* 0.524 and 0.994 respectively. This result agree with Tatsukawa *et al* *P.value* 0.645 (Tatsukawa *et al*., 2018) . Also similar result was accomplished by Xu *et al P.value* more than 0.05 (Xu *et al*., 2016). Also there were present study show no correlation between the mean level of IL-8 and gender in both case and control *P.value* was 0.334 and 0.341 respectively, this result confirmed by Tatsukawa *et al* which found no correlation between mean of IL-8 and gender *P.value* 0.119 (Tatsukawa *et al*., 2018).

In the present study there is no correlation between mean level of IL-8 and selected variables (Duration *P.value* 0.627, Treatment P.value 0.982, Blood transfusion P.value 0.486 and Jaundice P.value 0.339), similar result was found by Tatsukawa *et al* which found no correlation between IL-8 and treatment *P.value* 0.374 (Tatsukawa *et al*., 2018).

5.2. Conclusions:

- High plasma level of IL-8 in hepatitis B patients compared with control subjects with statistically significant differences (*P. values* 0.042).

- There was no correlation between IL-8 level and age and gender.

- There was no correlation between IL-8 level and duration of disease, treatment, blood transfusion and jaundice.

5.3. Recommendations:

1. The increase of sample size and patients population (chronic carriers, patient with liver cirrhosis and hepatocellular carcinoma) is likely to enhance our understanding of IL-8 roles and biological activities.

2. Furfure studies correlates between viral load and IL-8 level in hepatitis B infection.

3. Furfure studies should be done to evaluate the expression of IL-8 in liver tissues using PCR.

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APPENDICES

Appendix (1)

Sudan University of Sciences and Technology

Collage of Post Graduates

Evaluation of Interleukin-8 Levels in Sudanese Hepatitis B Patients

تقييم مستوى المادة الخلوية 8 في السودانين المصابين بالتهاب الكبد ب الفيروسي

_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: { }

Appendix (2)

IL-8 ELISA Kits

Appendix (3)

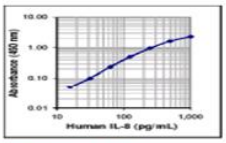
Assay Procedure Summary

1. Coat plate with 100 µL diluted Capture Antibody. Incubate overnight, 4°C
2. Wash 4 times. Add 200 µL 1X Assay Diluent A. Incubate 1 hr, RT, shaking
3. Wash 4 times. Add 100 µL diluted standards and samples. Incubate 2 hrs, RT, shaking
4. Wash 4 times. Add 100 µL diluted Detection Antibody. Incubate 1 hr, RT, shaking
5. Wash 4 times. Add 100 µL Avidin-HRP. Incubate 30 min, RT, shaking
6. Wash 5 times. Add 100 µL Substrate Solution C. Incubate 15 min, RT, in the dark
7. Add 100 µL Stop Solution
8. Read absorbance at 450 nm and 570 nm

For other technical resources, please visit: www.biolegend.com/support or email: techserv@biolegend.com

Typical Data

Standard Curve: This standard curve was generated at BioLegend for demonstration purposes only. A standard curve must be run with each assay.



Performance Characteristics

Sensitivity: The expected minimum detectable concentration of IL-8 for this set is 8 pg/mL.
Specificity: No cross reactivity was observed when this kit was used to analyze 14 human recombinant cytokines.

Troubleshooting

High Background:
- Background wells were contaminated.
- Matrix used had endogenous analyte.
- Plate was insufficiently washed.
- TMB Substrate Solution was contaminated.

No signal:
- Incorrect or no antibodies were added.
- Avidin-HRP was not added.
- Substrate solution was not added.
- Wash buffer contains sodium azide.

Low or poor signal for the standard curve:
- Standard was incompletely reconstituted or was stored improperly.
- Reagents were added to wells with incorrect concentrations.
- Plate was incubated with improper temperature, timing or agitation.

Signal too high, standard curves saturated:
- Standard was reconstituted with less volume than required.
- One or more reagent incubation steps were too long.
- Plate was incubated with inappropriate temperature, timing, or agitation.

Sample readings out of range:
- Samples contain no or below detectable levels of analyte.
- Samples contain analyte concentrations greater than highest standard point.

High variations in samples and/or standards:
- Pipetting errors may have occurred.
- Plate washing was inadequate or nonuniform.
- Samples were not homogenous.
- Samples or standard wells were contaminated.

BioLegend
The path to legendary discovery™

Human IL-8

ELISA MAX™ Deluxe Sets

Cat. No. 431504 (5 Plates)
431505 (10 Plates)
431506 (20 Plates)

BioLegend's ELISA MAX™ Deluxe Sets contain the components necessary for the accurate quantification of natural and recombinant human IL-8. These sets are designed for cost-effective and accurate quantification of human IL-8 in cell culture supernatant, serum, plasma or other biological fluids. They are sensitive, accurate, and robust.

It is highly recommended that this instruction sheet be read in its entirety before using this product. Do not use this set beyond the expiration date.

Materials Provided

1. Human IL-8 ELISA MAX™ Capture Antibody (200X)
2. Human IL-8 ELISA MAX™ Detection Antibody (200X)
3. Human IL-8 Standard
4. Avidin-HRP (1000X)
5. Substrate Solution C
6. Coating Buffer A (5X)
7. Assay Diluent A (5X)
8. Matrix Diluent A (for serum and plasma samples)
9. NUNC Maxisorp™ 96 MicroWell Plates
10. Instruction Sheet
11. Lot-Specific Instruction/ Analysis Certificate

Introduction

Human IL-8 is a member of the alpha (C-X-C) subfamily of chemokines. In response to proinflammatory stimuli, IL-8 is produced by monocytes, macrophages, T cells, neutrophils, and fibroblasts. IL-8 promotes neutrophil chemotaxis and degranulation leading to local inflammation in damaged or infected tissues.

Principle of the Test

BioLegend's ELISA MAX™ Deluxe Set is a sandwich Enzyme-linked Immunosorbent Assay (ELISA). A human IL-8 specific monoclonal antibody is first coated on a 96-well plate. Standards and samples are added to the wells, and IL-8 binds to the immobilized capture antibody. Next, a biotinylated anti-human IL-8 detection antibody is added, producing an antibody-antigen-antibody "sandwich". Avidin-horseradish peroxidase is subsequently added, followed by TMB Substrate Solution, producing a blue color in proportion to the concentration of IL-8 present in the 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.

For research purposes only. Not for use in diagnostic or therapeutic procedures.

Rev. 090111

Materials to be Provided by the End-User

- A microplate reader capable of measuring absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 2 µL to 1 mL
- Deionized (DI) water
- PBS (Phosphate-Buffered Saline): 8.0 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, add deionized water to 1 L; pH to 7.4, 0.2 µm filtered.
- Wash Buffer: PBS with 0.05% Tween-20. PBS: 8.0 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, add deionized water to 1 L; pH to 7.4. Add 0.5 mL of Tween-20, mix well. BioLegend Cat. No. 421601 is recommended.
- Wash bottle or automated microplate washer
- Stop Solution (2N H_2SO_4)
- Log-Log graph paper or software for data analysis
- Tubes to prepare standard dilutions
- Timer
- Plate Sealer
- Absorbent paper

Storage Information

- Store kit components at 4°C.
- After reconstitution of the lyophilized standard with 1X Assay Diluent A, aliquot into polypropylene vials and store at -70°C for up to one month. Avoid repeated freeze/thaw cycles.
- Prior to use, bring all components to room temperature (18-25°C). Upon assay completion return all components to appropriate storage conditions.

Health Hazard Warnings

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online for details (www.biolegend.com/support/#msds).
2. Substrate Solution C is harmful if ingested. Additionally, avoid skin, eye or clothing contact.
3. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum and/or plasma in accordance with NCCLS regulations.

Specimen Collection and Handling

Cell Culture Supernatant: If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at < -20°C. Avoid repeated freeze/thaw cycles.
Serum: Use a serum separator tube and allow clotting for at least 30 minutes, then centrifuge for 10 minutes at 1,000 X g. Remove serum layer and assay immediately or store serum samples at < -20°C. Avoid repeated freeze/thaw cycles. Serum specimens should be clear and non-hemolyzed.
Plasma: Collect blood sample in a citrate, heparin or EDTA containing tube. Centrifuge for 10 minutes at 1,000 X g within 30 minutes of collection. Assay immediately or store plasma samples at < -20°C. Avoid repeated freeze/thaw cycles. Plasma specimens should be clear and non-hemolyzed.

Reagent Preparation

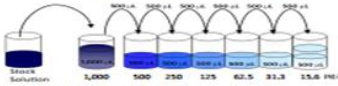
Do not mix reagents from different sets or lots. Reagents and/or antibodies from different manufacturers should not be used with this set. All reagents should be diluted immediately prior to use.

1. Dilute 5X Coating Buffer A to 1X with deionized water. For one plate, dilute 2.4 mL 5X Coating Buffer A in 9.6 mL deionized water.
2. Dilute pre-titrated Capture Antibody 1:200 in 1X Coating Buffer A. For one plate, dilute 60 µL Capture Antibody in 11.94 mL 1X Coating Buffer.
3. Dilute 5X Assay Diluent A to 1X with PBS (pH 7.4). For 50 mL, dilute 10 mL 5X Assay Diluent A in 40 mL PBS.
   NOTE: Precipitation of Matrix Diluent A may be observed when stored long term at 4°C. The precipitation does not alter the performance of the assay. If heavy precipitation is observed, it can be filtered to clarify the solution.
4. Lyophilized vials are under vacuum pressure. Reconstitute lyophilized standard with 0.2 mL of 1X Assay Diluent A. Allow the reconstituted standard to sit for 15 minutes at room temperature, then mix gently prior to making dilutions.
5. Prior to use, prepare 1,000 µL of the top standard from the stock solution in 1X Assay Diluent A (refer to Lot-Specific Instruction/Analysis Certificate) at a concentration of 1,000 pg/mL for measuring cell culture medium sample or 2,000 pg/mL for measuring serum or plasma samples.
6. Dilute the pre-titrated Biotinylated Detection Antibody 1:200 in 1X Assay Diluent. For one plate, dilute 60 µL Detection Antibody in 11.94 mL 1X Assay Diluent A.
7. Dilute Avidin-HRP 1:1000 in 1X Assay Diluent A. For one plate, dilute 12 µL Avidin-HRP in 11.99 mL 1X Assay Diluent A.

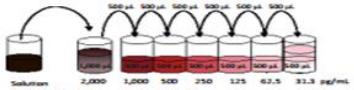
Assay Procedure

Do not use sodium azide in any solutions as it inhibits the activity of the horseradish-peroxidase enzyme.

1. One day prior to running the ELISA, dilute Capture Antibody in 1X Coating Buffer A as described in Reagent Preparation. Add 100 µL of this Capture Antibody solution to all wells of a 96-well plate provided in this set. Seal plate and incubate overnight (16-18 hrs) at 4°C.
2. Bring all reagents to room temperature (RT) prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.
3. Wash plate 4 times with at least 300 µL Wash Buffer per well and blot residual buffer by firmly tapping plate upside down on absorbent paper. All subsequent washes should be performed similarly.
4. To block non-specific binding and reduce background, add 200 µL 1X Assay Diluent A per well.
5. Seal plate and incubate at RT for 1 hour with shaking at 200 rpm on a plate shaker.
6. While plate is being blocked, prepare the appropriate sample dilutions (if necessary) and standards.
7. For measuring cell culture medium sample:
   - Prepare 1,000 µL of top standard at 1,000 pg/mL from stock solution in 1X Assay Diluent A (refer to Reagent Preparation). Perform six two-fold serial dilutions of the 1,000 pg/mL top standard with 1X Assay Diluent A in separate tubes. After diluting, the human IL-8 standard concentrations are 1,000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, and 15.6 pg/mL, respectively. 1X Assay Diluent A serves as the zero standard (0 pg/mL).



   - Wash plate 4 times with Wash Buffer.
   - Add 100 µL/well of standards and cell culture medium samples to the appropriate wells. If needed, samples can be further diluted with 1X Assay Diluent A before adding 100 µL/well diluted samples.
8. For measuring serum or plasma samples:
   - Prepare 1,000 µL of top standard at 2,000 pg/mL from stock solution in 1X Assay Diluent A (refer to Reagent Preparation). Perform six two-fold serial dilutions of the 2,000 pg/mL top standard with 1X Assay Diluent A in separate tubes. After diluting, the human IL-8 standard concentrations are 2,000 pg/mL, 1,000 pg/mL 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, and 31.3 pg/mL, respectively. 1X Assay Diluent A serves as the zero standard (0 pg/mL).



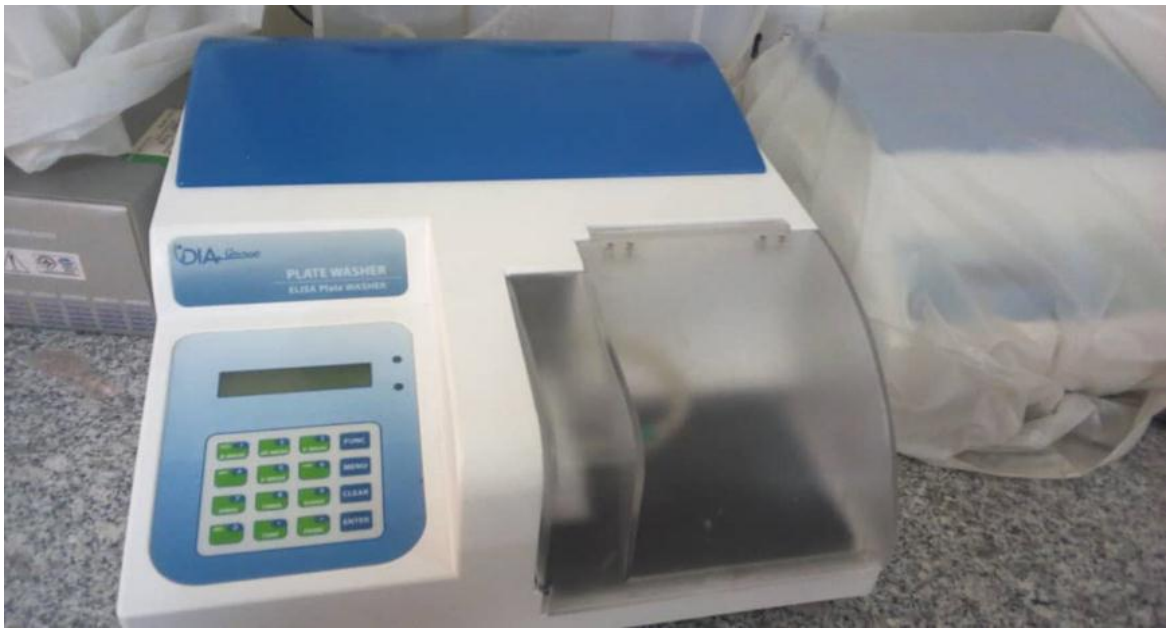
   - Wash plate 4 times with Wash Buffer.
   - Add 50 µL/well of Matrix provided to the standard wells. Add 50 µL/well of Assay Diluent A to the sample wells.
   - Add 50 uL/well of standards prepared above to the standard wells. Add 50 uL/well of serum or plasma samples to the sample wells. If needed, samples can be further diluted with Matrix provided before adding 50 µL/well diluted samples.
9. Seal plate and incubate at RT for 2 hours with shaking.
10. Wash plate 4 times with Wash Buffer.
11. Add 100 µL of diluted Detection Antibody solution to each well, seal plate and incubate at RT for 1 hour with shaking.
12. Wash plate 4 times with Wash Buffer.
13. Add 100 µL of diluted Avidin-HRP solution to each well, seal plate and incubate at RT for 30 minutes with shaking.
14. Wash plate 5 times with Wash Buffer. For this final wash, soak wells in Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.
15. Add 100 µL of TMB Substrate Solution C and incubate in the dark for 15 minutes*. Positive wells should turn blue in color. It is not necessary to seal the plate during this step.
16. Stop reaction by adding 100 µL of Stop Solution to each well. Positive wells should turn from blue to yellow.
17. Read absorbance at 450 nm within 30 minutes. If the reader can read at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

*Optimal substrate incubation time depends on laboratory conditions and the optical linear ranges of ELISA plate readers.

Calculation of Results

Plot the standard curve on log-log axis graph paper with analyte concentration on the x-axis and absorbance on the y-axis. Draw a best fit line through the standard points. To determine the unknown analyte concentrations in the samples, find the absorbance value of the unknown on the y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the x-axis and read the corresponding analyte concentration. If the samples were diluted, multiply by the appropriate dilution factor. The data is best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm. If a test sample's absorbance value falls outside the standard curve ranges, that test sample needs to be reanalyzed at a higher or lower dilution as appropriate.

Appendix (4)



(a) ELISA Washer

(b) ELISA Reader

Appendix (5)

ELISA micro plate