Sudan University of Sciences and Technology

College of Graduate Studies

Estimation of plasma Interleukin-10 Levels among neonatal sepsis patients in Khartoum State

قياس مستويات المادة الخلوية البلازمية10 وسط حديثي الولادة المصابين بالانتان الوليدي في ولاية الخرطوم

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الآية

بسم الله الرحمن الرحيم

وَقَضَىٰ رَبُّكَ أَلَّا تَعْبُدُوا إِلَّا إِيَّاهُ وَبِالْوَالِدَيْنِ إِحْسَانًا ۚ إِمَّا يَبْلُغَنَّ عِنْدَكَ الْكِبَرَ أَحَدُهُمَا أَوْ كِلَاهُمَا فَلَا تَقُلْ لَهُمَا أُفٍّ وَلَا تَنْهَرْهُمَا وَقُلْ لَهُمَا قَوْلًا كَرِيمًا ﴿23﴾ وَاخْفِضْ لَهُمَا جَنَاحَ الذُّلِّ مِنَ الرَّحْمَةِ وَقُلْ رَبِّ ارْحَمْهُمَا كَمَا رَبَّيَانِي صَغِيرًا ﴿24﴾

صدق الله العظيم

سورة الإسراء الآيات 23-24

Dedication

To my parents soul which they a strong and gentle soul they taught me to trust Allah , believe in hard work and that so much could be done with little ,fate wanted them not to complete the journey with me, my God have mercy on them.

To my respectful brother, sisters .

To my husband.

To my friends.

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

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Abstract

IL-10 is a molecule with immune-regulatory properties, secretion of it in sepsis could limit and ultimately terminate inflammatory responses , which called as anti-inflammation cytokine.

This is a case-control study that aimed to evaluate the IL-10 level in neonatal sepsis cases and in neonates control subjects in Khartoum state during the period from June 2020 to May 2021 .

Fifty two subjects, selected randomly in this study, with age varies from 1- 13 day, 26 subject were septic neonates (13 males ,and 13 females) ,26 were healthy neonates (13 males and 13 females).

Venous blood sample (1ml) was collected in EDTA container from each subject.

IL-10 concentration was measured using Enzyme Linked Immunosorbent Assay (ELISA) in lab of Institute of Endemic Diseases. The data was analyzed using Statistical Package Social Science programme (Version 20). independent sample t-test was used to compare between means. P. value significant when ≤ 0.05.

It was found the mean of IL-10 were 117.9±117 and 10.66±6.7 in the case group and control group respectively . IL-10 level was significantly elevated in neonatal sepsis than the control group (P. value 0.000). The difference in mean of IL-10 between males and females in the case group of this study was not statistically significant (P. value 0.952). There was no statistical correlation between IL-10 level and age ,and IL-

10 level and weight in study group (P. value 0.391 , 0.291) respectively .

There was no statistical correlation between level of IL-10 and duration (early / late) of sepsis (p. value 0.993) ,And level of IL-10 between term and pre term neonates (p. value 0.320).

The study concluded that in the studied population, IL-10 concentration may be useful as prognostic marker for neonatal sepsis.

مستخلص البحث

المادة الخلوية 10 هو جزيء له خصائص تنظيمية للمناعة ، وإفرازه في تعفن الدم يمكن أن يحد من الأستجابات الالتهابية ويؤدي لنهايتها ، والتي تسمى السيتوكين المضاد للالتهاب.

هدفت هذه الدراسة والتى هي عبارة عن دراسة الحالات والشواهد لتقييم مستوى المادة الخلوية 10 في حالات الإنتان الوليدي مقارنة مع حديثي الولادة الطبيعيين في ولاية الخرطوم خلال الفترة من يونيو 2020 إلى مايو 2021 .

تم اختيار اثنان وخمسين فردا عشوائيا لهذه الدراسة وكانت اعمارهم تتراوح من 1-13 يوما ,وهم عبارة عن 26 مريضا من مرضى الانتان الوليدي (13 ذكر و13 انثى) و26 من حديثي الولادة الطبيعيين(13 ذكر و13 انثى) .

سحبت 1مل عينة وريديه من كل مشارك في انبوبة تحتوى على مانعة تجلط EDTA.

قيس تركيز المادة الخلوية 10عن طريق فحص الممتز المناعي المرتبط بالانزيم في معمل معهد الأمراض المتوطنة . حللت البيانات باستخدام الحزمة الاحصائية للمجتمع (نسخه 20) استخدم اختبار T لمقارنه الاوساط وكانت القيمة المطلقة متوافقة عند اقل من 0,05 .

وجد أن الوسط الحسابي للمادة الخلوية 10 117,9± 117 , 10,66±6,7 لدى كل من مرضى الانتان الوليدي وحديثي الولادة الطبيعيين بالترتيب . لقد وجد ان هنالك ارتفاع ذو دلالة احصائية في معدل المادة الخلوية 10 لدى حديثي الولادة المصابين بالانتان الوليدي مقارنة بحديثي الولادة الطبيعيين(كانت القيم الاحتمالية 0,000). لا توجد دلالة وصفيه حسابية للمادة الخلوية 10 بين حديثي الولادة الذكور والاناث المصابين بالانتان الوليدي في هذه الدراسة (القيم الاحتمالية 0,952) ,كما لم يكن هنالك علاقة احصائية بين معدل المادة الخلوية 10 والعمر, والمادة الخلوية 10 والوزن في مجموعه الدراسة (القيم الاحتمالية 0,391 0,291) على التوالي .

ليس هنالك علاقة بين معدل المادة الخلوية 10و المدة (المبكرة / المتأخرة) للإنتان الوليدي(القيم الاحتمالية 0,993), ومعدل المادة الخلوية 10 بين حديثي الولادة مكتملي النمو والخدج (القيم الاحتمالية 0,320).

لقد توصلت هذه الدارسة الى ان تركيز المادة الخلوية 10 يمكن ان يستخدم كعلامة للتكهن بالانتان الوليدي

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List of abbreviations

ANC	**Absolute neutrophil count**
BHI	**Brain heart infusion**
CD	**Cluster of differentiation**
CKs	**Chemokines**
CLR	**C-type lectin receptors**
CONS	**Coagulase –negative Staphylococci**
CRP	**C-reactive protein**
CSF	**Cerebrospinal fluid**
DC	**Dendritic cell**
DNA	**Deoxyribo nucleic acid**
EDTA	**Ethylene Di-ammine Tetra acetic acid**
ELBW	**Extremely low birth weight**
ELISA	**Enzyme linked immunosorbent assay**
EOS	**Early-onset neonatal sepsis**
fasL	**Fas Ligand**
GA	**Gestational age**
GBS	**Group B *streptococci* .**
GI	**Gastro intestinal**
HIE	**Hypoxic –Ischemic encephalopathy**
HSV	**Herpes Simplex virus**
IFN	**INertferon**
IL	**Interleukin**
ILCs	**Innate lymphoid Cells**
JAK	**Janus Kinase**
KD	**Kilo Dalton**
LOS	**Late-onset neonatal sepsis .**
LPS	**Lipo poly-saccharide**

MAS	**Meconium Aspiration syndrome**
MSAF	**Meconium-stained amniotic fluid**
NICU	**Neonatal Intensive care Unit**
NK	**Natural killer cell**
NLRs	**NOD like receptors**
NOD	**Nucleotide binding oligomerization Domain containing protein**
P.V	**Probability value**
PCR	**polymeraze chain reaction**
PCT	**Procalcitonin**
Pg	**Pico gram**
PPROM	**Preterm prelabour rupture of the fetal membranes.**
PRRs	**Pattern recognition receptors**
RDS	**Respiratory Distress syndrome**
RDW	**Red cell Distribution width**
RIG-1	**Retinoic acid inducible gene-1**
RLR	**RIG-1 like receptors**
Rpm	**Rotate per minute**
RSV	**Respiratory syncytial virus**
SAA	**Serum Amyloid A**
SPSS	**Statistical package for social science**
TGF	**Tissue Growth Factor**
Th	**T-helper cell**
TLRs	**Toll-like Receptors**
TMB	**Tetra-methylbenzidine**
TNF	**Tumor necrosis factor**
Treg	**T-regulatory cell**

TTN	**Transient Tachypnea of the neoborn**
U.S.	**United state**
VLBW	**Very low birth weight**
VLOS	**Very late-onset neonatal sepsis.**
WBC	**white blood cell**

CHAPTER I
INTRODUCTION

Chapter I

INTRODUCTION

1.1.Introduction

The term neonatal sepsis is used to designate a systemic condition of bacterial, viral, or fungal (yeast) origin that is associated with haemodynamic changes and other clinical manifestations occurring in infants at 28 days of life and results in substantial morbidity and mortality (Edwards and Baker , 2004) .Despite years of clinical experience with the care of neonates with confirmed or suspected sepsis, challenges remain including the absence of a consensus definition of neonatal sepsis (Wynn *et al*., 2014). In 2010 worldwide, 7.6 million children less than 5 years old died, predominantly because of infectious causes including sepsis; neonatal deaths (in the first 28 days of life), accounted for 40% of the total lives lost(Liu *et al*.,2012). The prevalence of neonatal sepsis in Sudan was 17.5% and the mortality was 14.5% (Kheir and Khair , 2014).

Neonatal sepsis is classified into early or late according to the different ages at onset of infection during the neonatal period (Kliegman *et al*., 2011). The clinical relevance of this distinction is that early-onset disease is often due to organisms acquired during delivery while, late-onset disease is more frequently caused by organisms acquired from nosocomial or community sources (Robinson *et al*., 2008).

Neonates with bacterial sepsis might show non-specific signs and symptoms or focal signs of infection, including temperature instability, hypotension, poor perfusion with pallor and mottled skin, metabolic

acidosis, tachycardia or bradycardia, apnoea, respiratory distress, grunting, cyanosis, irritability, lethargy, seizures, feeding intolerance, abdominal distention, jaundice, petechiae, purpura, and bleeding (Shane *et al*.,2017).

In developed countries, *Streptococcus agalactiae* (GBS) and *Escherichia coli* were the most common pathogens isolated in severe cases (Le Doare and Heath , 2013). Early-onset neonatal sepsis(EOS) is caused by GBS, *E. coli, Streptococcus viridans*, *Staphylococcus aureus , and Haemophilus influenzae* (Weston *et al* .,2011).

Blood culture has been the gold standard for confirmation of the diagnosis but the sensitivity is low and result are usually not promptly obtained (Liu *et al*.,2014). Other study on various diagnostic markers like hematological indices, acute phase reactants, C-reactive protein, procalcitonin, cytokines, and cell surface markers among others (Shah and Padbury,2014).

Cytokines are relatively small molecules with short serum half-life(from minutes to a few hours)and play a central role in immune response in neonates with sepsis. During sepsis, cytokine levels may be observed in picograms per milliliter of plasma or in nanograms or even micrograms per milliliter (De Jong *et al*.,2010).

Cytokines are regulators of the immune response to infection and play a key role in regulating inflammation and trauma. There are two types of cytokines: Pro-inflammatory cytokines stimulate systematic inflammation, whereas anti inflammatory cytokines inhibit inflammation and enhance healing .Host immunosuppression may be responsible for late death in patients with sepsis (Chaudhry *et al*.,2013).

1.2. Rationale

Neonatal sepsis is along with high neonatal morbidity (1-10 per 1000 live birth) and mortality (15-50%), especially in preterm babies (Boskabadi and Zakerihamidi ,2017).

Early, accurate, and rapid diagnosis of neonatal sepsis remains a major diagnostic challenge in neonatology, revealing the need for reliable and timely diagnostic biomarkers to enable clinicians to efficiently diagnose sepsis risk during the early phases of sepsis (Mally *et al*.,2014).Bacterial cultures are time-consuming because that ,the recent interest has shifted to chemokines, cytokines and interferons whose circulating levels may be different in healthy and infected subjects ,to avoid unnecessary treatment of noninfected neonates (Prashant *et al*.,2013).

There are no published data about IL-10 among Sudanese neonatal sepsis patients.

1.3.Objectives

1.3.1.General objective

To evaluate plasma level of IL-10 among neonatal sepsis patients in Khartoum state hospitals during the period from June 2020 to May 2021 .

1.3.2.specific objectives

-To measure plasma level of IL-10 in neonatal sepsis patients(case group) and in healthy one (control group) using Enzyme linked immune sorbent assay .

-To compare between the result of IL-10 in neonatal sepsis patients and normal controls .

- To compare mean plasma levels of IL10 in neonates with early onset neonatal sepsis and late onset neonatal sepsis.

-To compare mean of plasma level of IL10 in term and preterm neonates among the study groups.

- To compare mean of plasma level of IL10 in study groups according to gender.

Chapter II

Literature Review

Chapter II

Literature Review

2.0.Background

Sepsis is a life-threating organ dysfunction caused by a dysregulated host response to infection (Singer *et al*.,2016). Neonatal sepsis is considered one of the major causes of morbidity and mortality among term and preterm infants in neonatal intensive care units (NICU) (Camacho-Gonzalez *et al*.,2013). May be classified with regard to its onset into : early onset sepsis (EOS); which occurs within the first 72 h of life, is generally associated with the acquisition of microorganisms from the mother and usually presents with respiratory distress and pneumonia. The source of the infection is commonly the maternal genital tract and the microorganisms, late-onset sepsis (LOS); usually presents after 72 h of birth, it may either be caused by peri or postnatally acquired organisms, while it usually occurs as a consequence of nosocomial transmission, and very late-onset sepsis (VLOS); is usually diagnosed in extremely low birth weight (ELBW) infants who remain hospitalized for several weeks after birth. The major factors increasing the sepsis risk in these infants include the intravascular catheters required for their care, prolonged exposure to antimicrobial agents, and ongoing immature host defense mechanisms (Ozkan *et al* ., 2014).

The most important neonatal factor predisposing to infection that could result in sepsis is prematurity or low birth weight. Preterm low birth weight infants have a 3–10 times higher incidence of infection than full-term normal birth weight infants. Immune dysfunction and an absence of transplacentally acquired maternal IgG antibodies in premature infants

might increase risk of infection,additionally, preterm infants often require prolonged intravenous access, endotracheal intubation, or other invasive procedures that provide a portal of entry or impair barrier and clearance mechanisms, placing them at increased risk for hospital-acquired infections. Furthermore, lower neonatal 25-hydroxyvitamin D concentrations have been associated with early-onset sepsis (Cetinkaya *et al* .,2015).

2.1. Risk factor of neonatal sepsis

2.1.1. Preterm prelabour rupture of the fetal membranes (PPROM)

Is defined as rupture of membranes prior to the onset of active labour, irrespective of gestational age (Gezer *et al*.,2013). precedes 30% of preterm births and is a risk factor for early onset neonatal sepsis. As PPROM is strongly associated with ascending vaginal infection, prophylactic antibiotics are widely used (Brown *et al*.,2018).

2.1.2. Respiratory Distress Syndrome (RDS)

Also known as hyaline membrane disease, is a common cause of respiratory disease in the premature infant. RDS is also seen in infants whose mothers have diabetes in pregnancy. RDS is caused by a deficiency of alveolar surfactant, which increases surface tension in alveoli, resulting in micro atelectasis and low lung volumes. Surfactant deficiency appears as diffuse fine granular infiltrates on radiograph. Pulmonary edema plays a central role in the pathogenesis of RDS and contributes to the development of air bronchograms, excess lung fluid is attributed to epithelial injury in the airways, decreased concentration of sodium-absorbing channels in the lung epithelium, and a relative oliguria in the first 2 days after birth in premature infants (Reuter *et al*.,2014).Normally, the newborn’s respiratory rate is 30 to 60 breaths per

minute. Tachypnea is defined as a respiratory rate greater than 60 breaths per minute (Edwards *et al*.,2013; Warren and Anderson,2010). Tachypnea is a compensatory mechanism for hypercarbia, hypoxemia, or acidosis ,both metabolic and respiratory (West ,2012).

2.1.3.Transient Tachypnea of the Newborn(TTN)

Results from failure of the newborn to effectively clear the fetal lung fluid soon after birth. TTN represents the most common etiology of respiratory distress in term gestation newborns and sometimes requires admission to the neonatal intensive care unit. TTN can lead to maternal-infant separation, the need for respiratory support, extended unnecessary exposure to antibiotics and prolonged hospital stays (Alhassen *et al*.,2020).

2.1.4.Meconium Aspiration Syndrome(MAS)

Meconium –stained amniotic fluid occurs(MSAF) when the fetus passes meconium before birth. Infants born through MSAF are at risk for aspiration of meconium in utero or immediately after birth. Any infant who is born through MSAF and develops respiratory distress after delivery, which cannot be attributed to another cause, is diagnosed as having MAS. Meconium is composed of lanugo, bile, vernix, pancreatic enzymes, desquamated epithelia, amniotic fluid, and mucus. Meconium is present in the gastrointestinal tract as early as 16 weeks' gestation but is not present in the lower descending colon until 34 weeks' gestation; therefore, MSAF is seldom seen in infants younger than 37 weeks' gestation (Yeh ,2010).

2.1.5.Hypoxic-ischemic encephalopathy (HIE)

The condition is typically diagnosed in newborns with neonatal acidemia, low Apgar scores, and clinical signs of encephalopathy, such as seizures or abnormal consciousness. Infants with HIE are at high risk for brain injury and neonatal death. Survivors often suffer from long-term neurodevelopmental disabilities such as cerebral palsy and cognitive impairmen (Shankaran *et al*.,2012).

2.2.Causes of neonatal sepsis

2.2.1.Bacterial causes of sepsis

2.2.1.1.GBS(*Streptococcus agalactiae*)

GBS are Gram-positive encapsulated bacteria for which 10 different serotypes have been identified; with serotype III strains responsible for the majority of disease (54%) (Imperi *et al*.,2011).The U.S. incidence of EOS overall from 2005 to 2008 was 0.76 to 0.77cases/1,000 live births ,Recent population-based surveillance studies in the United States revealed GBS as the etiological agent of EOS in 38 to 43% of all bacterial sepsis cases, with the incidence of neonatal GBS sepsis estimated to be 0.29 to 0.41/ 1,000 live births . The majority of these GBS EOS cases,73%,were in term neonates (Stoll *et al* .,2011 ; Weston *et al* .,2011).

2.2.1.2. *Escherichia coli*

A gram-negative rod that commonly colonizes the maternal urogenital and GI tracts, is considered the second most common cause of neonatal sepsis in term infants and the most common cause in VLBW neonates with rates of 5.09 per 1000 live births (Tsai *et al*.,2012). The antigenic structure of *E.coli* is represented by multiple antigens (O), (K) and (H) which in combination account for the genetic diversity of the bacteria.

Strains with the K1 antigen have been associated with the development of neonatal sepsis and meningitis as well as with increased risk of mortality when compared with K1 negative strains (Kaczmarek *et al*.,2012).

The most common organisms associated with early-onset neonatal sepsis are *Streptococcus agalactiae* (GBS) and *Escherichia coli*. In almost 400 000 live births from 2006 to 2009 at academic-based neonatal centers in the USA, 389 newborn infants had early-onset infection (0·98 cases per 1000 live births) with 43% due to GBS (0·41 per 1000 live births) and 29% to *E. coli* (0·28 per 1000 live births). Most infants with GBS infections were full term (73%) although 81% of those with *E. coli* infections were preterm; infection rates increased with decreasing birth weight. Case fatality rate overall was 16%, but it was inversely related to gestational age: 54% at 22–24 weeks, 30% at 25–28 weeks, 12% at 29–33 weeks, and 3% at more than 37 weeks' gestation. Although 9% of sepsis and 33% of infants with *E. coli* sepsis died, infants with a GBS the risk of death was not significantly higher for infants with sepsis associated with *E .coli* infection compared with sepsis associated with GBS infection after adjustment for gestational age (Stoll *et al*.,2011 ; Verani *et al*.,2010).

2.2.1.3. Gram-negative rods

Are less frequent causes of EOS but remain very important causes of LOS and are of increasing importance related to growing antimicrobial resistance concerns. Among the *Enterobacteriaceae* , *Enterobacter* spp., *Klebsiella spp*., and *Serratia spp*. are important causes of sepsis and possess polysaccharide capsules which contribute to their virulence by preventing opsonization, phagocytosis, and bacterial lysis. *Citrobacter* spp. and *Cronobacter sakasakiia* ccount for 5% of bacterial sepsis cases

in VLBW infants but are important due to their association with meningitis with brain abscesses and subsequent significant neurological sequelae (Hunter and Bean,2013).

2.2.1.4. Coagulase-negative Staphylococci (CONS)

Have emerged as the predominant pathogens of LOS, accounting for 53.2%–77.9% of LOS in industrialised countries and 35.5%–47.4% in some developing regions (Dong and Speer , 2014). late-onset sepsis is predominantly caused by *Staphylococci* species and most frequently related with low birth weight of infants and use of intravascular catheters, endo-tracheal intubation, assisted ventilation, surgery, contact with hand of colonized personnel and contact with contaminated equipment as the main risk factors for late onset of neonatal sepsis (Mohammad *et al* .,2011 ; Shaw *et al*.,2015).

2.2.2.Viral causes of neonatal sepsis

Viral infections, including herpes simplex virus (HSV), enteroviruses, and parechoviruses, are also implicated in early-onset neonatal sepsis and must be clinically differentiated from bacterial sepsis .There are other viruses associated with congenital infections, such as rubella virus, cytomegalovirus, lymphocytic choriomeningitis virus, and human immunodeficiency virus, for example. Additional seasonal viruses, including influenza virus, respiratory syncytial virus (RSV), adenoviruses, rhinoviruses, and rotaviruses, have been identified in hospitalized neonates, related primarily to horizontal transmission (Simonsen *et al* ., 2014).

2.2.3.Fungal causes neonatal sepsis

Approximately 2.5% of all bloodstream infections in VLBW neonates are estimated to be due to fungal etiologies(Kaufman ,2010).The risk for fungal sepsis is increased by colonization acquired vertically from maternal sources as well as horizontally from the NICU environment. A positive correlation exists between multiple sites of colonization and risk for invasive candidiasis(Leibovitz,2012).

EOS and LOS can also be caused by fungal infections, most often *Candida albicans* or *Candida parapsilosis*, found primarily in preterm VLBW infants(Voller and Myers,2016).

2.3.Immunopathology of neonatal sepsis

The innate immune system is considered to be a rapid, first-line of defense. Cells of the innate system comprise primarily myeloid cells such as macrophages, neutrophils, basophils, eosinophils, monocytes, and innate lymphoid cells. The adaptive immune system has the ability to acquire ‘immunological memory’ to a wide variety of antigens, thereby resulting in improved immune responses over subsequent pathogen encounters, cells of the adaptive system are primarily composed of B and T lymphocytes. The development of immunological memory by the adaptive immune system involves the process of affinity maturation, which occurs through hypermutation of nucleotide sequences in the hypervariable regions of genes encoding T cell and B cell receptors,most recent data indicate that the innate immune system can also undergo a form of immunological memory termed ‘training’. Similar to adaptive immune responses, this training alters functional responses to subsequent pathogen exposures, and evidence suggests that this training is mediated through epigenetic changes (Saeed *et al* .,2014). The immune system of neonates is not completely developed, and thus they are more prone to bacterial infection. In neonatal period, innate immune cells including

macrophages and dendritic cells (DCs) initiate an effective immune response. Cytokines produced by macrophages and DCs play an important role during host defense mechanisms in response to infectious pathogens (Cloherty *et al* ., 2012 ; Hotoura *et al*.,2011).

The increased susceptibility of the neonate to widespread bacteremia can be associated with a quantitative deficit in neonatal neutrophils. Due to lack of adaptive immunity, neonates primarily rely on innate, first-line defenses to protect them in the relatively immunodeficient state following birth, however, for many neonates the innate immune system does not mature fast enough to adequately defend against bacterial infection. TLRs bind to conserved molecular patterns on pathogens and initiate key events of the innate immune response. TLR4 is a transmembrane receptor for the gram negative bacterial product LPS. The expression of this receptor is markedly low in newborns and subsequently limits the extent to which bacterial-derived LPS can stimulate the monocyte production of inflammatory cytokines, including TNF-α, IL-1, and IL-6 (Melvan *et al*.,2010).

Monocytes have been better studied, partly for historical reasons and partly because of their relatively high abundance in cord blood. Like other immune cell types, monocytes recognize pathogens through PRRs designed to recognize conserved microbial structures. These comprise endosomal, cytoplasmic, and extracellular antimicrobial detectors such as Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-containing protein (NOD) and NOD-like receptors (NLRs), C-type lectin receptors (CLRs), and the retinoic acid-inducible gene I (RIG-I) and RIG-I-like receptors (RLRs) (Brubaker *et al*., 2015). Three main monocyte subsets have been defined in adult blood, according to their expression of the CD14 and CD16 cell surface markers**.** Before 29 weeks

of gestation, a majority of fetal monocytes display an immature phenotype characterized by low CD14 expression (Ziegler-Heitbrock , 2014) . These monocytes are likely to play a more predominant role in tissue remodeling than in aggressive immune responses (Lavoie and Levy,2016). Anti-microbial immune recognition and antigen presentation are also significantly impaired at this gestation even in "classical" high CD14-expressing monocytes, because of reduced receptor expression and intracellular signaling (Sharma *et al*.,2015 ; Krow-Lucal *et al* .,2014).

2.4.Epidemology of neonatal sepsis

For infants born at >34 weeks gestational age (GA), the incidence of EOS is 0.3–0.8/1,000 live births. Lower GA dramatically increases the risk for perinatal infection, as 4.8%–16.9% of preterm infants exposed to chorioamnionitis go on to develop culture-positive EOS, in contrast to only 0.47%–1.24% of similarly exposed infants born at ≥35 weeks GA (Polin *et al*.,2014; Benitz *et al*.,2015 ;and van Herk *et al*.,2016) , LOS has been shown to be associated with increased risk of bronchopulmonary dysplasia and neurological morbidities (Bassler *et* the incidence of LOS is inversely *al*.,2009 ; Haller *et al*., 2016). associated with birth weight (BW). Similarly, 36.3% of neonates with gestational age(GA) <28 weeks had at least one episode of LOS, as compared with 29.6%, 17.5% and 16.5% of moderately preterm (GA of 29–32 weeks), late preterm (GA of 33–36 weeks) and term infants (Tsai *et al*.,2014).

2.5.Clinical Symptoms of neonatal sepsis

Generally symptoms of EOS are respiratory distress, apnea, lethargy or irritability, temperature instability, and feeding difficulties. These symptoms are unspecific as many non-infected neonates display similar symptoms. During the first days of life, there is a dynamic adaption of different organ systems to extra-uterine life. A single-point, clinical assessment to diagnose EOS therefore seems impossible, some guidelines suggest that a respiratory rate > 50 or 60/min may be suggestive of an infection. However, in healthy infants the 95th percentile for the respiratory rate is 65/min at 2 h of age (Tveiten *et al*.,2016). Feeding difficulties are also notoriously difficult to assess during the first 1–2 days of life. A capillary refill time > 2 s is included as a sign of EOS in some sepsis criteria (Duvoisin *et al* .,2014; Koch *et al* ., 2015).

Clinical signs associated with LOS were increased respiratory support, capillary refill, grey skin and central venous catheter are the most important clinical signs suggestive of LOS in preterms. Clinical signs that are too non-specific to be useful in excluding or diagnosing LOS were temperature instability, apnoea, tachycardia, dyspnoea, hyper- and hypothermia, feeding difficulties and irritability (Bekhof *et al* ., 2013).

2.6. Diagnosis of neonatal sepsis

2.6.1.Hematological indices

Newman *et al* noted that a low white blood cell (WBC) count and absolute neutrophil count (ANC) with a higher band count closely correlated with the presence of EOS (Newman *et al* .,2010). Elevated red cell–distribution width (RDW) has been shown to be associated with increased mortality from sepsis in both adult and pediatric critical care populations (Han *et al*.,2018 ; Ramby *et al*.,2015).

2.6.2.C-reactive protein (CRP)

CRP is an acute-phase protein, released by the liver in response to stimulation by pro inflammatory cytokines. Elevation of CRP begins between 12 and 24 hours following the onset of infection, with levels peaking at 48 hours. Within the context of EOS, CRP has been shown to have a sensitivity of 9%–83%, with the majority of studies quoting values of 49%–68% when a cutoff value of 10 mg/L is used (Hofer *et al* .,2012). Specificity values are higher, consistently reported as >90%, even though elevations of CRP can be caused by uninfectious inflammatory processes, such as meconium aspiration and prolonged transition. In both LOS and EOS, accuracy of CRP as a diagnostic marker improves with serial measurements (Delanghe and Speeckaert , 2015; Perrone *et al* .,2017).

2.6.3.procalcitonin

is the hormone calcitonin, which is normally produced by the C cells of the thyroid gland, leading to massive release of PCT into the bloodstream depending on the severity of sepsis, Is a propeptide of calcitonin produced mainly by monocytes and hepatocytes that is significantly elevated during infections in neonates, children, and adults (Altunhan *et al* ., 2011). The procalcitonin level is more likely to be elevated during bacterial infections than during viral ones (Meem *et al* .,2011). and declines rapidly with appropriate therapy. However, a physiologic increase in the procalcitonin concentration occurs within the first 24 h of birth, and elevated levels in serum can occur under non infectious conditions (e.g., infants with respiratory distress syndrome, hemodynamic instability, and diabetic mothers). Procalcitonin is also useful for detecting neonatal nosocomial sepsis (Auriti *et al* ., 2012).

2.6.4.Serum amyloid A (SAA)

is an early acute phase reactant apolipoprotein and source of SAA is liver. The other sources of SAA are endothelial cells, monocytes, and smooth muscle cells. The synthesis of SAA is regulated by IL1, IL6, and TNFa (Yuan *et al*.,2013).

2.6.5.Blood cultures

Blood culture test for bacteria often referred to as the gold standard is the most reliable diagnostic test of neonatal sepsis. However, it can take 48–72 h for the results to be released. As a result, treatment should often begin before the laboratory results are released. An additional challenge is the fact that the blood culture test can be false negative for one in five subjects with sepsis (Ganatra *et al* ., 2010 ; Benitz ,2010).

Using aseptic technique about 2 -5 ml of blood was obtained and inoculated directly into Brain Heart Infusion broth (BHI) in a ratio of blood: BHI of 1:10 and transported to the Microbiology laboratory for incubation and subsequent processing, aerobic Blood culture was done; after 24 hrs incubation gram stain was done followed by blind subculture on 5% sheep blood agar, chocolate agar, MacConkey agar and MacConkey with 30μg/ml cefotaxime . Broth cultures were further re-incubated and then sub cultured after 48 hours then after 96 hrs with last subculture on day 7. Identification of bacteria was made by conventional physiological and biochemical methods. These included gram stain, catalase reaction, coagulase reaction, hemolytic activity on sheep blood agar plates, hippurate hydrolysis and CAMP tests for gram positive bacteria. In case of gram negative colonies morphology on blood and MacConkey agar, triple sugar iron agar reaction, indole, motility, citrate, urease, hydrogen sulphide production and VP test were used, Finally,

antimicrobial susceptibility of all isolates was determined by disk diffusion method according to Clinical Laboratory Standard Institute (Kayange *et al* .,2010).

2.6.6.Molecular Assays

Using mass spectrophotometric techniques to identify bacteria have the possibility of earlier identification (within 1 hour) of pathogens but modest sensitivities (66%–80%), poor yield when there is low bacterial density, and risk of misidentification of pathogens, especially in situations of polymicrobial growth are significant limitations. Molecular techniques to detect the presence of bacterial DNA have been utilized to enhance the diagnostic yield in neonatal sepsis, Polymerase chain reaction (PCR) allows detection of bacterial DNA. PCR is technique to amplify a single or a few copies of a DNA strand across multiple orders of magnitude, generating thousands to millions of copies of a particular DNA sequence (Jordan ,2010; Edmond and Zaidi ,2010; and Srinivasan and Harris ,2012).

2.7.Treatment of neonatal sepsis

Absorption is the pharmacokinetic process that governs how much of an administered drug enters the body. After a drug has been absorbed, it can then be delivered to other sites in the body, including the site of action. Drug metabolism can occur in a variety of tissues; however, the primary sites of metabolism are the liver, kidneys, and biliary tract(Buxton and Benet ,2011).

Antibiotic therapy should be initiated promptly when the diagnosis of neonatal sepsis is suspected or proven and appropriate cultures have been obtained. It is often prudent to administer empiric parenteral antibiotics; usually as the combination of a penicillin and an aminoglycoside

.Ampicillin is favored due to its activity against gram-positive infections, including GBS, *L. monocytogenes*, and some gram-negative coverage, gentamicin is often added for its activity against many gram-negative pathogens that are common causes of early-onset neonatal sepsis (e.g., *E. coli* and other *Enterobacteriaceae* species). If staphylococcal infection is suspected, the initial treatment should include a penicillinase-resistant penicillin or vancomycin. The macrolide antibiotic erythromycin features activity against most gram-positive bacteria, including strains of penicillin-resistant staphylococci. For the treatment of multidrug-resistant organisms, such as *Klebsiella pneumoniae* and extended-spectrum blactamase-producing *Enterobacteriaceae*, the carbapenem agents imipenem and meropenem may be considered . In cases of suspected or proven meningitis, cefotaxime may be preferable due to its superior cerebrospinal fluid (CSF) penetration (Stockmann *et al*.,2013).

There are several antiviral agents that can be used for the treatment of neonatal viral infections. Acyclovir (60 mg/kg/d IV divided every 8 hours) is the treatment of choice for term infants with herpes simplex virus (HSV) and varicella-zoster infections (Bradley and Nelson,2014). After parenteral therapy with acyclovir, it is recommended to give HSV suppressive regimen (300 mg/m^2/dose po tid), which improves neurodevelopmental outcomes of infants with central nervous system involvement (Kimberlin *et al*.,2011).

2.8.Cytokines

2.8.1.Defiinition and classification

Cytokines are pleiotropic proteins or small glycoproteins with a molecular weight less than 30 kDa (<200 amino acids) Cytokines are

produced by a number of cell types, such as leukocytes which regulate immunity, inflammation and hematopoiesis. They are categorized on the basis from which they are produced either from Th1 cells or Th2 cells. Recently a third subset of Th cells (Th17) and T regulatory cells (Treg) are categorized which show different cytokine profile from Th1 and Th2 cells . According to their secretion they are classified into lymphokines :cytokines that are secreted by T cells and regulate the immune response, proinflammatory cytokines :cytokines that amplify and perpetuate the inflammatory process, growth factors :cytokines that promote cell survival and result in structural changes in the airways , chemokines :cytokines that are chemotactic for inflammatory cells, and anti-inflammatory cytokines :cytokines that negatively modulate the inflammatory response (Gulati *et al*.,2016).

2.8.2.Cell produces cytokines

Cells of the innate immune system include mast cells, macrophages, neutrophils, dendritic cells (DCs), basophils, eosinophils, natural killer (NK) cells, and the growing set of innate lymphoid cells (ILCs) and innate-like T lymphocytes, and are responsible for immediate, early immune responses against pathogens. They act as effectors through the production of 'effector cytokines' or direct cytotoxic activity (Guo *et al*., 2012). soluble mediators secreted by different innate immune cells includes TNF, IFNγ, interleukins IL-1β, IL-4, IL-6, IL-10, IL-12, IL-18, CCL/4RANTES, and TGFβ (Lacy and Stow ,2011).

Th1 subset secreted IL-2, IFNγ, and TNF, and is responsible for many classic cell mediated function including activation of cytotoxic T lymphocytes and macrophages (Siransy *et al* ., 2018). Th2 subset secretes IL-4, IL-5, IL-6, IL-9, IL10, and IL-13, and regulates B-cell activity and differentiation (Owen *et al*., 2013).

2.8.3.Cytokines signaling

Cytokines are secreted glycoproteins that act as intercellular messengers to control the hematopoietic and immune systems and the inflammatory response ,cytokine consistent with their ability to mobilize cells to sites of infection and inflammation. Each cytokine binds to a specific receptor on the surface of its target cell. These receptors contain intracellular domains which are constitutively associated with members of the JAK (Janus Kinase) family of tyrosine kinases(Morris *et al*.,2018).

After interaction with their cognate antigen, naive $CD4^+$ T cells proliferate and, depending on the cytokine micro-environment, polarize towards different $CD4^+$ lineages, which then shape the immune response. $CD4^+$ lineages include T helper type 1 (Th1), which drives the immune response against intracellular pathogens, Th2, which promotes humoral responses, Th17 cells, which contribute to the elimination of extracellular pathogens, and regulatory T (Treg) cells, which prevent the development of autoimmunity .The differentiation towards each lineage is associated with the up-regulation of specific transcription factors that act as master regulators by controlling the expression of a panel of genes, conferring a specific phenotype (Zhu *et al*.,2010). There is accumulating evidence that $CD4^+$ T-cell lineages are not as stable as initially thought, but rather, in specific environments, secrete cytokines and co-express master regulators specific for other lineages (Murphy and Stockinger,2010).

The Th1 cells secrete high levels of interferon-γ (IFN-γ) and IL-2, and drive immunity against intracellular pathogens but also promote autoimmunity. Interleukin-12, in synergy with IL-18, drives Th1 differentiation, in large part via induction of T-bet (T-Box expressed in T cells), a master regulator transcription factor that controls the expression

of IFN-γ(Zhu and Paul ,2010). The Th2 cells secrete large amounts IL-4, IL-5, IL-9 and IL-13, and consequently promote the humoral response (Paul and Zhu,2010).

2.8.4. Type of cytokines

2.8.4.1. Tumor necrosis factor (TNF)

Is a key mediator in the inflammatory response which is implicated in the onset of a number of diseases (Cabal-Hierro and Lazo ,2012). The tumor necrosis factor (TNF) family includes TNF alpha (TNFa), TNF beta (TNFb), CD40 ligand (CD40L), Fas ligand (FasL), TNFa is mainly produced by macrophages, whereas TNFb is mainly produced by T lymphocytes. Other cells can also express TNFa and TNFb at 2013). low levels (Chu,

2.8.4.2 Interferon (IFN)

IFNs are a group of cytokines that induce an antiviral state. They are currently classified into three groups: type I, type II, and type III IFNs (Makowska *et al*.,2011). INF activate immune cells, such as natural killer cells and macrophages; they increase host defenses through up-regulating antigen presentation by virtue of increasing the expression of major histocompatibility complex (MHC) antigens (Espinosa *et al* ., 2017).

2.8.4.3. Tissue Growth factor(TGF)

TGF-β is so called because of its ability to induce a transformed characteristic or tumor-cell characteristic in normal cells. The TGF-β superfamily includes TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3),

activins , inhibins, growth factors, and differentiation factors. TGF-β family members are believed to be involved in the initiation, maintenance, and resolution of inflammatory responses(Halwani *et al* .,2010) .

2.8.4.4. Chemokines (CKs)

Are small molecules (8–12 kDa), usually soluble, not only specialized in the recruitment but also activation of immune cells. CKs need specific receptors coupled to G proteins. Chemo attraction of cells requires a gradient of concentration of chemokines. There are four types of CKs that are classified according to their amino acid sequence and the spacing between their two cysteine residues: CC, CXC, CX3C, and XC CKs. In contrast to other cytokines which have broad effects on many cells, CKs are usually cell specific . CKs induce not only leukocyte recruitment to the site of infection but also the release of immune cells from the bone marrow or spleen (Cai *et al*.,2010 ; Lionakis *et al*.,2013;and Chousterman *et al*.,2016).

2.8.4.5 Interleukins

Are the most important group of cytokines released during infectious processes. They encompass a large variety of proteins secreted by leukocytes and endothelial cells and that contribute to cell signaling and promote activation, proliferation, death, and/or motility of immune cells. They are artificially divided into Pro-inflammatory interleukins which are supposed to be responsible for cell activation, tissue damage, and necrosis while anti-inflammatory interleukins aim to dampen and finally reverse the inflammatory process(Weber *et al*.,2010).

2.8.4.5.1. Interleukin-1 family

Is a group of 11 cytokines that plays a central role in the regulation of immune and inflammatory responses to infections (Dinarello , 2011). 7 ligands with agonist activity (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β and IL-36γ), 3 receptor antagonists (IL-1Ra, IL-36Ra and IL-38) and 1 anti-inflammatory cytokine (IL-37) (Garlanda *et al*., 2013).

2.8.4.5.2. Interleukin-2 family

Also known as the common γ-chain family, is composed by ILs 2, 4, 7, 9, 15 and 21. All these ILs bind to the common γc receptor, also called CD132. These cytokines act as growth and proliferation factors for progenitors and mature cells (Sim and Radvanyi , 2014).

2.8.4.5.3. Interleukin-6 family

Comprises IL-6, IL-11, IL-27, IL-31(Jones and Jenkins ,2018).

2.8.4.5.4.Interleukin-10 family

Involved IL-19, IL-20, IL-22, IL-24 and IL-26 (Ouyang and O'Garra ,2019).

2.8.4.5.5. Interleukin-17 family

Consists of 6 cytokines which are structurally related, IL-17A (also called IL-17), IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25) and IL-17F(Li *et al*.,2020).

2.8.5.IL-10

Interleukin-10 (IL-10) and Transforming Growth Factor Beta (TGF-β)are Anti-inflammatory cytokines, which are important inflammatory mediators, by preventing excess proinflammatory response during sepsis .IL-10 is produced by different types of immune system cells such as monocytes, macrophages, T and B lymphocytes, and NK cells .This cytokine suppresses the production of proinflammatory mediators including TNF-α, IL-1, IL-6, IFN-γ, and GM-CSF in cells of the immune system. IL-10 has been associated with septic shock in both adults and children. high IL-6/IL10 ratio was found in patients with a worse prognosis. Similarly, a high IL-10/TNF ratio has also been associated with severe late-onset neonatal sepsis(Machado *et al*.,2014).

2.8.6.Relation between IL10 and neonatal sepsis

IL-10 is a potent immune regulatory molecule. The consequences of IL-10 on the immune response include: the down-regulation of key signaling receptors on antigen presenting cells such as CD40, CD80, CD86 and MHC II, decreased Mac-1 expression and inhibition of neutrophil oxidative burst , suppression of T cell proliferation and IL-2, IL-6 and IFN-c production, the maintenance of FoxP3 expression in regulatory T cells, and suppression of NK cell function (Kasten *et al*.,2010).

The levels of IL-10, even as a marker of anti-inflammation, are directly related to the degree of inflammation , demonstrating that pro- and anti-inflammation are closely related. High IL-10 levels are also associated with more important features of sepsis-induced immunosuppression(Hotchkiss *et al*.,2013).

IL-10 is a molecule with immune-regulatory properties. Secretion of IL-10 in sepsis could limit and ultimately terminate inflammatory responses , which called as anti-inflammation cytokine. Besides, high levels of IL-

10 could induce a state of functional immunoparalysis, leading to an incontrollable infection .IL-10 may play a role in the apoptosis of T-lymphocytes(Li *et al*.,2017).

2.9.Previous studies

In Brazil , Cancelier and his colleagues(2009) collected of cord blood from 120 newborns included 20 cases of proven and 20 cases of highly probable neonatal sepsis, and 80 controls . levels of IL-10, were higher in the sepsis group when compared with control(*P.value* less than 0.01)(Cancelier *et al*.,2009).

In Suez chanal , Egypt , Zeitoun and his colleagues(2010) evaluated IL-10 as diagnostic marker of early and late onset neonatal sepsis ,between 49 sepsis infant and 49 healthy control group .They found it was significantly higher in the serum of sepsis group compared with the control group (*P. value*=0.0001)(Zeitoun *et al*.,2010).

Campos and their colleagues(2010) ,evaluated serum IL10in 55 neonates who developed early sepsis,they observed there was appositive correlation between the level of IL10 between the course of early sepsis and the level observed in the umbilical cord ($P<0.001$) (Campos *et al*.,2010).

In Iran, Boskabadi and their colleagues(2011) evaluated serum IL10 as Early diagnosis of late neonatal sepsis, Eighty eight neonates they were categorized on the basis of their clinical presentation, laboratory parameters and blood culture results into: cases [definitive infection (with positive blood or/and cerebrospinal fluid _.(CSF) cultures) or clinical sepsis (clinical and laboratory evidence of infection, but without positive blood or CSF cultures)] and controls (physiologic hyperbilirubinemia or routine feeding). They found that serum IL-10 were significantly higher

in the order of definitive infection > clinical sepsis > healthy controls respectively (P >0.001)(Boskabadi *et al*.,2011).

In India, Sugitharini and colleagues(2013) estimated the plasma level of IL 10 in 179 samples (cases of neonatal sepsis and control),the result was significantly higher levels of IL10 in neonates with sepsis compared to normal (p=0.0077)(Sugitharini *et al*.,2013).

In Iran, Boskabadi and their colleagues(2013) evaluated serum IL10 as diagnostic markers of neonatal infection ,total of 84 infants (sepsis case = 41,healthy control =43),statistically significant differences were observed between control and case groups (P >0.001)(Boskabadi *et al*.,2013).

Khaertynov and his colleagues(2017) In Tatarstan, estimated the serum level of IL10 in 25 cases with a diagnosis of neonatal sepsis and eight healthy controls. The result was significantly different in these two cohorts the LOS and EOS (p = 0.003), as well as between the LOS cohort and healthy controls(p = 0.002) (Khaertynov *et al*.,2017).

In China ,study done by Ye and his colleagues (2017)serum inflammatory cytokine levels were evaluated in the early stage of neonatal sepsis and after antimicrobial treatment, there were significant differences in the IL-10 levels between the study and control groups (P < 0.001) (Ye *et al*.,2017).

In Mexican, Leal and his colleagues(2018) evaluated the diagnostic value of serum level of IL-10 in 96 neonates with high risk of developing sepsis .They found fifty presented sepsis (26 early and 24 late)was associated with high levels of IL-10 (*P. value*=0.02) (Leal *et al* ., 2018).

Observational study done by Hibbert and their colleagues(2020) in Australia to evaluate plasma cytokine profiles in very preterm infants with late-onset sepsis. The result was markedly higher concentrations of

IL10in infants with confirmed LOS compared to non LOS (Hibbert *et al*.,2020).

Chapter III

Materials and Methods

Chapter III

Materials and Methods

3.1.Study design

This was prospective case control study.

3.2. Study area and duration

Study was conducted in Soba and Ibrahim Malik hospitals in Khartoum state during the period from June(2020) to May(2021).

3.3. Study population

Study population consisted of 88 Sudanese individuals of age between 1-21 day , they were divided into 2 groups as follows :clinical sepsis group(case group) consist of neonates with positive blood culture and who were hospitalized for clinical suspicion of neonatal sepsis in neonatal intensive care units (NICU) and healthy control subject (control group)consist of healthy neonates.

3.4. Inclusion criteria

Term and preterm neonates, confirmed sepsis ,had sign of EOS or LOS hospitalized in NICU(case group),healthy neonates without any sign of infections (control group) were included in this study.

3.5. Exclusion criteria

All neonates with situation that affected cytokine level including congenital malformation, mother with immune disease.

3.6. Sample size

Eighty-eight (n=88)neonates were enrolled in this study. 44 blood samples were collected from septic neonates and 44 blood samples were collected from healthy neonates.

3.7. Data collection

Data was collected through review of collected data in file of each neonate ,through check list which includes demographic ,clinical, and laboratory data.

3.8. Ethical consideration

Ethical approval was obtained from scientific research Committee, College of Medical Laboratory Science, Sudan University of Science and Technology ,and informed consent from hospitals and neonates parent's verbal consent .

3.9.Sampling

One ml of venous blood was collected from patients and control in Ethylene di- amine tetra acetic acid(EDTA) container. Then the samples were centrifuged and plasma was separated in a sterile cryovial tube and stored at -20 ˚C until analysis. plasma level of IL-10 were measured using ELISA. (Biolegend's ELISA MAXTM).

3.10. Principle and procedures

3.10.1. Principle of the ELISA

Biolegend's ELISA MAXTM Deluxe Set is a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). A human IL-10 specific monoclonal antibody is first coated on a 96well plate. Standards and samples are added to the wells, and IL-10 binds to the immobilized capture antibody. Next, a biotinylated anti-human IL-10 detection antibody is added,

producing an antibodyantigen-antibody "sandwich". Avidin-horseradish preoxidase is subsequently added, followed by Tetra-methylbenzidine (TMB) Substrate Solution, producing a blue color in proportion to the concentration of IL-10 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. (www.biolegend.com).

3.10.1.1. ELISA Procedure

Day I 100 µL of diluted capture antibody solution was added to each well, the plate was sealed and incubated overnight between 2˚C and 8˚C .

Day II 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, sealed the plate and incubated 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; added 100 µL Diluted standareds and samples to the appropriate wells. sealed the plate and incubated at room temperature for 2 hours with shaking. then washed plate 4 times; added 100µL diluted detection antibody solution to each well, sealed the plate and incubated at room temperature for 1 hour with shaking. washed plate 4 times; added 100 µL diluted Avidin-HRP solution to each well, sealed the plate and incubated at room temperature for 30 minutes with shaking. washed plate 5 times; soaking for 30 seconds to 1 minute per wash. added 100µL of freshly mixed TMB substrate solution to each well and incubated in the dark for 30 minutes. Added 100µL stop solution to each well. Absorbance was read at 450 nm and 570 nm within 15 minutes.

The absorbance 570 nmm can be substracted from the absorbance at 450 nm. (www.biolegend.com.)

3.10.1.2. ELISA Washer principle

First the wash solution is pumped from the wash bottle, the solution is dispensed to the cuvette by short pins, and then the wash liquid is aspirated 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).

3.10.1.3. 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. Statistical analysis

Data was analyzed by using statistical package for social science {SPSS} (version 20) using Mean ± SD and independent T test for testing difference significance, correlation test to find out correlation and to obtain mean and stander deviation. Probability value (PV) less than or equal 0.05 was considered statistically significant.

Chapter IV

Results

Chapter IV

Results

4.1.Result

Fifty two volunteers of age between 1- 13 day were enrolled in this study 26 were neonatal sepsis patients with a mean age 5.23±3.06, 13(50%) were males ,and 13(50%) were females .26 were healthy neonate witha mean age 1.8±0.85, 13 (50%) were males and 13(50%)were females .

The mean level of IL-10(pg/ml) was 117.9±117, 10.66±6.7 in the case group and control group respectively. Statistically there was significant correlation in IL-10 level between case and control(P. value = 0.000)(Table 4-1).

Mean level of IL-10 in males and females of case group were 119.4 ±115 and 116.5 ±123.64 respectively ,(P. value =0.952),there was no statistical correlation between level of IL-10 and gender in case group (Table 4-2).

There was no statistical correlation between IL-10 level and age in study group (P. value= 0.391). Also no statistical correlation between level of IL-10 and weight of sepsis neonates, (P. value was 0.291).

Mean level of IL-10 in EOS and LOS patients was 117.8±126, 118.26 ±97 respectively, there was no statistical correlation between level of IL-10 and duration (early / late) of sepsis(p-value = 0.993) (Table 4-3).

Mean level of IL-10 in term and preterm neonate in study group were 96.35 ±111 , 143.1±123.5 respectively, there was no statistical correlation between level of IL-10 and maturity, (p. value= 0.320)(Table 4-4).

Table (4-1): comparison of IL-10 level between the case and control group:

IL-10	population	N	Mean	Std. Deviation
concentration	Case	26	117.9	117
	control	26	10.66	6.7

p. value = 0.000 (independent sample t-test)

Table (4-2): level of IL-10 in male and female of case group:

IL-10	gender	N	Mean	Std. Deviation
concentration	male	13	119.4	115
	female	13	116.5	123.64

p. value = 0.952 (independent sample t-test)

Table (4-3): level of IL-10 in EOS and LOS patients:

IL-10	Duration	N	Mean	Std. Deviation
concentration	EOS	19	117.8	126
	LOS	7	118.26	97

p-value = 0.993 (independent sample t-test)

Table (4-4): level of IL-10 in term and preterm neonate in study group:

IL-10	birth	N	Mean	Std. Deviation
Concentration	Term	14	96.35	111
	preterm	12	143.1	123.5

p-value = 0.320 (independent sample t-test)

CHAPTER V

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

Chapter V

Discussion, Conclusions and Recommendations

5.1.Discussion

Interleukin-10 (IL-10), has a central role in infection by limiting the immune response to pathogens and preventing damage to the host , IL-10 inhibits the development of TH1-type responses , also leads to the suppression of TH2 cell . In addition to an autocrine inhibitory effect on macrophages and DCs, IL-10 enhances the differentiation of IL-10-secreting TReg cells, thus providing a positive regulatory loop for its induction (Saraiva and O'Garra ,2010).

In the present study plasma level of IL-10 was measured in 26 neonatal sepsis patients and in 26 apparently health control. The result relieved that the mean level of IL-10 in case group was higher than control and the difference was significant (P. value 0.000). This result was supported by Cancelier *et al* (2009) who demonstrated that significantly elevated IL-10 level in sepsis case than healthy control P. value <0.01 (Cancelier *et al*.,2009).

The mean level of IL-10 in the present study agreed with Zeitoun *et al* (2010) result which showed that IL-10 level was significantly increased in sepsis case than healthy control P. value=0.0001 (Zeitoun *et al*.,2010). Another study conducted by Sugitharini *et al* (2013) in India found that the mean level of IL-10 was significantly higher in neonates with sepsis compared to normal P. value =0.0077 (Sugitharini *et al*.,2013). Boskabadi and their colleagues estimated serum level of IL10 in total of 84 infants (sepsis case = 41 , healthy control =43),statistically significant

differences were observed between control and case groups P. value =0.001 (Boskabadi *et al*.,2013). which supported my study.

In the present study there was no correlation between the mean level of IL-10 and duration of sepsis (EOS,LOS) P. value=0.993 . This result agree with Zeitoun *et al* P. value=0.2 (Zeitoun *et al*.,2010). On the contrary Khaertynov and their colleagues showed that subjects with LOS demonstrated a significant increase in serum levels of L-10(Khaertynov *et al*.,2017) this difference may be due to sample size. Also the present study showed no correlation between the mean level of IL-10 and term / pre term neonate maturity P. value =0.320 and gender in the study group.

5.2. Conclusions

High plasma level of IL-10 in neonatal sepsis patients compared with control subjects with statistically significant differences (P. values 0.000).

There was no correlation between IL-10 level and duration of sepsis ,age ,weight ,gender and maturity of neonates in study group.

5.3.Recommendations

- The sample size would be increased in future studies.

-IL-10 can use as prognostic marker of neonatal sepsis.

-Combination of two biomarkers at least should be used to detect the present of sepsis , example CRP and IL-10 should be tested.

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APPENDICES

Appendix (1)

Sudan university of Science and Technology

Collage of Post Graduates

Evaluation of interleukin 10 levels in Sudanese Neonatal sepsis patients

- Date: / /2020.

- ID number : ……

- Age :…… Day. Weight: ……/g

- Gender : Male ( ). Female : ( ).

- Causes of sepsis:

……
……
……
……
……
……
……
……

- Duration of Sepsis :

- Less than 24 hrs( ). - 24-72 hrs( ).

-More than 72 hrs ( ).

- Other disease :

……
……
……

Appendix (2)

IL-10 ELISA Kits

Appendix (3)

Human IL-10 ELISA MAX™ Deluxe Set

Certificate of Analysis

Product Name: Human IL-10 ELISA MAX™ Deluxe Set
Product Cat. No: 430604 (5 plates) / 430605 (10 plates) / 430606 (20 plates)
Lot No: B232253
Expiration Date: 28-Feb-2019

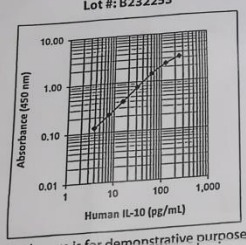
Contents Description	Quantity (5 plates)	Volume (per bottle)	Part No.	Lot No.
Human IL-10 ELISA MAX™ Capture Antibody (200X)	1 vial	300 µL	79029	B229580
Human IL-10 ELISA MAX™ Detection Antibody (200X)	1 vial	300 µL	79030	B229581
Human IL-10 Standard	2 vials	30 ng	79031	B232246
Avidin-HRP (1,000X)	1 vial	60 µL	79004	B231254
Substrate Solution A	1 bottle	30 mL	78570	B231227
Substrate Solution B	1 bottle	30 mL	78571	B231228
Coating Buffer A (5X)	1 bottle	30 mL	79008	B232549
Assay Diluent A (5X)	1 bottle	60 mL	78888	B230792
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 #: B232253



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

This is to certify that the product was manufactured under stringent process controls to ensure lot to lot consistency and complete lot traceability. The product has been tested and meets quality control specifications.

Signature: _____ (Quality Control) Date: 3/2/17

BioLegend is ISO 9001:2008 and ISO 13485:2003 Certified

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Phone: (858)-768-5800 | Fax: (877)-455-9587 | biolegend.com

ELISA MAX™ Deluxe Set Protocol

Materials to be Provided by the End-User

1. Phosphate-Buffered Saline (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 1.0 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. 423601 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 12 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 IL-10 Standard by adding 0.2 mL of 1X Assay Diluent A to make the 150 ng/mL standard stock solution. Allow the reconstituted standard to sit at room temperature for 15-20 minutes, then briefly vortex to mix completely.

To prepare 250 pg/mL top standard, perform an initial 1:10 dilution by adding 10 µL standard stock solution to 90 µL of 1X Assay Diluent A. Then add 16.7 µL to 983.3 µL of 1X Assay Diluent A. Perform six two-fold serial dilutions of the 250 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 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 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.
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 30 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/430604.pdf

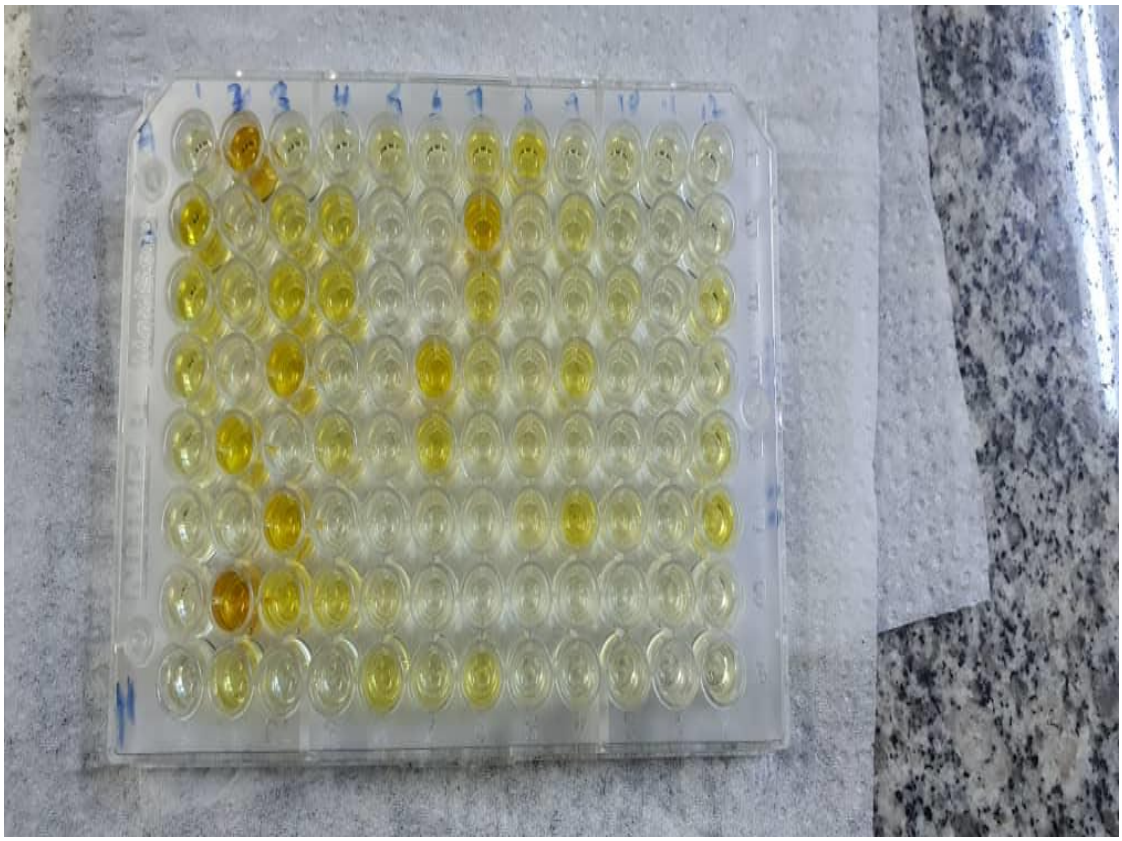
BioLegend®
Part No. 78517_V02

Appendix (4)

a- ELISA Washer

b-ELISA reader

Appendix (5)



ELISA micro plate