การเปลี่ยนแปลงระดับซีรัมไมโครอาร์เอ็นเอ122 ในการบาดเจ็บที่ตับเฉียบพลัน โดยการเหนี่ยวนำจากการบาดเจ็บที่ไตเฉียบพลันและติดเชื้อในกระแสเลือด ในหนูทดลองชนิด CD-1

ร้อยตรีหญิงธนภรณ์ พานิช

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2557

ALTERATION OF SERUM MICRO RNA-122 IN ACUTE LIVER INJURY INDUCED BY ACUTE KIDNEY INJURY AND SEPSIS IN CD-1 MOUSE MODEL

Sub Lieutenant Tanaporn Panich

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	ALTERATION OF SERUM MICRO RNA-122 IN ACUTE LIVER INJURY INDUCED BY ACUTE KIDNEY INJURY AND SEPSIS IN CD-1 MOUSE MODEL
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Field of Study	Medical Microbiology
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ธนภรณ์ พานิช : การเปลี่ยนแปลงระดับซีรัมไมโครอาร์เอ็นเอ122 ในการบาดเจ็บที่ตับเฉียบพลัน โดยการเหนี่ยวนำจากการบาดเจ็บที่ไตเฉียบพลันและติดเชื้อในกระแสเลือด ในหนูทดลองชนิด CD-1 (ALTERATION OF SERUM MICRO RNA-122 IN ACUTE LIVER INJURY INDUCED BY ACUTE KIDNEY INJURY AND SEPSIS IN CD-1 MOUSE MODEL) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. นพ. ดร.อัษฎาศ์ ลีฬหวนิชกุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. พญ. ดร.ณัฏฐิยา หิรัญกาญจน์, 51 หน้า.

ไมโครอาร์เอ็นเอ-122 เป็นตัวบ่งชี้ทางชีวภาพตัวใหม่ของโรคตับวายเฉียบพลันโดยตรง และกำลังเป็นที่สนใจในขณะนี้ แต่มีการศึกษาน้อยในโรคตับวายที่ไม่ได้เกิดจากการบาดเจ็บบริเวณตับโดยตรง ดังนั้น คณะผู้วิจัยจึงทดลอง โดยใช้หนู CD-1 ทำให้เกิดตับวายที่เกิดจากภาวะไตวายเฉียบพลันซึ่งเกิดภาวะการบาดเจ็บที่ตับโดยอ้อมภาวะไตวายเฉียบพลันทำโดย 1) การผูกท่อไตไว้ทั้งสองข้าง (BUO) และการตัดไตออกทั้งสองข้าง (BiNX) และ2) การติดเชื้อในกระแสเลือด(CLP) ซึ่งเหนี่ยวนำให้เกิดตับวาย หลังจากนั้นเจาะเลือดทุก 6, 24 ชั่วโมง และเก็บชิ้นเนื้อตับเพื่อนำมาดูพยาธิสาพ ในขณะเดียวกันทำให้เกิดตับวายโดยตรงด้วยการ ปิดกั้นหลอดเลือดไม่ให้เลือดไปเลี้ยงตับ ณ ช่วงเวลาหนึ่ง แล้วปล่อยกลับคืนสู่สภาพปกติ อันเป็นการทำให้เกิดการบาดเจ็บที่ตับโดยตรง แล้วทำการเจาะเลือดและเก็บชิ้นเนื้อตับที่ 0.5, 1 ชั่วโมง วัดระดับ ALT, miR-122, ไซโตไคน์, ครีเอตินีน (sCr) ในซีรัม และพยาธิสภาพในตับ ในภาวะเซลล์ตับบาดเจ็บโดยอ้อม ณ 6 ชั่วโมง พบว่า ระดับ ALT, ไซโตไคน์ เพิ่มสูงกว่าระดับปกติ 1.5 เท่า แต่ไม่พบการแสดงออกของ miR-122 ทั้งที่มีพยาธิสภาพเกิดขึ้นจากการตรวจพบการสร้างแวคิลโอลในเซลล์ตับ แต่ miR-122 ถูกตรวจพบว่ามีการแสดงออกเพิ่มขึ้นที่ 24 ชั่วโมงหลังการผ่าตัด ซึ่งมีระดับ cytokines และ ALT สูงกว่าที่ 6 ชั่วโมง การสะสมของcytokines อาจส่งผลให้มีการแสดงออกของ miR-122 การทดลองบ่มเซลล์ตับกับ cytokines และวัดระดับmiR-122 ที่ 24 ชั่วโมง พบว่ามีการแสดงออกเพิ่มขึ้น อนึ่งในสัตว์ทดลองกลุ่มที่ตับมีการบาดเจ็บโดยตรง พบค่า ALT เพิ่มสูงมากถึง 5 เท่า และ 22 เท่า ที่เวลา 0.5 และ 1 ชั่วโมง ตามลำดับ พร้อมทั้งมี miR-122 เพิ่มขึ้นและพยาธิสภาพที่ตับชัดเจน miR-122 มีความไว 68% และความจำเพาะ 70% เมื่อใช้ ALT> 40U/L ในการวินิจฉัยภาวะตับอักเสบ โดยสรุป miR-122 อาจจะมีข้อจำกัดในกรณีตับบาดเจ็บโดยอ้อม ที่มีค่า ALT น้อยกว่า 100 U/L

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5687124520 : MAJOR MEDICAL MICROBIOLOGY
KEYWORDS: MIR-122 / ACUTE KIDNEY INJURY-INDUCED LIVER INJURY / SEPSIS-INDUCED LIVER INJURY

TANAPORN PANICH: ALTERATION OF SERUM MICRO RNA-122 IN ACUTE LIVER INJURY INDUCED BY ACUTE KIDNEY INJURY AND SEPSIS IN CD-1 MOUSE MODEL. ADVISOR: ASADA LEELAHAVANICHKUL, M.D., Ph.D, CO-ADVISOR: PROF. NATTIYA HIRANKARN, M.D., Ph.D, 51 pp.

MicroRNA-122 (miR-122) is an interesting and well-known candidate biomarker of direct liver injury but less data about correlation in indirect liver injury. Here, we used CD-1 mice to induced indirect acute liver injury after 1) acute kidney dysfunction induced by bilateral ureter obstruction (BUO) and bilateral nephrectomy (BiNX) 2) cecal ligation and puncture (CLP), in comparison with a direct liver injury model induced by ischemic and reperfusion of liver (liver I/R). Liver was collected and staining for study histology and serum biomarkers; alanine transaminase (ALT), serum creatinine (sCr), pro and anti-inflammatory cytokines were measured at different time points. Serum cytokines, ALT increased approximately 1.5x of baseline at 6 h in indirect liver injury models without miR-122 expression. Then, miR-122 was detected at 24h with high cytokines, ALT and hepatocyte vacuolization in histopathology. By the different time-points of miR-122 and cytokines detection, we hypothesized that cytokines induced miR-122 and test in vitro. HepG2 cells were incubated with cytokines for 24h showed the up-regulation of miR-122. In contrast, in direct liver injury (liver I/R), ALT was increased 5 folds and 22 folds at 0.5h and 1h, respectively. The sensitivity and specificity of miR-122 in comparison with ALT>40U/L as a standard of liver injury were 68% and 70%, respectively. In conclusion, miR-122 might had a limitation in detecting liver injury with ALT less than 100 U/L.

Field of Study: Medical Microbiology
Academic Year: 2014

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

AFP	Alpha-fetoprotein
Ago	Argonaute
AIN	Acute interstitial nephritis
AKI	Acute kidney injury
ALI	Acute liver injury
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate amino transferase
ATN	Acute tubular necrosis
BiNX	Bilateral nephrectomy
BUO	Bilateral ureter obstruction
CINC	Cytokine-induced neutrophil chemoattractant
CLP	Cecal ligation and puncture
CUTL1	Cut-like homeobox 1
ELISA	Enzyme-linked immunosorbent assay
ENA-78	epithelial-derived neutrophil-activating peptide 78
GFR	Glomerular filtration rate
GGT	Gamma glutamyl transferase
H&E	Hematoxylin and Eosin
HCV	Hepatitis C virus

HNF	Hepatocyte nuclear factor
ICAM-1	Intracellular adhesion molecule-1
IL	Interlukin
I/R	Ischemic and reperfusion
KC	Keratinocyte –derived cytokine
LETs	Liver-enriched Transcription Factor
LPS	Lipopolysaccharide
MIP-2	Macrophage inflammatory protein-2
miR-122	MicroRNAs-122
PAF	Platelet-activating-factors
PAMPs	Pathogen-associated molecule pattern
qRT-PCR	Quantitative real-time PCR
RISC	RNA-inducing silencing complex
sCr	serum creatinine
SIRS	Systemic inflammatory response syndrome
TNF-alpha	Tumor necrosis factor alpha
UO	Urine output

CHAPTER I INTRODUCTIONS

Acute kidney injury (AKI) or acute renal failure is a clinical syndrome which characterized by rapid loss of kidney function from numerous etiologies and diagnosed by 1). an accumulation of metabolism waste products, such as urea,creatinine, *p*-cresol, etc., as refer to "uremic toxins" or 2). a decrease in the urine output or 3). both criteria. AKI is commonly occurred in critical ill patients and related with a large number of mortality and morbidity rates. [1]. AKI causes various of organ dysfunction and sometimes refers to the "renal and extra renal organ cross-talk"or "AKI distant organs effect"[2-5]. Several patho-physiologies of AKI-induced distant organs injury are explained through abundance of cytokines, reactive oxygen species induction, increase vascular permeability and distant organs dysfunction [6-8]. It is interesting that liver and small intestine are interconnected by the portal circulation and work in tandem to propagate multi-organs dysfunction after AKI. Liver injury is one of effected organ in the kidney-gastrointestinal interaction. Several pro-inflammatory cytokines such as IL-17A, IL-6, TNF-alpha in intestinal leads to loss of membrane integrity, bacteria in intestine spread via hepatic portal vein caused of liver injury. High cytokines concentration in AKI because of loss of clearance function and increase production are another one cause of kidney induced distant organ dysfunction [2]. Intensive care unit patients with AKI had high mortality rates from AKI-induced distant organ dysfunction [9]. Previous study revealed that renal ischemia/reperfusion (IR) injury and bilateral nephrectomy (BiNX) in rats cause of liver damaged due to a cytokines accumulation and hence recommended AKI patients should be monitored liver injury [10]. The earlier detected of liver damage histology at 6 h in BiNX model was compelling topic [10, 11]. Moreover, Liver injury has been known to occurs frequently in patients with sepsis.

Sepsis is a systemic response to the microbial (bacteria, virus, fungus) invasion which does not depending on species of the organisms. According to the conference of American College of Chest Physician (ACCP), Society of Critical Care Medicine (SCCM), Surgical Infectious Society and European Society of Intensive Care Medicine assign infection in blood stream in four conditions by severity including (systemic inflammatory response syndrome or SIR), sepsis severe sepsis, septic shock respectively. In the first phase of infection, innate immune response plays role to destroy pathogen by recognizing some parts of pathogens as refer to "Pathogen

associated molecular pattern (PAMP)" leading to several effecter responses, one of response is cytokine secretion. Pro-inflammatory cytokines are secreted for clearance pathogen and stimulated other immune cells. Hyper-cytokinemia mainly occurs with sepsis and AKI due to an increase production and a defect in renal clearance, respectively. Sepsis induced acute liver injury (ALI) is reported in approximately 12-20% of sepsis patients [12, 13]. During sepsis, liver involves in several inflammatory processes by clearing bacteria/ bacterial products (e.g., endotoxin), vasoactive substances, and inflammatory mediators. In addition, the stimulated liver produces and releases high amounts of various cytokines, bioactive lipids, and acute phase proteins. These mechanism have been implicated that causing liver damage but the contribution of sepsis induced ALI remain unclear [14].

In the present, alanine transminase (ALT), also known as the serum glutamic-pyruvic transaminase [15], also found in muscle ,liver, heart but ALT is the predominant in cytosol hepatocyte. Clinical routine laboratory using ALT to detect liver injury [16]. However, ALT is a protein that possibly degrade from sample with an inappropriate processes and can be increased form extrahepatic organ injury [15, 17, 18]. MicroRNA-122 or miR-122 is stable and novel liver injury biomarker [19]. The high stability of miRNA is well-known in the research field [20-22].

MicroRNAs (miR) are single stranded RNA approximately 20 – 24 nucleotides in length. MicroRNAs are derived from non-protein coding RNA or intron of protein-coding gene. miRNA/RISC complex is latest form of miRNA biogenesis. The complex mediated gene expression at post-translation step by binding to target mRNA at 3'UTR leading to mRNA degradation or inhibit proteins translation [23]. MicroRNAs are specifically convinced in several cell function such as development, differentiation, metabolism also in disease, and stable in various tissue and body fluid [24, 25]. One miRNA can regulate hundreds of target mRNA and many study report that miR levels differently express in target tissue during disease or injury. In addition, cell-free microRNAs also found in body fluid accompany to organ injury because of apoptosis, necrosis then leakage of contained-microRNAs membrane vesicle. Previous study exhibit the alteration of microRNAs levels in blood circulation after organ injury. Moreover miRNA have a unique position among RNA and stability for use as clinical biomarker to detect organ injuries [26]. MiR-122 mostly found in liver and function as stimulate cell differentiation, regulate lipid metabolism, facilitate HCV replication and inhibit HBV

amplification [27-31]. Previous evidences used miR-122 as drug target of HCV treatment and biomarker for detect liver damage from various causes such as drug-induced liver injury, hepatitis B virus, liver cirrhosis [19, 32-35]. *Laterza OF, et al* measured miR-122 for determined liver injury in human, the results show that miR-122 levels represent severity of liver injury with ALT> 10,00 U/L[35]. Then a positive correlation of miR-122 with the obvious direct liver injury is known but the data of in-direct liver injury with low ALT level is limited. Then several models of indirect liver injury were used to explore serum miR-122. Indirect liver injury models include AKI bilateral nephrectomy (BiNX ; an abrupt loss of kidney function), bilateral ureter obstruction (BUO ; an abrupt loss of excretory function but has an intact renal blood perfusion) and a sepsis model (cecal ligation and puncture [CLP]) were used this study. For a comparison, we used a direct liver injury model, liver ischemic reperfusion injury (liver I/R) for an equivalent of a positive control model.

Hypothesis

1. Serum miR-122 is earlier detected in indirect acute liver injury.
2. Cytokine accumulation induced miR-122 expression in hepatocyte cell line.

Objective

1. To determine correlation between miR-122 and ALT in indirect liver injury.
2. To determine correlation of cytokines in indirect liver injury with miR122 expression.

CHAPTER II LITERATURE REVIEWS

Sepsis

Sepsis is condition of illness from immune response to invading microbial leads to inflammation in a whole body [36]. The symptoms were effected from immune response to pathogen. ICU patients with AKI had high mortality rates in United State has about 750,000 annual cases of sepsis [12, 35]. The mortality rates of sepsis patients were higher than 70%. Moreover, sepsis also causing acute kidney injury in ICU patient in approximately 6% of patients [13].

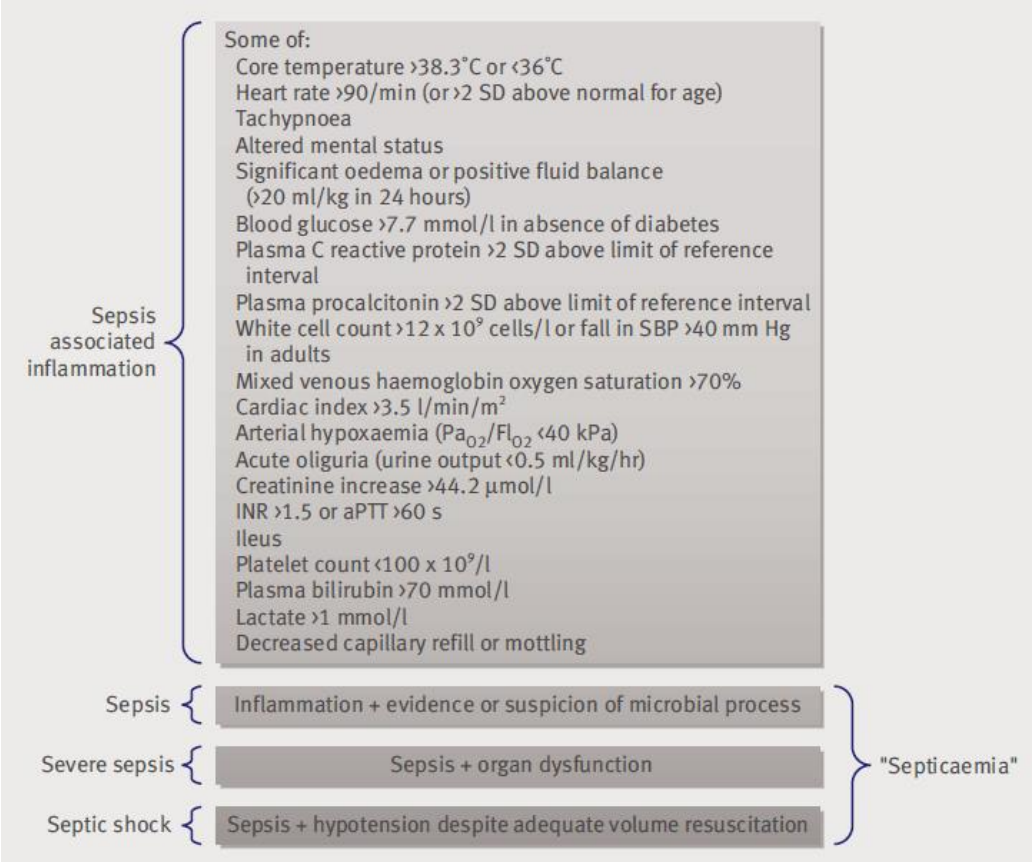


Figure 1 Definition of sepsis, severe sepsis, septic shock [36].

Gram positive and gram negative bacteria have similar cytoplasm but different external structures which classified by gram's stain. Lipopolysaccharide (LPS) or

endotoxin is high abundant in gram negative bacterial cell wall [37]. LPS has an important role in sepsis and result in a rapid disease progression.

Sepsis is a non-specific host immune response to micro-organisms (bacteria, virus, fungus) [36]. The definition of sepsis was identified by clinical symptoms with evidences of microbial infection. Sepsis with other organs dysfunction contribute to severe sepsis. When severe sepsis combine with hypotension or need fluid resuscitation called septic shock. Septicemia is the illness characterized by the presence of pathogenic microorganism in blood circulation, this term can used in general for sepsis, severe sepsis and septic shock [38].

Bacteria identification in sepsis were gram-negative bacteria and gram-positive about 60% and 40% of cases, respectively [39]. Studied of bacterial components and structures were important for the understanding of mechanisms of immune responses.

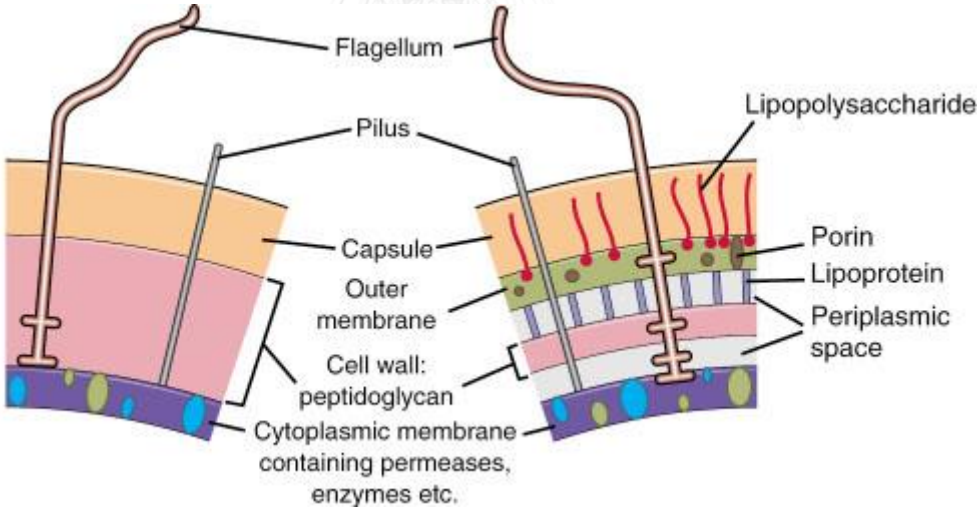


Figure 2 Characteristic cell wall structures of gram-positive (left) and gram-negative (right) bacteria [37].

The complete Structure of LPS contains Lipid A, inner core, outer core and O antigen.

- Lipid A: A subgroup of fatty acid which located at the most inner region molecules. Lipid A is a part of outer membrane responsible for immune activation.
- Inner core: contained 2 types of carbohydrate molecules include 3-deoxy-D-manno-oct-2-ulosonic acid (KDO) which is the inner core bases and heptose (L-glycero-D-manno-heptose) attach in KDO.
- Outer core: consisted of hexose residues (D-glucose, D-mannose, D-galactose, *etc)* which added in the inner are at last position of heptose residue. Moreover, at least three hexose can bind with other carbohydrate residue include beta1⟶3 which bind to O antigen and cleaved the third hexose.
- O Antigen: The primary structure composition of LPS which variation in different bacteria. It is the cell wall antigen which important in immunogenicity and used to identify serogroups of bacteria e.g. *Escherichia coli*, *Salmonella enterica*, and *Vibrio cholerae*.

On the other hand, gram positive bacteria have a thick peptidoglycan layer in cell membrane and lipoteichoic acid, these can produce exotoxin leads to septic shock. Exotoxin can bind with major histocompatibility class II (MHC-II) of CD-4 T-cell and activated T-cell to released pro-inflammatory lymphokines.

Sepsis immune response

Immune responses to sepsis were divided to 2 part; innate and adaptive immune response. Innate immunity is the first line defense against pathogen invasion before adaptive immune response activation [40]. Both of the innate and adaptive immune system interact with pathogen by pattern recognition receptor recognized some part of pathogen [pathogen-associated molecular patterns (PAMPs)] and released cytokines and chemokines to clear pathogen (cell to cell interaction). Important PAMPs include peptidoglycan and lipopolysaccharide (LPS) from bacterial cell wall of gram positive and negative, respectively. Binding of PAMPs with receptor activated immune cells to released pro-inflammatory cytokines and destroy microbials.[39]. The innate immune system also contributes of leukocyte migration to injured tissue. Leukocytes released tissue factor lead to thrombin formation and

inflammatory responses [41]. In sepsis animal model such as CLP, CASP with the very rapid disease progression, innate immune response is predominate [42].

Innate immunity to extracellular bacteria

The principle of mechanisms include complement activation, phagocytosis and the inflammatory response.

- Complement activation: Some part of structural cell membranes such as peptidoglycan and lipopolysaccharide can activate alternative pathway. Lectin pathway activated trough MBL receptor bind with mannose on bacterial surface.
- Activation of phagocyte and inflammation: Receptor on phagocytic cell membrane (mannose receptor, surface receptor, scavenger receptor) recognized bacteria which promote phagocytosis to microbe. In parallel, Fc receptor and complement receptor also bind with opsonized bacteria.

Innate immunity to intracellular bacteria

Bacteria were phagocytosed by WBC but some of them can replicate inside phagocytic cells. Thus, the host cells required other mechanisms to destroy bacteria. NK-cells and phagocytosis were main mechanisms**.** The interaction mediated by cytokines (IL-12 and IFN-gamma) [41].

Sepsis induced liver injury

Neutrophil is one of immune cells which caused tissue injury after ischemia-reperfusion (I/R) injury in various organs including liver. Due to a high mortality of white blood cells and ability to releasing or producing influent cytotoxic mediator, neutrophil were recruited to site of inflammation to kill microbial. It is hypothesized that neutrophil accumulate in liver vascular system in response to inflammatory mediators and lead to liver injury in sepsis [39].

Acute Kidney Injury

Recently, AKI is used instead of acute renal failure to emphasis that continuum of kidney injury happen to the loss of kidney excretory function [43]. AKI was diagnosed by increased of uremic toxin (blood urea nitrogen and creatinine) or decreased urine output or both followed a consensus definition by the Acute Dialysis Quality Initiative as RIFLE criteria[44].

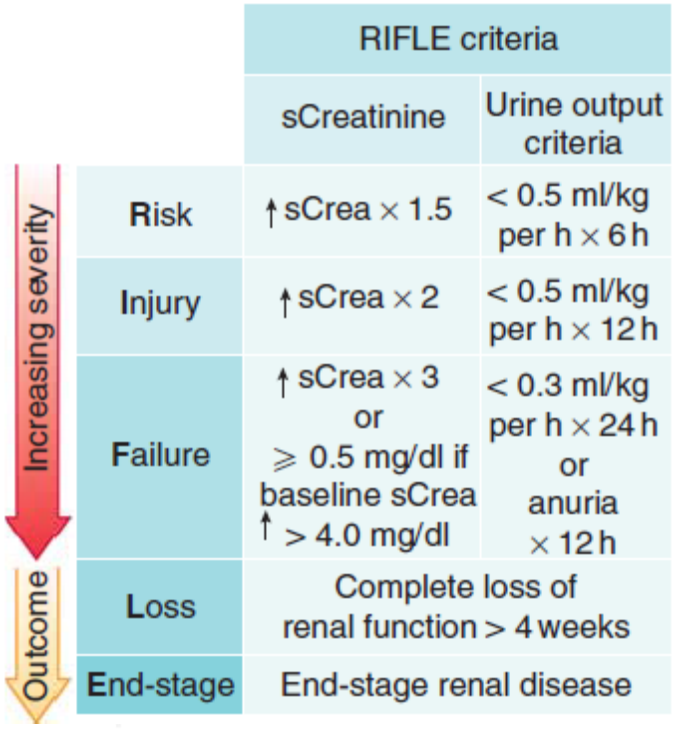


Figure 3 RIFLE criteria classification for AKI [9].

Then 3 years later, RIFLE criteria was re-classified by Acute Kidney Injury Network (AKIN) by change name from Risk, Injury, Failure to stage 1, 2 ,3 ant cut two last stage off the RIFLE criteria.

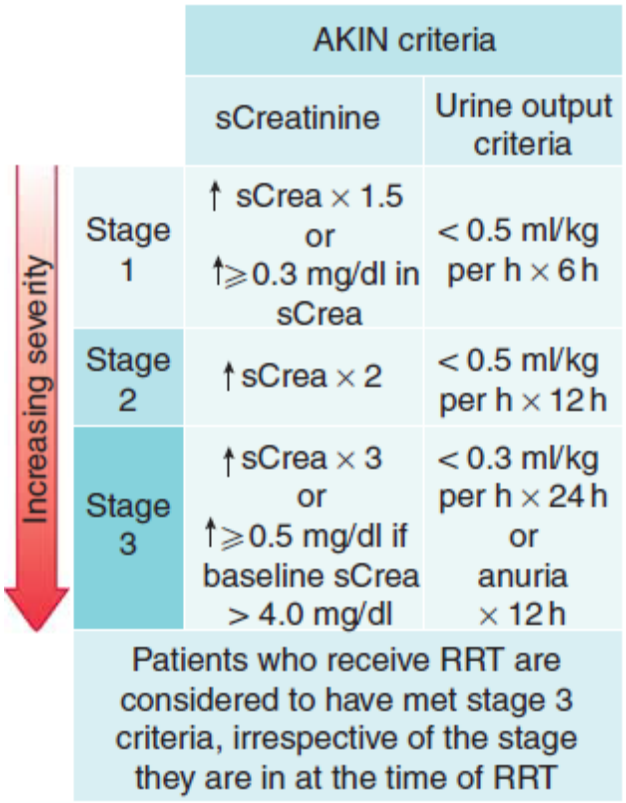


Figure 4 The AKIN classification of AKI [9].

AKIN criteria was considered after obtain adequate hydration and exclusion of other AKI etiologies because this criteria depend on serum creatinine 2 values within 48 h. If serum creatinine increase at least 0.3 mg/dl at 48 h after first admit, patient will diagnosed as AKI.

Etiologies of acute kidney injury

AKI frequently occured in patients in intensive care unit. The mortality rate of AKI with sepsis still as high as 30-80%. The etiologies of AKI were divided to 3 types as follow.

- Pre-renal: It is the condition that reduced blood flow to renal from several causes include reduced of effective extracellular fluid volume, decreased cardiac output from myocardial dysfunction, peripheral vasodilatation from sepsis lead to insufficient blood flow through kidney, some drugs e.g NSAID

caused severe renal vasoconstriction and occlusion of renal artery all of causes lead to decreased urine output but kidney function become normal when the condition was resolved.

- Post-renal: This condition occurs from ureter obstruction by crystal (uric acid, oxalic acid, methotrexate, acyclovir, triamterene, sulfonamide) or proteins (light chains, myoglobin, hemoglobin) causing renal tubule obstruction. In addition, extra renal obstruction is mostly found at ureter, pelvic obstruction, bladder obstruction, urethral obstruction.
- Intrinsic renal: Kidney functions were defected from intrinsic factors include acute tubular necrosis (ATN), acute interstitial nephritis (AIN), acute glomerular disease, renal vascular disorders.

AKI and extra-renal organ dysfunction

ICU patients with AKI often progress to multi-organ dysfunction with the mortality rate approximately 50% [1, 2, 45]. This review was summarized organ cross talk between kidney and lung, heart, brain, liver.

Kidney-lung interaction: After renal ischemic reperfusion injury (I/R) and BiNx, acute lung injury mediated by inflammatory cytokines accumulation (IL-6, IL-1beta, TNF-alpha and IL-8). High inflammatory cytokines lead to increased lung vascular permeability and neutrophil recruitment as demonstrated by vascular congestion on tissue histology [3, 8].

Kidney-gastrointestinal interaction: Renal I/R and BiNX lead to overproduction of IL-17A and inflammation in small intestine [46]. IL-17A is one of pro-inflammatory cytokines effected to intestinal barrier by loosed of membrane integrity and made villous endothelial and epithelial cell injury. Neutrophils and bacterial products in intestine translocated to liver which interconnected with small intestine through hepatic portal vein caused liver inflammation, neutrophil infiltration, hepatic cell necrosis and apoptosis [47].

Kidney-heart interaction: Clinical studied of heart failure in AKI patient thought that acute cardiac decompensation effected to hemodynamics mechanism loss function from kidney. The Myocytes apoptosis and impaired cardiac function from cytokines increased (TNF-alpha, IL-1 and ICAM-1)

demonstrated in renal I/R model. TNF-alpha plays important role in apoptosis of AKI induced heart dysfunction. Anti-TNF-alpha antibody reduced myocytes apoptosis. Study of histology found a lots of neutrophil infiltration to cardiac tissue.

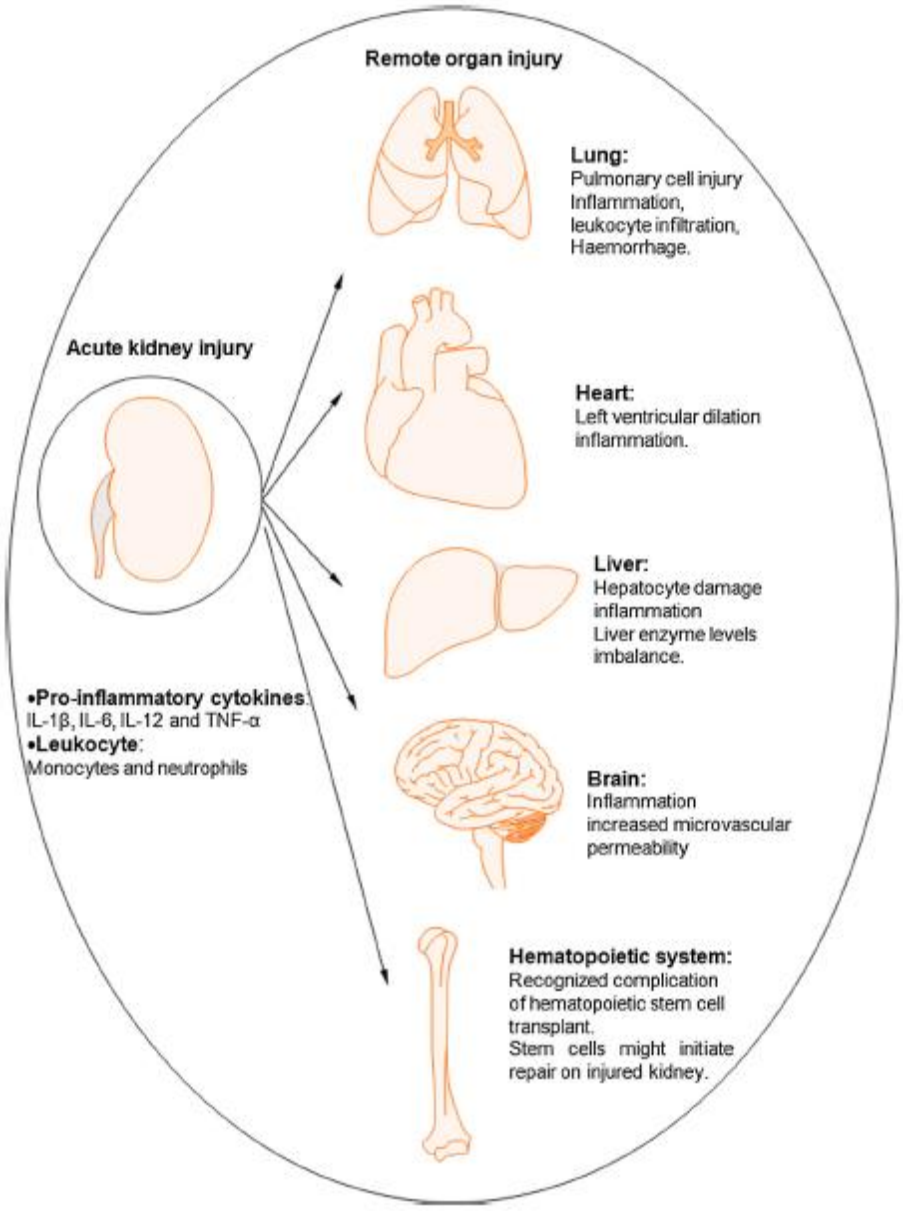


Figure 5 Distant organ effect of acute kidney injury [48].

Kidney-brain interaction: In animal models of AKI, uremic toxins altered brain biochemical especially Ca concentration, water manipulation and neurotransmitter. It was suppressed and decreased motor activity. In addition, AKI

also increased vascular permeability to disrupt blood brain barrier and enhanced inflammation by increased inflammatory cytokines, KC and GM-CSF, microglial cells and neuronal pyknosis but no apoptosis appearance observed in histology [26]

The coexistent between AKI and extrarenal organ dysfunction causes higher mortal rates. Therefore, the earlier detection of organ injury is important to identify disease progression and develop new therapeutic to prevent and attenuated deleterious effect from AKI.

Acute liver injury

From all of the internal organ, liver is the largest and It has a lot of important and various functions including to

- Biochemical function: Plasma protein was synthesized from amino acid in blood circulation flowed into hepatic cells and manufactured in rough endoplasmic reticulum then released to blood circulation via exocytosis. Heme, ammonia and purine metabolisms; heme, amino acid and purine were metabolized to porphyrin, ammonia and uric acid respectively.
- Nutrients metabolism: Liver changes absorbed amino acid to energy. Liver is an essential organ for glucose metabolism. Lipid in form of chylomicron and free fatty acid were absorbed to liver, there had enzymes lipoprotein, lipase to digested glycerol, fatty acid and stored in form of triglyceride. Carbohydrate also digested and stored as glycogen.
- Biotransformation: Drug and toxic substance were transformed in liver via oxidation, reduction and/or conjugation to non-toxic substance via structure alteration as follow mechanism cytochromes P-450, glucuronyltransferase, Sulfotransferase, GSH - S – Transferase.
- Bile acid metabolism: Constituent of liver bile were electrolyte, bile salt, lecithin, cholesterol and bilirubin from red blood cell. Bile acid wad secreted from liver and storage in gall bladder before flow into bile duct.

  As previous describe liver showed a vital role in various system if liver injury it could effected to many organ, thus identified liver injury was important to attenuated liver injury.

Neutrophil-mediated liver injury

Many studies about mechanism of hepatic injury divided to two phase of hepatic injury after ischemic reperfusion. Firstly was neutrophil-independent phase which mediated by reactive oxygen species. And the second phase name was neutrophil-dependent phase, TNF-alpha had important role in this phase. Previous studies of Lisa M. *et cal.* show that TNF-alpha was released after hepatic ischemic/reperfusion (I/R), sepsis or inflammation. ENA-78 was important to recruited

neutrophil to tissue and ICAM-1 was important in neutrophil recruitment in process of transendothelial migration and parenchymal cells adherence. Mechanism of neutrophil recruitment involved three steps. The first with sequestration of neutrophil in blood circulation, after that neutrophil was migrated from vasculature through endothelial cells to liver and adhered to parenchymal cells. As a result of hepatocytes injury. These investigator suggested that liver-derived TNF induced inflammatory response and recruited neutrophil to inflammatory site lead to hepatic injury via increase of ENA-78 and ICAM-1. In addition, various inflammatory mediators which caused neutrophil accumulation in live vasculature such as TNF-alpha, IL-1beta, IL-8, keratinocyte-derived cytokine (KC), macrophage inflammatory protein-2 (MIP-2), cytokine-induced neutrophil chemoattractant (CINC), activated complement factors, platelet-activating-factors (PAF) can increased CD11b/CD18 expression. CD11b/CD18 is one of beta2-integrin family in adhesion molecule.

Biomarker for liver disease

Biological marker or biomarker has been defined as alteration of cellular, biochemical, molecular and biological characteristics which can be measured, evaluated and differentiated between normal and pathologic condition. In addition, biomarkers can be used for examined response of patients to drug therapies and determine prognosis of diseases.The biomarkers were in form of biological media include human cells or tissue, body fluid such as serum or plasma, urine which produced by disease organ or body response to disease. In practice, biomarker used for screening and assessing risk of disease before diagnosis, then determine staging, grading, and selection treatment to patient during diagnosis. In addition biomarker was used to monitor prognosis and choose supplementary treatment, or monitor recurrent diseases.

Ideal biomarker should have approach to measurement easy and safety to both patients and technicians. Cost of test should be inexpensive. Biomarker should be consistent among genders, different countries and genetics. It should have high sensitivity and specificity by positive in patients who had disease and negative in patients without disease, respectively. Moreover, ideal biomarker should be a specific product in target tissue and can differentiate pathologies of disease. The sensitive

biomarker should indicate initial of disease before clinical symptom presentation or significantly expression after tissue injury proportional to severity of pathology and long half-life. The alteration of biomarker should not express in non-correlate condition with disease. The approach to measure biomarker should early detect, simple, accuracy, inexpensive and non-invasive. Data from measurement translate to correlate pre-clinical result and clinical result.

There are various methods to evaluate liver injury and monitor stage of disease with hepatic function. Laboratory test of liver function test include alanine aminotransferase (ALT), aspartate amino transferase (AST), ratio of aminotransferases (AST/ALT ratio), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), serum bilirubin, 5' nucleotidase, ceruloplasmin and alpha-fetoprotein which are end product of metabolism [49] The level changes when liver function abnormality or liver injury occur, liver enzyme (alanine aminotransferase ; ALT) is commonly used in routine laboratory.

Alanine aminotransferase is an enzyme mainly produce in liver. Virtually, ALT also found in extra hepatic organs such as muscle, heart but in liver cells are the highest concentration of all organs. It can be used as biomarker of liver injury because ALT exist in cytosol is soluble state and leakage to extracellular when liver cells damaged. ALT is cytoplasmic catalysing the transamination reaction as follow [16].

L-glutamic acid + a-ketoglutarate -> oxaloacetic acid + L-alanine

Normal values of ALT in serum/plasma is 7-56 U/L. Elevated ALT levels up to 100 U/L were defiined as liver had mild and/or moderate injury [50]. ALT was significantly increased after liver injury at 6-8 h and peak value within 24-36 h. Patients who had ALT up to 500 U/L patients should be carefully observed because high ALT levels implicated high severity of diseases such as hepatitis, liver injury from hypoxia, toxin-induced liver injury. However, elevated ALT correlated with hepatocellular disease but absolute peak of ALT did not parallel with liver cells injury. Recent study found that ALT associated with metabolic syndrome by elevated ALT reduced insulin sensitivity and increased free fatty acid, triglyceride accumulation lead to non-alcoholic steatohepatitis.

Aspartate aminotransferase (AST) is catalytic transamination reaction. AST has two isoforms; mitochondrial form and cytosol form with genetics different. AST is an enzyme mostly found in heart but also found in other tissue such as liver, kidney, skeletal muscle with lower concentrations. AST normal value is 0- 35 U/L, elevated AST detected in myocardial infarction, chronic liver disease which are derived from mitochondrial isoenzyme.

AST/ALT ratio has been used to identified cirrhosis and post necrosis cirrhosis, alcoholic hepatitis and non-alcoholic steatohepatitis, liver fibrosis, chronic hepatitis C infection, Wilson's disease. The impaired liver function associated with mean ratios of 1.17, 1.3 was found in cirrhosis and post necrosis cirrhosis respectively. In liver fibrosis and chronic hepatitis C the ratio was more than 1. In addition, AST/ALT ratio was used to differentiate non-alcoholic steatohepatitis from alcoholic hepatitis by ratio was show 0.9 and 2.6 in each disease respectively.

Alkaline phosphatase (ALP) is hydrolase enzymes responsible for phosphatase removal. ALP found in various organ but high concentration in liver, bile duct and bone marrow. Thus, ALP was used for detect liver disease and bone disorder. The liver is major source of ALP in conditions of liver injury, damaged cells release ALP to extracellular matrix. This test also used to detect bile ducts obstruction If one or more of them were obstructed the levels of ALP will be increased. ALP released when bone marrow when bone cell has activities such as bone growth, repaired or any condition that effect to bone. This test may be used to detect, for example, bone pathology, bone malformation and also monitor treatment of Paget's disease or illness in bone disorder conditions. However, ALP increased didn't determine source of pathology between liver or bone, GGT test or 5'-NT were used to differentiate between liver and bone disease. GGT and 5'-nucleotidase levels are increased in liver disease but not in bone disorders.

Gamma glutamyl transferase (GGT), an microsomal enzyme which found in cell membrane and responsible for extracellular catabolism of glutathione (GSH). The enzyme produce in many organs such as liver, intestine, pancrease, renal tubule and epithelial cells of biliary duct. Liver is major source of GGT which is detected in serum. Normal level of serum GGT is 0 – 85 U/L it was effected by many factors include alcohol, lipid in plasma and fat in body, blood glucose and other drugs.

Mostly, GGT was used to indicated alcohol taking and liver dysfunction. High levels of GGT were result from free radical and deplete threat of glutathione. Previous study reported that high levels of GGT increased risk to cardiovascular disease and metabolic syndrome. Cyseinyl-glyceine, the products of GSH hydrolysis by GGT producing, can originate superoxidide anion radicals via free iron interacted. This effect could be increased atherogenesis by LDL oxidation. There are many evidence about elevated GGT associated with fatty liver which cause hepatocellular damage leading to simulate GGT synthesis. For instead, liver fat excessive could increase oxidative stress, leading to overconsumption of GSH with increased GGT synthesis to compensation. Eventually, a higher GGT production could be secondary to a low grade hepatic inflammation induced by hepatic steatosis

5' Nucleotidase (NTP) is an intrinsic membrane glycoprotein which disseminated throughout the tissues of the body. It help the hydrolysis of the phosphate group from 5'-nucleotides, resulting in corresponding nucleosides. Normal values of 5-NT is 0 to 15 U/L. Elevated 5-NT found in obstructive jaundice, parenchymal liver disease, hepatic metastasis and bone disease. NTP is accurate biomarker for early detection of hepatic primary or secondary tumors.

Ceruloplasmin is a liver synthesized globulin. It function as acute phase protein. It binds with the copper and serves as copper containing protein in blood. Like other plasma protein, serum ceruloplasmin levels drop in patients with hepatic disease, wilson's disease (WD) because of reduced synthesizing capabilities and increased in chronic active liver disease (CALD). Thus, cerulopasmin can be used for differentiate between CALD and WD in routine laboratory.

Alpha-fetoprotein (AFP) also called as alpha-1-fetoprotein, alpha-fetoglobulin, or alpha fetal protein is major protein produced by the yolk sac and the liver during fetal development encoding by AFP gene but it was repressed after birth. It was used as a tumor marker to detect and diagnosis liver, ovarian and testes cancer. AFP levels were increased in patients with hepatocellular carcinoma and ovarian cancer and/or embryonal cell carcinoma. Lung and gastrointestinal tract cancer also found high levels of AFP. There have many evidences reported that detected AFP usually correlated with cancer stage that found lower level in initial stage and higher in final stage.

As previous incidents, the high sensitivity and specificity of screening test can be facilitated to early detection liver injury lead to reduced mortality rate. Although the success of patient safe life, the early detection was interfered by many problems such as over diagnosis, insufficient specific marker, low compliance and analytical tools for find new biomarker in short supply. Limitation of biomarkers move researcher forward to used high-throughput technologies platforms to investigate a new candidate biomarker. These technologies were beneficial for evaluated genomics data, transcriptomics data, proteomics data and metabolomics data. Molecular biomarker has been candidate because the rapid growth of molecular biology and the expanding of laboratory technology. Various type of molecular biomarker discovered by high-throughput technic include DNA biomarkers, RNA biomarkers, Protein biomarkers. These provided potential approach to know spectrum of diseases with distinct applications in epidemiology analyzed, clinical trials, diseases prevention, diagnostic and manage of diseases.

MicroRNA-122

MicroRNA is an interesting molecular biomarker because of it can regulate gene at post-translational step by one gene can be effected to hundred genes. Simply to detect and rarely confounder factor unlike protein based assay. There had various protocols to extract microRNA some people used small RNA, Total RNA and quantified by qRT-PCR or microarray, both two method also require normalized gene, U6 is mostly used as house-keeping gene. U6 is small nuclear RNA (snRNA), non-coding RNA act as major effector molecule associated with RNA splicing.

MicroRNAs (miRNAs) are small non-coding RNA, approximately 20 - 22 nucleotides [35]. Mature miRNAs function as regulatory molecules to repress protein production at post-translational modification[25]. Biosynthesis of miRNAs initial with transcribed of double-stranded primary RNA as hair-shaped approximately 2000 nucleotides in length which is cleaved by RNase II-type (RNA polymerase II). Enzyme Drosha combind with DGCR8 as microprocessor complex to generate the precursor miRNAs (pre-miRNAs) size 60 – 70 nucleotides. Then, pre-miRNAs was transport across nuclear membrane through cytosol by exportin-5. Another RNase II-type enzyme Dicer and TRBP2 in cytoplasm responsible for pre-miRNAs cleavage into 22 nucleotides RNA duplex. Only one strand represents for mature miRNA. Mature miRNAs were grouped with Argonaute (Ago) proteins to form the RNA-induced silencing complex which recognized target gene at 3'-UTR to repress protein expression. The mechanism was, microRNA bound to complementary bases on messenger RNA (mRNA) at 3'-UTR affect to interrupt protein production and mRNA degradation thus protein synthesis was decreased. Normally, miRNA release to extracellular fluid via several process such as microparticles, exosomes and protein bound miRNA. Microparticles were a small vesicle (size 100-100nm) budding from cell membrane and contain cell components including miRNA. Another cell-derived vesicle is exosome, generate from multi vesicular bodies in cytoplasm then fuse with plasma membrane or secretory pathway and secrete to extralcellular in form of exosome size (30-100 nm) [51]. MiRNA can released to extra cellular fluid by protein binding such as Ago-2 to form a complex[52, 53]. Several studies report that mi-RNA can change biological process in to adjacent cells and play role in cell-to-cell communication.

In condition of cell stress from several causes leads to cell apoptosis and or necrosis directly effect to miRNA levels in blood. As part of miRNA release to blood circulation but miRNA measurable not only in blood, cell-free miRNA also found in all part wide array of body fluid and more stable than exogeneously added RNA.

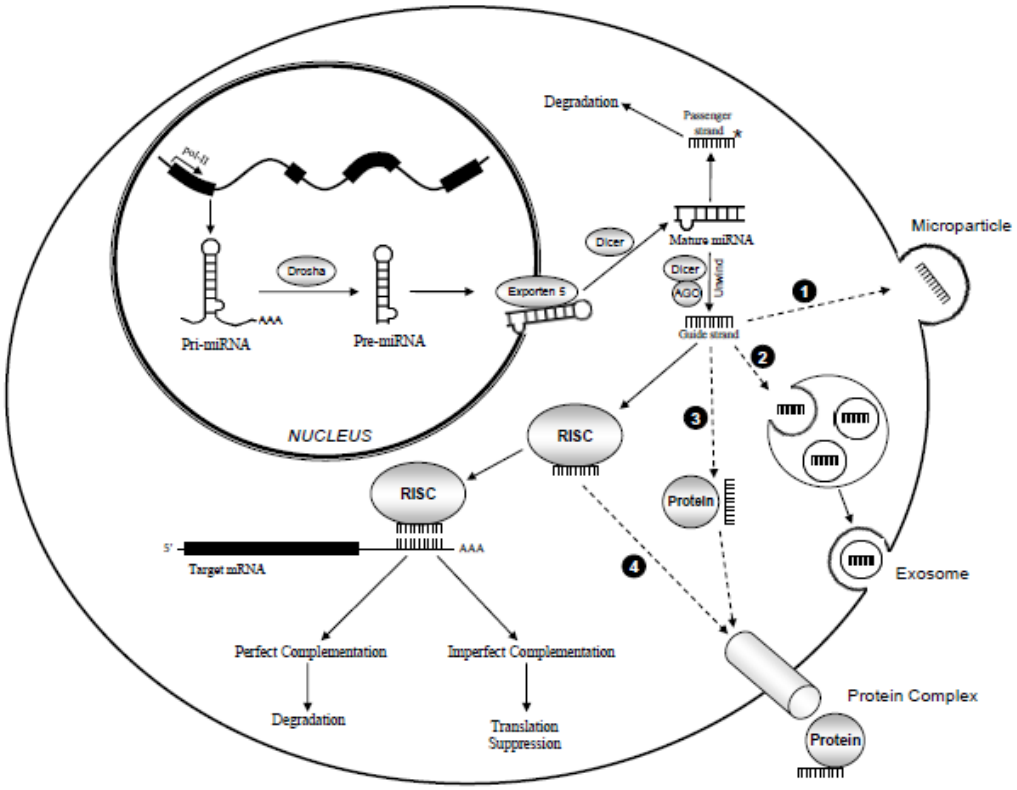


Figure 6 Biogenesis of microRNAs and extracellular release [53].

As previous describe, miRNAs play role in essential cell mechanism such as metabolic regulation, apoptosis, cell proliferation, cell differentiation and maturation including cell development and cell growth [31, 54]. Hence, alterations of miRNAs production has been associated with various disease include metabolic disease, immune disorder disease and correlation between levels of miRNAs expression with cancer has been remarkable. Due to scientists assume that human cells has miRNAs amount up to 1,000 types or more which regulate cells function.

It's surprising that dysregulation of miRNA networks were correlated with many disease. Several evidences show that progress of disease might be contribute from alter miRNAs expression. In hepatocytes, miR-122 was found at 50,000 copies per cell or 70% of total liver miRNA population r[55]. Many studies reported that miR-

122 plays a key role in liver development, differentiation, homeostasis and function[56].

Roles of miR-122 in liver development, liver-enriched transcription factors (LETs) include hepatocyte nuclear factor (HNF) 1alpha, HNF3beta, HNF4alpha, CCAAT/EBP (enhancer-binding protein binding to miR-122 promoter then activate miR-122 expression. One of target miR-122 is cut-like homeobox1 (CUTL1), gene transcriptional suppressor which specific at terminal differentiation, down regulate of CUTL 1 leads to hepatocyte differentiation. Moreover, miR-122 act as positive feedback to stimulate HNF6 in conditional of hepatocyte differentiation [56]. Studies of anti-sense mediated inhibition of miR-122 show the delay liver development in animals implied that miR-122 has important role in liver development and differentiation

Recently, silence miR-122 in normal mice leads to decrease LDL cholesterol, liver steatosis and increase HDL cholesterol. Silence miR-122 in high fat diet fed mouse reduction of long-lasting hepatic steatosis, decrease cholesterol synthesis and increase fatty acid oxidation. Because of some genes associated with fatty acid synthesis, oxidation were regulated by miR-122. Moreover, miR-122 was an inhibitor in mevalonate pathway, function associated with cholesterol and isospenoid.

Interestingly, Hepatitis C virus (HCC) have miR-122 binding site including within 5’ NCR (non-coding region), 25 nucleotides from 3’ nucleotide and 5’ end within 3’ NCP. MiR-122 leads to viral replication through 5’ interact with miR-122 by enhance ribosome with viral RNA. MiR-122 facilitates virus replication in Hek (human embryonic kidney epithelial cells). Structure studies found that miR-122 stimulates open conformation of HCV IRES at 5’NCR leads to facilitates HCV repletion. Ago associated with the viral 5’-UTR secondary structure with present of miR-122 and RISC-like complex enhance stability of HCV viral genome and protect this genome from host 5’ exonuclease. MiR-122-RISC complex protect mRNA degradation more than induce miR-122 to destroy mRNA. As previous review suggest that increase of miR-122 imply to increase viral replication because of treatment patient HCV with miR-122 antogomir inhibit viral replication and quantified amount miR-122 by RT-PCR found that miR-122 level decrease more than 300 fold. Thus, miR-122 possibly an ideal drug target of HCV treatment. In hepatitis B virus infection, miR-122 inhibit

HBV replication and expression via downregulate cyclin G1 and enhance P53 to inhibit HBV transcription.

Moreover, levels of miR-122 has been increase in serum when liver injury. The piece of evidence from *Omar F et al.* show that plasma miRNA function as a sensitive and specific biomarker of tissue injury by treat of liver toxicants, muscle toxicants, stroke-induced rat then quantified amount of miR-122 (liver specific), miR133a (muscle specific) and miR124 (brain specific) by qRT-PCR after treatment [57]. The comparative levels of tissue from experiment show highest levels of miR-122, miR-133a and miR-124 in liver tissue, muscle and brain respectively and results of plasma miRNAs were correlated with tissue. In addition, the detection of miR-122 sensitivity and specificity was demonstrated in experiments of *Sofie et al.* in animal models drug induced liver injury, then measure levels of miR-122, liver enzyme and liver histology. Rat with exposed liver toxic substance show high levels of miR-122, liver enzyme and consistent of liver histology[34]. Although response of miR-122 was parallel with other marker but miR-122 was earlier detect than standards of liver injury marker. Recently, circulating miRNAs are attractive to use as biomarker because of the high abundance and tissue specific properties, differential increase in population with different phenotype [58]. and miRNAs easily amplified for detection by PCR-based method. All of reason make miRNAs as interesting candidate biomarkers for tissue injury.

Hepatic miRNA alteration in different disease of liver injury imply that hepatic injury is cause of miRNA release to blood circulation. There are benefits of miRNA to serve as candidate biomarker include; detect alteration of miR-122 prior ALT, data less vary than animal to animal response. That's interestingly that microRNAs are organ specific, stable and use simply mechanism to detected.

CHAPTER III MATERIALS AND METHODS

PART 1

Animal models of indirect liver injury

Animal models

In this experiment, using thirty eight CD-1 mice. Their ages were 6-week-olds and weight approximately 34±3g. These mice were kept on 12-hour light/dark cycle, temperature (22±1°C), relative humidity (40-50%) and ventilation (15 air changes/h) in conventional room.

Animal experiments were controlled in accordance with National Institutes of Health (NIH) criteria to the used and treatment were approved by Animal Experimentation Ethics Committee of Chulalongkorn University Medical School (8/2556).

To prepare mice for experiment, fifty-four mice were separated to adapt environments for 1 week before surgical. In the experiment, male CD-1 mice mice were perform in 5 group of 6 mice each.

1. Sham operation
2. Bilateral nephrectomy (BiNX)
3. Bilateral ureter obstruction (BUO)
4. Cecal ligation and puncture (CLP)
5. Liver ischemic and reperfusion (I/R)

All surgical protocols were follow National Institutes of Health (NIH) guideline. Mice were anesthesia with isoflurane. Then, opened abdomen to operation and closed two layers with nylon 6-0. There were no post-operative fluid/ antibiotics administration in all models. Data of sham at the different time-points were combined due to the reciprocal value in each model. Renal vessel and ureter in sham group was operate by ventral laparotomy and closure in two layers

Liver injury induced by acute kidney injury model

There are two acute kidney injury (AKI) models was used in this experiment.

In BiNX, The operation were perform through abdomen incision. To create BiNX models renal vessels was cut and clamp after that both kidneys were removed and adrenal glands were remained. The ureters were also cut between the bladder and kidney and clamped.

In BUO, both ureters were tied at 0.5 cm lower position from renal pelvis and ligated between proximal and distal part. BUO mice without intact proximal stump at the sacrifice time were excluded.

Liver injury induced by cecal ligation and puncture sepsis model

CLP represent human disease of rupture appendicitis. It was a gold standard in sepsis studied. Cecum in mice were tied with silk 2-0 and puncture through with 23-gauge needle. In negative control, mice had only ventral laparotomy to identified internal organs and closed.

Animal models of direct liver injury

Liver injury induced by liver ischemic reperfusion model

Mice anesthesized by isoflurane had abdominal incision and induced liver portal triad occlusion by vascular clamp for 50 mins. This process leads to liver hypoxia observed from pale liver. After that, we removed vascular clamp and closed the abdomen in two layers. In control group, abdominal incision was done then then re-opened and closed again at 50 mins later to mimic with experimental groups.

Collection and preparation of serum samples.

When mice were euthanized at 0.5 h and 1 h after liver I/R and sham of liver I/R. In parallel, mice in other models and reciprocal sham were euthanized at 6 and 24 h, blood were collected through cardiac puncture. For ensuring uniformity, all sample were processed identically. Blood was allowed to clotted at room temperature, then centrifuge at 8,000 ×g for 10 min at 4°C to separate serum. Serum

was collected for measuring serum parameters then kept in -80°C until used. Sample with notable hemolysis were discarded.

Serum creatinine, alanine transaminase and cytokine measurement

Serum creatinine (Scr) and alanine transaminase (ALT) were measured with colorimetric detection by QuantiChrom™ Creatinine Assay kit (DICT-500, Hayward, CA, USA) and ALT IFCC MOD™ kit (Diasys Diagnostic Systems, Holzheim, Germany), respectively. Serum TNF-alpha, IL-6, IL-1beta and IL-10 were determined by ELISA (ReproTech, NJ, USA)

Liver histology

Left lobe hepatic tissue was collected from euthanized mice at 0.5 h, 1 h after liver I/R and BiNX, BUO, CLP at 6 h and 24 h. Hepatic tissue was sectioned and fixed in 10% formalin solution for a minimum 48 h at room temperature. After 48 h of fixation, tissue was moved into 70% ethanol for long term storage. Fixed tissue was embedded in paraffin block then using microtome for tissue sectioned at 4 μm. Hematoxylin and eosin colors was stained into tissue then examined liver histology under microscopic with 400x high power field and counting hepatic cells vacuolization from ten fields.

PART 2

Cytokines induced miR122 in Hepatoma cell lines

Hepatoma cell line experiments

Human Hepatoma cell, HepG2, was cultured and maintained at 37°C in a humidified atmosphere of 5% CO_2 95% air in dulbecco's modified eagle medium (DMEM) supplemented with 10% Fetal bovine serum (FBS). HepG2 was treated for 24 h with 100 ng/ml at 50 µl of each cytokine including: TNF-alpha, IL-6, IL-1beta and IL-10 (R&D System Inc., Minneapolis, MN, USA) and vehicle control for HepG2 cell 100,000 cell/ well. After treated, microRNA (miRNA) was extracted by miRNAeasy kit (Qiagen, Valencia, CA, USA) and kept at -80°C until measured.

Preparation of RNA and PCR amplification

miRNA quantified using a Nanodrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington,U.S.A). Ten nanograms of total RNA were conversed to cDNA using Taqman® reverse transcription reagents (Invitrogen™ Applied Biosystem®, Branchburg, New jersey USA.) mix with RT primer. PCR primer using hsa-miR-122 575µl, 20X (assay ID 02245) and RNU6B 1gki50 µl, 5X (assayed ID 001903) was used for control of miRNA assay. The sequence for primers were as follow :

miR122	UGGAGUGUGACAAUGGUGUUUG
RNU6B	CGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTTT

All PCR primers were obtained from invitrogen (Applied Biosystems®). RT reactions were performed at 16°C for 30min, 42°C for 30min, 85°C for 5 min and then maintained at 4°C.

Quantitative real-time polymerase chain reaction

Following the RT reactions, 3 µl of cDNA was used for a polymerase chain reaction (PCR) using 10 µl of the TaqMan Universal PCR mastermix Part Number 4304437 (Applied Biosystems® Branchburg, New Jersey USA.) and 1 µl of miR122, RNU6B probe.

Real-time PCR was perform in ABI step one plus real-time pcr system (Applied Biosystems®) and thermal profile are denaturation at 94°C for 2min followed by 1 cycle of denaturation at 94°C for 20 s, 60°C for 30 s and 35 cycles of annealing at 72°C for 30 s, followed by a final elongation step at 72°C for 10min. Data are expressed in relative to the reference genes using Rui Shi method[57, 59]. All samples were using RNU6B snRNA for normalization purposes. Moreover, U6snRNA was used as the internal control.

Analysis relative change of miR122 expression using $2^{-\Delta\Delta}$CT assay[60]. C_T value can provide from realtime PCR instrument. Calcultation of $2^{-\Delta\Delta}$CT using :

$$\Delta\Delta CT = (CT_{miR122} - CT_{RNU6B})\text{Treated} - (CT_{miR122} - CT_{RNU6B})\text{vehicle control}$$

Statistical analysis

SPSS 16.0 software system (SPSS Inc., Chicago, IL, USA) was used to perform statistical analyzed. Data are presented as mean± S.D. (all SDs were < 5%), with $p < 0.05$ considered significant. Correlation between treated and untreated group was calculated by student's t-test. P-value <0.05 was considered to be statistically significant

CHAPTER IV RESULTS

PART 1

Alteration of Scr, ALT, cytokines, miR-122 level and liver histology in liver injury model

MiR-122 expression after ALT increased in the indirect liver injury.

In the first part of experiments, indirect acute liver injury was conducted from acute kidney injury models and sepsis model (BiNX, BUO, CLP), after that serum creatinine (sCr, kidney injury biomarker), Alanine transminase (ALT, liver injury biomarker), cytokines and miR-122 was measured at two time points includes 6 h, 24 h. The results showed at 6 h sCr levels were increased in BiNX and BUO ($P<0.01$) compared with sham at 0 h and sCr levels increased higher at 24 h after operation. Moreover, sCr in BiNX was elevated faster than BUO from the data BINX versus BUO at 6 show were 1.11±002 versus 0.61±0.03 mg/dL and the comparison at 18 h after first measured were 2.41±0.03 versus 1.47±0.01 mg/dL (Figure 7A, 7B). Levels of sCr also elevated in sepsis model (CLP) at 6 h, 24 h, but less amounts than acute kidney injury models (BiNX, BOU) (Figure 7C). In liver I/R model sCr was measured parallel to other but it was measured at 0.5 h and 1 h (Figure 7D).

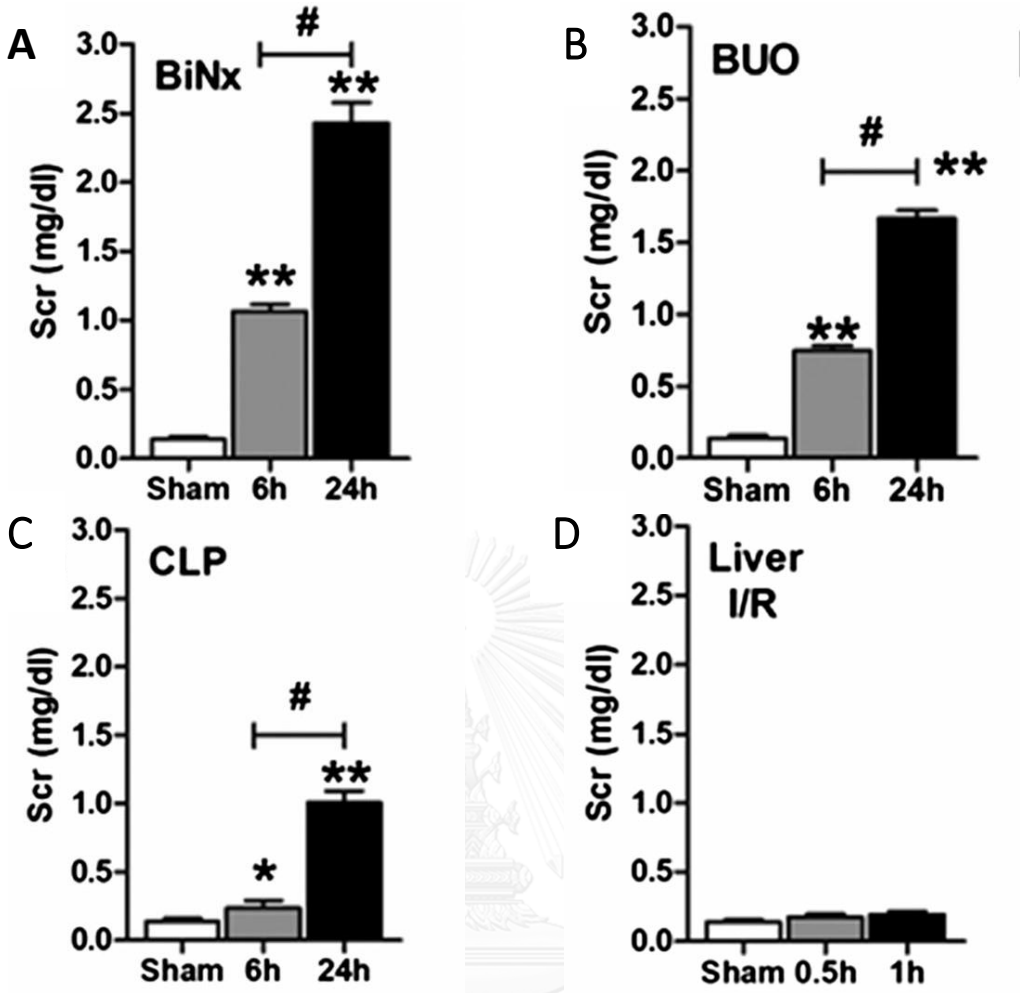


Figure 7 Serum creatinine levels at 6 h and 24 h of BiNX, BUO, sepsis CLP and Liver I/R at 0.5 h and 1 h. Scr increased as early as 6 h in all models of indirect liver injury. (n = 5-7 /sham, 6 h and 24 h in each model). *P <0.05 versus 0h, **P <0.01 versus 0 h, #P <0.01

At the same time, ALT also measured in each models at 6 h and 24 h for indirect liver injury, 0.5 h and 1 h for direct liver injury. ALT was early observed at 6 h in CLP, BiNX (101±13 U/L) and BUO (69±6 U/L) (Figure 8A,B,C). Liver I/R was detected injury and show higher ALT levels at 1 h (Figure 8D).

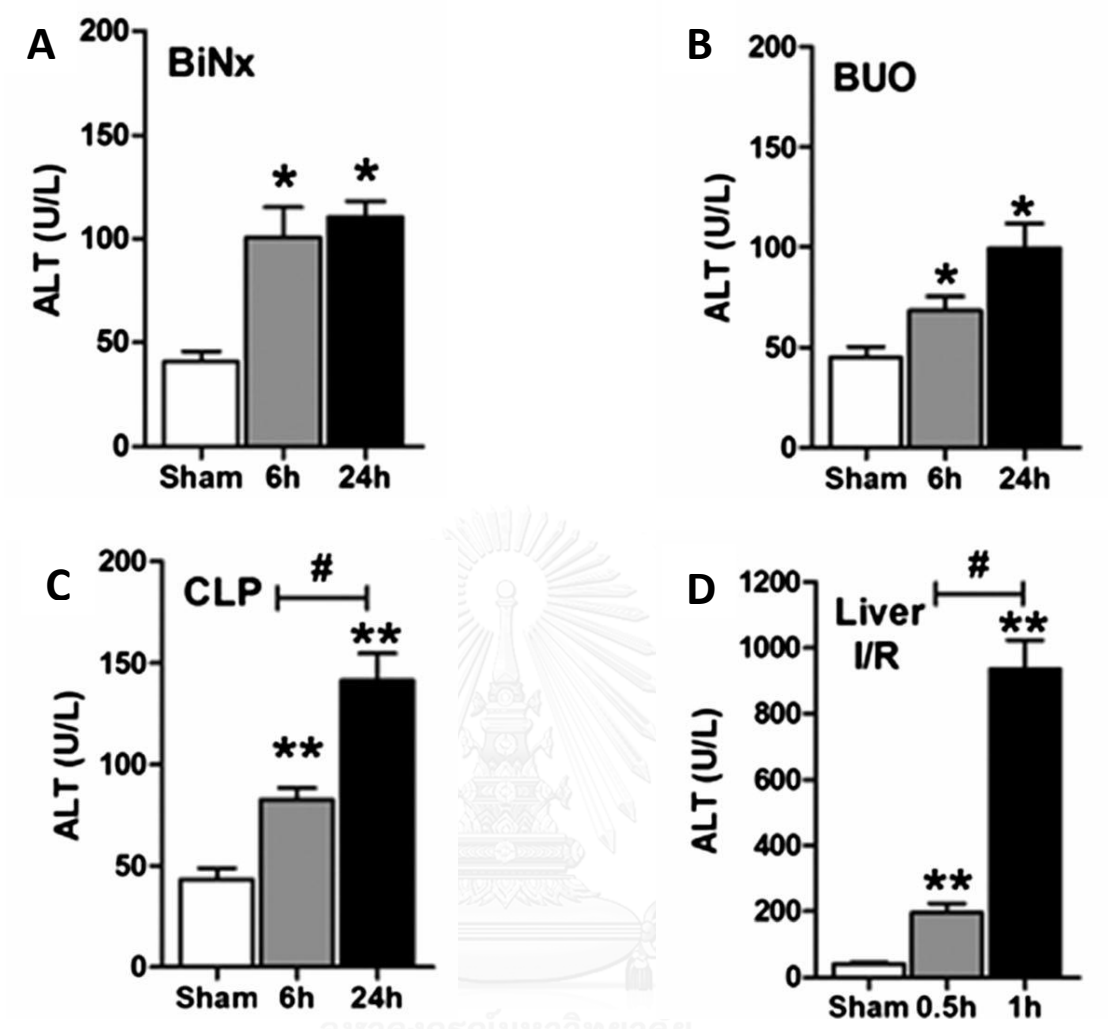
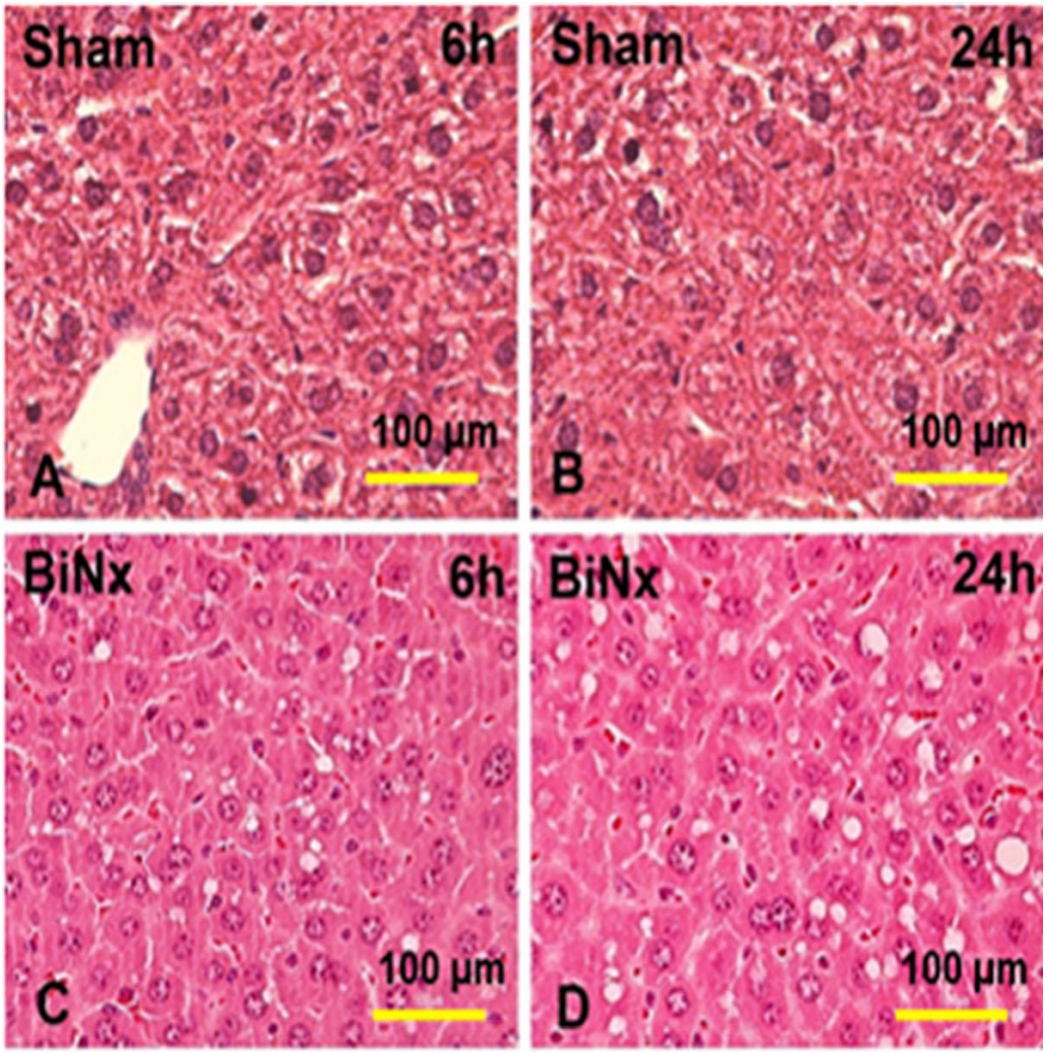


Figure 8 Alanine transaminase levels in BiNX, BUO, sepsis CLP at 6 and 24 h, Liver I/R at 0.5 h and 1 h. ALT increased as early as 6 h in all models of indirect liver injury. (n = 5-7 /sham, 6 h and 24 h in each model). *P <0.05 versus 0h, **P <0.01 versus 0 h, #P <0.01

Liver tissues were embedded in paraffin and stained with hematoxylin and eosin for study liver histology. Hepatocyte vacuolization, a liver injury indicator, was early detected at 6 h in BiNX(Figure 9C) and vaculolization was prominently at 24 h. BUO and CLP found vacuolization appearance at 24 h after surgery (Figure 9D, E, F). In contrast, CLP model was found liver histological changes with vacuolization appearance and leukocyte influx at 6 h post operation to confirmed liver damage (Figure 9G, H). Appearance of hepatocyte vacuolization was detected in liver I/R at 1 h

(Figure 9I, J). The hepatocyte vacuolization of all liver injury models were counted under microscopic and calculated for quantitative analysis of liver histology (Figure 10).



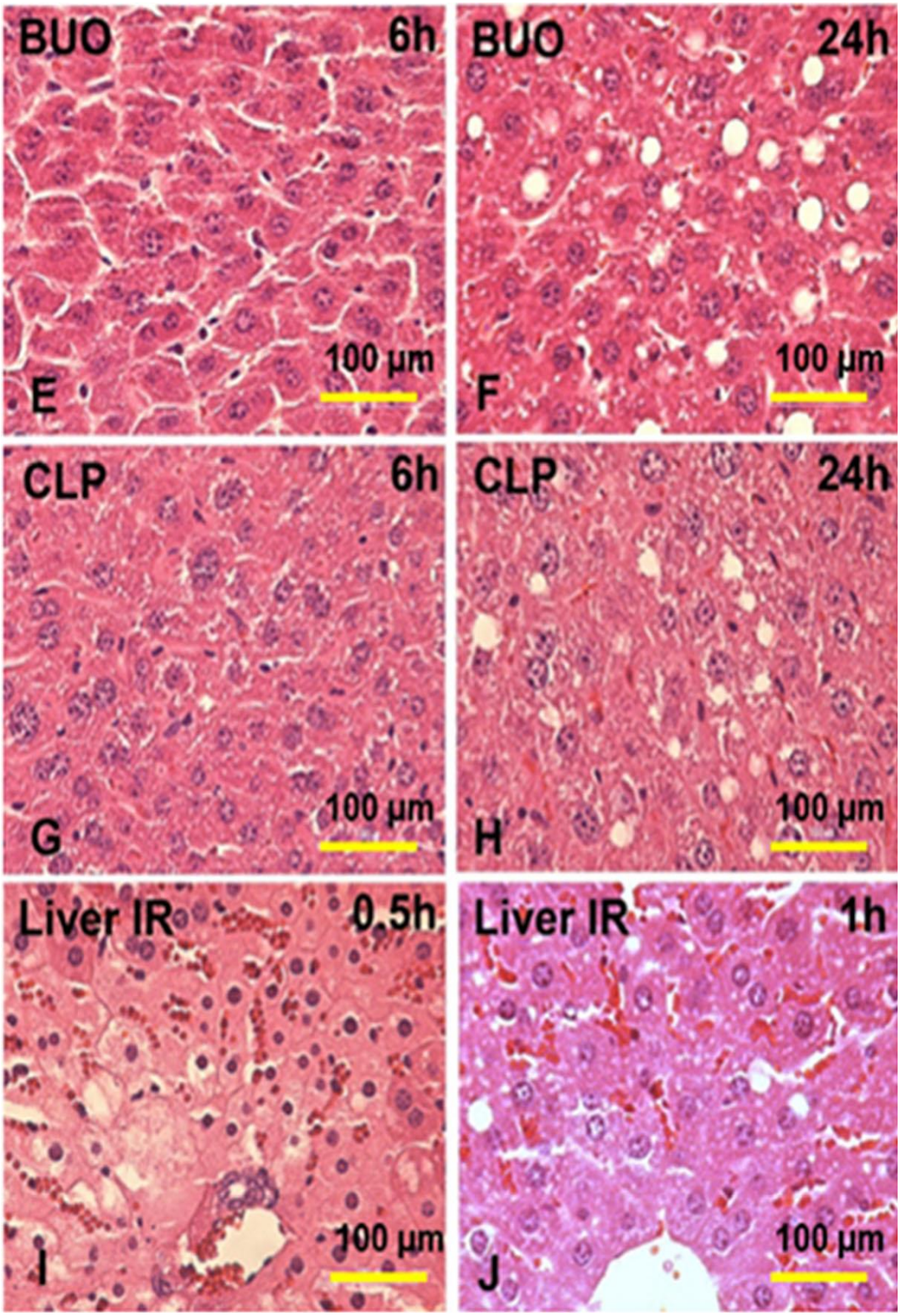


Figure 9 Liver histology of BiNX, BUO and CLP at 6 h, 24 h and liver I/R at 0.5 h, 1 h.

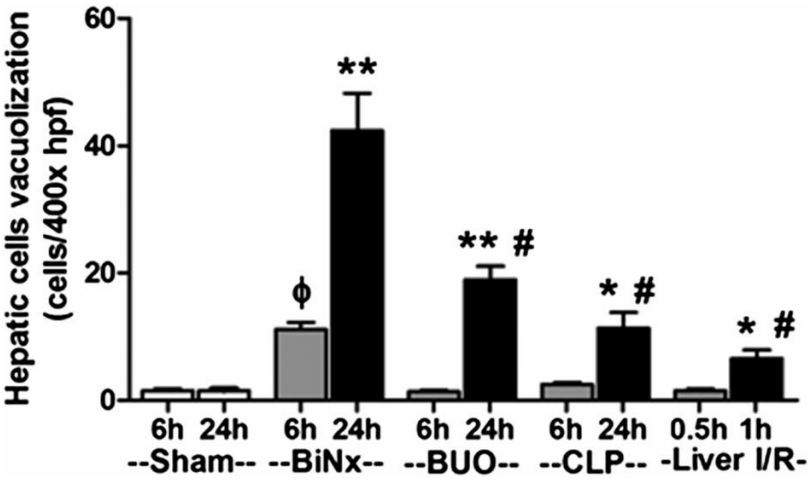


Figure 10 Hepatic cells vacuolization quantified from liver histology.

Serum from BiNX, BUO, CLP models were separated by centrifugation to extract miR-122. Serum miR122 of BiNX was not upregulate at 6 h after surgery but liver injury biomarker and hepatocyte vacuolization were detected. Serum miR-122 at 24 h after BiNX, BUO were compared with sham 0 h, the results showed that miR-122 significantly increased ($P<0.05$) simultaneously with high ALT levels and prominent hepatocyte vacuolization (Figure 11A, B). The levels of miR-122 in CLP models similar with BiNX and BUO, It did not increased until 24 h post-surgery with an appearance of hepatocyte vacuolization. MiR-122 increased together with ALT (141±13 U/L) (Figure 11C). Liver I/R, a direct liver injury model with high ALT, can detected miR-122 upregulate as early as 0.5 h and levels of miR-122 still elevated at 1 b after ischemia and reperfusion (Figure 11D). The results show that high severity of liver correlated with miR-122 expression. Thus, miR-122 and ALT levels were paralleled with liver damage histology.

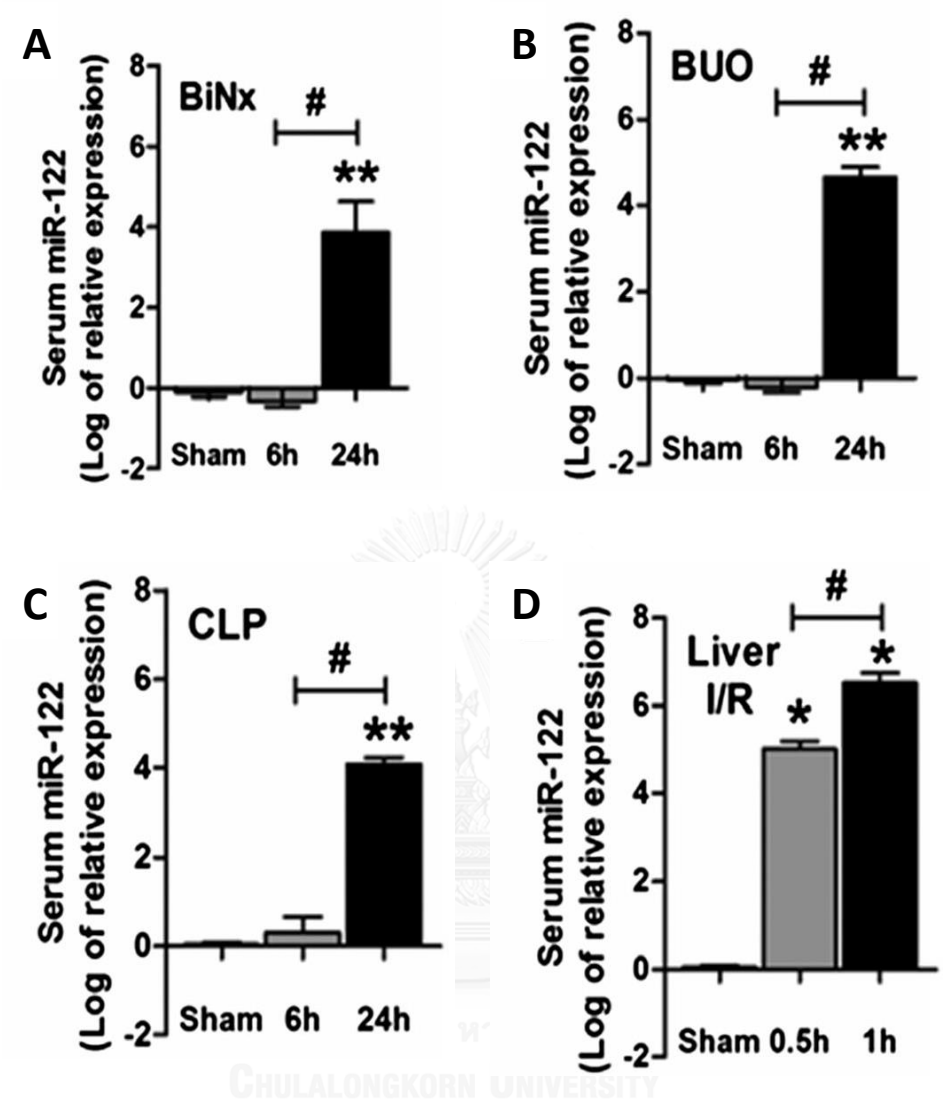


Figure 11 Log of serum miR-122 expression in each models of liver injury..
miR-122 elevated at 24 h in indirect liver injury models but rapidly increased at 0.5 h in liver I/R (n = 5-7 /sham, 6 h and 24 h in each model). *P<0.05 versus 0 h, **P < 0.01 versus 0 h, #P <0.01

Level of miR-122 was positively correlated with ALT in liver I/R injury model.

The pathology of liver I/R models at 0.5 h found the change of nuclei and cytoplasmic in liver cells. Moreover, the section of liver I/R histology was markedly increased of infiltrated neutrophil and vascular abrasion (Figure 9I). At 1 h of liver I/R, liver histology more severe than 0.5 h owing to a large number of cell injuries with alteration of nucleus, cytoplasm morphology, in addition the histology was even more

leukocyte influx and vacuole in hepatocyte than at 0.5 h (Figure 9J). ALT and miR-122 levels were positively correlated with the histopathology in the liver I/R model (Figure 12).

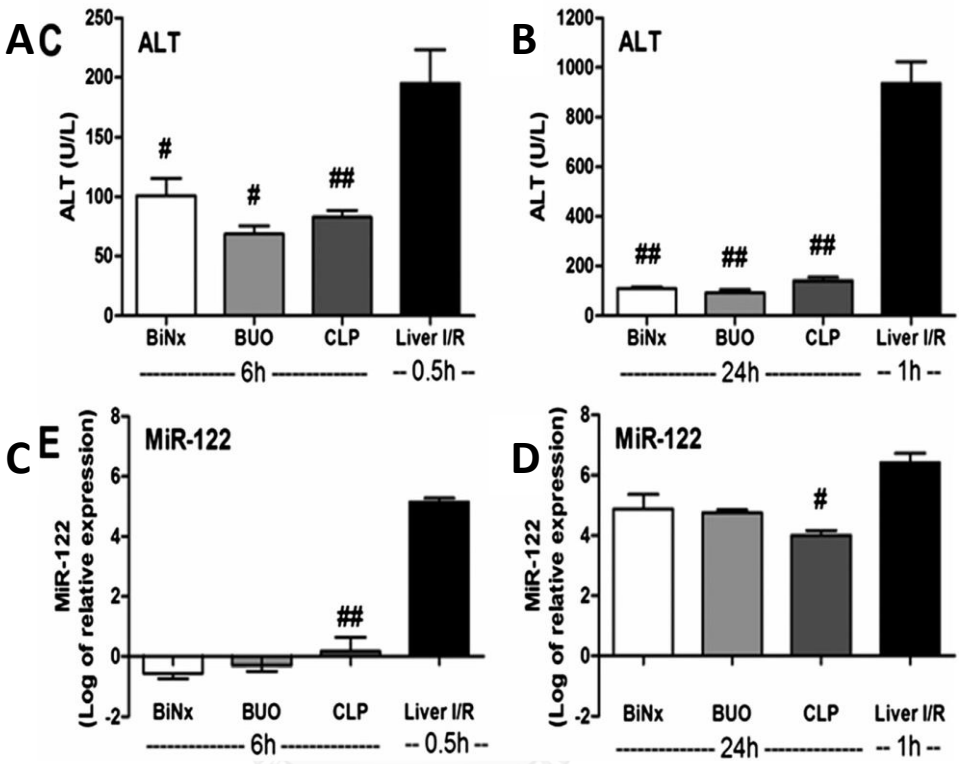


Figure 12 ALT and miR-122 were compared in each liver injury models . miR-122 and ALT alteration were rapidly detect at 6 h in indirect liver injury and 0.5 h in direct liver injury. The higher amount were detected at second timepoint correlate with severity of injury. (n=5–7/sham, 6 h and 24 h in each model). *P<0.05 versus sCr, **P<0.01 versus sCr, #P<0.05 versus liver I/R, ##P<0.01 versus liver I/R

Indirect liver injury model serum cytokines were detected at 6 h.

Pro-inflammatory cytokines (TNF-alpha, IL-6, IL-1beta) and anti-inflammatory cytokines (IL-10) elevated at 6 h of indirect liver injury (Figure13). In contrast, miR-122 did not increase at 6 h in direct liver injury models but miR-122 up regulation was detected at 24 h after surgery (Figure 12C, D). In the liver I/R model serum ALT, miR-122 and cytokine increased as early as 0.5 h simultaneously with appearance of

hepatic cell vacuolization even more prominent of liver damage (Figure 8D, 9I, 9J, 11D).

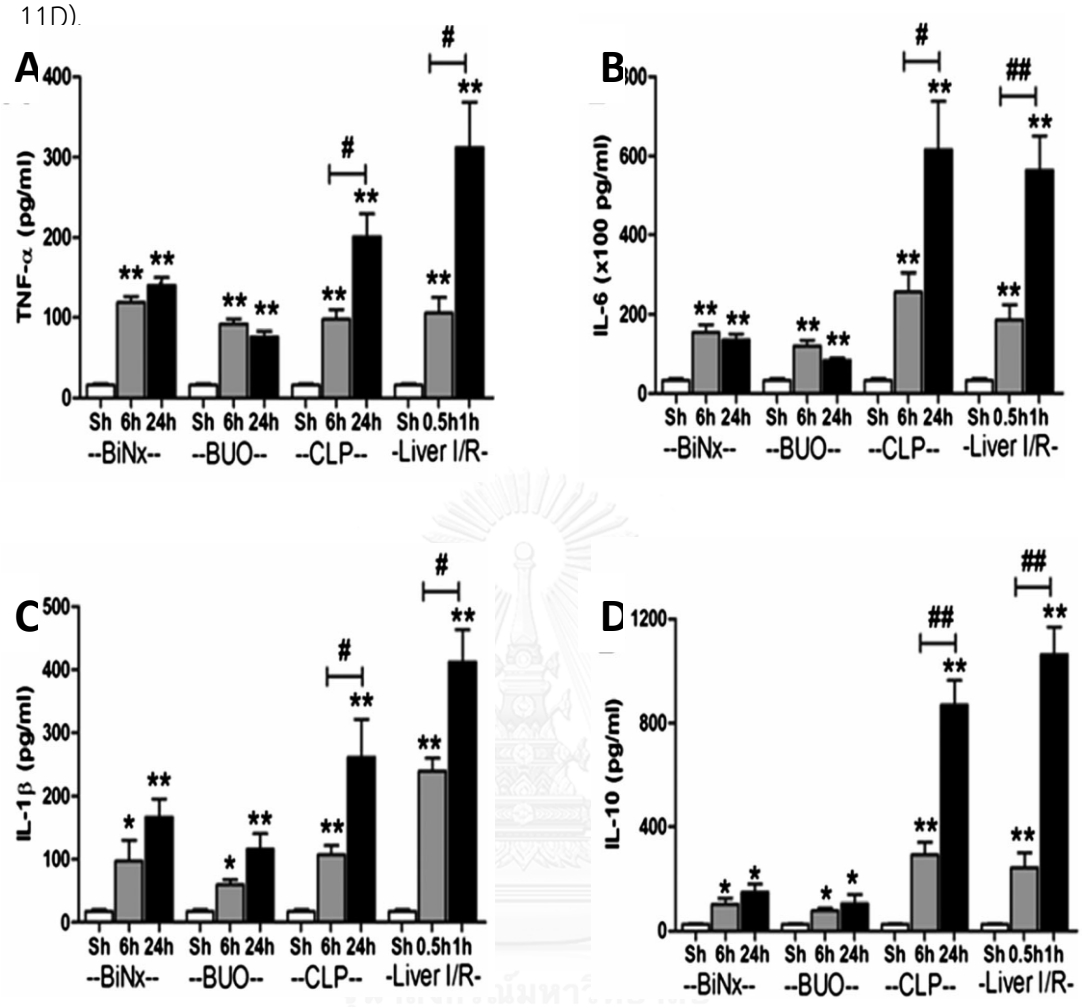


Figure 13 pro-inflammatory cytokines and anti-inflammatory cytokine levels in each liver injury models. Pro- and anti-inflammatory cytokines rapidly elevated 6 h in BiNX , BUO, CL, and 0.5 h in liver I/R. (n = 5 mices/group). *P <0.05 versus 0 h, **P < 0.01 versus 0 h, #P < 0.05.

miR-122 exhibits a good correlation with ALT (ALT> 100 U/L)

The relation between ALT and miR-122 when ALT lower than 100 U/L are r^2 = 0.0257 and *P* = 0.3299 respectively (Figure 14B). In higher amount ALT, ALT > 100 U/L, either from direct or indirect liver injury, there were a good correlations in miR-122 and ALT levels which had r^2=0.59, P=0.995 (Figure 14A, C). However, there was no data of ALT <100 U/L in direct liver injury.

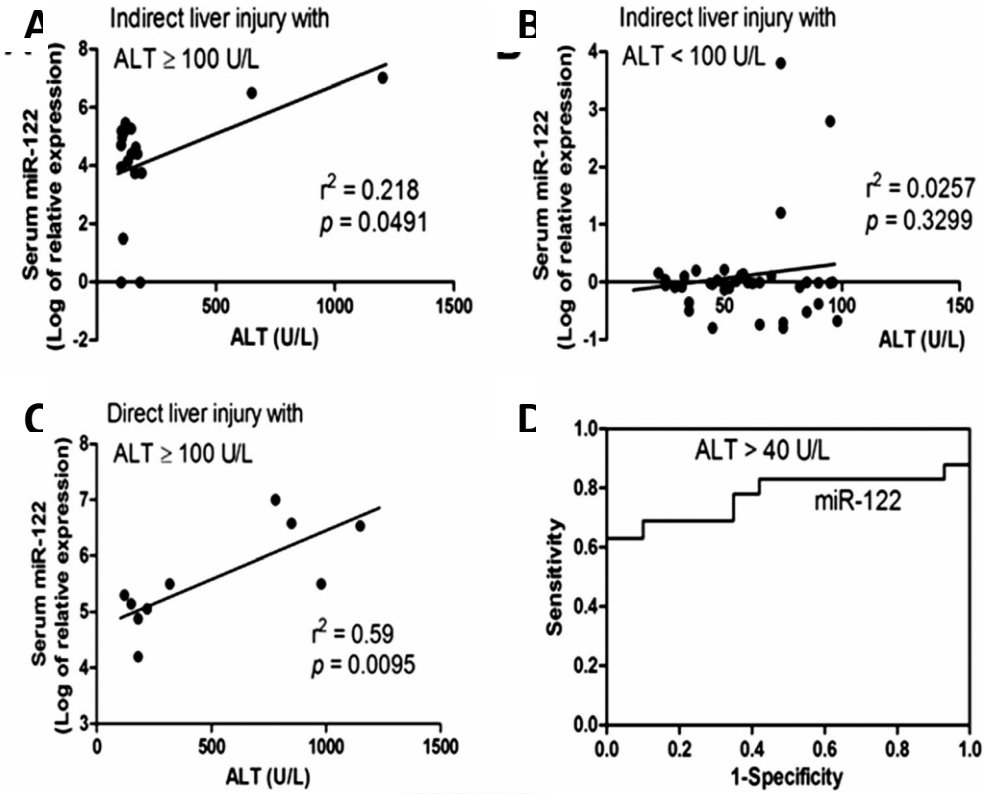


Figure 14 Correlation graph of ALT and miR-122 in indirect and direct liver injury. miR-122 is positively correlated with ALT more than 100 U/L. The correlation of miR-122 and ALT when more than 100 U/L (A) or lower than 100 U/L (B) in indirect liver injury models (BiNX, BUO and CLP) (n – 18 and 39 respectively) and this correlation of direct liver injury with ALT higher than 100 U/L (C) (n=10) was also demonstrated. In addition, the ROC curve of miR-122 in diagnosing liver injury with ALT cut off more than 40 U/L (D).

ROC curve (receiver operating characteristic) was used for analyzed miR-122 sensitivity and specificity. ALT of more than 40 U/L, the routine cut-off values, was used as a gold standard. The ROC between miR-122 and ALT was shown in Figure 14D, area under curve was 0.78. The analyzed miR-122 sensitivity was 68%.. The specificity was 70% indicated some limitation of miR-122.

PART 2

Effect of cytokines to miR-122 production in hepatocyte cell line

miR-122 production in human hepatoma cell line induced by cytokines.

Role of cytokines to miR-122 expression was tested in HepG2 cell line by incubated HepG2 cells with pro-inflammatory and anti-inflammatory cytokines for 24 h. HepG2 cells which treated by pro-inflammatory cytokines and anti-inflammatory cytokines had high miR-122 production (Figure 15). HepG2 cells incubated with TNF-alpha had miR-122 levels compared with other inflammatory cytokines. Thus, miR-122 production, at least in part, induced by cytokines aggregation.

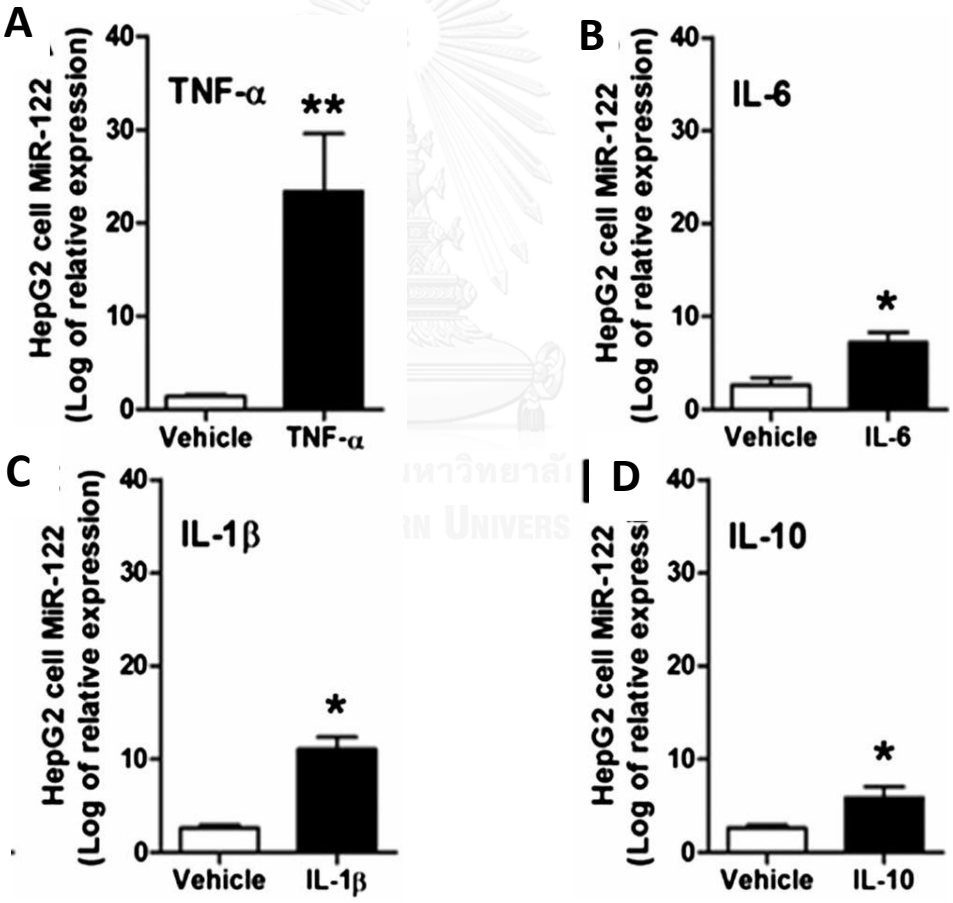


Figure 15 Inflammatory cytokines in the HepG2 cells induced the production of miR-122. miR-122 levels in HepG2 cell post incubation with tumor necrosis factor (TNF)-alpha (A), interlukin (IL)-1beta (B), IL-6 (C) and IL-10 (D). *P < 0.05 versus vehicle control, **P <0.01 versus vehicle control.

CHAPTER V DISCUSSIONS

PART 1

Alteration of Scr, ALT, cytokines, miR-122 level and liver histology in liver injury model

There were various biomarkers to detect liver injury, circulating miR-122 was a candidate biomarker for liver injury because of a high abundance and the tissue specific properties [33]. Several evidences showed good correlation of miR-122 in direct liver injury but little is known in indirect acute liver injury. Thus, the correlation between ALT and miR-122 in indirect liver injury was assessed in animal models of acute liver injury induced from other organs injury such as acute kidney injury (BiNX , BUO) and sepsis model (CLP), in parallel, direct liver injury model (liver I/R) was used as a comparison.

miR-122 detection in indirect and direct liver injury models

There were many reports of BiNX caused liver damage. Thus, in this experiment two different methods were used to inducing AKI. First is BiNX by remove two kidney, another was BUO by induced ureters occlusion but kidneys were remain. BUO model still have renal perfusion. The physiologic difference between two models was to see if interested biomarkers metabolize through kidney or not. The markers that metabolized through kidney will be lower if kidney is absent. For an example, cystatin C will be higher in BiNX compared with BUO [61]. However, miR-122 levels of BiNX and BUO showed no significant different level implied that miR-122 did not metabolize through renal tubular cell.

There were low ALT levels in BUO and BiNX, ALT were two fold and three fold higher than baseline at 6 h and 24 h, respectively. Serum creatinine levels also increase in both of AKI model. In addition, liver vacuolization, histological characteristic of liver injury, was prominent at 6 h in BiNX and at 24 h in both BiNX and BUO.

ALT levels in CLP model also detected at 6 h and 24 h thus sepsis also induce liver injury. Ischemia was one of the important causes of organs failure in sepsis but

did not directly to the large main vessel. Then sepsis caused indirect liver injury. In contrast, the directly occlusion of liver blood supplies in liver I/R model lead to liver hypoxia and very severe injury. Serum creatinine, ALT, miR-122 and liver histology were measured parallel at 0.5 h and 1 h in direct liver injury of I/R because of the severity of model. The follow-up time-point at 6 and 24 h resulted in the high mortality rate of the mice. Then the time-point of the measurement in liver I/R was at 0.5 and 1 h. The results showed there was only slightly alteration of serum creatinine in each time points but ALT, miR-122 increased in correlation with liver histopathology injury. As expected, liver I/R with more direct liver injury showed highest ALT among all models. But, among indirect liver injury models, BiNX showed highest hepatic cells vacuolization at 6 h ($P<0.05$). Other indirect liver injury models showed prominent liver cell vacuolization at 24 h. Figure 10 show hepatic cells vacuolization count was significantly increased at 24 h of all models.

Serum miR122 of BiNX did not increase at 6 h after surgery but liver injury biomarker and hepatocyte vacuolization were detected, hence miR-122 increase slower than ALT and liver histology alteration.

With all liver injury models induced by several results the renal dysfunction or liver ischemia or combination of several factors (sepsis), it was interesting that all models were able to induce miR-122 production. The correlation of miR-122 and ALT in indirect liver injury show limited correlation in low level of ALT (ALT <100 U/L).

Of note, AKI 2 models which both of models lead to acute zero glomerular filtration rate. The difference between these two models was the presentation of kidneys. In BiNX, kidneys were removed but in BUO, kidneys were still existed. We used two different AKI models for investigate whether miR-122 biomarker metabolized through kidney. A previous study showed that serum cystatin C, a biomarker of AKI [61], was absorbed by kidneys. Comparison levels of cystatin C in BiNX and BUO found that cystatin C increased in BiNX but decreased in BUO. In this experiments, serum miR-122 was not different in both BiNX and BUO indicate that miR-122 didn’t metabolized through kidney.

MiR-122 increased late, at 24 h, in indirect liver injury models despite high cytokines at 6 h

High cytokines in AKI and sepsis were mainly cause of organ injury due to the lower excretion and the higher production in AKI condition. Cytokine accumulation have previously been reported to cause liver damage. In BiNX, BUO and CLP model, ALT and cytokines was detected at 6 h but not miR-122 which detectable at 24 h. The up regulation of miR-122 after cytokine elevation was possible that inflammatory cytokines might induce miR-122 production.

Serum miR-122, for the detection of liver injury in indirect liver injury.

Normally, ALT of healthy people and rodents were less than 40 U/L[50]. As previous describe, ALT seems to correlated well with miR-122 when ALT > 100 U/L. But with ALT < 100 U/L, miR-122 showed a less correlation. More importantly, miR-122 increased later than liver histological change. Then the usefulness of miR-122 in indirect liver injury with low ALT level might be limited. More studies needed.

However, despite of miR-122 was detected later than ALT in indirect liver injury models, miR-122 showed high abundance in hepatocyte and still might be important as new therapy in several diseases [55].

PART 2

Effect of cytokines to miR-122 production in hepatocyte cell line

Cytokine which increased earlier in indirect liver injury might responsible for miR-122 production at 24 h. This concept was confirmed by *in vitro* experiment, HepG2 cells incubated with individual cytokines (TNF-alpha, IL-1beta, IL-6 and IL-10) for 24 h. The increased of miR-122 production was found in treated cells compare with vehicle control and TNF-alpha induction resulted in the highest miR-122 than other group (Figure 15A). It seems to be TNF-alpha had high potency to induced miR-122 production in HepG2 cells. Nevertheless, this result is a proof of concept that cytokines accumulation was responsible for serum miR-122 production.

CHAPTER VI CONCLUSIONS

All of BiNX, BUO and CLP models were representative of indirect liver injury condition. On contrary, liver I/R was used for a direct liver injury. Liver injury was detected by increased of serum ALT, miR-122 and hepatocyte vacuolization in histology. Both of indirect and direct liver injury induced miR-122 production at different time point.

We demonstrated the correlation between miR-122 and ALT in indirect and direct liver injury. In the indirect liver injury, ALT can be detected as early as 6 h and higher level at 24 h. But miR-122 was detectable at 24 h but not 6 h. Interestingly, miR-122 elevation slower than ALT. But at 24 h of indirect liver injury (high ALT level), ALT correlated well with miR-122. We concluded that miR-122 did not has any benefit over ALT when ALT less than 100 U/L. This is a limitation of miR-122 as liver injury biomarker despite the good stability of miR-122 in the test samples. In indirect liver injury, cytokines were one factor which plays an important role for miR-122 production. According to the *in vivo* experiments, high cytokines levels were demonstrated after indirect liver injury models. Increased miR-122 in the indirect liver injury models might due to cytokine accumulation. Results from *in vi*tro experiments concluded that incubation of several inflammatory cytokines results in miR-122 production in hepatocytes.

On the other hand, in a direct liver injury model, miR-122 was positively correlated with ALT as previously showed in several publications. MiR-122 increased very early (at 0.5 h after liver I/R) and enormously higher at 24 h after I/R. The correlation of miR-122 and ALT was very good in this direct liver injury. Then the translation of miR-122 measurement in obviously liver injury in patient condition is interesting.

All in all, this experiment can conclude that the measurement of miR-122 in serum for liver injury detection is an interesting assay which help to handle samples easier than protein biomarker due to the stability of miR. However, in this experiments show the limitation of miR-122 in the detection of indirect liver injury with less ALT level. The adoption of miR-122 assay for the routine laboratory of liver injury biomarker needs more confirmatory studies.

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APPENDIX

VITA

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