Master of Science

Time-course changes in the expression levels of microRNA as a biomarker in mouse drug induced liver injury (DILI) model

The Graduate School of

University of Ulsan

Department of Medical Science

Da Som Jeong

Time-course changes in the expression levels of microRNA as a biomarker in mouse drug induced liver injury (DILI) model

Supervisor: Woo-Chan Son

A Dissertation

Submitted to
the Graduate School of the University of Ulsan.
In partial Fulfillment of the Requirements
for the Degree of

Master of Science

by

Da Som Jeong

The Graduate School
of the University of Ulsan
Department of Medical Science
February 2022

Time-course changes in the expression levels of microRNA as a biomarker in mouse drug induced liver injury (DILI) model

This certifies that the master's thesis of Da Som Jeong is approved.

_____

Committee Chairman Dr. Chang Ohk Sung

_____

Committee Member Dr. Jong Hoa Ok

_____

Committee Member Dr. Woo-Chan Son

The Graduate School

of the University of Ulsan

Department of Medical Science

February 2022

Abstract

Liver is a major site of metabolism and exposed to various kinds of xenobiotic substances. Therefore, liver frequently becomes a target organ of hepatotoxicants and finally results in drug-induced liver injury (DILI). However, the diagnosis and treatment for DILI is complicated. Therefore, there is an urgent need for more specific biomarker for DILI which have better diagnostic and prognostic potential. microRNA (miRNA) is a small non-coding RNA that regulates gene expression, and mediates normal homeostasis, development, physiology, and even disease pathogenesis. Therefore the possible correlation of miRNA and DILI is needs to be investigated for excavation of potential miRNA biomarkers.

In this study, acetaminophen (APAP), isoniazid (INH), and Tamoxifen (TAM) are used to induce liver injury in CD-1 (ICR) mice. The expression levels of pre-selected miRNAs extracted from liver tissue and levels of serum biomarkers are measured at 5 timepoints throughout a day (0, 2, 6, 12, and 24 h). Histopathological analysis was also conducted at same timepoints. Notably, over the time-course of liver injury, miRNAs from tissue showed changes in expression levels. miRNAs have been reported to mediate apoptosis or cellular protection showed significant changes in expression at early to middle phase. On the contrary, miRNAs regulate inflammatory pathway or immune response changed at middle to late phase.

At present, the serum ALT and AST levels are used to determine the degree of liver injury and the diagnosis for DILI. However, there have been shortcomings of these biomarker in DILI diagnosis. Although ALT is liver specific, AST is affected by other organs than liver. Moreover, the exact stages of liver injury, for example, metabolization, apoptosis, initiation of innate immune system, or inflammation and hepatocyte necrosis. Therefore, more specific, and fast biomarkers for DILI is needed in order to differentiate other liver diseases from DILI.

In conclusion, the present study suggests several miRNA biomarkers in three DILI models at different timepoints. Particularly miR-677, which is seemed to be involved in the inflammatory response, showed significant changes in all three DILI models. These results are associated with inflammatory cell infiltration in histopathological analysis. The exact function of miRNA-1839 in liver injury has not been studied and its function in other organs are still unclear. Recent study suggested correlation with liver cell apoptosis, but it could have other functions in liver injury models.

Keywords: Drug-induced liver disease, microRNA, acetaminophen, isoniazid, tamoxifen

Contents

List of Tables

List of Figures

1. Introduction

Liver is a major site of metabolism and exposed to various kinds of xenobiotic substances. Drug metabolism in the liver leads to production of toxic metabolites, and liver frequently becomes a target organ (Leise, Poterucha, & Talwalkar, 2014). Acetaminophen, anesthetics such as halothane, antituberculosis agents such as isoniazid, non-steroidal anti-inflammatory drugs (NSAIDs), and diverse xenobiotics produce toxic effects to liver. In addition, a plethora of traditional medical therapies and herbal remedies may also be hepatotoxic. Drug-induced liver injury (DILI) is an adverse effect caused by those xenobiotics. DILI has been provoked cessation of drug development, restrictions on drug usage, and even withdrawal of approval drugs (Endo, Yano, Fukami, Nakajima, & Yokoi, 2014). Since DILI is one of the dominant cause of acute liver failure (ALF), early and precious diagnosis of DILI is important (Guicciardi, Malhi, Mott, & Gores, 2013). However, diagnosis and management of DILI is challenging because the mechanism of DILI is still unclear (Hoofnagle & Björnsson, 2019). The incidence of DILI is uncommon and differential diagnosis and exclusion of other liver diseases are complicated. Nonetheless, discontinuation of medication and intensive therapy are required for DILI patients. If rapid and accurate diagnosis and effective treatment are not adjusted, DILI could be worsened, and patients may require liver transplantation. DILI and ALF even ends in death (Sarges, Steinberg, & Lewis, 2016). However, it is hard to differentiate the severity or grade of DILI with current biomarkers, but late diagnosis is directly connected to poor prognosis (Vliegenthart, Antoine, & Dear, 2015). Therefore, more specific, and advanced biomarkers are required for better diagnosis and prognosis for DILI patients.

DILI in human is typically classified into three types, which is direct, idiosyncratic, and indirect hepatotoxicity. Types can be sub-categorized as acute or chronic, and either as hepatitis, cholestatic, or mixed pattern of injury (David & Hamilton, 2010). The hepatitis pattern is characterized by hepatocyte necrosis and is associated with a poor prognosis. There are three types of acute cholestatic drug-induced injury. The types are bland cholestasis is the result of abnormal biliary secretion which is not accompanied by significant hepatocellular damage, cholestatic hepatitis (mixed type) refers to cholestasis with concomitant hepatic parenchymal damage, and acute cholestasis which is defined by the presence of bile duct injury or cholangiolitis (Erlinger, 1997). Hepatotoxic drugs may cause chronic cholestasis through two additional mechanisms. Those mechanisms are the obliteration of bile ducts, also known as the vanishing bile duct syndrome, and the other is extrahepatic biliary obstruction, known as secondary sclerosing cholangitis (Chitturi & Farrell, 2001). The types and characteristics of

DILI types are summarized in table 1 (Hoofnagle & Björnsson, 2019).

Table 1. Summarization of types of DILI

Variable	Direct hepatotoxicity	Idiosyncratic hepatotoxicity	Indirect hepatotoxicity
Frequency	Common	Rare	Intermediate
Dose-related	O	X	X
Predictable	O	X	△
Time to onset	Rapid (days)	Variable (days to years)	Delayed (months)
Phenotypes	Acute hepatic necrosis, serum enzyme elevations, sinusoidal obstruction, acute fatty liver, nodular regeneration	Acute hepatocellular hepatitis, mixed or cholestatic hepatitis, bland cholestasis, chronic hepatitis	Acute hepatitis, immune-mediated hepatitis, fatty liver, chronic hepatitis
Implicated agents	Acetaminophen, Niacin, Aspirin, Cocaine, Amiodarone (IV), Methotrexate (IV), cancer chemotherapy	Amoxicillin-clavulanate, Cephalosporins, Isoniazid, Nitrofurantoin, Minocycline, Fluoroquinolones, Macrolide antibiotics	Antineoplastic agents, Glucocorticoids, Monoclonal antibodies, Protein kinase inhibitors
Causes	Intrinsic hepatotoxicity due to high dose of xenobiotics	Idiosyncratic metabolic or immunologic reaction	Indirect action of agent on liver of immune system

MicroRNAs (miRNAs) are short, non-coding RNAs consisted of about 18 – 25 nucleotides. miRNAs regulate gene expression at post-transcriptional level. miRNAs target mRNA by imperfectly base-paring to partially complementary 3'-UTR regions and promoting a reduction in their translation (Guo, Ingolia, Weissman, & Bartel, 2010). This reaction is followed by target mRNA de-adenylation and de-capping, and it results in more rapid degradation of mRNA degradation (L. Wu, Fan, & Belasco, 2006). Over 30% of mammalian genes are regulated by miRNAs through those mechanism (Lewis, Burge, & Bartel, 2005). miRNAs have also been shown to be integral in regulating not only just normal homeostasis, development, and physiology but also disease pathogenesis (initiation, progression, or recovery of the disease) (D. Wu & Murashov, 2013). Moreover, drug-metabolizing enzymes, such as those from the CYP family genes, are targeted by certain miRNAs (Mohri et al., 2010). These aspects of miRNA highlights the potential of miRNAs as mechanistic biomarkers of pathogenesis.

Several miRNAs have been explored as potential biomarkers and mediators in the progression of pathogenesis for liver injuries (Sita-Lumsden, Dart, Waxman, & Bevan, 2013). The current

golden standard biomarkers for liver injury is serum ALT and AST level. However, those biomarkers are not sufficient for distinctive diagnosis of DILI. Since serum ALT and AST levels are affected by the injury of other organs, so not selective for liver injury. Therefore, gene expression variants would be more preferable for the classification of hepatotoxicants and diagnosis for liver injury. Recently, several reports concerning miRNA expression in relation to toxicological phenomena have been published and suggest that aberrant miRNA expression is involved in the progression of diseases, including cancer, heart disease, viral infections, and inflammatory diseases (Giusti, D'Ascenzo, & Dolo, 2013). It has been reported that miRNA expression levels differ significantly in various diseases, indicating the potential for their use as biomarkers. The liver-enriched miRNA species, such as miR-122, demonstrate high expression levels in DILI model. Especially for miR-122, which is associated with liver biology and disease, have been valid for potential biomarker for diagnosis of hepatotoxicity (Antoine et al., 2013). miR-122 also has correlation with cell cycle progression, hepatocellular carcinogenesis (B. Wang, Wang, & Yang, 2012), lipid metabolism (Tsai et al., 2012), and fibrosis (Arataki et al., 2013).

However, miRNA expression in time-course changes has not been comprehensively studied caused by various xenobiotics and drugs. Moreover, various kinds of miRNA should be suggested in order to clearly distinguish DILI type and suggest more specific biomarkers for liver injury. In this study, time-course changes of selected miRNAs and correlation with serum biomarkers and histopathological findings were investigated. Additionally, kinetics of miRNAs were identified to suggest miRNAs as new biomarkers for liver injury models.

2. Materials and methods

Animals

A total of 45 male CD-1 (ICR) mice, 5 weeks old, and weighing 27-30 g, were used in this study. The mice were obtained from ORIENT BIO (Seongnam, Korea). All mice were kept in a separate standard plastic cage in specific pathogen free (SPF) facility under controlled light and temperature conditions with free access to food and water ad libitum. All the animal care were approved by the Institutional Animal Care and Use Committee (IACUC) of No. 2020-12-113 (Asan Institute for Life Science, Seoul, Korea).

DILI models

After 1 week of adaptation period, mice were randomly divided into 3 groups for 15 mice each. For APAP group, mice were intraperitoneally (i.p.) administered 500 mg/kg APAP suspended in normal saline, 10 ml/kg. For INH group, mice were orally administered 200 mg/kg INH in normal saline, 10 ml/kg. TAM group were intraperitoneally injected 200 mg/kg TAM dissolved in normal saline, 10 ml/kg. Blood and liver tissue samples were obtained before treatment, 2, 6, 12 and 24 h after administration of each chemical (n=3 per time point/group). Samples were collected under isoflurane anesthesia at indicated time points. Blood was collected from the caudal vena cava. Collected blood samples in SST tube (BD Bioscience, Cat no.365967) were centrifuged at 3,000 rpm for 10 min at 4 , and the serum was collected and stored at -80 until use. Medial lobe with gall bladder and left lobe from liver were fixed in 10 % neutral buffered formalin. The remaining portion of liver tissue were stored in RNAlater™ Stabilization Solution and preserved at -80 .

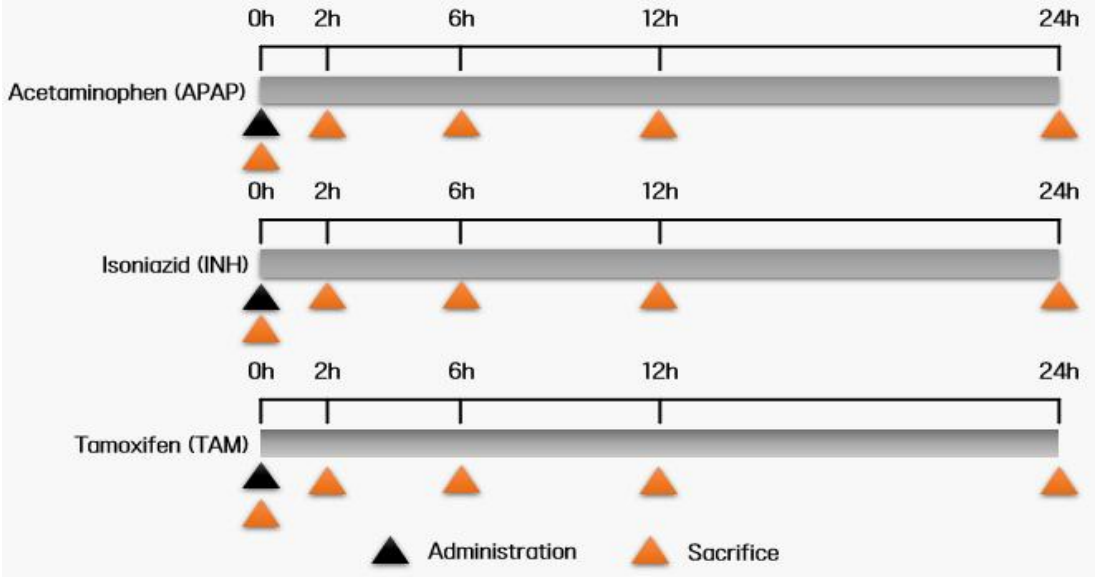

Figure 1. Study design for APAP, INH and TAM. Blood and liver tissue samples were collected before treatment, 2, 6, 12, 24 h after treatment. 3 mice were sacrifice for samples at each time point per group.

Biochemical assay

Serum Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), Total Bilirubin (TBIL), Triglyceride (TG) and Cholesterol (CHOL) levels were determined using a standard automatic analyzer (Hitachi 7180, Hitachi Co., Ltd, Japan)

Total RNA isolation and reverse transcription

Total RNA extraction from livers of mice was performed using miRNeasy Micro Kit (QIAGEN, Germany) according to the manufacturer's protocol. The total RNA concentration was measured using Nanodrop 2000 (Thermo Fisher Scientific, USA). RNA quality and integrity were analyzed using Nanodrop 2000 and performing 1% agarose gel electrophoresis. Total RNA was reverse transcribed into cDNA using TaqMan™ Advanced miRNA cDNA Synthesis Kit (A28007, Applied Biosystems™, USA) according to manufacturer’s instruction.

miRNA Real-time quantitative PCR (RT-qPCR) analysis

RT-qPCR analysis of individual samples were performed in a total volume of 20 μL, comprised of 2X TaqMan™ Fast Advanced Master Mix (4444964, Applied Biosystems™, USA) 10 μL, 20X TaqMan™ Advanced miRNA Assay (A25576, Applied Biosystems™, USA) 1 μL, RNase-free water 4 μL and 0.1X cDNA template 5 μL. The RT-qPCR reactions were performed in MicroAmp™ Optical 384-Well Reaction Plate with Barcode (4309849, Applied Biosystems™, USA) on a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems™, USA). The RT-qPCR cycling condition were as follows : Enzyme activation at 95 for 20 seconds, followed by 40 cycles of denature at 95 for 1 second and anneal / extend at 60 for 20 seconds. The miRNA Assay sequence are presented in Table 1. Hsa-mir191-5p and has-mir-361-5p were used as endogenous controls according to manufacturer's instruction. Relative miRNA expression fold change was calculated using 2- ΔΔ Ct method (Livak & Schmittgen, 2001). All experiments were conducted in triplicate.

Table 2. List of miRNA Assay sequences used in this experiment

Assay Name	Assay ID	Target Sequence
has-miR-191-5p	477952_mir	CAACGGAAUCCCAAAAGCAGCUG
has-miR-361-5p	478056_mir	UUAUCAGAAUCUCCAGGGGUAC
mmu-miR-34a-5p	mmu481304_mir	UGGCAGUGUCUUAGCUGGUUGU
mmu-miR-107-3p	mmu478254_mir	AGCAGCAUUGUACAGGGCUAUCA
mmu-miR-125b-5p	mmu480907_mir	UCCCUGAGACCCUAACUUGUGA
mmu-miR-155-5p	mmu480953_mir	UUAAUGCUAAUUGUGAUAGGGGU
mmu-miR-378a-5p	mmu482643_mir	CUCCUGACUCCAGGUCCUGUGU
mmu-miR-677-3p	mmu482058_mir	GAAGCCAGAUGCCGUUCCUGAGAAGG
mmu-miR-677-5p	mmu482059_mir	UUCAGUGAUGAUUAGCUUCUGA
mmu-miR-1839-3p	mmu481561_mir	AGACCUACUUAUCUACCAACAGC
mmu-miR-1839-5p	mmu480961_mir	AAGGUAGAUAGAACAGGUCUUG
mmu-miR-122-5p	mmu480899_mir	UGGAGUGUGACAAUGGUGUUUG
mmu-miR-134-5p	mmu480922_mir	UGUGACUGGUUGACCAGAGGGG
mmu-miR-200a-3p	mmu478490_mir	UAACACUGUCUGGUAACGAUGU
mmu-miR-200a-5p	mmu478752_mir	CAUCUUACCGGACAGUGCUGGA

Pathological examination

Formalin-fixed median lobe and left lobe were sectioned and dehydrated in graded ethanol, cleared in xylene in a Shandon Excelsior ES tissue processer (Thermo Fisher Scientific). Processed liver samples were embedded in paraffin on the EG1150H paraffin-embedding station (Leica Biosystems, Wetzlar, Germany). The paraffin blocks were cut into sections 3㎛ and stained with hematoxylin and eosin using Autostainer (Leica Biosystems). Histological examination of the liver was scored using semi-quantitative method. The lesions were graded as minimal (1), slight (2), moderate (3), and marked (4).

Statistical analysis

The data were presented as the mean ± standard error of the mean. Statistical differences between groups (untreated – treated) were determined using Student's t-tests. Statistical analysis were conducted using IBM SPSS software 27 (SPSS, Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant difference.

3. Results

Acetaminophen (APAP)

Biochemical assay

The serum ALT and AST levels (Figure 2) were dramatically increased at 2 and 6 h after APAP injection compared to control group. 2h after APAP treatment, the mean serum AST level was peaked, and increased by 5.8-fold compared to untreated control group. The mean serum ALT level was peaked at 6h after treatment and was 3.2-fold higher than control group. There were no significant changes in ALP, TBIL, TG and Cholesterol.

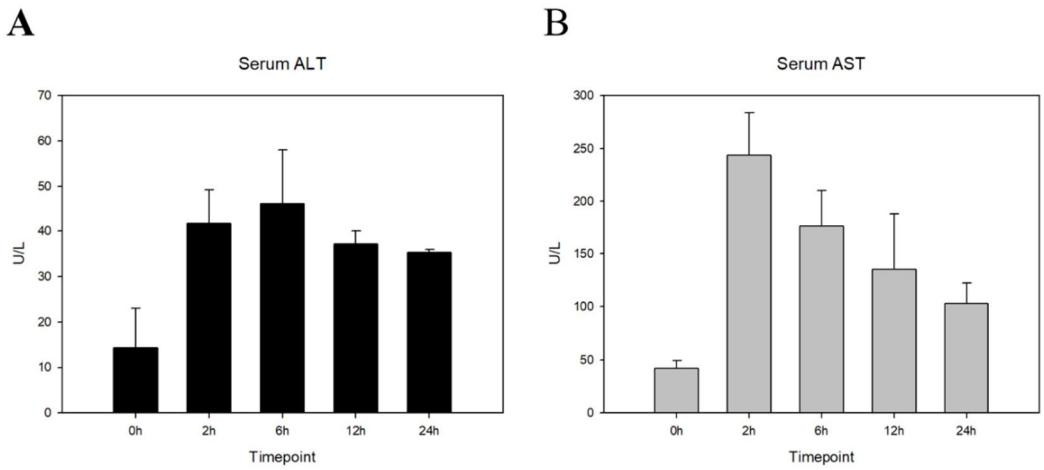

Figure 2. Results of serum chemistry analysis in APAP treated group. Serum ALT level were highest at 6h, while serum AST level peaked at 2h.

Liver weight and histological analysis

There were no significant difference of absolute and relative liver weight after APAP treatment in a time-dependent manner (Figure 3).

A variable grade of liver injuries were observed. The findings were characterized by inflammatory cell infiltration, hepatocyte degeneration, hepatocyte vacuolation and focal or multifocal hepatocyte necrosis (Figure 4). The liver injury score was significantly higher at 12 and 24 h after APAP administration (Figure 5). However, evidences of liver injury were observed from 2 h onwards. There were notable focal or multifocal area of hepatocyte necrosis at 12 and 24 h. No treatment related lesions were observed in the control animals.

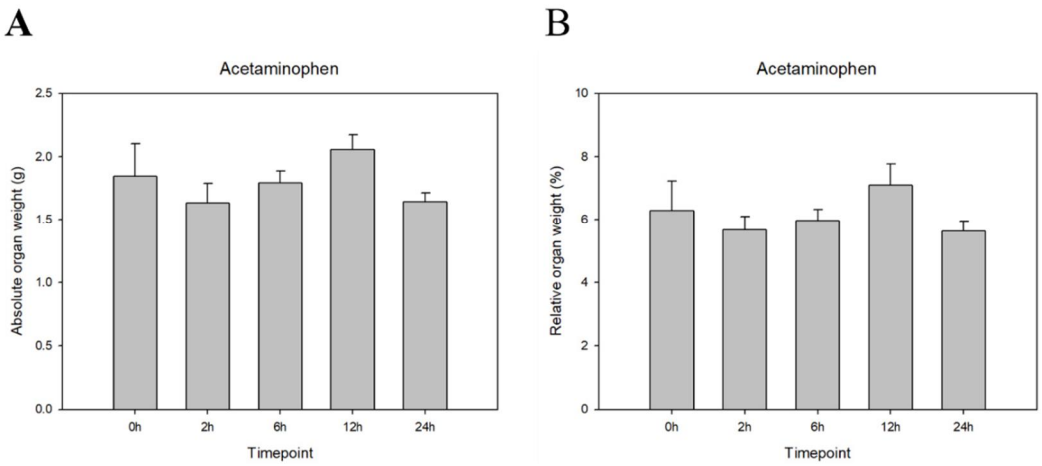

Figure 3. Absolute liver weight (A) and relative liver weight (B) in APAP treated group.

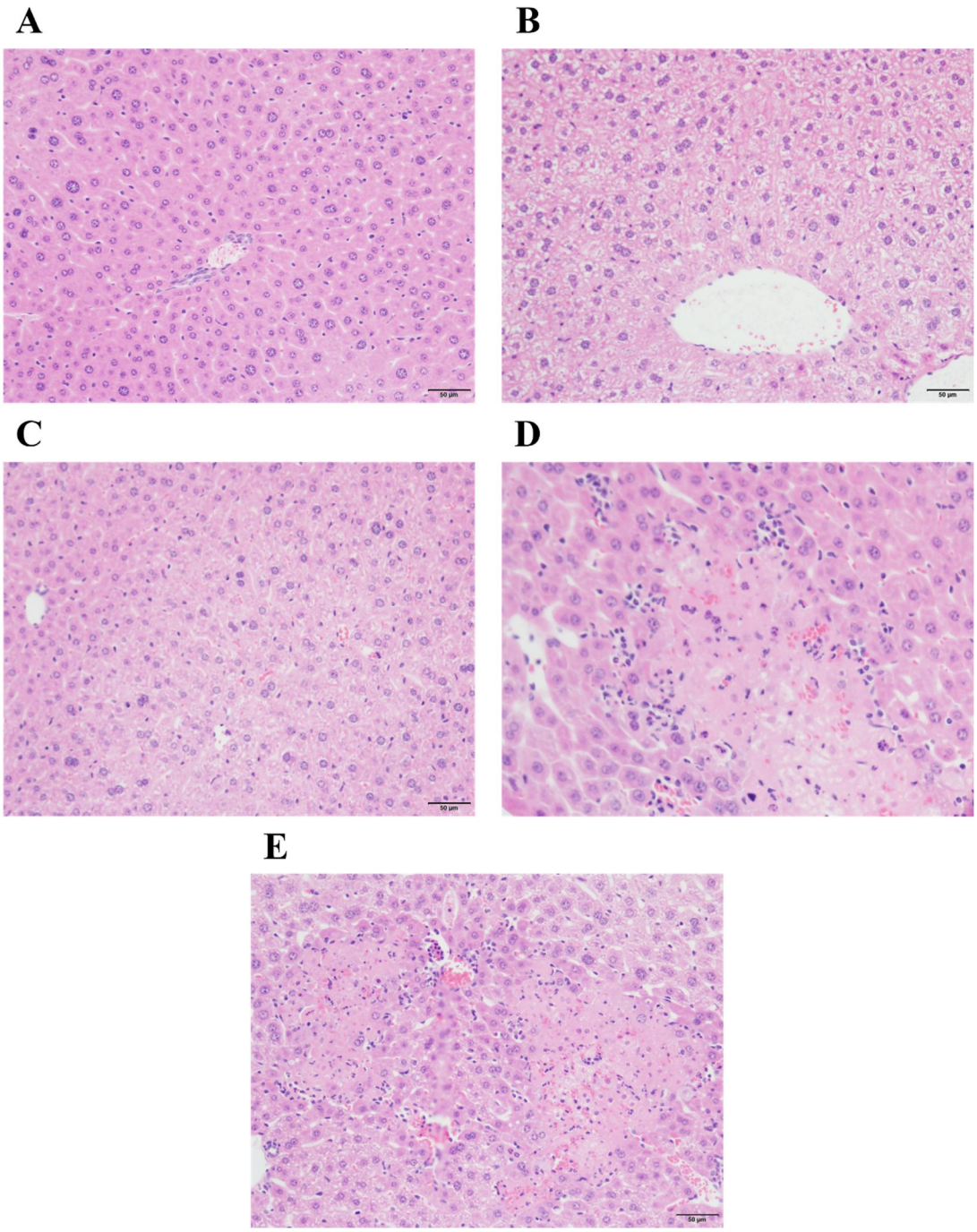

Figure 4. Representative hematoxylin and eosin-stained liver sections from APAP treated groups. (A) control mice, (B) 2 h, (C) 6 h, (D) 12 h, and (E) 24 h after APAP administration (original magnification, x200).

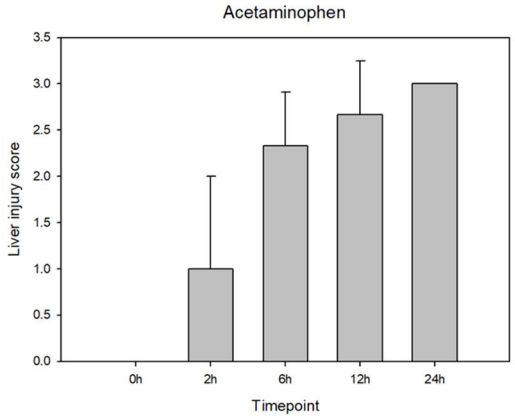

Figure 5. Liver injury score from APAP treated group at various timepoints throughout a 24 h period after administration of APAP. Histological examination of the liver was scored using semi-quantitative method.

RT-qPCR analysis

The Ct value of endogenous control was not significantly different between tests. However, the Ct value of 361-5p was more constant than 191-5p at each timepoints.

Expression levels of pre-selected microRNAs were observed in the mice liver tissue. The 107-3p levels were peaked at 2 h, with 2.5-fold increase compared to the untreated control (Figure 7A). The 182-5p levels were peaked at 2h and 12h, with 2.4-fold ($p < 0.005$) and 3.6-fold ($p < 0.05$) increase for each timepoints (Figure 7B). The 200a-3p level showed significant increasement at 2h (2.2-fold, $p < 0.05$) and the fold change decreased at later timepoints (Figure 7C). The 378b level showed similar expression pattern to 200a-3p, although the fold change was not statistically significant (Figure 7D). The 677-5p level kept increasing over 24 h period (Figure 7E). The 677-5p level peaked at 24h, with 6.8-fold ($p < 0.05$). The level of 1839-5p was up-regulated. The highest fold change was 2.8-fold ($p < 0.05$) at 12h.

In contrast, the 122-5p level was down-regulated over 24 period (Figure 8A). The 122-5p level showed significant reduction, with 0.5-fold decrease at 6 and 12 h ($p < 0.005$). The level of 677-3p showed similar pattern (Figure 8C). The expression level was lowest at 24 h, with 0.7-fold ($p < 0.005$). The 200a-5p level was up-regulated at 2 h, but the expression level was decreased at later timepoint, with 0.7-fold ($p < 0.05$) decrease at 24 h.

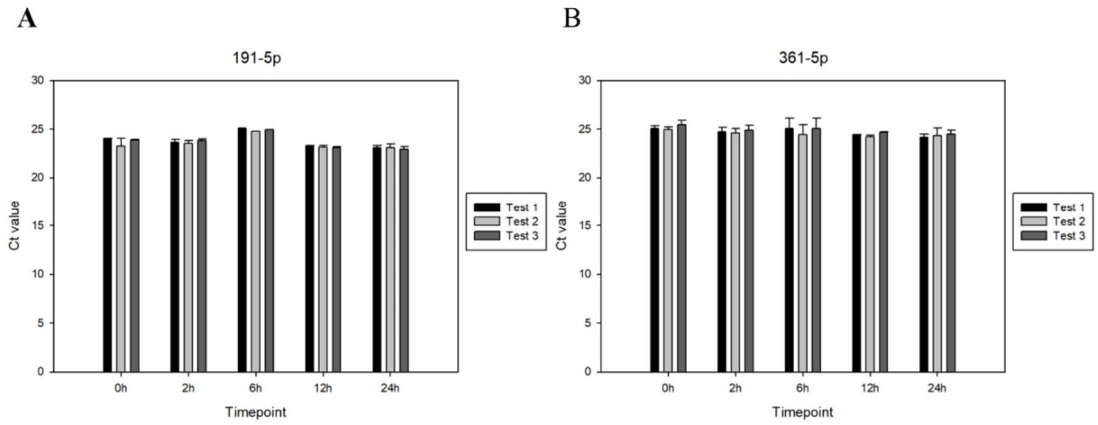

Figure 6. Ct values for each endogenous control in APAP treated group. (A) The Ct value of 191-5p ranged from 22.8 to 25.1. (B) In contrast, the Ct value of 361-5p ranged from 24.2 to 25.0.

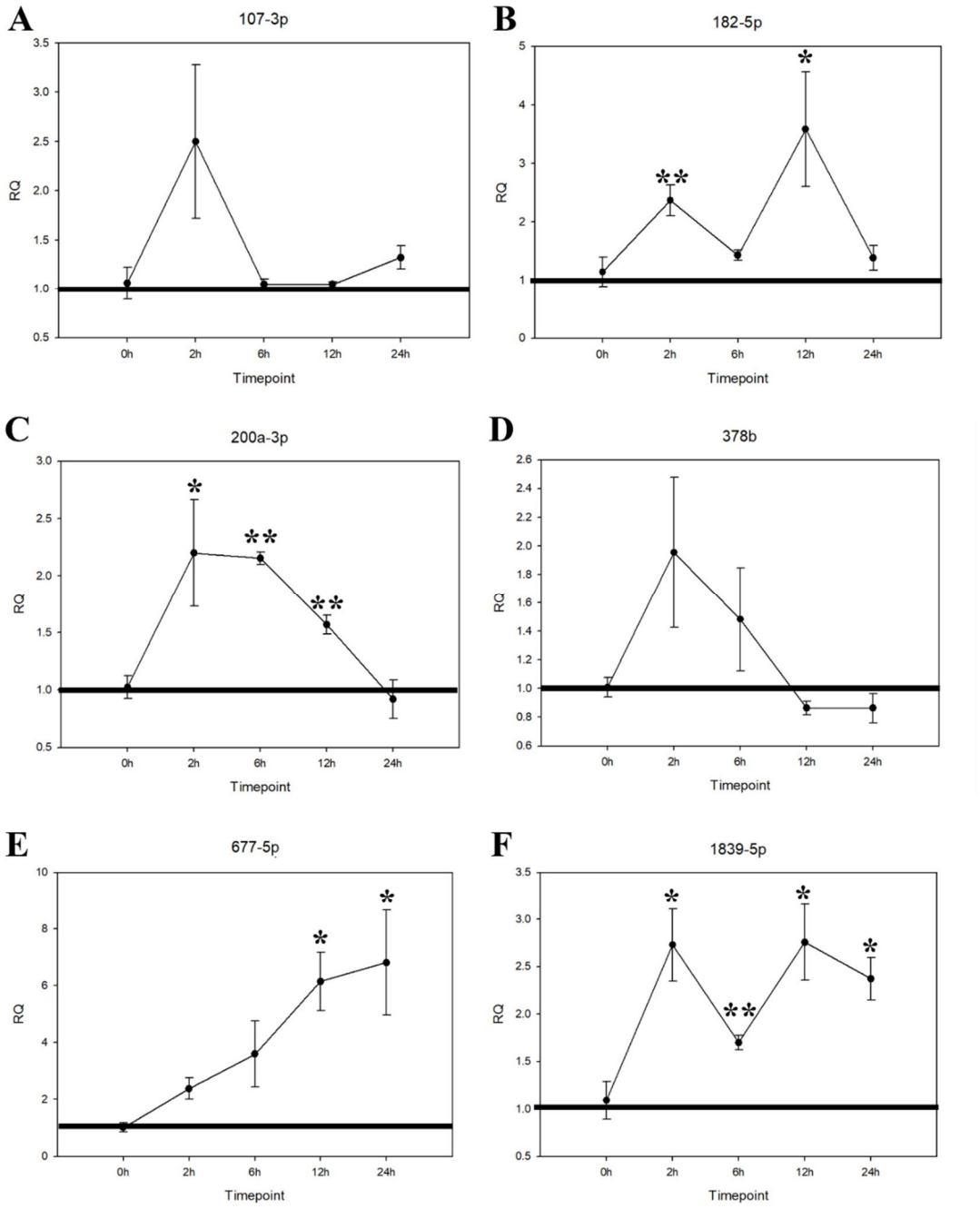

Figure 7. Time-course up-regulated changes in the liver miRNA expression level at various timepoints throughout a 24 h period after administration of APAP. 107-3p (A), 182-5p (B), 200a-3p (C), 378b (D), 677-5p (E), and 1839-5p(F). *P < 0.05, and **P < 0.005.

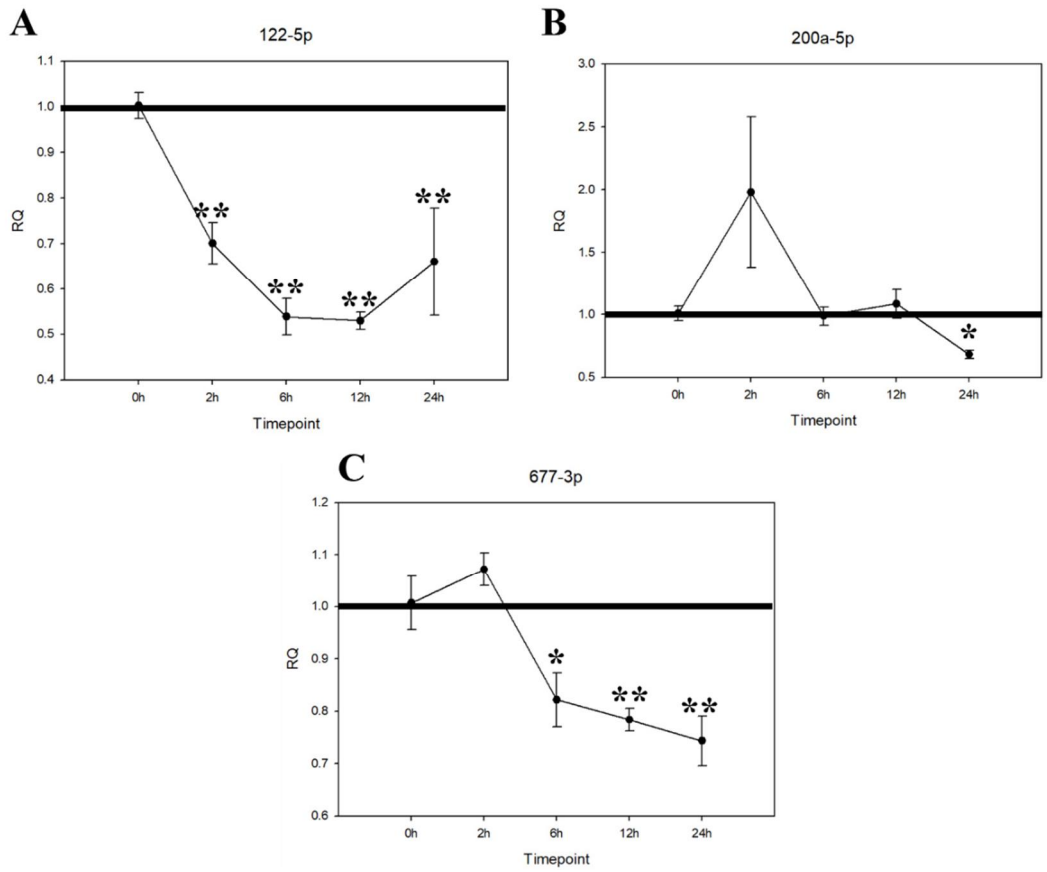

Figure 8. Time-course down-regulated changes in the liver miRNA expression level at various timepoints throughout a 24 h period after administration of APAP. 122-5p (A), 200a-5p (B), and 677-3p (C). *P < 0.05, and **P < 0.005.

Isoniazid (INH)

Biochemical assay

The serum ALT and AST levels (Figure 9) were increased at 2, 6, and 12h after INH injection compared to control group. 2h after INH treatment, the mean serum AST level was peaked, and increased by 5.8-fold compared to untreated control group. The mean serum ALT level was peaked at 12h after treatment and was 3.5-fold higher than control group. There were no significant changes in ALP, TBIL, TG and Cholesterol.

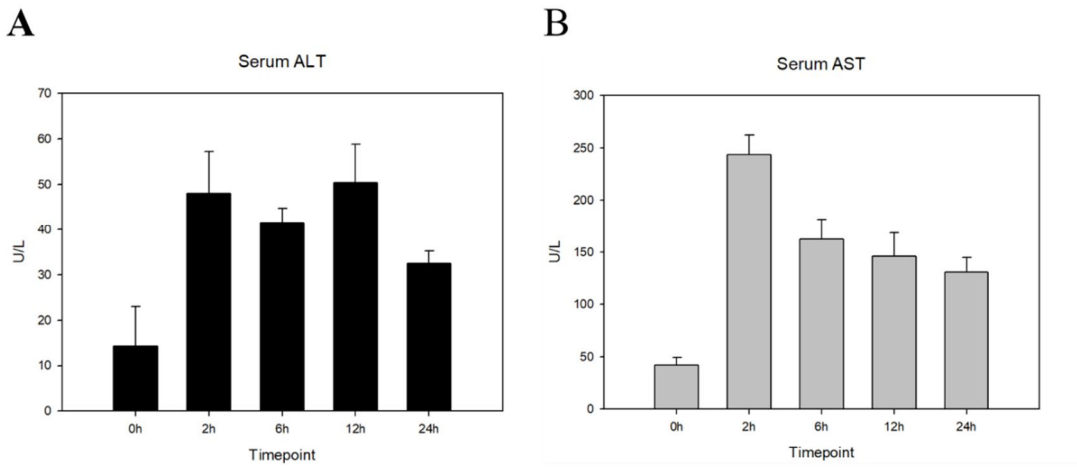

Figure 9. Results of serum chemistry analysis in INH treated group. Serum ALT level were highest at 12h, while serum AST level peaked at 2h.

Liver weight and histological analysis

The absolute liver weight was increased at 6 h after INH treatment by 20%. There were no significant difference of absolute and relative liver weight at other timepoints (Figure 10).

A variable grade of liver injuries were observed. The findings were characterized by inflammatory cell infiltration, hepatocyte degeneration and hepatocyte vacuolation (Figure 11). The liver injury score was significantly higher at 6 and 24 h after INH administration (Figure 12). However, evidences of liver injury were observed from 2 h onwards.

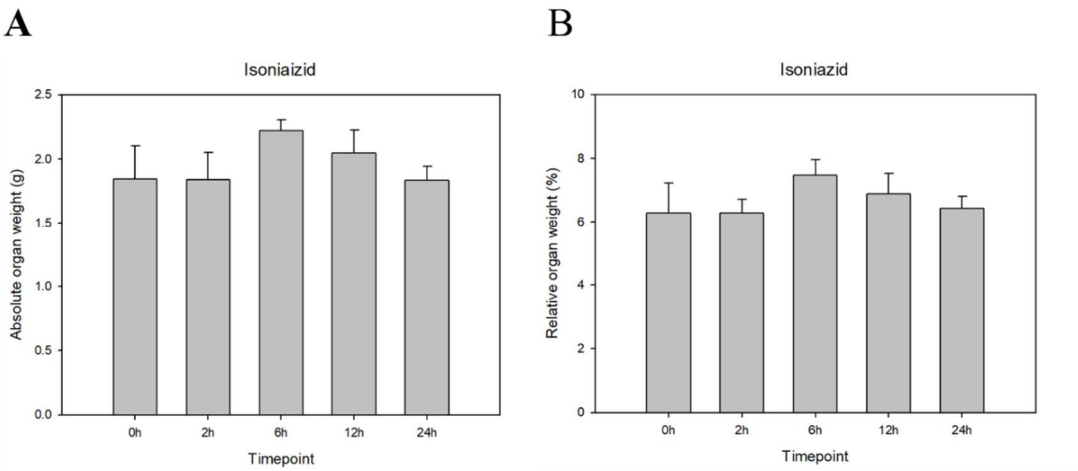

Figure 10. Absolute liver weight (A) and relative liver weight (B) in INH treated group.

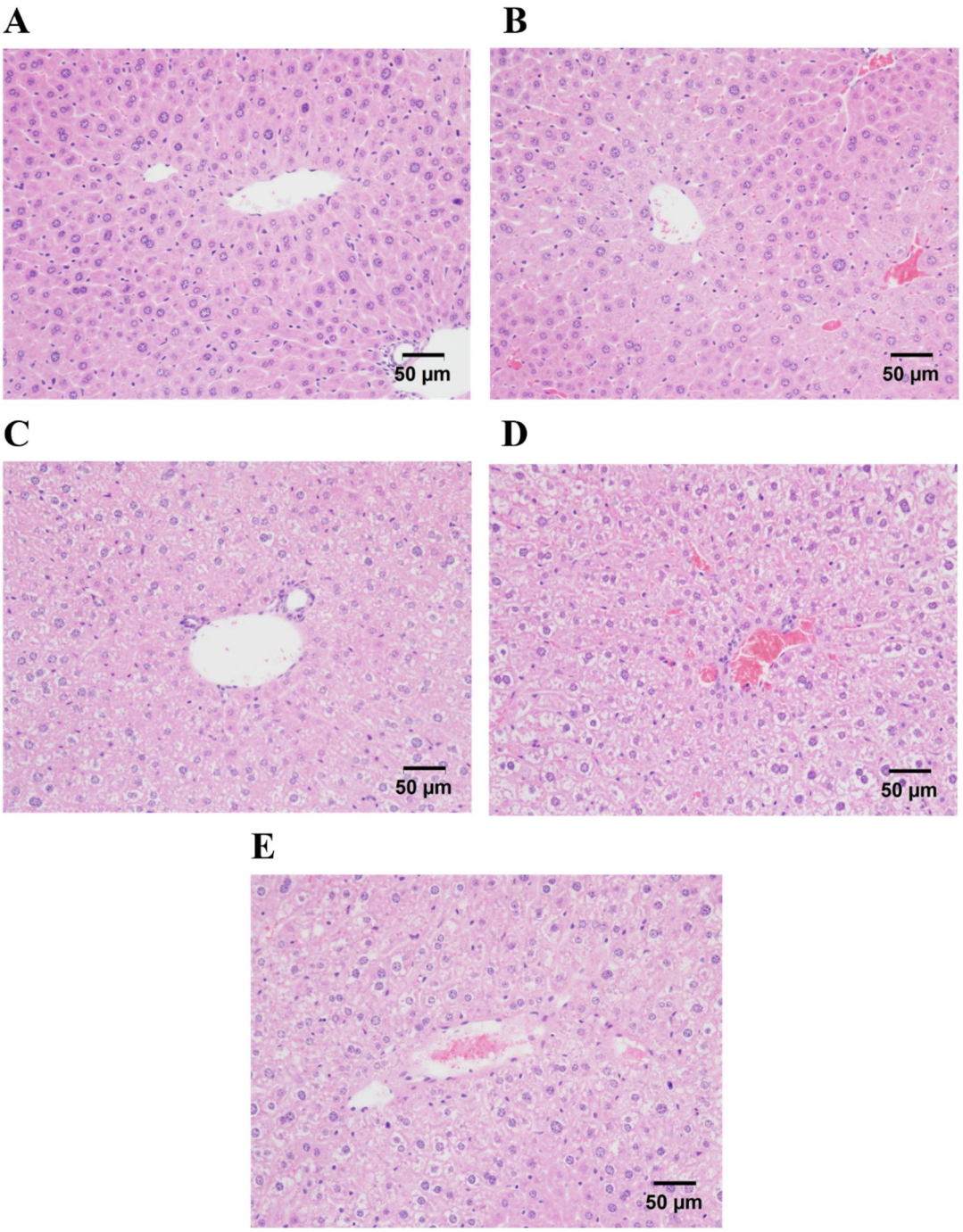

Figure 11. Representative hematoxylin and eosin-stained liver sections from INH treated groups. (A) control mice, (B) 2 h, (C) 6 h, (D) 12 h, and (E) 24 h after APAP administration (original magnification, x200).

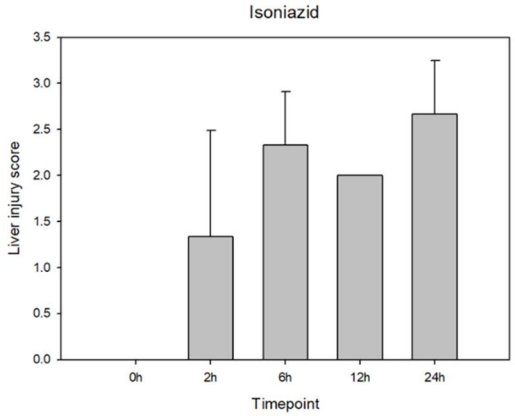

Figure 12. Liver injury score from INH treated group at various timepoints throughout a 24 h period after administration of INH. Histological examination of the liver was scored using semi-quantitative method.

RT-qPCR analysis

The Ct value of endogenous control was not significantly different between tests. However, the Ct value of 361-5p was more constant than 191-5p at each timepoints.

Expression levels of pre-selected microRNAs were observed in the mice liver tissue. The 34a-5p levels were peaked at 12 h, with 1.9-fold ($p < 0.005$) increase compared to the untreated control (Figure 14A). The 107-3p levels were peaked at 12h and 24h, with 1.4-fold ($p < 0.05$) increase (Figure 14B). The 122-5p level showed increasement at 2h (1.3-fold, $p < 0.005$) and the fold change decreased at later timepoints (Figure 14C). The 134-5p level peaked with 2.2-fold ($p < 0.005$) at 12h (Figure 14D). The expression of 155-5p showed similar pattern and peaked with 1.6-fold ($p < 0.005$) at 12h (Figure 14E). The 1839-5p level kept increasing over 24 h period (Figure 14F). The 1839-5p level peaked at 24h, with 1.3-fold ($p < 0.05$).

The 200a-5p level increased at 2h (1.4-fold, $p < 0.05$). In contrast, the expression was down-regulated with 0.5-fold ($p < 0.05$) at 12h (Figure 15A). The 677-3p showed opposite pattern (Figure 15B). The expression of 677-3p was down-regulated at 2h (0.6-fold, $p < 0.005$) but the expression was up-regulated at 12h (1.2-fold, $p < 0.05$). The 677-5p level kept decreasing over 24 h period (Figure 15C). The expression of 1839-3p showed similar pattern (Figure 15D). It peaked at 12h with 0.5-fold ($p < 0.05$).

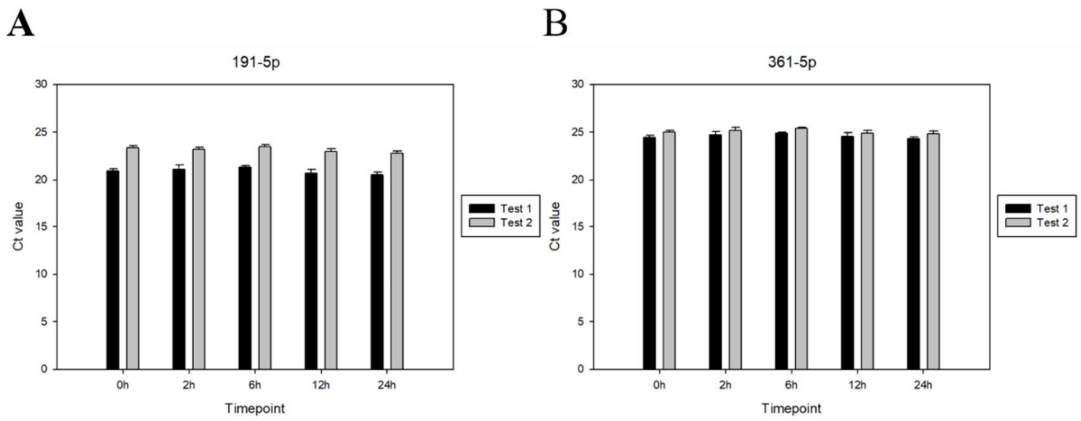

Figure 13. Ct values for each endogenous control in INH treated group. (A) The Ct value of 191-5p ranged from 20.5 to 23.5. (B) In contrast, the Ct value of 361-5p ranged from 24.3 to 25.4.

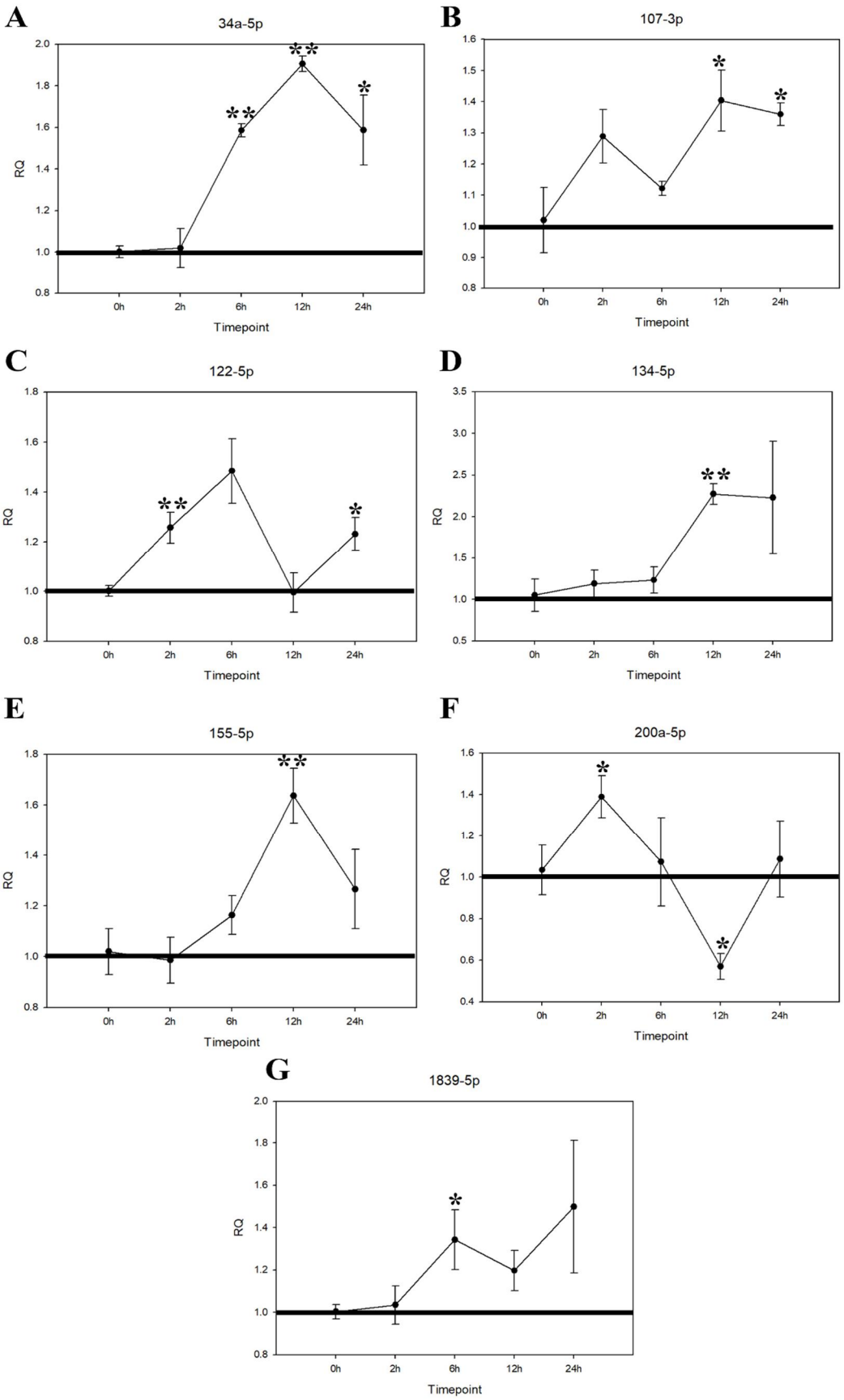
A
34a-5p
RQ
Timepoint
0h
2h
6h
12h
24h
B
107-3p
C
122-5p
D
134-5p
E
155-5p
F
200a-5p
G
1839-5p

Figure 14. Time-course up-regulated changes in the liver miRNA expression level at various timepoints throughout a 24 h period after administration of INH. 34a-5p (A), 107-3p (B), 122-5p (C), 134-5p (D), 155-5p (E), 200a-5p (F), and 1839-5p (G). *P < 0.05, and **P < 0.005.

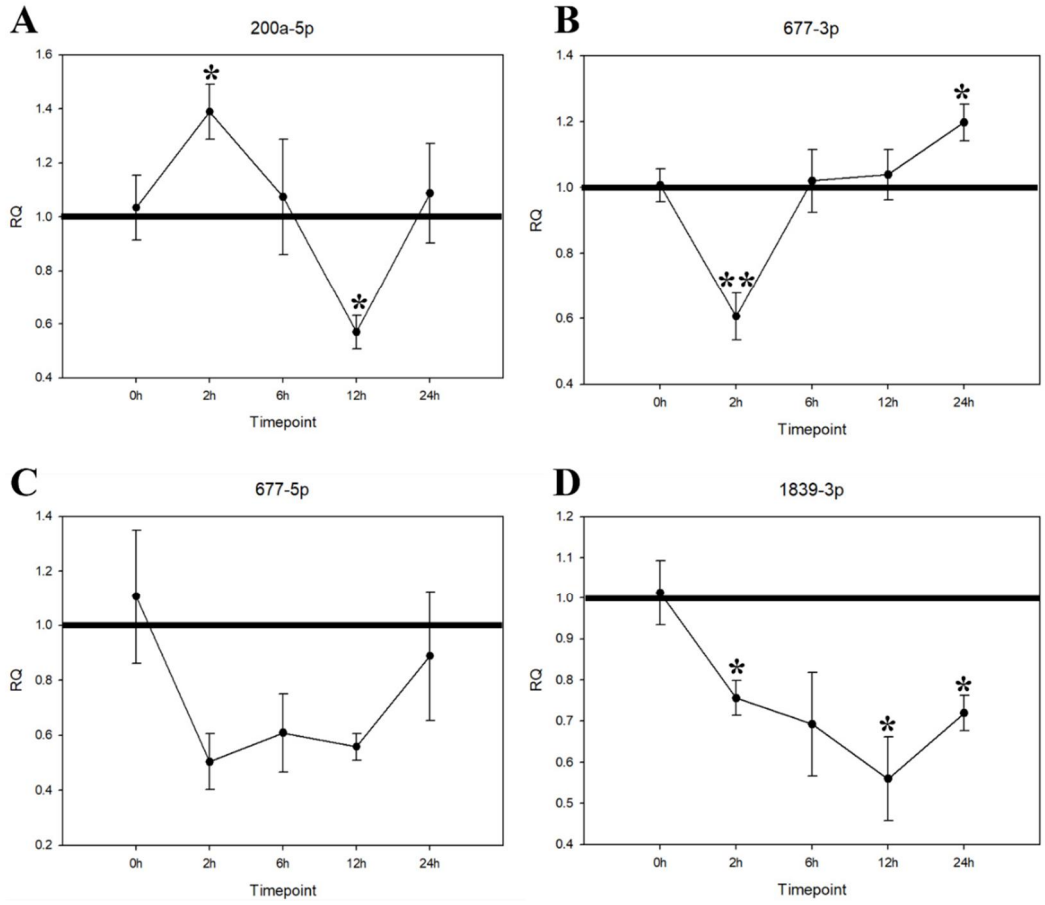

Figure 15. Time-course down-regulated changes in the liver miRNA expression level at various timepoints throughout a 24 h period after administration of INH. 200a-5p (A), 677-3p (B), 677-5p (C), and 1839-3p (D). *P < 0.05, and **P < 0.005.

Tamoxifen (TAM)

Biochemical assay

The serum ALT and AST levels (Figure 16) were increased at 2h after TAM injection compared to control group. 2h after INH treatment, the mean serum AST level was peaked, and increased by 5.2-fold compared to untreated control group. The mean serum ALT level was peaked at 2h after treatment and was 4.6-fold higher than control group. There were no significant changes in ALP, TBIL, TG and Cholesterol.

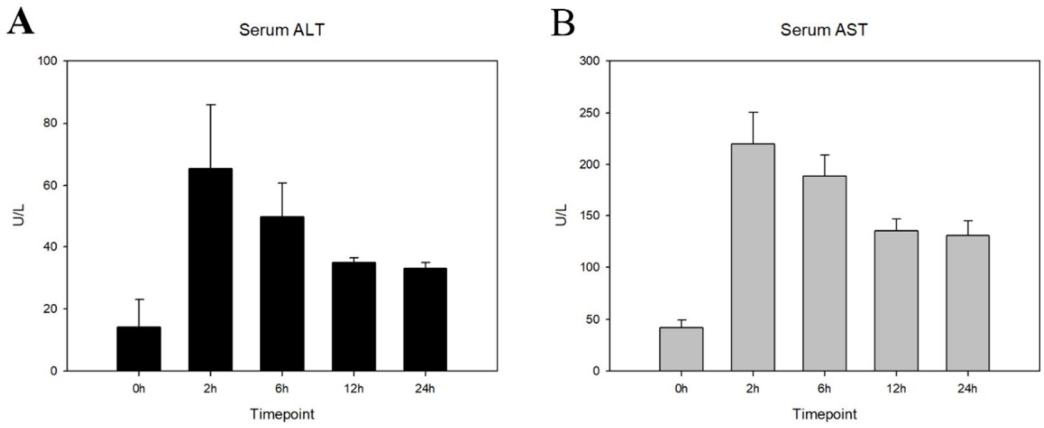

Figure 16. Results of serum chemistry analysis in TAM treated group. Serum ALT level (A)and serum AST level (B) peaked at 2h.

Liver weight and histological analysis

There were no significant difference of absolute and relative liver weight (Figure 17).

A variable grade of liver injuries were observed. The findings were characterized by inflammatory cell infiltration, hepatocyte degeneration, hepatocyte vacuolation and focal or multifocal hepatocyte necrosis (Figure 18). The liver injury score was significantly higher at 12 and 24 h after TAM administration (Figure 19). However, evidences of liver injury were observed from 2 h onwards. However, evidences of liver injury were observed from 2 h onwards. There were notable focal or multifocal area of hepatocyte necrosis at 24 h. No treatment related lesions were observed in the control animals.

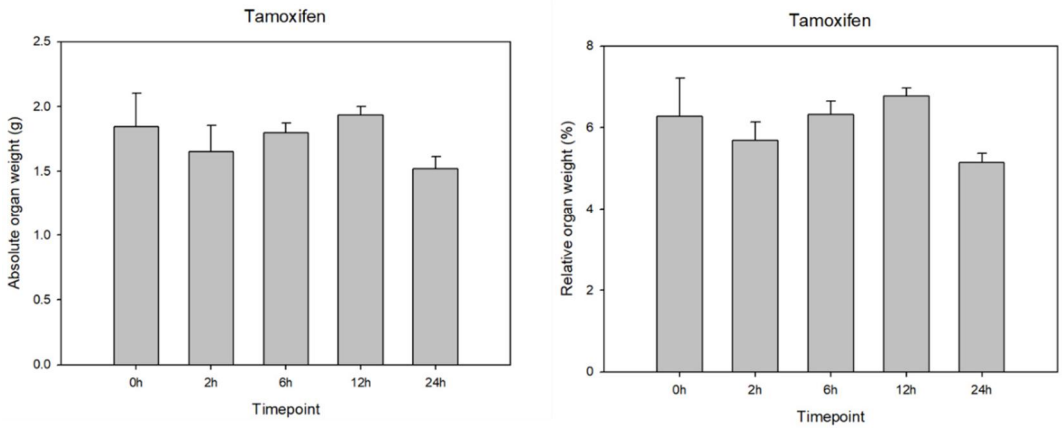

Figure 17. Absolute liver weight (A) and relative liver weight (B) in TAM treated group.

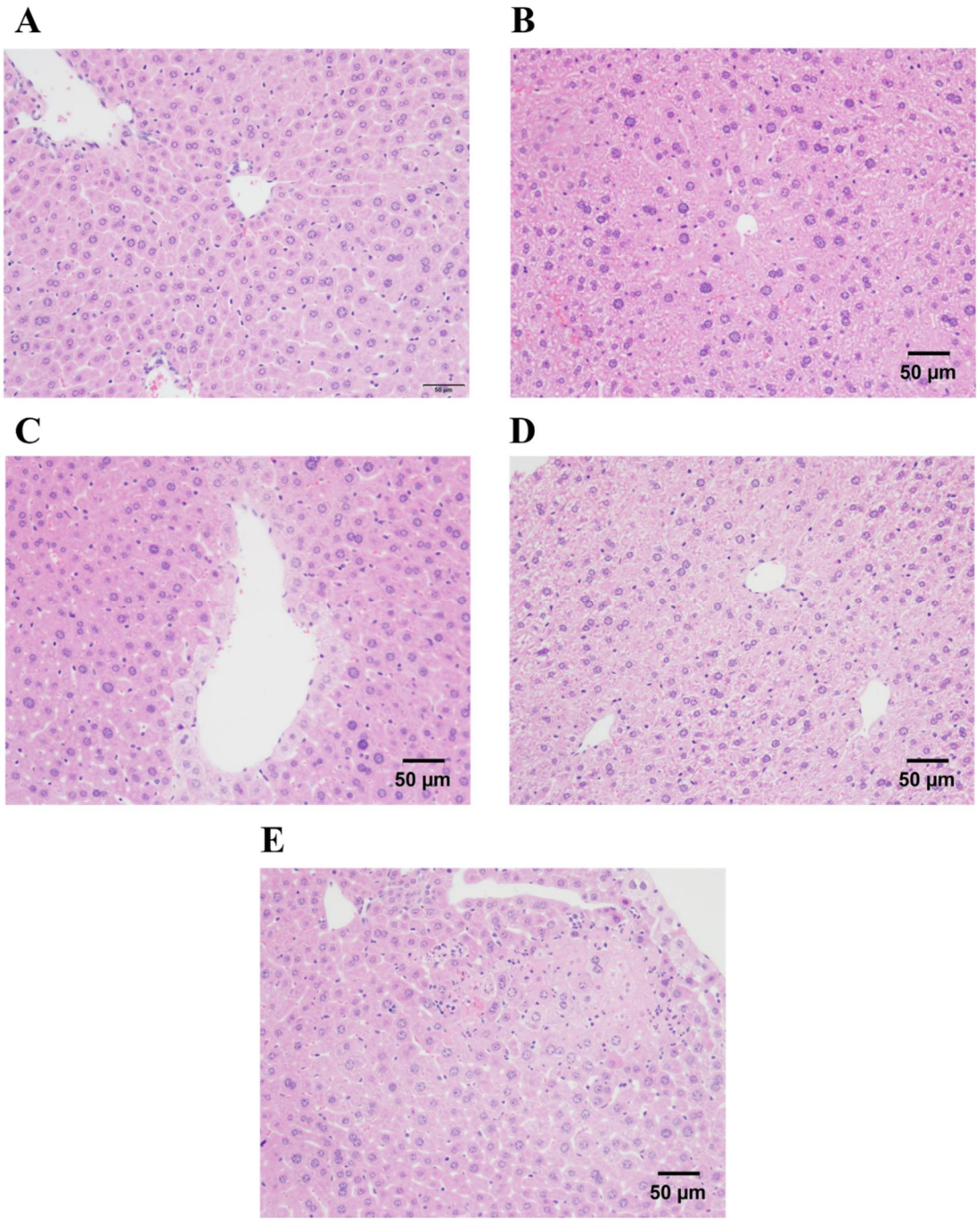

Figure 18. Representative hematoxylin and eosin-stained liver sections from TAM treated groups. (A) control mice, (B) 2 h, (C) 6 h, (D) 12 h, and (E) 24 h after TAM administration (original magnification, x200).

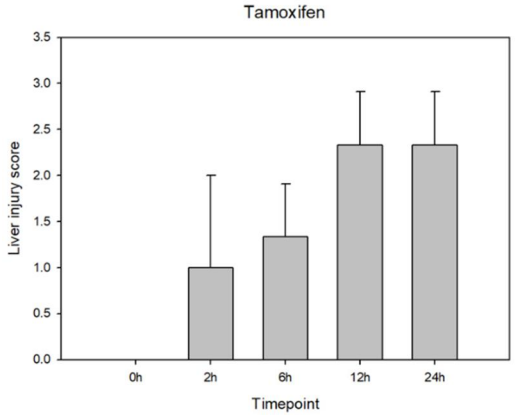

Figure 19. Liver injury score from TAM treated group at various timepoints throughout a 24 h period after administration of TAM. Histological examination of the liver was scored using semi-quantitative method.

RT-qPCR analysis

The Ct value of endogenous control was not significantly different between tests.

Expression levels of pre-selected microRNAs were observed in the mice liver tissue. The 107-3p levels were peaked at 24h, with 1.5-fold ($p < 0.05$) increase compared to the untreated control (Figure 21A). The expression pattern of 378a-5p and 1839-3p showed similar pattern (Figure 21B, C). The levels of 378a-5p and 1839-3p decreased at 2h, with 0.9-fold and 0.8-fold each. The 1839-5p level kept increasing over 24 h period (Figure 21D). The expression level of 1839-5p peaked with 2.3-fold ($p < 0.005$) at 24h.

The 34a-5p level was down-regulated, and the expression level was lowest 0.5-fold ($p < 0.005$) at 12h (Figure 22A). The 125b-5p level peaked at 2h and 12h (Figure 22B), with 0.6-fold each ($p < 0.05$). The expression pattern of 155-5p and 677-3p was similar to 34a-5p (Figure 22C, D). The expression level was peaked at 12h both, with 0.5-fold ($p < 0.005$) and 0.6-fold ($p < 0.005$) each. The 677-5p level significantly decreased at 2h (Figure 22E), with 0.2-fold ($p < 0.05$). However, the expression value returned to normal at 24 h.

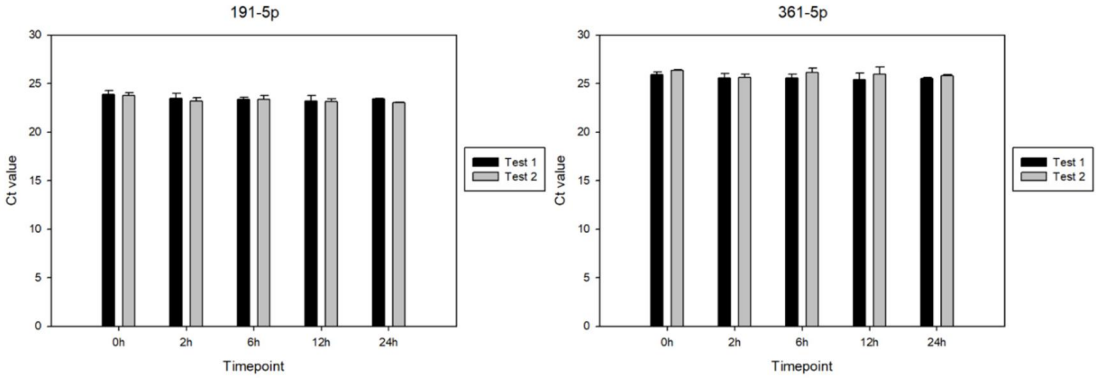

Figure 20. Ct values for each endogenous control in TAM treated group. (A) The Ct value of 191-5p ranged from 23.0 to 23.9. (B) The Ct value of 361-5p ranged from 25.3 to 26.1.

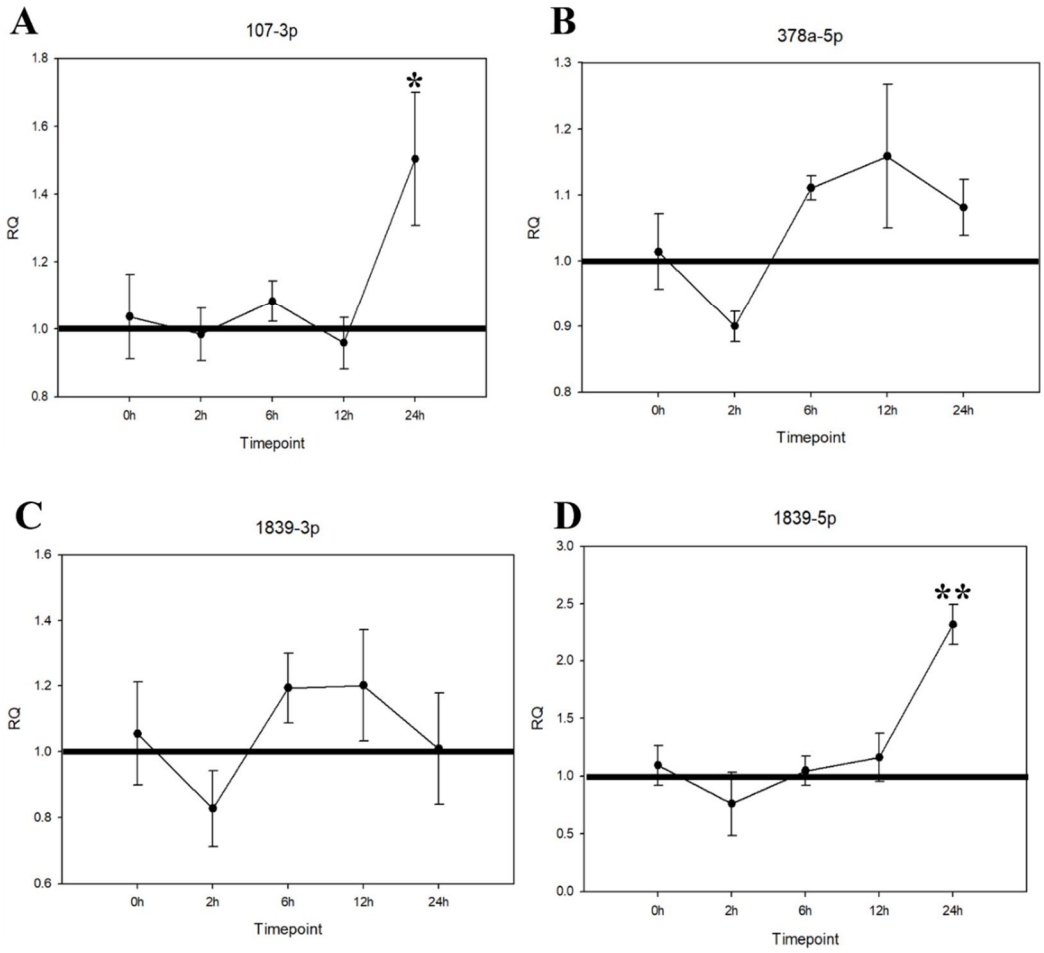

Figure 21. Time-course up-regulated changes in the liver miRNA expression level at various timepoints throughout a 24 h period after administration of TAM. 107-3p (A), 378a-5p (B), 1839-3p (C), and 1839-5p (D). *P < 0.05, and **P < 0.005.

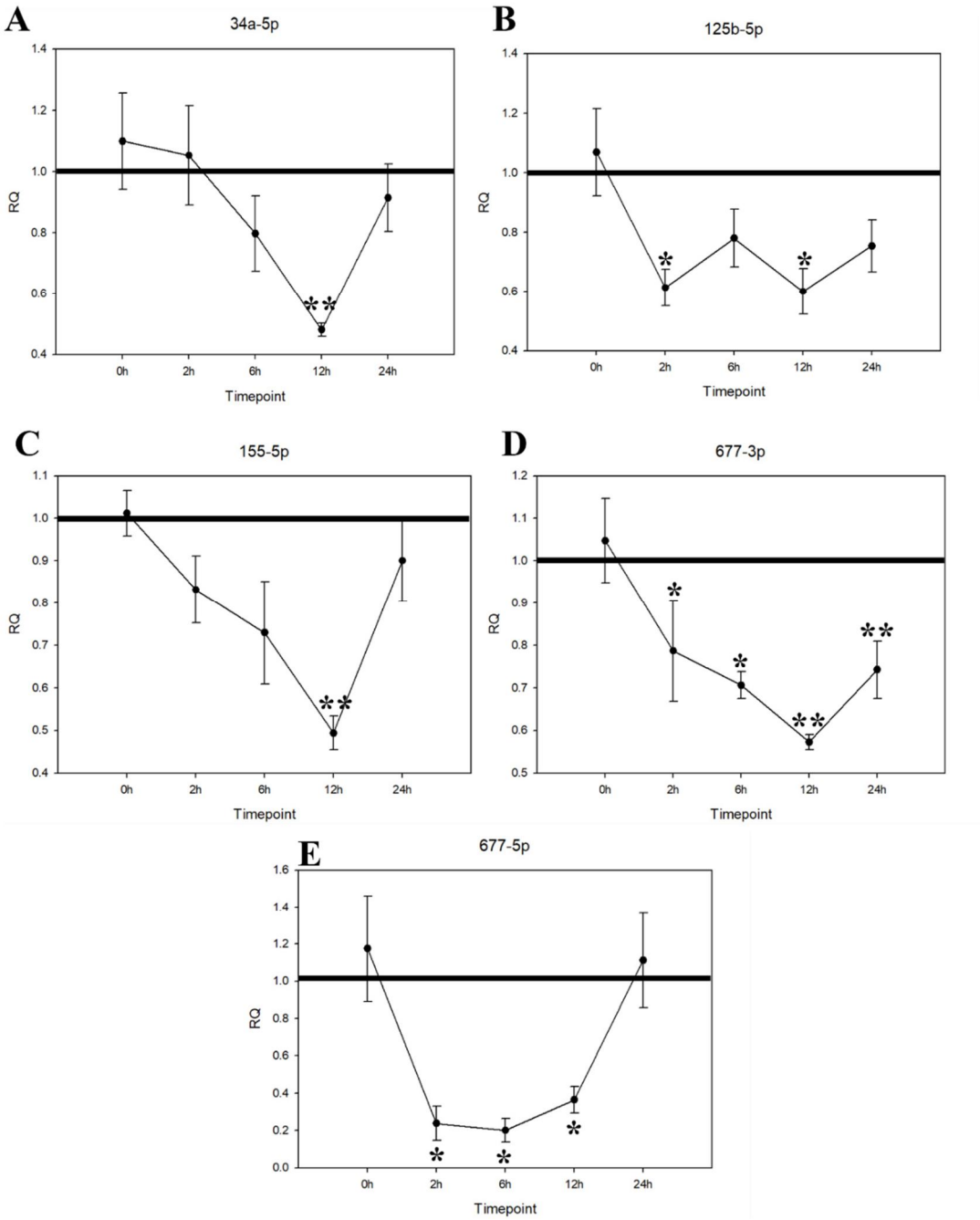

Figure 22. Time-course down-regulated changes in the liver miRNA expression level at various timepoints throughout a 24 h period after administration of TAM. 34a-5p (A), 125b-5p (B), 155-5p (C), 677-3p (D), and 677-5p (E). *P < 0.05, and **P < 0.005.

Time-course changes

miRNA expression

In each DILI model, some miRNAs showed similar pattern while some microRNAs showed different expression levels between DILI models. For time-course changes, the expression pattern at 6 and 12 h were closely connected. However, the expression at 2 and 24 h were independently existed. Therefore, in order to compare the aspects of miRNA expression between different DILI models and each timepoints, selected timepoints were re-grouped as early, middle, and late stages. The kinetics of miRNA expression pattern is summarized in the table 3.

Table 3. Summaries of kinetics of miRNA expression

Group (Treatment)	Changes	Early (2 h)	Middle (6, 12 h)	Late (24 h)
Acetaminophen (APAP)	Up	107-3p, 182-5p, 200a-3p, 200a-5p, 378b, 667-3p, 677-5p, 1839-5p	182-5p, 200a-3p, 378b, 677-5p, 1839-5p	107-3p, 182-5p, 677-5p, 1839-5p
	Down	122-5p	122-5p, 667-3p	122-5p, 200a-5p, 378b, 667-3p
Isoniazid (INH)	Up	677-3p, 677-5p, 1839-3p	200a-5p, 677-5p, 1839-3p	677-5p, 1839-3p
	Down	-	378a-5p, 1839-3p	107-3p, 378a-5p, 1839-5p
Tamoxifen (TAM)	Up	125b-5p, 155-5p, 378a-5p, 677-3p, 677-5p, 1839-3p	34a-5p, 125b-5p, 155-5p, 677-3p, 677-5p	125b-5p, 378a-5p, 677-3p, 677-5p
	Down	677-3p, 677-5p, 1839-3p	200a-5p, 677-5p, 1839-3p	677-5p, 1839-3p

4. Discussion

microRNAs (miRNAs) are proved to be integral in regulating normal biological homeostasis, development, and physiology (Lewis et al., 2005). Some miRNAs are involved in disease pathogenesis in terms of disease initiation, progression, or resolution. Moreover, certain miRNAs are targeting drug metabolizing enzymes, such as CYP family genes in liver. Those aspects of miRNAs reinforces the possibility of miRNAs as mechanistic and pathogenetic biomarkers. Tissue and serum miR-122 is considered as most potential and specific biomarker of liver injury.

In order to find specific biomarkers for reflecting the hepatotoxicity induced by acetaminophen (APAP), isoniazid (INH), and tamoxifen (TAM), 15 miRNAs were pre-selected by preliminary study. In the present study, comprehensive and time-dependent analysis of miRNA expression during three types of drug induced liver injury (DILI). Each mouse model of APAP, INH and TAM induced liver injury was established and changes in tissue miRNA expression levels at different time points were analyzed in order to excavate specific miRNA for each drug models and timepoints. There were some notable findings regarding the changes of the candidate miRNAs showing dynamic changes in correlation with liver damage and serum ALT and AST levels. Serum ALT and AST levels were peaked at 2 – 12 h after drug administration. In contrast, histopathological liver damage score was highest at 12 – 24 h after drug administration. For the expression patterns of miRNAs, selected timepoints were defined as early, middle, and late stages for comparison between each drug models.

APAP is known to be metabolized by CYP enzymes, especially CYP2E1 (Hinson, Roberts, & James, 2010). APAP is converted into metabolites, such as N-acetyl-phenzoquinoneimine (NAPQI), which is well known to cause hepatotoxicity (Kang et al., 2008). The antioxidant glutathione (GSH) converts NAPQI into reduced form, which is harmless. However, after depletion of GSH, residual amounts of NAPQI binds to mitochondrial proteins (Prescott, 1979). After binding, cytotoxic protein adducts are formed and it leads to hepatocyte necrosis. Due to hepatocyte necrosis, innate immune system is activated, and immune cells are recruited to the site of necrosis, causing inflammation or apoptosis. Selected endpoints were 2 h (metabolism), 6 h (hepatocyte necrosis and immune response activation), 12 and 24 h (inflammation and immune response), according to previous study (Mossanen & Tacke, 2015).

After APAP administration, focal or multifocal hepatocellular necrosis and significant ALT and AST increasement were observed. ALT and AST peaked at 2 and 6 h after APAP treatment. At early phase, miR-200a-3p was up-regulated. miR-200a has been proved to induce

autophagy and enhance apoptosis (Zhao et al., 2018), which has correlation to hepatocyte injury due to metabolites. At middle phase, miR-182-5p and miR-1839-5p were up-regulated, while 122-5p was significantly down-regulated. miR-122 has been considered most potential and powerful biomarker for liver injury, since miR-122 is localized in liver, and highly specific for the liver (Lagos-Quintana et al., 2002). Therefore, the possible function of miR-122 has been studied in many literatures. miR-122 has been implicated in basic liver physiology, disease and regeneration (Szabo & Bala, 2013). In the previous studies, miR-122 were down-regulated in APAP treated mouse (K. Wang et al., 2009), and this study showed same results. In contrast, exact function of up-regulated miRNAs are not well studied. miR-182 is considered to be involved in apoptosis and the downstream of pro-inflammatory cytokines, TNF-α, and IL-6 (Tahamtan, Teymoori-Rad, Nakstad, & Salimi, 2018). miR-1839 has not been studied in liver, and its function in other organs (including liver) has not been proved well. Some studies using microarray, it is suggested that miR-1839 might be involved in IRE1α signal pathway, in order to initiate apoptosis (Weingartner et al., 2021). Those kinetics of miRNA at middle phase and mechanism of APAP induced liver injury has correlation to hepatocyte injury (apoptosis) and inflammatory response. In contrast to serum biomarkers, histopathological changes were evident at later timepoints . At late phase, 200a-5p and 677-3p were down-regulated and 677-5p was up-regulated in contrast. The exact function of miR-677 in liver is not clearly examined, but miR-677 is considered to be involved in the inflammation pathway, as IL-1β, TNF-α and NF-κB (Piccinin et al., 2019). The expression of miRNA at late phase has correlation to increased inflammatory and immune response, and decreased apoptosis or autophagy reaction.

INH and the metabolite of INH is associated with INH-induced liver injury (P. Wang, Pradhan, Zhong, & Ma, 2016). INH is acetylated by N-acetyltransferase (NAT) 2 and hydrolyzed to form isonicotinic acid (INA) and Hz. Hz and acetylated Hz (AcHz) are further oxidized to reactive metabolite and cause hepatotoxicity (Delaney & Timbrell, 1995). This response is meditated by CYP2E1 (Sarich, Adams, Petricca, & Wright, 1999). Moreover, INH conjugates several endogenous metabolites and disturb homeostasis of endogenous metabolites (Khan et al., 2016), including bile acids, cholesterol, and triglycerides (Cilliers et al., 2010).

AST and ALT peaked at 2 h and 12 h for each after INH treatment. Absolute liver weight were increased by 20% at 6 h, while liver injury score was highest at 24 h. Hepatocyte degeneration and inflammatory cell infiltration was observed. At early phase, 200a-5p was up-regulated. In contrast, 677-3p was down-regulated. miRNA expression and serum levels supposed to have

correlation with hepatocyte apoptosis due to the metabolites of INH. At middle phase, 34a-5p was significantly up-regulated. miR-34a and its function is well studied in liver injury models. miR-34a is implicated in promoting apoptosis and p21 expression (Dongiovanni, Meroni, Longo, Fargion, & Fracanzani, 2018). 107-3p was up-regulated and it promotes hepatic lipid accumulation, inducing hyperglycemia and impairing glucose tolerance (Bhatia, Pattnaik, & Datta, 2016). This miRNA might have correlation with disturbance of homeostasis of endogenous metabolites, although there was no significant difference in serum triglyceride or total bilirubin level. 122-5p, however, was appeared to be up-regulated. In contrast, previous studies using INH administrated mouse model, 122-5p showed down-regulation (Song et al., 2016). It was indicated that the damaged cells within the liver tissue resulted in the transport or released of cellular miR-122 into the peripheral circulation (K. Wang et al., 2009). Therefore, the expression level of miR-122 was up-regulated at serum and down-regulated at liver tissue. However, recent study have found that miR-122 can be up-regulated due to liver injury in non-alcoholic fatty liver disease (NAFLD) model (Jianling Zhang & Huang, 2021). As miR-122 takes protective role in pathogenesis of liver disease, it could be up-regulated at some timepoints for disease improvement and tissue regeneration (Szabo & Bala, 2013). The up-regulated 134-5p is involved in apoptosis and caspase-3 and 9 proteins in heart (Xiao, Wang, & Sun, 2019). This miRNA might have correlation with hepatocyte apoptosis or degeneration as well. 155-5p, which have been well studied in liver damage models, is able to influence TNF-α expression levels in order to increase inflammatory injury (Jingli Zhang, Zhu, Shi, & Zheng, 2021). It is also associated with the immune and hematopoietic systems (Ceppi et al., 2009). miR-155 is supposed to be an inflammatory response mediator, in consideration of its known function and histopathological evidences. 200a-5p and 1839-3p were down-regulated at middle phase. At late phase, 1839-5p was up-regulated.

The hepatotoxicity of TMX has been attributed to impaired mitochondrial β-oxidation of fatty acids and the generation of reactive oxygen species (ROS) (Farrell, 2002). TMX causes reduction in phosphorylation efficiency and effect in mitochondrial membrane integrity (Ribeiro, Santos, & Custódio, 2014). These deleterious impact results in mitochondrial dysfunction and a subsequent increase in ROS production. Even worse, metabolic activation of TMX also contributes to the overproduction of ROS (Desai et al., 2002). TMX also elevates liver lipid synthesis causing lipid metabolism increasement and inflammatory cytokine such as TNF- α, IL-1β and IL-6 (Sanchez-Spitman et al., 2019). However, there was no significant difference in serum triglyceride levels.

At 24 h after TAM treatment, focal or multifocal hepatocyte necrosis was observed. Liver injury score was highest at 12 and 24 h. Serum ALT and AST levels peaked at 2 h. There was no significant changes of miRNA expression at early phase. In contrast, at middle phase, 34a-5p, 125b-5p, 155-5p, 677-3p and 677-5p were observed to be down-regulation. 125b-5p, takes similar cytoprotective role as 122-5p at liver. miR-125b prevents liver injury and improves hepatocyte survival (Yang et al., 2016). Those miRNA expressions supposed to have correlation with hepatocyte injury due to ROS accumulation and mitochondrial damage. At late phase, both 107-3p and 1839-5p were up-regulated. 107-3p may have association with increased lipid synthesis as it promotes hepatocyte lipid accumulation.

At present, the serum ALT and AST levels are used to determine the degree of liver injury and the diagnosis for DILI. However, there have been shortcomings of these biomarker in DILI diagnosis. Although ALT is liver specific, AST is affected by other organs than liver. Moreover, the exact stages of liver injury, for example, metabolization, apoptosis, initiation of innate immune system, or inflammation and hepatocyte necrosis. Therefore, more specific, and fast biomarkers for DILI is needed in order to differentiate other liver diseases from DILI. Previous studies have demonstrated that miR-122 is a liver-specific biomarkers and allows early detection of liver injury (Sanjay & Girish, 2017). However, more biomarkers for DILI can be suggested for precise diagnosis and establish effective treatment strategies. In present study, miRNAs have been reported to mediate apoptosis or cellular protection showed significant changes in expression at early to middle phase. On the contrary, miRNAs regulate inflammatory pathway or immune response changed at middle to late phase.

In conclusion, the present study suggests several miRNA biomarkers in three DILI models at different timepoints. Particularly miR-677, which is seemed to be involved in the inflammatory response, showed significant changes in all three DILI models. These results are associated with inflammatory cell infiltration in histopathological analysis. The exact function of miRNA-1839 in liver injury has not been studied and its function in other organs are still unclear. Recent study suggested correlation with liver cell apoptosis, but it could have other functions in liver injury models.

However, further studies should be conducted in order to use miRNA as new biomarkers for DILI. For consolidation of miR-677 ana miR-1839 function in liver injury models, additional studies in gene or protein levels must be conducted for clarification of its mechanism. Since miRNAs can regulate various kinds of gene, there could be unknown functions witch effect liver injury. Moreover, additional studies for verification of the correlation of miRNA with

other liver biomarkers. Due to the lack of previous studies and reports about various kinds of miRNA, so this hypothesis needs further validation in more studies in various kinds of hepatotoxicant models.

Furthermore, this study was limited to acute hepatitis and other types of DILI (cholestatic hepatitis, fatty liver, or chronic hepatitis) were not clear. Therefore, longer term or repeated studies are required to find out and validate specific biomarkers for each DILI type.

This study observed expression changes of miRNA in mouse liver tissue, which is an invasive biomarker. Liver biopsy may be required in human, and this could be an improper biomarker for early diagnosis for DILI. Therefore, miRNA assay form mouse blood or plasma should be conducted for the sake of non-invasive and more safe biomarkers for DILI diagnosis in human.

Although miRNA still needs further and deeper studies in order to use as a biomarker, but discovery of more miRNA correspondent to DILI will contribute not only precise and rapid diagnosis but also possible future therapeutic intervention for DILI patients.

5. Reference

Antoine, D. J., Dear, J. W., Lewis, P. S., Platt, V., Coyle, J., Masson, M., . . . Moggs, J. G. (2013). Mechanistic biomarkers provide early and sensitive detection of acetaminophen-induced acute liver injury at first presentation to hospital. *Hepatology, 58*(2), 777-787.

Arataki, K., Hayes, C. N., Akamatsu, S., Akiyama, R., Abe, H., Tsuge, M., . . . Imamura, M. (2013). Circulating microRNA-22 correlates with microRNA-122 and represents viral replication and liver injury in patients with chronic hepatitis B. *Journal of medical virology, 85*(5), 789-798.

Bhatia, H., Pattnaik, B. R., & Datta, M. (2016). Inhibition of mitochondrial β-oxidation by miR-107 promotes hepatic lipid accumulation and impairs glucose tolerance in vivo. *International Journal of Obesity, 40*(5), 861-869. doi:10.1038/ijo.2015.225

Ceppi, M., Pereira, P. M., Dunand-Sauthier, I., Barras, E., Reith, W., Santos, M. A., & Pierre, P. (2009). MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. *Proceedings of the National Academy of Sciences of the United States of America, 106*(8), 2735-2740. doi:10.1073/pnas.0811073106

Chitturi, S., & Farrell, G. (2001). *Drug-induced cholestasis.* Paper presented at the Seminars in gastrointestinal disease.

Cilliers, K., Labadarios, D., Schaaf, H., Willemse, M., Maritz, J., Werely, C., . . . Donald, P. (2010). Pyridoxal-5-phosphate plasma concentrations in children receiving tuberculosis chemotherapy including isoniazid. *Acta Paediatrica, 99*(5), 705-710.

David, S., & Hamilton, J. P. (2010). Drug-induced Liver Injury. *US gastroenterology & hepatology review, 6*, 73-80. Retrieved from https://pubmed.ncbi.nlm.nih.gov/21874146 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3160634/

Delaney, J., & Timbrell, J. (1995). Role of cytochrome P450 in hydrazine toxicity in isolated hepatocytes in vitro. *Xenobiotica, 25*(12), 1399-1410.

Desai, P. B., Nallani, S. C., Sane, R. S., Moore, L. B., Goodwin, B. J., Buckley, D. J., & Buckley, A. R. (2002). Induction of cytochrome P450 3A4 in primary human hepatocytes and activation of the human pregnane X receptor by tamoxifen and 4-hydroxytamoxifen. *Drug Metab Dispos, 30*(5), 608-612. doi:10.1124/dmd.30.5.608

Dongiovanni, P., Meroni, M., Longo, M., Fargion, S., & Fracanzani, A. L. (2018). miRNA Signature in NAFLD: A Turning Point for a Non-Invasive Diagnosis. *International Journal of Molecular Sciences, 19*(12), 3966. Retrieved from https://www.mdpi.com/1422-0067/19/12/3966

Endo, S., Yano, A., Fukami, T., Nakajima, M., & Yokoi, T. (2014). Involvement of miRNAs in the early phase of halothane-induced liver injury. *Toxicology, 319*, 75-84. doi:10.1016/j.tox.2014.02.011

Erlinger, S. (1997). Drug-induced cholestasis. *Journal of hepatology, 26*, 1-4.

Farrell, G. C. (2002). Drugs and steatohepatitis. *Semin Liver Dis, 22*(2), 185-194. doi:10.1055/s-2002-30106

Giusti, I., D'Ascenzo, S., & Dolo, V. (2013). Microvesicles as potential ovarian cancer biomarkers. *BioMed research international, 2013*.

Guicciardi, M. E., Malhi, H., Mott, J. L., & Gores, G. J. (2013). Apoptosis and necrosis in the liver. *Compr Physiol, 3*(2), 977-1010. doi:10.1002/cphy.c120020

Guo, H., Ingolia, N. T., Weissman, J. S., & Bartel, D. P. (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature, 466*(7308), 835-840. doi:10.1038/nature09267

Hinson, J. A., Roberts, D. W., & James, L. P. (2010). Mechanisms of acetaminophen-induced liver necrosis. *Adverse drug reactions*, 369-405.

Hoofnagle, J. H., & Björnsson, E. S. (2019). Drug-Induced Liver Injury - Types and Phenotypes. *N Engl J Med, 381*(3), 264-273. doi:10.1056/NEJMra1816149

Kang, J. S., Wanibuchi, H., Morimura, K., Wongpoomchai, R., Chusiri, Y., Gonzalez, F. J., & Fukushima, S. (2008). Role of CYP2E1 in thioacetamide-induced mouse hepatotoxicity. *Toxicology and applied pharmacology, 228*(3), 295-300.

Khan, S. R., Morgan, A. G., Michail, K., Srivastava, N., Whittal, R. M., Aljuhani, N., & Siraki, A. G. (2016). Metabolism of isoniazid by neutrophil myeloperoxidase leads to isoniazid-NAD+ adduct formation: a comparison of the reactivity of isoniazid with its known human metabolites. *Biochemical pharmacology, 106*, 46-55.

Lagos-Quintana, M., Rauhut, R., Yalcin, A., Meyer, J., Lendeckel, W., & Tuschl, T. (2002). Identification of Tissue-Specific MicroRNAs from Mouse. *Current Biology, 12*(9), 735-739. doi:https://doi.org/10.1016/S0960-9822(02)00809-6

Leise, M. D., Poterucha, J. J., & Talwalkar, J. A. (2014). Drug-induced liver injury. *Mayo Clin Proc, 89*(1), 95-106. doi:10.1016/j.mayocp.2013.09.016

Lewis, B. P., Burge, C. B., & Bartel, D. P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell, 120*(1), 15-20.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods, 25*(4), 402-408. doi:10.1006/meth.2001.1262

Mohri, T., Nakajima, M., Fukami, T., Takamiya, M., Aoki, Y., & Yokoi, T. (2010). Human CYP2E1 is regulated by miR-378. *Biochemical pharmacology, 79*(7), 1045-1052.

Mossanen, J. C., & Tacke, F. (2015). Acetaminophen-induced acute liver injury in mice. *Lab Anim, 49*(1 Suppl), 30-36. doi:10.1177/0023677215570992

Piccinin, E., Arconzo, M., Graziano, G., Vacca, M., Peres, C., Bellafante, E., . . . Moschetta, A. (2019). Hepatic MicroRNA Expression by PGC-1α and PGC-1β in the Mouse. *International Journal of Molecular Sciences, 20*(22), 5735. Retrieved from https://www.mdpi.com/1422-0067/20/22/5735

Prescott, L. F. (1979). Paracetamol poisoning. Prevention of liver damage. *Med Chir Dig, 8*(5), 391-393.

Ribeiro, M. P., Santos, A. E., & Custódio, J. B. (2014). Mitochondria: the gateway for tamoxifen-induced liver injury. *Toxicology, 323*, 10-18. doi:10.1016/j.tox.2014.05.009

Sanchez-Spitman, A. B., Swen, J. J., Dezentje, V. O., Moes, D. J. A. R., Gelderblom, H., & Guchelaar, H. J. (2019). Clinical pharmacokinetics and pharmacogenetics of tamoxifen and endoxifen. *Expert Review of Clinical Pharmacology, 12*(6), 523-536. doi:10.1080/17512433.2019.1610390

Sanjay, S., & Girish, C. (2017). Role of miRNA and its potential as a novel diagnostic biomarker in drug-induced liver injury. *Eur J Clin Pharmacol, 73*(4), 399-407. doi:10.1007/s00228-016-2183-1

Sarges, P., Steinberg, J. M., & Lewis, J. H. (2016). Drug-Induced Liver Injury: Highlights from a Review of the 2015 Literature. *Drug Saf, 39*(9), 801-821. doi:10.1007/s40264-016-0427-8

Sarich, T. C., Adams, S. P., Petricca, G., & Wright, J. M. (1999). Inhibition of isoniazid-induced hepatotoxicity in rabbits by pretreatment with an amidase inhibitor. *Journal of Pharmacology and Experimental Therapeutics, 289*(2), 695-702.

Sita-Lumsden, A., Dart, D. A., Waxman, J., & Bevan, C. (2013). Circulating microRNAs as potential new biomarkers for prostate cancer. *British journal of cancer, 108*(10), 1925-1930.

Song, L., Zhang, Z., Zhang, J., Zhu, X., He, L., Shi, Z., . . . Feng, F. (2016). Ratio of microRNA-122/155 in isoniazid-induced acute liver injury in mice. *Experimental and therapeutic medicine, 12*(2), 889-894. doi:10.3892/etm.2016.3375

Szabo, G., & Bala, S. (2013). MicroRNAs in liver disease. *Nature reviews. Gastroenterology & hepatology, 10*(9), 542-552. doi:10.1038/nrgastro.2013.87

Tahamtan, A., Teymoori-Rad, M., Nakstad, B., & Salimi, V. (2018). Anti-Inflammatory MicroRNAs and Their Potential for Inflammatory Diseases Treatment. *Frontiers in immunology, 9*, 1377-1377. doi:10.3389/fimmu.2018.01377

Tsai, W.-C., Hsu, S.-D., Hsu, C.-S., Lai, T.-C., Chen, S.-J., Shen, R., . . . Tsai, T.-F. (2012). MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis.

The Journal of clinical investigation, 122(8), 2884-2897.

Vliegenthart, A. D., Antoine, D. J., & Dear, J. W. (2015). Target biomarker profile for the clinical management of paracetamol overdose. *Br J Clin Pharmacol, 80*(3), 351-362. doi:10.1111/bcp.12699

Wang, B., Wang, H., & Yang, Z. (2012). MiR-122 inhibits cell proliferation and tumorigenesis of breast cancer by targeting IGF1R.

Wang, K., Zhang, S., Marzolf, B., Troisch, P., Brightman, A., Hu, Z., . . . Galas, D. J. (2009). Circulating microRNAs, potential biomarkers for drug-induced liver injury. *Proceedings of the National Academy of Sciences of the United States of America, 106*(11), 4402-4407. doi:10.1073/pnas.0813371106

Wang, P., Pradhan, K., Zhong, X.-b., & Ma, X. (2016). Isoniazid metabolism and hepatotoxicity. *Acta Pharmaceutica Sinica B, 6*(5), 384-392. doi:https://doi.org/10.1016/j.apsb.2016.07.014

Weingartner, M., Jebbawi, F., Wang, J., Stücheli, S., Gottstein, B., Beldi, G., . . . Odermatt, A. (2021). Albendazole reduces endoplasmic reticulum stress induced by Echinococcus multilocularis in mice. *bioRxiv*, 2021.2002.2003.429530. doi:10.1101/2021.02.03.429530

Wu, D., & Murashov, A. K. (2013). Molecular mechanisms of peripheral nerve regeneration: emerging roles of microRNAs. *Front Physiol, 4*, 55. doi:10.3389/fphys.2013.00055

Wu, L., Fan, J., & Belasco, J. G. (2006). MicroRNAs direct rapid deadenylation of mRNA. *Proceedings of the National Academy of Sciences of the United States of America, 103*(11), 4034. doi:10.1073/pnas.0510928103

Xiao, J.-M., Wang, J.-J., & Sun, L.-L. (2019). Effect of miR-134 against myocardial hypoxia/reoxygenation injury by directly targeting NOS3 and regulating PI3K/Akt pathway. *Acta cirurgica brasileira, 34*(8), e201900802-e201900802. doi:10.1590/s0102-865020190080000002

Yang, D., Yuan, Q., Balakrishnan, A., Bantel, H., Klusmann, J.-H., Manns, M. P., . . . Sharma, A. D. (2016). MicroRNA-125b-5p mimic inhibits acute liver failure. *Nature communications, 7*, 11916-11916. doi:10.1038/ncomms11916

Zhang, J., & Huang, H. (2021). miR-122-5p/KIF5B/AMPK/AKT regulatory network regulates the progression of NAFLD. *American journal of translational research, 13*(2), 696-707. Retrieved from https://pubmed.ncbi.nlm.nih.gov/33594319 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7868843/

Zhang, J., Zhu, L., Shi, H., & Zheng, H. (2021). Protective effects of miR-155-5p silencing on IFN-γ-induced apoptosis and inflammation in salivary gland epithelial cells. *Experimental and therapeutic medicine, 22*(2), 882-882.

doi:10.3892/etm.2021.10314

Zhao, Y.-x., Sun, Y.-y., Huang, A.-l., Li, X.-f., Huang, C., Ma, T.-t., & Li, J. (2018). MicroRNA-200a induces apoptosis by targeting ZEB2 in alcoholic liver disease. *Cell Cycle, 17*(2), 250-262. doi:10.1080/15384101.2017.1417708

국문 요약 (Summary in Korean)

간은 다양한 외부 유래 물질들의 대사의 주요 부위로서, 주로 독성 반응의 표적이 되곤 한다. 이로 인해 간에 독성학적인 영향을 주는 약물들은 약인성 간 손상(drug-induced liver injury, DILI)를 야기하곤 한다. 개발중인 약물 또는 이미 시판 허가되었던 약물들이 DILI를 유발하는 것으로 알려지면, 해당 약물의 허가를 취소하거나 시장에서 철수하기도 하였다. 그럼에도 불구하고 해당 약물의 특성이나 효능이 다른 약물에 비해 뛰어나기 때문에 DILI의 위험에도 불구하고 계속해서 사용중인 약물 또한 존재한다. 하지만 DILI는 급성 간 부전(acute liver failure)를 야기할 수 있는 문제를 안고 있으며, 적절한 순간에 치료가 이루어 지지 않을 경우 치명적인 결과를 야기할 수 있다. 이에 DILI의 신속하고 정확한 진단이 중요하다. 하지만 DILI의 진단 및 치료는 매우 복잡하며, DILI의 유형이 다양하고 이를 유발하는 약물 또한 다양하기 때문에 병태생리학적 기전 또한 명확하게 알려지지 않았다. 이에 각각의 유형의 DILI를 진단하기 위한 새로운 바이오마커의 필요성이 대두되었다. microRNA (miRNA)는 작은 비발현 RNA 분자로, 유전자 발현 조절 기능을 한다. 이를 통해 정상적인 기능과, 발달 및 생리학적 기능에 관여할 뿐만 아니라 병인론적인 기전에도 영향을 미친다. 따라서 miRNA와 DILI의 연관성에 대해 분석하여, miRNA를 새로운 DILI의 바이오마커로 제시하고자 하였다.

본 시험에서 정상 CD-1 (ICR) 마우스에 acetaminophen (APAP), isoniazid (INH), 및 Tamoxifen (TAM)을 투여하여 간 손상 모델을 제작하였다. 투여 후 하루동안 총 5개의 타임포인트를 설정하여 (0, 2, 6, 12, 24 시간) 사전 시험에서 선정한 miRNA의 발현 정도와 혈청의 ALT 및 AST 수치, 그리고 간의 조직병리학적인 검사를 실시하였다. 시험 결과, 전체 시간 동안 각 약물별로 발현되는 miRNA의 양상이 다르게 관찰되었다. 특히 세포자멸사 또는 세포의 보호 작용에 관여하는 miRNA들은 약물 투여 초기 및 중간기에 유의미하게 변화하였다. 이와 반대로 염증반응 또는 면역반응에 관여하는 miRNA들은 중간기 또는 후기에 유의미하게 변화하였다.

현재 ALT 및 AST가 간의 손상 정도와 DILI의 진단에 사용되고 있지만. 이러한 바이오마커들의 한계 및 단점으로 인해 DILI의 진단이 곤란하였다. 또한 단순 ALT와 AST의 수치 변화만으로는 간 손상 과정에서 발생하는 대사, 세포자멸사, 면역 반응 또는 염증 반응 등의 다양한 양상을 분석할 수 없다. 따라서, 더 특이적이고 빠른 바이오마커들이 DILI와 기타 간 질환을 감별진단 하고 효과적인 치료를 진행하기 위해 필요시 되어 왔다. 본 연구의 결과로 세 종류의 약물에 반응하여 시간대에 따라 다르게 발현하는 miRNA들의 바이오마커로써의 가능성을 확인할 수 있었다. 특히 이전 시험들에서 간 및 간 손상 모델에서의 기능이 정확하게 분석되지 않은 새로운 바이오마커의 가능성을 지닌 miRNA들로 miR-677과 miR-1839를 확인할 수 있었다.

핵심어: 약인성 간 손상, 마이크로RNA, 아세트아미노펜, 이소니아지드 ,타목시펜