INVESTIGATIONS ON THE PROTECTIVE ROLE OF LOW-TO-MODERATE ALCOHOL CONSUMPTION ON HIGH FAT DIET-INDUCED NON-ALCOHOLIC FATTY LIVER DISEASE

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STATEMENT OF AUTHENTICITY

This thesis is submitted in fulfilment of the requirements for the postgraduate research degree, Doctor of Philosophy (PhD) at the School of Science and Health, Western Sydney University, NSW, Australia, under the primary supervision of Dr Srinivas Nammi and associate supervisors, Prof Dennis Chang and Prof Rajeswara Rao Pragada. To the best of my knowledge, the work presented in this thesis is original except as acknowledged in the text. I further declare that I have not previously submitted this research work either in whole or in part to this or at any other institution.

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Date: 25/01/2019

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To Science

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ABBREVIATIONS

Abbreviation	Full term
ACC	Acetyl CoA Carboxylase
ADH	Alcohol dehydrogenase
ALDH2	Aldehyde dehydrogenase
ALT	Alanine amino transferase
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
AST	Aspartate amino transferase
ATP	Adenosine triphosphate
BAC	Blood alcohol concentration
CAMKK	Calcium/calmodulin dependent protein kinase
CPT	Carnitine palmitoyl transferase
CYP2E1	Cytochrome P450 2E1
FFA	Free fatty acids
FOXO	Fork head transcription factor
HCC	Hepatocellular carcinoma
HDL	High-density lipoprotein
HFD	High fat diet
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
LCAT	Lecithin–cholesterol acyltransferase
LDL	Low-density lipoprotein
LKB1	Liver kinase B1
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NEFA	Non-esterified free fatty acids
PGC	Proliferator activated receptor
PP2A	Protein phosphate 2A
PPAR	Peroxisome proliferator-activated receptor
ROS	Reactive oxygen species
SIRT1	Sirtuin 1
SREBP	Sterol regulatory element-binding protein
TC	Total cholesterol
TG	Triglycerides
TNF	Tumor necrosis factor
VLDL	Very low-density lipoprotein

ABSTRACT

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome and is defined as the accumulation of fat in the liver of patients who do not consume excessive alcohol. NAFLD has the potential to progress through the inflammatory phase of non-alcoholic steatohepatitis (NASH) to fibrosis, cirrhosis, and in some cases to liver failure or hepatocellular carcinoma (HCC). The pathogenesis of NAFLD was described as a two-hit model. The first hit consisting of hepatic lipid accumulation, from sedentary lifestyle, high fat diet, obesity, and insulin resistance. The second hit activates inflammatory pathways, oxidative stress, mitochondrial dysfunction with associated fibrogenesis. High fat diet (HFD)-induced model was used for our studies as NAFLD model which also mimics to human NAFLD. Apart from the nutritional factors, several lifestyle activities such as alcohol consumption, smoking and physical inactivity were implicated in the pathogenesis of metabolic disorders. Notably, heavy alcohol consumption (>50 g/day) was implicated in the pathogenesis of metabolic disorders leading to late-stage complications, including atherosclerosis and fatty liver disease. Heavy alcohol consumption leads to alcoholic fatty liver disease, but in contrast to the alcoholic fatty liver, evidence shows that moderate alcohol has beneficial effects on protection from type 2 diabetes, protection against the risk of major cardiovascular disease events such as myocardial infarction and coronary artery disease in healthy populations.

Starting from this background, the primary aim of the present study was to determine the effects of low, moderate and high alcohol (1 g/kg/day, 2 g/kg/day, 6 g/kg/day) doses on the development of NAFLD in Sprague Dawley (SD) rats. It was hypothesized that low and moderate alcohol intake may protect the liver from developing NAFLD in HFD-fed rats.

Objectives

The proposed research project is based on the hypothesis that long-term low and moderate alcohol protects the liver from progression to NAFLD. The general objective of this thesis is to investigate the *in vivo* and *in vitro* effects of alcohol on NAFLD induced by HFD-fed rat model. The specific objectives of this thesis are (i) To elucidate the serum and histopathological changes observed by alcohol treatments on *in vivo* model, (ii) To elucidate the possible mechanism(s) of action of low and moderate alcohol on controlling HFD-induced NAFLD in the liver, (iii) elucidate the possible mechanism(s) of action of low and moderate alcohol on controlling FFA-induced NAFLD in HepG2 cells, (iv) evaluating the effect of alcohol on FFA-induced NAFLD in HeLa cells (*LKB1-deficient cells*) (v) evaluating the effect of alcohol on FFA-induced NAFLD in HepG2 cells in absence of AMPK, (vi) evaluating the effects of alcohol on FFA-induced NAFLD in HepG2 cells in absence of SIRT1.

Methods

In the present study, the protective effect of low and moderate alcohol on NAFLD were investigated in HFD-induced NAFLD rat model. The rats were weight matched and divided into six groups, each consisting of five to six rats. Treatment to the groups were done as following, standard diet to normal control,

and HFD to disease control and HFD with alcohol doses of 1 g/kg/day, 2 g/kg/day and 6 g/kg/day for 15 weeks. At the end of the treatment, blood was collected from cardiac puncture and serum was extracted for serum biochemical estimations and liver tissue was collected for histopathological studies and various protein estimations involved in the lipid metabolism pathways.

An *in vitro* model for finding the protective effect of low and moderate alcohol in FFA-treated HepG2 cells. HepG2 cells were treated with low (10 mM) and moderate alcohol (20 mM) with free fatty acids (FFA) 1 mM (Oleic acid 0.66 mM and palmitic acid 0.33 mM) with final concentration of 1% serum-free BSA. Followed by some *in vitro* studies by inhibiting the AMPK, SIRT1 and LKB1 in separate studies for evaluating the individual role of AMPK, SIRT1 and LKB1 in protecting the HepG2 cells from FFA-induced NAFLD. The underlying molecular mechanism(s) were further investigated by focusing on the expression of various proteins (by western blot technique) involved in lipid metabolism.

Results

The marked rise in total cholesterol, LDL cholesterol, VLDL cholesterol, triglycerides, free fatty acids, ALT, AST in serum, as well as hepatic cholesterol, triglycerides of HFD-fed rats were significantly reduced by low and moderate alcohol treatment. Furthermore, the major proteins such as AMPK-α1, p-AMPK-α1, LKB1, p-LKB1, SIRT1, ACC, p-ACC and SREBP1 were quantified. Low and moderate alcohol groups with HFD-diet have shown an increased expression of AMPK-α1, p-AMPK, LKB1, p-LKB1 proteins. Besides, the results of the *in vitro* experiments resembles the results observed in *in vivo* studies. Furthermore, the results of the LKB1 inhibitory studies indicate that LKB1 is essential for the

activation of AMPK-α1 and SIRT1. In contrast, the SIRT1 and AMPK-α1 inhibitory studies indicate that low-to-moderate alcohol regulate the expression of SIRT1 and AMPK-α1 independent of each other.

Conclusion

Overall, this study demonstrated that (i) chronic feeding of HFD to rats caused hepatic steatosis by accumulation the lipid in the liver which mimics the initial stage of NAFLD, (ii) Long-term feeding of low and moderate alcohol improved serum and hepatic lipid profile by inhibiting the progression to NASH, (iii) Low and moderate alcohol feeding over a long-term increases lipid metabolism by increasing AMPK-α1, SIRT1 and LKB1 proteins, (iv) alcohol groups with FFA treated HepG2 cells produced a similar result to that *in vivo* studies, (v) HeLa cells failed to reduce lipid synthesis proteins involved, due to the lack of LKB1 an upstream kinase for activating AMPK, (vi) Low and moderate alcohol increase SIRT1 in HepG2 cells(AMPK inhibited), likewise increase AMPK-α1 and LKB1 in FFA-treated HepG2 cells (SIRT1 inhibited). Thus, this thesis concludes that alcohol at optimal doses protects the liver from NAFLD.

Future directions

Future studies are required to find out the role of low to moderate alcohol on individual downstream proteins involved in regulating the lipid metabolism pathways. Furthermore, a more detailed studies are required on how low to moderate alcohol regulates the inflammatory pathways and oxidative stress, which play a major role in the pathogenesis of NAFLD. In addition, the role of alcohol metabolism pathways needs to be evaluated for the doses used *in vitro*

studies. Apart from HepG2 cells, all the *in vitro* studies need to be done in VA-13 cell lines for in-depth understanding of alcohol dehydrogenase-mediated alcohol metabolism in its protection against NAFLD.

CHAPTER-1

GENERAL INTRODUCTION

1.1 Non-alcoholic fatty liver disease(NAFLD)

Non-alcoholic fatty liver disease (NAFLD) is an increasingly recognized disease in which lipid accumulates in the liver in the absence of alcohol [1]. It is a clinico-pathological entity that comprehends liver disease spectrum which progresses from fatty liver to non-alcoholic steatohepatitis (NASH), fibrosis, and then to cirrhosis.

1.1.1 Progressive stages involved in fatty liver disease

NAFLD describes a range of conditions caused by a build-up of fat within liver cells. It is helpful to divide NAFLD into four stages for easy identification as below[1]:

1.1.1.1 *Fatty liver disease (FLD or hepatic steatosis)*

Fatty liver is defined by the presence of triglycerides (TG) accumulation in hepatocytes conventionally defined as the presence of lipid droplets within the cytoplasm in more than 5% of hepatocytes.

1.1.1.2 Non-alcoholic steatohepatitis (NASH)

NASH is a subcategory of NAFLD and is defined as the presence of hepatic steatosis and inflammation with hepatocyte injury.

1.1.1.3 Liver fibrosis

Any form of persistent hepatitis, including steatohepatitis, may eventually cause scar tissue (fibrosis) to form within the liver. When fibrosis first develops, often there are many liver cells that continue to function quite well.

1.1.1.4 Liver cirrhosis

This is a serious condition where normal liver tissue is replaced by a lot of fibrosis. The structure and functions of the liver are badly disrupted. It is, in effect, like a severe form of liver fibrosis. Many liver conditions can lead to cirrhosis, including NAFLD. Severe cirrhosis can lead to liver failure.

1.2 Etiology and pathogenesis of NAFLD: The nature and mechanisms

NAFLD etiology and its progression is clearly multifunctional, complex and incompletely understood. NAFLD is associated with many different agents and conditions. These may be due to acquired insulin resistance, metabolism errors, medical conditions or surgeries associated with weight loss, various drugs and toxins [2]. Most cases of NAFLD are related to “Western lifestyle” i.e., nutrient abundance coupled with a sedentary lifestyle; however, genetic predisposition plays an important role, if not decisive, role in determining which individuals are at increased risk for development of NAFLD and for its progression.

1.2.1 Genetic factors in NAFLD

Over the years, a number of studies have implicated the role of genetic predisposition in NAFLD. It is clear that how ethnic differences play a role in affecting NAFLD, especially explained on the basis of diet or socioeconomic differences in NAFLD progression. A recent study shows a higher incidence of NASH in Hispanic origin in the US population relative to whites and low rate of incidence in African Americans, despite a higher rate of obesity [3, 4]. The mixed

racial heritage population in the US would be useful in future to identify them using racial origin genetic markers. This kind of accumulating data may ultimately be used for screening population and/or public health intervention strategies. The South Asian population is the next high risk for development of NAFLD [5, 6].

In recent studies, sample population obtained with well-defined NAFLD have been used for genome scans to discover gene variants that are more common in NAFLD patients than in normal population. Some groups examined single nucleotide polymorphism (SNP) variants in candidate genes and implicated in the regulation of lipid metabolism or relation with risk factors for NAFLD [7].

1.2.2 Environmental factors in NAFLD

Exposure to environmental factors, especially dietary factors, is likely to contribute to the generation and accumulation of intrahepatic lipids [8]. Some studies suggest that specific dietary fats, such as trans-unsaturated fats, contribute to hepatic steatosis [9]. Contrarily, monounsaturated lipids such as oleic acid, linoleic acid, 33 or n-3 fatty acids decrease intrahepatic lipid accumulation and improve postprandial triglyceride levels, possibly by increasing peroxisomal activity and reduces damage by reactive oxygen species(ROS) [10]. The monosaccharide fructose is another dietary factor that contributes to hepatic steatosis. In a case-controlled study, sugar-sweetened beverage consumption was associated with hepatic steatosis, and this association was independent to the degree of obesity [11]. In addition, consumption of total fructose was known to be associated with NAFLD in general and NASH in particular [12]. Excess calorie intake leads to

obesity and is associated with insulin resistance which is in-part due to decreased adiponectin (Figure 1.1). All these factors contribute to NAFLD development indirectly by incresing the *de novo* lipogensis and directly by increasing the FFA flux to the liver via decreased inhibition of lipolysis.

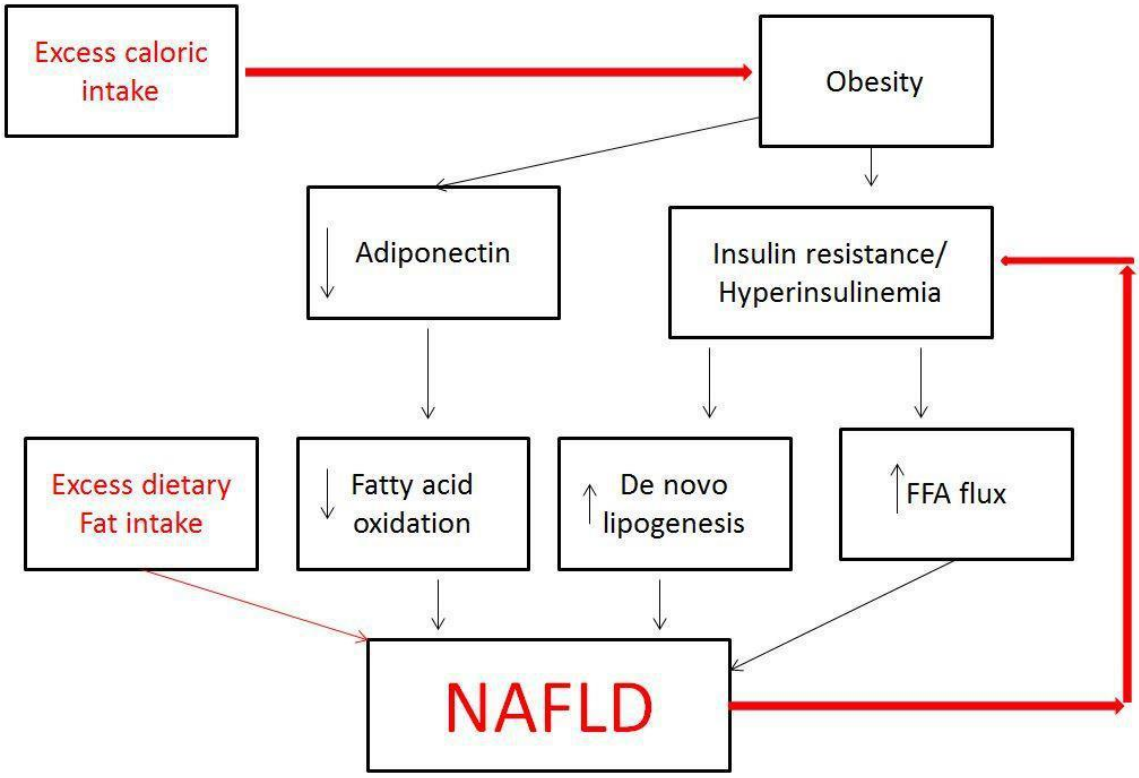

Figure 1.1 Schematic representation of mechanisms of high fat diet-induced NAFLD (*Adapted and modified from [13]*)

1.2.3 Pathogenesis of NAFLD

The pathogenesis of NAFLD is based on 2-hit hypothesis. The 'first hit', hepatic steatosis is a prerequisite for histological diagnosis of NAFLD. Several mechanisms may lead to steatosis, including (1) increased fat supply such as high-

fat diet and excess adipose lipolysis; (2) decreased fat export in the form of very low density lipoprotein-triglycerides; (3) decreased -oxidation of free fatty acids; and (4) increased *de novo* lipogenesis (*DNL*) [14]. All these factors increases the vulnerability of the liver to various possible 'second hits' such as inflammatory cytokines, oxidative stress, mitochondrial dysfunction which lead to steatohepatitis and/or fibrosis [15]. However, the insights on the role of free fatty acids (FFA) in promoting liver injury lead to modification of the theory. Hepatic fat accumulation was observed when there is an influx of FFA to the liver in obesity and insulin resistance. These FFA either undergo β-oxidation or are esterified with glycerol to form triglycerides, leading to hepatic fat accumulation. There is now substantial evidence that FFA can directly cause toxicity by activation of inflammatory pathways or by increasing oxidative stress [16].

1.2.3.1 Hepatic steatosis / Lipid accumulation

Hepatic steatosis results from an imbalance in lipid flux. Factors that contribute towards hepatic fat accumulation include an increase in lipid supply from increased adipose tissue lipolysis and/or high dietary fat intake, a decrease in lipid oxidation, an increase in hepatic *de novo* lipid synthesis and decreased hepatic very low density lipoprotein (VLDL) secretion [17]. Lipid utilization in the liver for energy occurs by FFA oxidation which is inhibited by increased levels of malonyl-CoA, as a result of insulin mediated activation of sterol regulatory element binding protein-1c (SREBP-1c) (Browning [18]. A second possible fate of hepatic lipid is the export that happens in the form of lipoproteins. Apo lipoprotein (apo) B facilitates

lipid export from the liver by incorporating triglyceride before being secreted. Hence hepatic fat accumulation can occur as a result of increased fat synthesis, increased fat delivery, decreased fat export, and/or decreased fat oxidation [19]. Modern diets containing *trans*-fatty acids (found in processed oils) and fructose which is a common sweetener, may also contribute to the pathogenesis of NAFLD. In humans, a high fructose diet is associated with increased insulin resistance and more severe hepatic inflammation and fibrosis in subjects with NAFLD[20].

1.2.3.2 The role de novo lipogenesis (DNL) in NAFLD

In liver Hepatic de novo lipogenesis (DNL) is a fundamental biosynthetic pathway contributing to the lipids that are stored and secreted by hepatocytes [21]. The increase in lipogenesis in liver could increase the steatotic nature of NAFLD. Several studies have shown that diets enriched in both saturated fat and simple sugar carry a high risk of hepatic steatosis, at least in part, through enhanced DNL[22, 23]. In NAFLD patients about 59% of TG in livers were from FA flux (possibly from lipolysis in adipocytes), 26% from DNL and 15% from the diet [24]. This is supported by data analysing the FA composition of TGs in subjects with and without NAFLD, showing increased levels of saturated FAs in subjects with NAFLD pointing toward DNL as the source, due to the major product of DNL being saturated FAs [25]. In hepatic insulin resistance, DNL regulate a transcription factor sterol regulatory element-binding protein-1 (SREBP1). DNL enzymes are also regulated by glucose through the action of a second transcription factor, carbohydrate response element-binding protein (ChREBP). Together, SREBP1 and

ChREBP promote hepatic DNL in response to high carbohydrate feeding, hyperglycaemia, and hyperinsulinemia[19].

1.2.3.3 The role of *sterol regulatory element-binding proteins (*SREBPs) in NAF*LD*

The sterol regulatory element-binding proteins (SREBPs) are a family of membrane-bound transcription factors synthesized in the endoplasmic reticulum (ER) as precursors with 125 kD. Proteolytic cleavage then allows the accumulation of active SREBP in the nucleus. SREBP-1c, the predominant isoform in the liver is one of three SREBP isoforms (SREBP-1a, SREBP-1c, and SREBP-2) and preferentially affects the transcription of genes that regulate *de novo* lipid synthesis, although SREBP2 regulates [26]. SREBP1a is expressed only at very low levels in the liver of adult mice, rats, and humans. SREBP1c. Enzymes that catalyze the synthesis of fatty acids, TG, and NADPH required for fatty acid synthesis are regulated by SREBP-1c. The typical genes regulated by SREBP-1c are ATP-citrate lyase, acetyl-coenzyme A (acetyl CoA) carboxylase, fatty acid synthase, ELOVL6 (elongation of long chain fatty acids family member 6), stearoyl-CoA desaturase, glycerol-3-phosphate acyl transferase, malic enzyme, and glucose 6-phosphate dehydrogenase [27]. *In vivo* transgenic model demonstrated that overexpression of SREBP-1c in the liver, which leads to the development of hepatic steatosis due to the increase in lipogenesis [28]. Increased rates of hepatic fatty acid synthesis contribute to the development of hepatic steatosis in rodent models of insulin-resistance and obesity. In ob/ob mice deletion of SREBP-1c gene results in an approximately 50% reduction of hepatic triglycerides, which indicates a significant

role of SREBP-1c in the hepatic steatosis exhibited in the ob/ob mouse, a model of insulin resistance [29].

1.2.3.4 The role of carbohydrate regulatory element-binding protein (ChREBP) in NAFLD

Carbohydrate regulatory element-binding protein (ChREBP), is a transcription factor that is independently activated by glucose rather than insulin. In contrast to SREBP1c, ChREBP is activated by the postprandial rise in glucose delivery to hepatocytes. It activates liver pyruvate kinase, which generates pyruvate, a source of acetyl-CoA, from phosphoenolpyruvate, as well as genes involved in fatty acid synthesis [30]. When glucose is abundant, adenosine monophosphate kinase (AMPK) is suppressed, leading to nuclear translocation of ChREBP and induction of glucose-responsive genes. ChREBP activity is activated by dephosphorylation [31]. ChREBP target genes include not only enzymes of glycolysis and lipogenesis that predispose to hepatic steatosis, but also glucose 6-phosphatase (G6PC), which catalyses the final reaction in glucose production, and glucokinase regulatory protein (GCKR), which inhibits hepatic glucose uptake [32]. Transcriptional induction of G6PC and GCKR manifests as hepatic glucose intolerance or insulin resistance (IR) [33]. Inhibition of ChREBP in liver of ob/ob mice, lipogenesis and triglyceride (TG) synthesis are decreased. As a result, the improvement of blood glucose levels was done by the restored inhibition of genes from the gluconeogenic pathway (G6Pase and PEPCK) by insulin. Correction of hepatic steatosis also leads to decreased levels of plasma TG and non-esterified fatty acids (NEFA). Therefore, insulin sensitivity is restored in skeletal muscles and

glycogen synthesis is enhanced, therefore contributing to the decrease in blood glucose concentrations was observed [34].

1.2.3.5 The role of AMPK in NAFLD

The liver plays a dominant role in the maintenance of energy storage and glucose homeostasis, knowledge on physiology as well as physiopathology of hepatic energy metabolism is required for our understanding of whole-body metabolism. The synthesis and utilization of hepatic carbohydrate, lipid and protein are tightly regulated according to our daily needs. AMPK is considered as a cellular energy sensor and is important to acquire knowledge on the mechanism by which hepatic AMPK coordinates hepatic energy metabolism. AMPK has been implicated as a key regulator of physiological energy dynamics by limiting anabolic pathways and by facilitating catabolic pathways. Activation of hepatic AMPK leads to increased fatty acid oxidation and simultaneously inhibition of hepatic lipogenesis, cholesterol synthesis and glucose production. The identification of AMPK targets in hepatic metabolism should be useful in developing treatments to reverse metabolic abnormalities of type 2 diabetes and the metabolic syndrome. The positive effects of AMPK are indicated by arrows and the inhibitory effects of AMPK action was indicated by T lines(Figure 1.2).

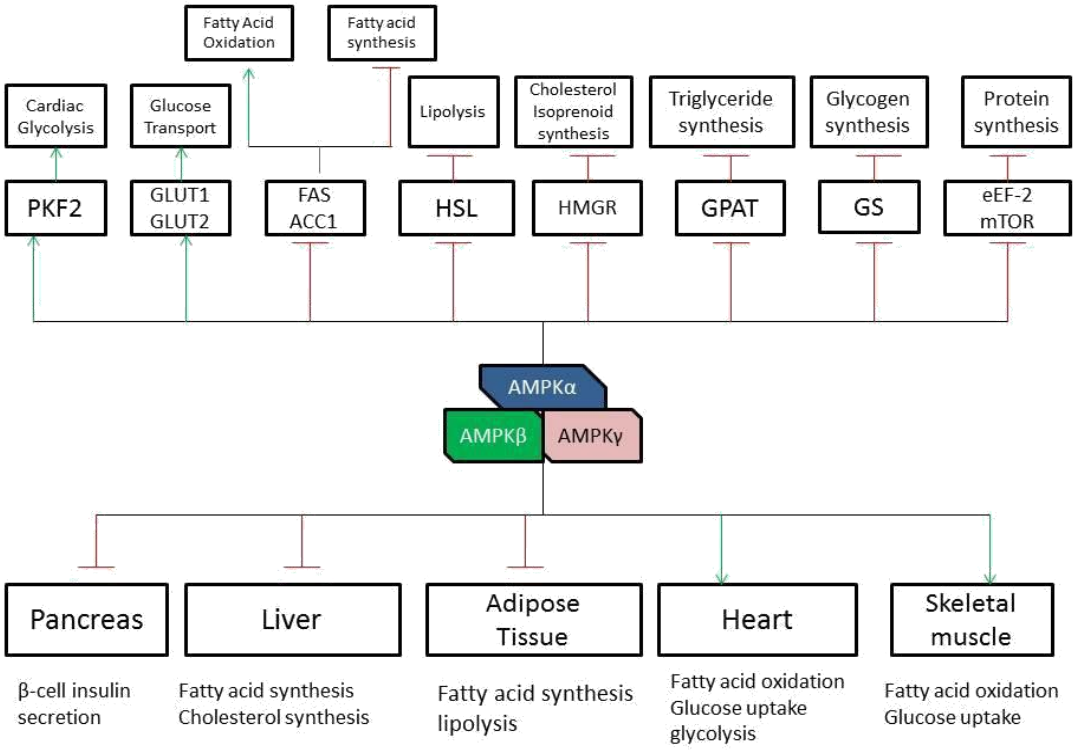

Figure 1.2 Physiological targets of AMPK (*Adapted and modified from[35]*)

AMPK protein plays a critical role in sensing energy availability at the cellular level. Upon exposure to low glucose or decreased energy stores, AMPK inhibits mRNA translation and protein synthesis of pathways that are nonessential in the short term. In turn, during times when food is plentiful, AMPK activity is inhibited, mRNA translation is up-regulated, and the cells and organism can grow in size [36, 37]. With caloric excess, there is fatty acid excess and insulin resistance fuelling hepatic triacylglycerol synthesis and steatosis. Excess calorie intake and lower adiponectin reduces AMPK activation, promoting hepatic stellate cell proliferation and generation of reactive oxygen species in the liver, leading to conversion from hepatic steatosis to steatohepatitis and ultimately cirrhosis. Through similar

pathways, lower adiponectin levels reduce AMPK in podocytes to promote podocyte foot process effacement and albuminuria [38].

Various studies on humans and animal models have suggested that efforts to enhance insulin sensitivity might improve fatty liver disease. It has been demonstrated that hepatic AMPK activation abolishes hyperglycaemia in diabetic *ob*/*ob* and STZ-induced diabetic mice by suppression of gluconeogenesis [39]. Similarly, adiponectin restores insulin sensitivity and decreases hepatic steatosis by lowering TG content in liver of obese mice [40]. The adiponectin activation is linked to activation of hepatic AMPK, ultimately leading to decreased fatty acid biosynthesis and increased mitochondrial fatty acid oxidation [41]. The role of AMPK has been confirmed by the decrease in liver TG content in lean and obese rodents during AICAR infusion and treating with direct AMPK activator[42]. Recent studies demonstrated that resveratrol improves insulin sensitivity and protects against lipid accumulation in diabetic and high fat feed animals with activation of hepatic AMPK [43]. Activation of AMPK and adiponectin may inhibit HSCs proliferation and hepatic fibrosis via multiple molecular mechanisms and drugs activating hepatic AMPK may have an additional rationale in their antifibrogenic properties. In addition, evidence suggest that SIRT1 and AMPK regulate each other, share similar signalling pathways, and modulate many common targets [44]. SIRT1 is a nicotinamide adenine dinucleotide(NAD^+) dependent class III histone deacetylase and acts as part of the de-acetylation reaction producing nicotinamide [45]. The de-acetylation of proteins and histones results in an up or down regulation of gene transcription and protein function.

1.2.3.6 The role of SIRT1 in NAFLD

Liver is one of the key organs where SIRT1 plays a pivotal role in the regulation of lipid metabolism and inflammation. The regulatory action of deacetylation activity of SIRT1 has been shown to have a positive impact on the pathophysiological mechanisms of NAFLD. The effects of SIRT1 are: healing activity on insulin sensitivity, there by ameliorating regulation, its antihyperlipidemic activities on lipid homeostasis via the adipose tissue of liver and skeletal muscles, and anti-inflammatory activities.

Accumulating evidence suggest that SIRT1 and AMPK regulate each other, share similar signalling pathways and modulate many common targets [44]. Studies in cultured hepatic cells and in animal liver have provided evidence that SIRT1 is able to stimulate AMPK activity via modulation of liver kinase B1 (LKB1), an upstream AMPK kinase [46]. On the other hand, activation of AMPK via LKB-1 leads to increased cellular NAD+ levels, which subsequently activates SIRT1 signalling [47, 48]. Thus, this unique SIRT1-AMPK axis participates in regulating various lipid metabolism and inflammation pathways [48].

1.2.3.7 The role of LKB1 *in NAFLD*

Liver kinase B1 (LKB1), known as a serine/threonine kinase, has been identified as a critical cancer suppressor in many cancer cells. It was first discovered in 1998 in Peutz-Jeghers Syndrome [49]. Initial studies, on LKB1 identified a little similarity to other protein kinases. LKB1 can regulate the activity of at least 14 different targets, thus controlling a wide range of biological processes. It

is a master upstream kinase of AMP-activated protein kinase (AMPK)-related protein kinases, and possesses versatile biological functions [50].

AMPK plays a central role in the regulation of whole-body energy metabolism, and its activation is beneficial for protecting the body from metabolic diseases, such as type 2 diabetes and obesity. Mutations of AMPK can lead to cardiac hypertrophy and arrhythmia. Recent findings have identified that LKB1 is an important upstream kinase of AMPK cascade in mammalian cells [51]. LKB1 can phosphorylate Thr172 on the activation loop of AMPK catalytic subunit and subsequently activates AMPK. In addition, inhibition of LKB1 activity in cells simultaneously abolishes the activation of AMPK by different stimuli [52]. LKB1-deficient murine embryonic fibroblasts show nearly complete loss of Thr172 phosphorylation and downstream AMPK signalling in response to different AMPK activators [53].

1.2.3.8 The role of peroxisome proliferator-activated receptor (PPAR)α in NAFLD

Peroxisome proliferator-activated receptor (PPAR) α is a ligand-activated transcription factor that is abundantly expressed in liver, which regulates lipid, glucose and energy homeostasis and controls body weight and vascular inflammation. PPARα is expressed at high levels in tissues that catabolize fatty acids, notably liver, skeletal muscle, and heart and at lower levels in other tissues, including pancreas [54]. In a clinical study with 125 subjects with suspected NAFLD, liver PPARα mRNA expression was found to be lower in subjects with NASH compared to subjects without NASH. Furthermore, a progressive decrease in PPARα expression was observed with increasing NAFLD Activity Score and

fibrosis stage. A rodent study with PPARα -null mice fed with HFD an increased steatosis, oxidative stress, and inflammation when compared to normal wild type mice, demonstrate that inhibition of PPARα functions may increase susceptibility to high fat–induced NASH. Administering PPARα agonists to rats not only prevents of MCD diet induced steatosis by preventing intrahepatic lipid and lipoperoxide accumulation, but also reverses hepatic fibrosis by decreasing the expression of fibrotic markers and reducing the number of stellate cells [55, 56]. An increased PPARα plays a role in modulation of hepatic steatosis by upregulating FA oxidation, reduction in the toxicity of FAs, and its anti-inflammatory effect.

1.3 Current therapeutic strategies for NAFLD

To date, no single therapy has been approved for treating NAFLD, but a growing consensus suggests that only patients with NASH require treatment and only they should be the targets of future clinical trials. Even so, patients with other forms of liver disease may be at risk for other complications of metabolic syndrome, and treatment for the underlying components of metabolic syndrome should be addressed. Several pharmaceutical agents have been used for the treatment of NASH; however, initial management must be focused on lifestyle modification and the reversal of conditions associated with NAFLD [57].

1.3.1 Lifestyle modification and weight loss

Life style modification including increased physical activity and weight loss management could be achieved by dietary control. The pathophysiological basis for this approach is that weight reduction results in the loss of white adipose

tissue, which decreases Insulin resistance. Exercise can also improve muscular insulin sensitivity, which may improve the impact of IR on NAFLD. A few trials have also shown a significant reduction in ALT values in patients with biopsy proven NAFLD where they are put on calorie-restricted diets with or without exercise [57-59].

In addition to exercise and diet, few studies have investigated the use of medication for weight loss. A clinical trial using orlistat, an enteric lipase inhibitor, showed a mean decrease in body weight and significant reductions in serum transaminase levels in obese patients with NASH [60]. A recent study from the *Swedish Obese Study Group* suggests that bariatric surgery can be associated with improved long-term outcomes in terms of cardiovascular risk factors such as diabetes mellitus, hypertriglyceridemia and hypertension compared to a conventional weight loss group[61]. In fact, another study by Dixon et al. showed that NASH resolved in 82% of patients undergoing laparoscopic adjustable gastric banding (LAGB) [62].

1.3.2 Bariatric surgery

NAFLD *per se* is not an indication for bariatric surgery [63]. But now a days, among morbidly obese patients an increasing bariatric surgery was an option for controlling NAFLD. Bariatric surgery-induced weight loss appears to have beneficial effects on lipid profile, hepatic steatosis, steatohepatitis, as well as reducing long-term mortality[64]. From a meta-analysis of clinical trials have confirmed that NAFLD patients undergoing bariatric surgery reported that steatosis resolved in 91.6 %, steatohepatitis improved in 81.3 %, and fibrosis in 65.5 % of cases [65]. Clearly, surgical intervention is not a panacea for all

patients with NASH, and before any further recommendations are made, a more robust data from random clinical trials are needed.

1.3.3 Treating insulin resistance

Drugs that enhance insulin action are a logical approach to treat NAFLD because insulin resistance is present in most patients [66, 67]. Metformin, thiazolinediones, rosiglitazone and pioglitazone, have been tested in several pilot studies. Metformin is now positioned as the first-line therapy, because of its low cost, weight-reducing effect, preventive effect on cardiovascular event, and safety profiles. Unfortunately, metformin has no data regarding improvement in liver enzymes and histology in NASH/ NAFLD, although it is associated with a reduced incidence of HCC and extrahepatic malignancies. An improvement in serum aminotransferase levels and hepatic steatosis was observed in rosiglitazone patients, but it failed to control the inflammation or fibrosis [68]. Pioglitazone significantly improved aminotransferase levels, steatosis, ballooning, and inflammation in patients with NASH who had impaired glucose tolerance or T2DM. The NASH improved with pioglitazone in 73% compared with 24% of placebo-treated patients (p<0.001) and there was a trend toward improvement in fibrosis [69].

1.3.4 Anti-oxidant drugs

Increased oxidative stress has been reported in both patients with NAFLD and patient with cardiovascular risk factors. Thus, oxidative stress represents a shared pathophysiological disorder between the two conditions. Therefore,

therapeutic efforts have been made using several pharmacological agents proven to be anti-oxidants such as vitamin E, vitamin C and betaine [70]. Oxidative stress is considered to be one of the key mechanisms leading to hepatocellular injury and disease progression. Vitamin E is currently the most widely assessed antioxidant and has been investigated as a potential treatment for NASH. Based on the available evidence, vitamin E (RRR-α-tocopherol) is currently only recommended in NASH adults without diabetes or cirrhosis and with aggressive histology [71].

1.3.5 Hepatoprotective agents

Historically speaking, several compounds have been claimed to have a theoretical protective effect on the liver cells, which in some patients could be clinically relevant and had been applied in practice either on empiric, local experience, or traditional management basis. Some of these products are available as nutritional supplements and may have a convincing rationale, whereas some others such as “essential phospholipids (EPL) may have evidence furnished on research basis. These medicines included N-acetyl cysteine, choline bitartrate, artichoke extract, dandelion root, taraxacin and inulin, turmeric (curcumin), milk thistle extract (silymarin), essential phospholipids, UCDA, vitamin E, S-adenosyl methionine, Ganoderma spores, traditional Chinese medicine, including Gansu and some herbal compounds [72]. The myriad of hepatoprotective effects exerted by the hydrophilic natural bile acid ursodeoxycholic acid (UDCA) led investigators to test this agent in both experimental models and patients with NAFLD [73]. Some conceptual support for the potential usefulness of UDCA therapy in NAFLD comes from the demonstration that animals with fatty liver exhibit mild cholestatic features

[73, 74]. Promising effects were seen in rodents and in pilot human trials [75-77], but two randomized controlled trial (RCTs) involving 27 and 166 patients, respectively, did not show positive effects either in liver chemistry or in histology [78, 79]. Phosphatidylcholine (PC), a main component of EPL, is one of the most important support nutrients for the liver and is considered as a universal building block for cell membranes, which regulate most of the activities that make up life. PC helps recovery and maintenance of the consistency of the hepatocytes; it activates the phospholipid-depending enzymes and improves lipid metabolism by accelerating synthesis of lipoproteins in the liver [80]. EPL treating 324 patients of NAFLD or NAFLD associated with comorbid clinical conditions such as T2DM and hyperlipidaemia. It was concluded from the study that there was a significant improvement for all symptoms, general and gastrointestinal, and a significant reduction of the elevated transaminases associated with NAFLD disease [81].

1.4 Alcohol

The term alcohol is conventionally used to refer ethyl alcohol (alcohol) which has a molecular formula of C_2H_6O and molecular weight of 46.07 g/mol. Alcohol will be present in any yeast habitat and also commonly be found in overripe fruit [82]. Alcohol is also produced during the germination of many plants as a result of natural necrobiosis [83]. It is a colour less, volatile, flammable liquid with a specific gravity of 0.785. It is the principal type of alcohol found in alcoholic beverages and produced by the fermentation of sugars by yeast. Apart from its many industrial

uses, alcohol is a psychoactive drug and one of the oldest recreational drugs used by humans.

1.4.1 Pharmacokinetics of alcohol

Alcohol is a commonly used recreational drug that has many important health consequences. Therefore, it is imperative to understand the pharmacokinetic properties of alcohol and the factors that influence the kinetics of alcohol.

1.4.1.1 Absorption

Oral administration of alcohol determines the rate and proportion at which alcohol reaches the blood and body tissues. Alcohol is a small water soluble molecule that can cross cell membranes, it is absorbed from both the stomach (20 %) and the upper small intestine (80 %) [84, 85]. The rate of absorption vary significantly in both intra individual and inter individual even at standardised conditions [86]. The speed of absorption is also influenced by variation in portal blood flow; because alcohol crosses the biological membrane by passive diffusion thus good blood flow will maintain the concentration gradient and promote absorption [87]. The rate of alcohol absorption depends on whether food is consumed at the same time and the macronutrients within that food. Foods containing fat and protein slow gastric emptying and therefore consumption of alcohol with these foods can slow alcohol absorption [88]. The rate of alcohol absorption is also determined by the type of drink consumed. Drinks with alcohol content between 20-30% are absorbed quickest. Whereas, drinks with a higher alcohol content are absorbed more slowly, because an alcohol content over 30%

irritates the gastric mucosa increasing mucus secretion and decreasing gastric emptying. Thus, drinks with alcohol content above 30% can cause a faster rise in BAC if served diluted with a mixer; especially carbonated drink can also increase the rate of absorption than if they are served without dilution [84].

1.4.1.2 Distribution

Alcohol enters the portal vein and the liver before entering the general circulation. Thus the bioavailability of alcohol is reduced by first pass metabolism. Majority of alcohol oxidation occurs via oxidation by alcohol dehydrogenase (ADH) in the liver hepatocytes. But a small portion of alcohol oxidation occurs in gastric mucosa by gastric ADH [87]. The absorbed alcohol which escapes first pass metabolism enters the systemic circulation and is rapidly distributed throughout the body tissues via the blood plasma until an equilibrium between the blood alcohol content and tissue concentration is reached [85]. The pharmacokinetics of alcohol will be influenced by the total body water, because it determines the volume of distribution available for alcohol distribution within the body. Therefore body composition is an important consideration in pharmacokinetic studies because both body size and composition will have a significant impact on the volume of distribution [89].

1.4.1.3 Metabolism

Liver is the main organ responsible for metabolizing alcohol. In human's, elimination of alcohol is done by various mechanisms. A small portion of absorbed alcohol i.e.,(2-5%) is excreted unchanged in the urine, sweat or breath [90, 91].

The majority of alcohol (90%) is eliminated via ADH, the major pathway of oxidative. In general alcohol metabolism is achieved by both oxidative pathways, (ADH, cytochrome P450, and catalase enzymes) and non-oxidative pathways.

1.4.3.1 Oxidative Pathways

The process of alcohol oxidation involves at least three distinct enzymatic pathways [92]. They are

- Alcohol dehydrogenase ADH
- Microsomal alcohol oxidizing system (CytochromeP450 2E1(CYP 2E1))
- Catalase

Each of these pathways produces a specific metabolic and toxic disturbances and all these results in the production of acetaldehyde, a highly toxic metabolite.

ADH, cytochrome P450 2E1 (CYP2E1), and catalase all contribute to oxidative metabolism of alcohol (Figure 1.3).

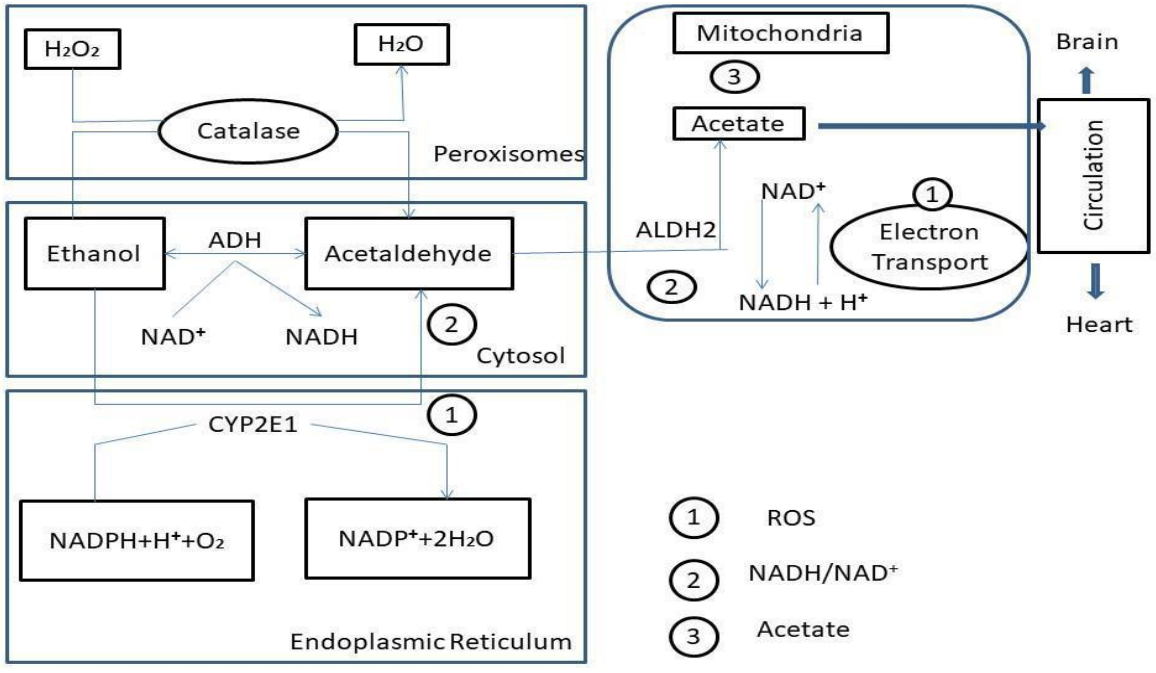

Figure 1.3 Oxidative pathways of alcohol metabolism *(Adapted and modified from[93])*

Alcohol Dehydrogenase (ADH)

Alcohol metabolism by the oxidative pathway is majorly involved by ADH (present in cytosol), an enzyme with different variants (isozymes). ADH converts alcohol to acetaldehyde. This reaction involves an intermediate carrier of electrons, nicotinamide adenine dinucleotide is an (NAD^+) which is reduced by two electrons to form NADH [94]. In humans five classes of ADH has been categorized based on the kinetic and structural properties (Table 1.1). At higher concentrations, alcohol is eliminated at the higher rate because of the presence of enzyme systems with higher activity levels (K_m) [2] , such as class II ADH, β_3-ADH(encoded by ADH4 and ADH1B genes respectively) and CYP2E1 [95]. Alcohol oxidation causes damage from the by-products of alcohol metabolism, such as free radicals and acetaldehyde.

Cytochrome P450

The Oxidation of alcohol in liver is also contributed by cytochrome P450 isozymes, including CYP2E1, CYP1A2, and CYP3A4 which are predominantly present in the liver microsomes. During chronic alcohol consumption, CYP2E1 is induced and plays an important role in metabolizing alcohol to acetaldehyde at elevated alcohol concentrations. In addition, alcohol oxidation that occurs in other tissues such as brain is CYP2E1-dependent, where ADH activity is low. CYP2E1 also produces reactive oxygen spices (ROS), including hydroxyethyl, superoxide anion, and hydroxyl radicals, which increase the risk of tissue damage.

Catalase

It is another enzyme which is located in peroxisomes, is capable of oxidizing alcohol *in vitro* in the presence of hydrogen peroxide(H_2O_2) generating the enzyme complex NADPH oxidase or the enzyme xanthine oxidase. It is considered as a minor pathway of alcohol oxidation ,except in the fasted state [96].

Table 1.1 Nomenclature for ADH genes and enzyme subunits [adapted and modified from [94]]

Class	Gene Nomenclature		Protein	K_m mM	V_{max}	Tissue	Alcohol catalytic efficiency
	New	Former					
I	ADH1A	ADH1	α	4.0	30	Liver	Low
	ADH1B*1	ADH2*1	β1	0.05	4	Liver, Lung	High
	ADH1B*2	ADH2*2	β 2	0.9	350		High
	ADH1B*3	ADH2*3	β 3	40.0	300		Low
	ADH1C*1	ADH3*1	γ 1	1.0	90	Liver, stomach	High
	ADH1C*2	ADH3*2	γ 2	0.6	40		High
II	ADH2	ADH4	Π	30.0	20	Liver, Cornea	Low
III	ADH3	ADH5	χ	>1,000	100	Most Tissues	Very low
IV	ADH4	ADH7	Σ(μ)	30.0	1,800	Stomach	High
V	ADH5	ADH6	Not identified	?	?	Liver, Stomach	Nit identified

The ADH1B and ADH1C genes have several variants with differing levels of enzymatic activity. K_m is a measurement used to describe the activity of an enzyme. It describes the concentration of the substance upon which an enzyme acts that permits half of the maximal rate of reaction. V_{max} is a measure of how fast an enzyme can act. It is expressed in units of products formed per time.

1.4.3.2 Non-oxidative Pathways

Alcohol metabolism through non-oxidative pathways occurs to a minimal extent. Its products may have both diagnostic and pathological properties. Non-

oxidative metabolism of alcohol is done by at least two pathways. When alcohol reacts with fatty acids it leads to formation of molecules called fatty acid ethyl esters (FAEEs) which are weak organic acids that play a functional role in human cells. The other non-oxidative pathway results in formation of a type of fat molecule containing phosphorus (phospholipid) known as phosphatidyl alcohol (Figure 1.4). After alcohol ingestion, FAEEs are detectable in serum and other tissues and persist a longer time after alcohol is eliminated. The metabolic pathways of alcohol metabolism are correlated. Alcohol oxidation inhibition by compounds that inhibit ADH, CYP2E1, and catalase result in an increase in the non-oxidative metabolism of alcohol and increased production of FAEEs in the liver and pancreas [97].

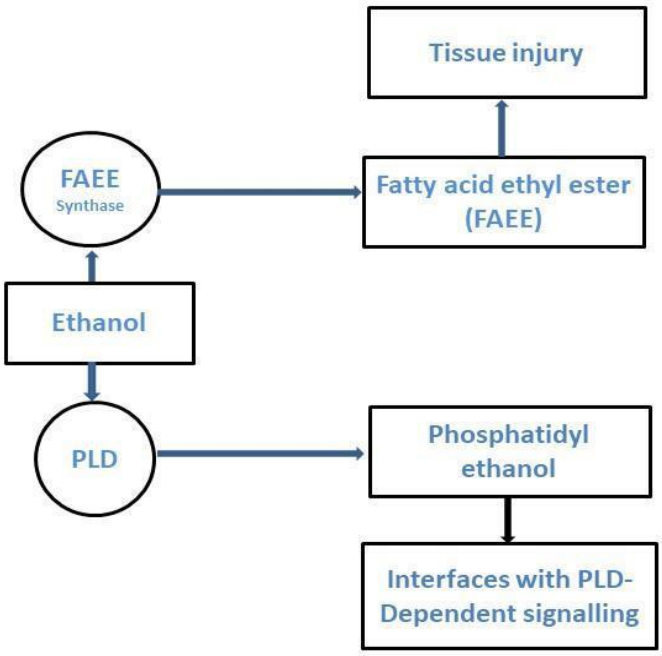

Figure 1.4 Non-oxidative pathways of alcohol metabolism
(Adapted and modified from [94])

1.4.1.4 Excretion

About 95% of ingested alcohol in the body will be eliminated through metabolism done by liver. The remaining alcohol in the body is eliminated through breath, urine, sweat, faeces and saliva. The body uses several different metabolic pathways in oxidation of alcohol to acetaldehyde to acetic acid to carbon dioxide and water [98]. Fair consistent rate of alcohol metabolism takes place in healthy people. A person will eliminate one average drink or 5oz (15ml) of alcohol per hour which is influenced by several factors. The rate of elimination tends to be higher when the blood alcohol concentration in the body is very high. Also, chronic alcoholics may (depending on liver health) metabolize alcohol at a significantly higher rate than the average. Finally, the body's ability to metabolize alcohol quickly tend to diminish with age [98].

1.4.2 Pharmacodynamics of alcohol

Alcohol affects a number of physiological systems. The pharmacological effects of alcohol depend upon the concentration of alcohol that is being consumed by the people. The clinical response ranges from mild anxiolytic effect, disinhibition of behaviour, to sedation and respiratory depression, depending on the dose.

1.4.2.1 Standard drink and drinking patterns

A clear understanding of what constitutes a ‘standard drink’ is essential for the accurate interpretation and application of alcohol guidelines. The present guidelines follow the previous Australian drinking guidelines, which define standard

drink as containing 10 g of alcohol (equivalent to 12.5 mL of alcohol). The notion of a standard drink issued widely, but the definition varies from country to country [99] . Accordingly, the alcohol drinking pattern as defined below is divided into (i) light alcohol drinking; (ii) moderate alcohol drinking; (iii) heavy alcohol drinking; and (iv) binge alcohol drinking

Light alcohol drinking: Alcohol consumption up to ONE standard drink per day (ONE Standard drink = 10 g of alcohol) by an average 60 kg adult.

Moderate alcohol drinking: Alcohol consumption up to TWO standard drinks per day by an average 60 kg adult.

Heavy alcohol drinking: Alcohol consumption more than TWO standard drinks per day by an average 60 kg adult.

Binge alcohol drinking/single occasion of drinking: Drinking up to FIVE or more standard alcohol drinks in a single occasion [100]

1.4.2.2 Blood alcohol concentration

The levels of alcohol in your bloodstream is referred to as blood alcohol concentration (BAC). It is also called blood alcohol content, or blood alcohol level and is most commonly used as a metric of alcohol intoxication for legal or medical purposes. BAC is determined by how quickly alcohol is absorbed, distributed, metabolized, and excreted.

BAC is usually expressed as a percentage of alcohol in the blood in units of mass of alcohol per volume of blood. For humans in most of the countries the legal BAC levels are 0.5 mg/ml [101]. The effects of alcohol on various tissues depend

on its concentration in the blood (blood alcohol concentration [BAC]) over time. The effects of alcohol with different BAC's is mentioned below Table 1.2. The factors effecting the BAC levels are empty stomach, body fat, sex, inexperience

Table 1.2 BAC Vs effects of alcohol [102]

BAC Level	Effect of Alcohol
BAC = 0.02 to 0.03%	No loss of coordination, slight euphoria and loss of shyness. Depressant effects are not apparent.
BAC = 0.04 to 0.06%	Feeling of wellbeing, relaxation, lower inhibitions, and sensation of warmth. Euphoria. Some minor impairment of reasoning and memory, lowering of caution.
BAC = 0.07 to 0.09%	Slight impairment of balance, speech, vision, reaction time, and hearing. Euphoria. Reduced judgment and self-control. Impaired reasoning, memory, and sense of cautiousness.
BAC = 0.100.125%	Significant impairment of motor coordination and loss of good judgment. Speech may be slurred; balance, vision, reaction time, and hearing will be impaired.
BAC = 0.130.15%	Gross motor impairment and lack of physical control. Blurred vision and major loss of balance. Euphoria is reducing, and dysphoria is beginning to appear.
BAC = 0.160.20%	Dysphoria predominates, nausea may appear. The drinker has the appearance of a "sloppy drunk." May vomit.
BAC = 0.25%	Needs assistance in walking; total mental confusion. Dysphoria with nausea and some vomiting. Death has occurred at this level, and it is considered a medical emergency.
BAC = 0.30%	Loss of consciousness.
BAC = 0.40% +	Onset of coma, possible death due to respiratory arrest.

The Blood alcohol concentrations similar to our alcohol doses from previously published papers are as follows. The BAC concentration of doses similar to the doses used in our studies (1.2 g/kg and 1.6 g/kg) showed a maximum concentration of 0.15 mg/dL at 20 minutes after alcohol administration and reached to base line by 160 minutes [103]. Dilley et al. (2018) demonstrated that the BAC at

different doses (1.16 g/kg/BW, 2.44 g/kg/BW, 3.38 g/kg/BW) via intra gastric infusion, the low alcohol rats (1.16 g/kg/BW) showed a peak BAC 1.1 mg/ml at 60 minutes and reached base line by 240 minutes. Whereas, the moderate alcohol group showed a peak of 1.5 mg/ml at 150 minutes of BAC and remained elevated through 240 minutes [104]. Based on the above alcohol doses and BAC concentrations in rodents via different routes of alcohol administration was similar to the standard drink that was recommended for humans.

1.4.2.3 Benefits of alcohol consumption

Two decades ago, the first quantitative estimate of long-term safe consumption of alcohol based on public health perspective was presented. The proposed acceptable daily intake (ADI) for a healthy and nondependent male was set at 0.1 g/kg bodyweight. Lemmens et al (1995) [105] demonstrated the found empirical evidence for four types of dose-response curves for the effects of alcohol consumption on health : (a) a linear curve, (b) a convex or exponential curve, (c) a curve with a threshold, and (d) a U-shaped risk curve. Particular attention is often focused on the U- or J-shaped curve that suggests that light-to-moderate drinking produces a protective effect. Such an inverse relationship indicates a reduction of risk for both light and moderate consumers and an excess risk not only for excessive users.

The psychological benefits of drinking include the reduction of tension, self-consciousness, stress, fear, pain, and depression, and the increase in affective expression and good feelings. Several studies show that small doses of alcohol are

associated with improvements in mood and decreases in depression and tension [106].

Cardiovascular disease

A numerous studies has demonstrated a U-shape and J-shape relation between alcohol intake, cardiovascular disease and all cause of mortality [107, 108]. Friesema et al (2007) has demonstrated that non alcoholics and former drinkers were less healthy than the moderate drinkers [109]. The levels of alcohol consumption that have been associated with protective effects range as widely as from one drink every other day to about five drinks per day [110]. There are indications that low-level consumption may be particularly protective if alcohol is taken with meals [111].

The protective effect has been demonstrated across all age ranges [112, 113] although it appears stronger for older people,[114, 115] and it is present for both men and women [107, 116, 117]. The cardioprotective effects of moderate alcohol might be due to increase in HDL-cholesterol levels [118] .

Type 2 diabetes

Low to moderate alcohol consumption was associated with reduced risk of T2DM among normal weight and overweight individuals. However, there was no significant association between alcohol consumption and risk of T2DM in obese individuals. Analyses categorised alcohol into various groups, there appeared to be an approximate U-shaped relationship between alcohol consumption group and risk of T2DM in the full model and in the normal and overweight groups but not the

obese group. A study of 84,941 female nurses reported a U-shaped association between alcohol consumption and risk of T2DM over 16 years of follow up in those with a body mass index (BMI) < 25 kg/m^2 but an inverse association in the overweight and the obese [119]. However, in the same study carried out 6 years earlier in 109,690 nurses aged 25 to 42 years, they reported an inverse relationship between alcohol intake and incidence of T2DM in both the obese (BMI ≤ 30 kg/m^2) and non-obese[120].

A randomized controlled clinical trial concludes that in post-menopausal women drinking at 30g/d (2 drinks per day) of alcohol has beneficial effects on insulin and inulin sensitivity in normal women [121]. Schrieks etal (2015) from his meta-analysis and systemic review concludes that moderate alcohol consumption may decrease fasting insulin concentration among non-diabetic subjects, which was more among women [122]. It has also been hypothesized that regular moderate alcohol consumption promotes insulin sensitivity of skeletal muscle, resulting in a protective effect for risk of T2DM [123].

Protection against cognitive loss

Low to moderate alcohol consumption in older women is associated with better cognitive performance than not drinking. Differences were seen across all the cognitive domains that were tested, including global cognition, speed of information processing, and verbal memory. Approximately 11,000 women were followed up with serial cognitive testing over 2 years. Those with low alcohol intake (up to 7 U per week) performed better than non-drinkers on tests of general cognitive function and verbal memory and had a slower rate of decline, although those with higher

alcohol intakes did not differ significantly in cognitive performance from non-drinkers [124]. Another study followed up a community sample of more than 1,200 older people (61% women) for 7 years and found low to moderate alcohol intake to be associated with attenuated decline in MMSE score and trail making but no effects on memory (delayed recall)[125]. If low to moderate alcohol protects against cognitive decline, it might be hoped that this would lead to a reduced risk of dementia. One study (833 subjects) has claimed benefit in terms of reduced risk of Alzheimer's disease with wine (up to 3 U per day) but a trend toward greater risk with spirits or beer [126]. There are a number of potential mechanisms by which low to moderate alcohol intake might protect against cognitive decline in older people. The alterations in lipids [127], lower fibrinogen levels [128] and inhibition of platelet aggregation [129] that occur with low to moderate alcohol intake might be expected to reduce the risk of ischemic cerebrovascular events. The hypothesized mechanism by which low to moderate alcohol intake might protect against cognitive decline in older people is many alcoholic beverages contain antioxidants that could theoretically protect against vascular and Alzheimer's pathology by reducing oxidative stress [130].

1.5 Experimental models of NAFLD

1.5.1 In vivo models of NAFLD

The clinical spectrum of NAFLD develops over years and results from an inter play of several risk factors including over nutrition and /or inappropriate dietary pattern, decreased energy expenditure, which are influenced by modern

sedentary life styles and genetic susceptibility , all leading to multiple molecular alterations in the humans [131]. Researchers have attempted to introduce a suitable rodent model (mice and rat) of NAFLD imitating at least the most important pathogenic and histological features of NAFLD. Apart from NAFLD model, animal models are designed for different stages of disease progression like NASH or even fibrosis and cirrhosis. Furthermore these models should also display metabolic abnormalities like overweight, insulin resistance, impaired glucose tolerance, dyslipidemia and altered adipocytokines profiles as well as the increased bacterial endotoxin levels frequently found in patients with NAFLD [131].

1.5.1.1 Dietary rodent models of NAFLD

High fat diet-induced NAFLD: Animals' feeding with HFD 30-60% fat is commonly used rodent model for human relevant NAFLD. However, the amount of fat, the composition of the HFD and the duration of the diet regime may cause different responses with respect to obesity, impaired glucose tolerance, dyslipidemia, increased lipogenesis (SREBP1c, LXR, and PPARγ), production of proinflammatory cytokines depending on the species, strain and gender [132, 133]. In HFD feeding rat model (71% energy from fat, 11% from carbohydrates and 18% from protein), the histopathology of these rats resembles to human NASH, increased insulin levels, insulin resistance, hepatic intracellular lipid accumulation, oxidative stress, collagen type I and α1 (I) procollagen mRNA upregulation, an increase in TNF levels and abnormal mitochondria was seen in these rats [134]. There are inter strain differences in susceptibility of rats to HFD. Male rats of three different strains (Wister, Lewis, and Sprague-Dawley) were fed with HFD (71% of kcal fat) for 3

weeks showed that all three stains develop steatosis affecting >66% of liver cells, but the histological patterns were different [135]. In all the strains microvesicular, mixied, and macrovesicular were found, but Sprague-Dawley rats exhibited highest degree of fibrosis, hepatocytes damage, and reduced blood flow velocity in central veins than rest. The effect of HFD seems to be more pronounced in rats than mice as the pathology better resembles that seen in human NASH [136]. Unlike various other animal models, animals fed an HFD mimic both the histopathology and pathogenesis of human NAFLD, as they have the hallmark features observed in human NAFLD patients, including obesity and IR. The degree of hepatic steatosis, however, seems to depend on various factors, including rodent strain.

Fructose rich diet-induced NAFLD: A shift in increased consumption of mono and disaccharides, primarily sucrose (50% fructose) and fructose (i.e., candy, soft drinks), is reported to be a risk factor for the development of NAFLD in humans. Ingestion of fructose promotes *de novo* lipogenesis, ATP depletion, the formation of reactive oxygen species and insulin resistance [20, 23]. In mice, the addition of 30% fructose to drinking water caused a fourfold increase in the levels of hepatic triglycerides and a marked increase in steatosis and weight over 8 weeks [137]. The C57BL/6 mice accessed to different, mono and disaccharides in drinking water revealed that fructose had the most damaging effect on the liver despite having the least impact on body weight gain [46]. With regards to NAFLD, no published data have shown that fructose alone can alter metabolic parameters, but biochemically it has been argued that fructose has potent metabolic attributes and can promote insulin resistance [138]. However, previous publications reported that

mice fed with high fat and high carbohydrate diet with fructose 55% in drinking water for 16 weeks develop NASH like phenotype with significant fibrosis as well as obesity [139]. In summary, HFD closely resembling not only the pathological and molecular alterations but also the dietary patterns found in humans with NAFLD.

Methionine and choline deficient (MCD) diet-induced NAFLD: The MCD diet, one of the most commonly used dietary models of NAFLD and NASH, this model produces more significant cell injury and cell death compared to other NAFLD models. The MCD diet contains high sucrose and fat (40% sucrose and 10% fat), this diet is lack of choline and methionine, which are essential for hepatic β-oxidation and very low density lipoprotein (VLDL) [140]. Choline is an essential substance that is involved in many metabolic reactions in rats (e.g., methylation or transport of lipids). Methionine can be used for the synthesis of choline, when the diet is lacking choline. Moreover, lack of methionine may be partially responsible for the decrease of glutathione synthesis. The absence of both choline and methionine substantially disturbs the formation of phosphatidylcholine, which is essential for the normal formation of VLDL and its secretion from the liver [141]. Rodents fed a MCD diet rapidly develop hepatic steatosis within 1 to 2 weeks due to enhanced uptake of fatty acids and decreased secretion of VLDL [142]. Despite the fact that with MCD diet rodents develop significantly faster and more pronounced liver damage, this dietary feeding regiment does not reflect several causative features of human NASH [143]. The metabolic profile of this model is opposite to that of typical human NASH, as plasma triglycerides and cholesterol levels are decreased,

which is opposite of that seen in NASH patients. These changes do not reflect the pathophysiology of NASH in humans.

1.5.1.2 Genetic rodent models of NAFLD

SREBP transgenic mice: Sterol regulatory element binding proteins (SREBPs) are important transcription factor that regulate hepatic cholesterol homeostasis. SREBPs activate the expression of more than 30 genes regulating the synthesis and uptake of cholesterol, fatty acids, triglycerides and phospholipids, as well as the NADPH cofactors required to synthesize these molecules [144, 145]. In liver three SREBPs (SREBP-1a, SREBP-1c and SREBP-2) regulate the production of lipids for export as lipoproteins and as bile. The mammalian genome encodes three SREBP isoforms, designated. Over expression of SREBP-1c model creates a model of congenital lipodystrophy in which severe insulin resistance and diabetes develop secondary to impaired adipose differentiation [146]. On 8^{th} day of age marked hepatic steatosis occurs in these mice , when fed with standard diet, results in dysregulation of the bio synthetic pathways which generate cholesterol and fatty acids resulting in hepatic steatosis, lobular inflammation and perivenular and pericellular fibrosis develop at 20 week [147]. However, due to decrease in white adipose tissue mass questionable whether this model can be used as a model for typical NAFLD/NASH because visceral fat characteristically increases in human NAFLD/NASH and exhibits low levels of circulating leptin, its applicability is restricted in a similar fashion as ob/ob mice [133, 148].

ob/ob mice: This model has been extensively studies and is used as a model for various metabolic diseases, among them NAFLD [149-151]. *ob/ob* mice carries a mutation in the leptin gene, which leads to leptin deficiency, subsequently leading to a hyperphagic, obese, inactive and show hyperglycaemia, insulin resistance, hyperinsulinemia and diabetic phenotype accompanied by the development of NAFLD [152, 153]. In *ob/ob* mice, fat is redistributed from adipose tissue to the liver and other non-adipose tissue. Fat accumulation in the liver induces hepatocyte lipotoxicity and lipoapoptosis. Early studies of *ob/ob* mice demonstrated that the hepatic steatosis enhances the vulnerability towards pro-injurious stimuli including endotoxin (lipopolysaccharide) or TNF[154, 155]. *ob/ob* mice develop spontaneous hepatic steatosis [156], however development of steatosis to steatohepatitis do not occur spontaneously, but require some kind of second hit such as dietary intervention such as methionine and choline diet (MCD) or HFD or challenge with small dose of endotoxin [157, 158]. Interestingly, ob/ob mice are protected against MCD diet induced fibrosis despite developing similar necro-inflammatory lesions as their genetic controls [159].

db/db mice: Contrary to what is seen in ob/ob mice, db/db mice carry a spontaneous mutation in the leptin receptor (ob-Rb) gene [160]. Although these mice have normal or elevated levels of leptin, due to the mutation confers resistance to the effects of leptin. These mice are hyperphagic and develop obesity, hyperglycemia, hyperinsulinemia, insulin resistance and develop macrovasicular hepatic steatosis [161, 162]. These mice develop to NASH when a “second hit" such as an MCD diet is added [160, 162]. Hyperleptinemia, due to loss of the leptin

receptor in db/db or fa/fa mice resembles the human condition more closely. when these mice are feed with MCD diet , significant liver fibrosis was observed compared to ob/ob mice, due to an important role of the short-form leptin receptor in hepatic fibrogenesis in NAFLD [163].

1.5.2 In vitro models of NAFLD

1.5.2.1 Monolayer cultures of primary hepatocytes

Primary hepatocyte cell lines are the most relevant *in vivo* like liver based *in vitro* model used extensively in basic research of liver functions, pathophysiology, pharmacological, disease and other related subjects [164]. Isolation of primary hepatocytes from a freshly removed liver tissue is done by two step collagenase perfusion technique which was introduced by Berry and Friend in 1969 [165] which was later modified by many and the most common method was described by [166]. Oleic and palmitic acid causes liver steatosis, combined action of oleic and palmitic acid causes more steatosis when compared individually [167, 168]. Interleukin-17, a pro inflammatory cytokine , exacerbated the accumulation of lipid droplets in hepatocytes [169]. Interestingly, an increased presence of triglycerides based lipid drop lets and VLDL secretion without change in apolipoprotein B has been absorbed in human hepatocytes treated with oleic or eicosapentaenic acid which makes these cell lines a suitable *in vitro* model for the study of liver steatosis [170]. The cons with primary hepatocytes are , they lose their liver specific functionality and morphology in culture, moreover their availability is limited, phenotypic instability and limited life spam [171]. CYP activity especially CYP1A2 and CYP3A4 was down regulated, when triglycerides accumulate which may affect

the metabolism of administered drugs [172]. Disturbances in gene expression mainly in those involved an antioxidant process, heat shock proteins, NO synthase, methionine adenosyltransferase [173, 174].

1.5.2.2 Monolayer culture of liver-based cells lines / Immortalized cell lines

Cell lines derived from hepatocarcinoma has some advantages and disadvantages over primary hepatocytes. The cell lines are being attractive due to their highly availability, easy handling and unlimited life span, whereas the draw backs are genetic instability and a phenotype that strongly differs from primary hepatocytes [175]. The mostly used human hepatocyte cell lines are HepG2, HUH7, H4IIE, PAV-1, and LX2 which are used to explore the molecular events in NAFLD. HepG2, which are originated from Human HCC, can retain several biochemical functions including the potential to secrete lipoproteins [176, 177] . Accordingly, using HepG2 cell lines is an appropriate model for studying the human lipid metabolism. Triglycerides and intra cellular accumulation of lipid droplets are exhibited when HepG2 cell lines are exposed to different concentration of oleic acid and palmitic acid [167, 178, 179]. However, these cell lines lack CYP activity, which is important for xenobiotic metabolism. Therefore characterized for drug metabolizing is potential for Hepg2 cell is essential before used in *in vitro* studies [180]. Thus a number sub-clones, such as HepG2/C3A, which express functional CYP activity and that display accumulation of TGs after exposure to oleic acid either alone or combined with plamatic acid [181]. The HepaRG cell lines which were derived from the female suffering from Hepatocellular carcinoma. These cells

quickly recover the bipotent progenitors and actively divide until they reach confluence, when seeded in low density. These cells express major liver specific functions, including CYP activity, are functionally stable at confluency and have an indefinite growth potential [171]. These cell lines have great potential in invite liver steatosis research, as they accumulate lipid droplets following exposure to stearic acid, palmitic acid, oleic acid linoleic acid, eicosapentaenoic acid, docosahexaenoic acid and amiodarone [182].

1.6 Rational and objectives

From literature, it has been confirmed that NAFLD is a spectrum, ranging from NAFL to NASH, advance fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). It is also associated with the traditional metabolic conditions: obesity, diabetes mellitus, and dyslipidaemia. Given the role of dyslipidaemia in the progression of NAFLD and the associated metabolic disorders therapeutics interventions to improve lipid metabolism are important in the management of NAFLD. As limited drug are available for protecting the liver from dyslipidaemia such as anti-oxidants, hepatoprotective agents and targeting insulin resistance and changes in lifestyle modifications and weight loss.

On the other hand, alcohol use on limited basis have beneficial effects on health. From the previous studies it was confirmed that light and moderate alcohol activates the molecules that control the lipid synthesis pathways in rodents and clinical studies [183, 184]. The chronic therapeutic intervention with low and moderate alcohol to alleviate dyslipidaemia by increasing the AMPK-α a “master switch” controlling various cellular processes of glucose and lipid homeostasis and

SIRT1 proteins which decreases the lipogenesis and increase the lipolysis pathways. A very few studies were reported on rodents feeding HFD with low and moderate alcohol regulating insulin resistance, there are no specific studies on HFD induced NAFLD treated with low and moderate alcohol doses. Hence, the specific objectives of the project were to:

- Investigate the *in vivo* effects of low and moderate alcohol on protecting the liver from HFD-induced NAFLD in animal model.
- Elucidate the possible mechanism(s) of action of low and moderate alcohol on HFD-induced NAFLD
- Investigate the *in vitro* effects of low and moderate alcohol on protecting the HepG2 cells from FFA-induced NAFLD in animal model.
- Elucidate the possible mechanism(s) of action of low and moderate alcohol on HFD-induced NAFLD
- Elucidate the role of AMPK, SIRT1 and LKB1 in protecting the HepG2 cells mechanism(s) of action of low and moderate alcohol on HFD-induced NAFLD

Thus, in this study, low and moderate alcohol was examined in high fat diet induced NAFLD in Sprague Dawley rats. The proposed research project was based on the hypothesis that low and moderate alcohol feeding may improve lipid metabolism and protect the liver from NAFLD.

CHAPTER-2

EFFECT OF LOW AND MODERATE ALCOHOLON HFD-INDUCED NAFLD: AN OBSERVATIONAL STUDY

2.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome and is defined as the accumulation of fat in the liver of patients who do not consume excessive alcohol [185]. It is estimated that about 24% of world population are affected with NAFLD [186]. The prevalence of NAFLD across geographical locals was assessed by utilizing ultrasonography and proton NMR spectroscopy [187], whereas studies based on elevated liver enzymes systematically underestimated the true prevalence [186]. Even though NAFLD is highly prevalent in all countries, but the highest rates are reported from South America (31%) and the Middle East (32%), followed by Asia (27%), the USA (24%) and Europe (23%), whereas NAFLD is less common in Africa (14%) [186]. The prevalence of NAFLD is constantly increasing (15% in 2005 to 25% in 2010) and similarly the rate of NASH in the same timeframe has almost doubled (59.1% versus 33%) [186]. NASH is now considered the second most common indication for liver transplantation in the USA after chronic hepatitis C, and is still growing [188].

The pathogenesis of NAFLD is described as a two-hit model. The first-hit consisting of hepatic lipid accumulation, due to sedentary lifestyle, high fat diet consumption, obesity, and insulin resistance [185]. The second-hit activates inflammatory cytokines, oxidative stress, mitochondrial dysfunction with associated fibrogenesis [189]. However, the insights on the role of free fatty acids (FFA) in promoting liver injury lead to modification of the theory. Hepatic fat accumulation was observed when there is an influx of FFA to the liver in obesity and insulin

resistance. These FFA either undergo β-oxidation or esterified with glycerol to form triglycerides, leading to hepatic fat accumulation. There is now substantial evidence that FFA can directly cause toxicity by activation of inflammatory pathways or by increasing oxidative stress [16]. This two-hit model has lost some favour as it was believed to be too simplistic to fully describe the intricacy of human NAFLD where a multitude of factors are acting in concert with one another in a genetically predisposed individual [189]. Evidence based on the results of various studies performed in recent years suggest apart from genetic predisposition, alterations of the intestinal barrier function, and over-nutrition, but also certain dietary patterns (e.g. a diet rich in fat and sugar or iron/animal-derived protein) may be responsible in the development of NAFLD [190].

For better understanding and investigation in the progression of NAFLD, three types of NAFLD animal models were developed such as genetic, nutritional, or a combination of genetic and nutritional factors. Nutritional factor-induced NAFLD models include methionine- and choline-deficient diet, high-fat diet (HFD), high-cholesterol and high-cholate diet, cafeteria diet, and high-fructose diet [191]. Apart from the nutritional factors, several lifestyle activities such as alcohol consumption, smoking and physical inactivity were implicated in the pathogenesis of metabolic disorders. Notably, heavy alcohol consumption (>50 g/day) was implicated in the pathogenesis of metabolic disorders leading to late stage complications, including atherosclerosis and fatty liver disease [192, 193]. Thus, alcoholic liver disease is one of the major causes of chronic liver diseases, the

threshold of alcoholic consumption causing an adverse effect on the liver and metabolic dysfunction is still controversial [194, 195].

In contrast, population-based studies suggest that moderate alcohol consumption (<20 g on 1–3 days/week) may even decrease the odds to develop NAFLD [196, 197]. These data are in line with the findings of others who reported that moderate drinkers (<20 g/day) had a lower risk of being diagnosed with non-alcoholic steatohepatitis (NASH) and also fibrosis than lifetime non-drinkers [198]. Results of human studies suggest that the beneficial effect of moderate alcohol intake may be associated with increased levels of adiponectin [199].

Several epidemiological studies have reported an association between regular low-to-moderate alcohol consumption and lower risk of mortality and morbidity from life-threatening diseases [200]. It was shown that low-to-moderate alcohol consumption is associated with lower risk of development of insulin resistance [201, 202] and type 2 diabetes [184, 203] and protection against the risk of major cardiovascular disease events such as myocardial infarction and coronary artery disease in healthy population [204, 205] and in patients with type 2 diabetes [206, 207], and hypertension [208, 209]. Other health benefits of regular light-to-moderate alcohol consumption include lower risk of dementia and cognitive impairment [124, 210] osteoporosis [211] and cancer [212].

However, the molecular mechanisms involved in the beneficial effects of moderate intake of alcoholic drinks have not yet been fully understood as the results obtained in animal experiments are inconsistent. Indeed, chronic alcohol consumption (≤21 g/kg body weight /day) has been observed to show protective

effect on NAFLD in *ob*/*ob* mice [213]. In contrast, results of other studies suggest that moderate to high consumption of plain alcohol may actually add to the progression of NAFLD [214, 215] and that this could even be dose dependent [216, 217]. Furthermore, it was argued that the protective effect found in human studies might not be resulted from the intake of alcohol itself but rather from other factors associated with the intake of alcohol (e.g. intake of resveratrol in red wine or hops ingredients in beer or even changes in lifestyle [218-221].

Starting from this background, the primary aim of the present study was to determine the effects of low, moderate and high alcohol (1 g/kg/day, 2 g/kg/day, 6 g/kg/day) on the development of HFD-induced NAFLD in Sprague-Dawley rats. It was hypothesized that low and moderate alcohol intake may protect the liver from developing NAFLD in HFD-fed rats.

2.2 Materials and Methods

2.2.1 Chemicals used

Alcohol (100%) was purchased from Chem-Supply (Gilman, SA, Australia) while the high fat diet (60% fat with 2% cholesterol) was supplied by Speciality Feeds (Glen Forrest WA, Australia). The diagnostic kits for triglycerides, total cholesterol, HDL cholesterol, glucose, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) used in this study were obtained from Pointe Scientific (Canton, MI, USA) whereas non-esterified free fatty acids kit was obtained from Wako Diagnostics (Osaka, Japan). Rat insulin EIA kit was purchased from IBL International (Hamburg, Germany).

2.2.1.1 10% neutral buffered formalin

Sodium dihydrogen phosphate, monohydrate ($NaH_2PO_4.H_2O$) (4 g) and disodium hydrogen phosphate, anhydrous (Na_2HPO_4) (6.5 g) were added to 100 mL of water, pH was adjusted to 7.0 and made up the final volume to 1 litre.

2.2.2 Animals and diets

Adult male Sprague-Dawley rats (198 g-235 g) obtained from the Animal Resource Centre (Canning Vale, WA, Australia) were used in the studies. The rats were housed in a well ventilated animal facility maintained at an ambient temperature of 24 2 C having 50-60% relative humidity with 12-h light/dark cycle and provided with standard pellet diet and water *ad libitum*. Both the standard diet (AIN93G) and high-fat diet (HFD) (SF12-017) were supplied from Speciality Feeds (Glen Forrest, WA, Australia). The standard diet contained (in weight percentage) approximately: 60% carbohydrate, 17.5% protein, 5% fat, 7% crude fibre, with a digestible energy of 16.1 MJ/kg and the 60% HFD contained (in weight percentage) approximately: 8.6 g sucrose, 20.0 g casein (acid), 10 g canola oil, 40 g cocoa butter, 10 g clarified butter, 5 g cellulose, plus 2% cholesterol with a digestible energy of 24.1 MJ/kg. All procedures involving animals are conducted by the terms of the Institutional Animal Ethics Committee of the Western Sydney University (Project code: A9387) following the NHMRC guidelines on the “Australian Code of Practice for the care and use of animals for scientific purposes.

2.2.3 Experimental design and treatments

The rats were weight-matched and divided into six groups, each consisting of five to six rats. The normal and the high-fat diet (HFD) control groups received the standard diet and the high-fat diet respectively and treated with vehicle (water) by oral gavage twice daily for 15 weeks. The low, moderate, and high alcohol groups received HFD along with respective alcohol doses (1 g/kg/day, 2 g/kg/day, 6 g/kg/day) twice daily around 9 am and 5 pm by oral gavage for a period of 15 weeks. The daily food intake from each cage was determined daily at around 9 am while the body weight of each animal was recorded daily at around 4 pm. At the end of the 15-week treatment schedule, animals were kept on overnight fasting for 12 hours, animals were euthanized with an intraperitoneal injection of ketamine (75 mg/kg), and xylazine (10 mg/kg). Blood was collected from the cardiac puncture, serum was extracted from the blood and kept at -20 ºC until analysis for various biochemical parameters. The livers were dissected out, weighed and snap frozen in liquid nitrogen and stored at -80 C for molecular analysis.

2.2.4 Biochemical estimations

2.2.4.1 Body weight and food intake

The daily food intake from each cage was determined daily at around 9 am while the body weight of each animal was recorded daily at around 4 pm up to 15 weeks.

2.2.4.2 Determination of serum triglycerides

Serum triglycerides were estimated using an enzymatic colorimetric assay based on GPO-DAOS method *[222]* following the manufacturer's instructions (Point

Scientific, USA) with absorbance measured at 550 nm using Ultrospec 2100 Pro UV/VIS spectrophotometer.

2.2.4.3 Determination of serum total cholesterol

Total cholesterol in serum was estimated using an enzymatic colorimetric assay based on cholesterol esterase and oxidase, in a single reagent to determine total cholesterol *[223]* following the manufacturer's instructions (Point Scientific, USA) with absorbance measured at 550 nm using Ultrospec 2100 Pro UV/VIS spectrophotometer.

2.2.4.4 Determination of serum HDL cholesterol

Serum HDL cholesterol was estimated using an enzymatic colorimetric assay based on the cholesterol oxidase method after removal of other lipoproteins with phosphor tungstate-magnesium *[224]*, following the manufacturer's instructions (Point Scientific, USA) with absorbance measured at 550 nm using Ultrospec 2100 Pro UV/VIS spectrophotometer.

2.2.4.5 Determination of serum LDL and VLDL cholesterols

Serum LDL and VLDL cholesterols were calculated using Friedwald's equation [225].

LDL = Total cholesterol- [HDL + (Triglycerides/5)]

VLDL = Triglycerides/5

2.2.4.6 Determination of serum non-esterified free fatty acids (NEFA)

Serum *NEFA* were estimated using an enzymatic colorimetric assay based on acyl-CoA synthetase, acyl-CoA oxidase (ACS-ACOD) *[226]* method following the manufacturer's instructions (Wako Diagnostics, Japan) with absorbance measured at 550 nm using Ultrospec 2100 Pro UV/VIS spectrophotometer.

2.2.4.7 Determination of serum glucose

Serum glucose was measured using an enzymatic colorimetric assay based on glucose oxidase-peroxidase (GOD-POD) *[227]* method following manufacturer's instructions with absorbance measured at 500 nm using Ultrospec 2100 Pro UV/VIS spectrophotometer.

2.2.4. 8 Determination of serum insulin

Serum insulin was measured using an enzyme-immuno assay (EIA) [228] method following the manufacturer's instructions with absorbance measured at an excitation of 414 nm Thermo Multiskan microplate reader.

2.2.4.9 Homeostatic model assessment of insulin resistance (HOMA-IR)

HOMA-IR, a measure of insulin resistance index *[229]* was calculated from the real-time fasting serum glucose and fasting insulin concentrations of different groups of rats using the mathematical HOMA-IR formula: HOMA-IR = (fasting serum insulin in U/ml X fasting serum glucose in mg/dL)/405 .

2.2.4.10 Determination of aspartate amino transferase (AST)

Serum AST was measured using an enzymatic colorimetric assay based on oxidation of L- aspartate to L-malate and NADH to NAD^+ in presence of (AST-MDH) *[230, 231]* method following manufacturer's instructions with absorbance measured at 340 nm using Shimadzu UV-1201 UV/VIS spectrophotometer.

2.2.4.11 Determination of alanine amino transferase (ALT)

Serum ALT was measured using an enzymatic colorimetric assay based on oxidation of L- aspartate to L-malate and NADH to NAD^+ in presence of (AST-MDH)
[232] method following manufacturer's instructions with absorbance measured at 340 nm using Shimadzu UV-1201 UV/VIS spectrophotometer.

2.2.4.12 Determination of hepatic lipids

Total lipids were extracted from the liver by the modified method of Hara and Radin et al (1978) [233]. Briefly, 75-100 mg aliquots of liver tissue was homogenized in 20 volumes of isopropanol, shaken in an orbital shaker for 45 minutes and centrifuged at 3000 *g* for 15 minutes and the supernatant was analysed for hepatic total cholesterol and triglycerides using commercial diagnostic kit.

2.2.5 Histological analysis of liver

2.2.5.1 Haematoxylin and eosin staining

The liver was fixed in 10% neutral-buffered formalin, dehydrated in increasing concentrations of alcohol, cleared with xylene and embedded in paraffin. Liver sections (3-5 µM) were prepared using the paraffin blocks in fully automated rotary microtome (Leica RM2255, Leica Biosystems, Germany) and stained with haematoxylin and eosin (H & E). Briefly, deparaffinised and re-hydrated sections by 2 changes of xylene for 10 minutes and 5 changes of absolute alcohol for 5 minutes respectively. Thereafter, sections were dipped in 95% alcohol for 2 minutes and 70% alcohol for 2 minutes and wash briefly in distilled water. After that, sections were stained with Harris Hematoxylin solution for 8 minutes and Wash in running tap water for 5 minutes. Afterwards, sections were dipped in 0.2% ammonia water followed by washed in running tap water for 5 minutes and rinsed in 95% alcohol (10 dips). Thereafter, sections were counterstained in eosin-phloxine solution for 1 minute. The sections were cleared in 2 changes of xylene and mounted in xylene based mounting medium*[234]*. The sections were examined for assessment of histopathological features such as inflammation cells, macro vesicular steatosis hepatic ballooning, using a binocular Olympus CX31 microscope

2.2.5.2 Oil Red O staining

Oil Red O staining was done by using frozen sections. Samples were sectioned at 5µm thickness and mount on the slides for air drying, slides were washed with propylene glycol for 5 minutes and stained with oil red O staining. The

slides were washed with running tap water for 3 minutes, mounted with glycerine *[235, 236]* were examined for assessment of lipid droplets using a binocular Olympus CX31 microscope.

2.2.6 Data and statistical analysis

All the results are expressed as means ± SEM. To examine the quantitative differences among the experimental groups, the respective data were subjected to analysis of variance (ANOVA) using GraphPad Prism-7.03 (GraphPad Software Inc., California, CA) statistical programme. Post hoc comparisons were made using Dunnett's multiple comparisons test. In all tests, $p<0.05$ value was used as the criterion for statistical significance.

2.3 RESULTS

2.3.1 Body weight & Food intake

The changes in the mean body weight of the different experimental groups of rats over the 15-weeks treatment period are shown in Figure 2.1. There was no significant difference in the initial body weight (from 198.1 ± 3.0 g to 236.1 ± 8.2 g; n=4-6) among the groups and continued up to week 5 (402.8 ± 2.5, 391.3 ±8.1, 399.5 ±11.3, 399.9 ± 6.5, 425 ± 22.3). During the 15-week feeding with HFD, the growth rate of the HFD-fed control group (582.4 ± 13.1 g) was not significantly different ($p=0.57$) to that of standard chow-fed rats (543.4 ± 9.5 g), as shown in Figure 2.1. From week 6, the low alcohol-treated group (n=5) exhibited a decrease ($p=0.36$) in body weight when compared to rest of the alcohol treated groups and this has continued up to end of week 15 (532.0 ± 26.5 g). At the end of 15 weeks there was no significant difference ($p=0.99$ to 0.32) in body weights were observed in moderate alcohol-treated group (n=6) (590.2 ± 19.8 g) and high alcohol-treated group (n=4) (635.125 ± 40.5 g) when compared to the HFD-fed control group. The average amount of food consumption in HFD-fed control group (34.1 ± 2.8 mg/24h/g BW) was significantly lower ($p<0.001$) than that in standard chow-fed normal group (62.6 ± 4.6 mg/24h/g BW) (Table 2.1). However, their calculated energy intakes were similar in terms of digestive energy units (Kcal). The feeding efficiency [body-weight gain (g)/ energy intake (kcal)] in these groups was compared to that of HFD control. The effects of low, moderate and high alcohol with HFD on liver weight and liver index are shown in Table 2.1. The HFD group (n=6) showed a significant difference ($p<0.01$) in liver weights and ($p<0.001$) with liver index when compared to normal control group (n=5). Whereas, the low alcohol-

treated (p=0.05), moderate alcohol-treated (p=0.99), and heavy alcohol-treated (p=0.96) groups did not exhibit any significant difference in liver weights when compared to HFD control group. No significant difference in liver index was observed in low alcohol-treated (p=0.08), moderate alcohol-treated (p=0.52), and heavy alcohol-treated (p=0.10) groups compared to the HFD-fed control group.

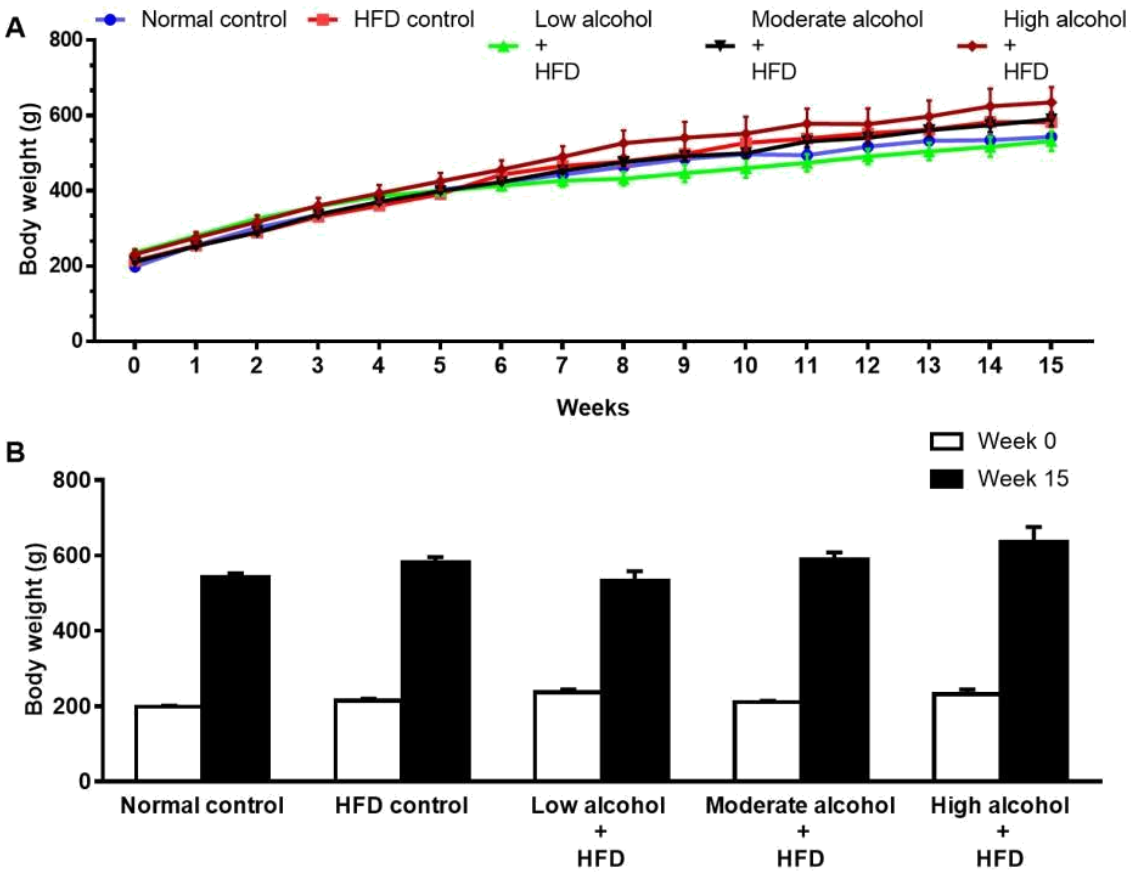

Figure 2.1 Effect of low, moderate and high alcohol on body weight change in HFD-fed rats for 15 weeks.

(A) Daily recordings of the mean body weight changes of the experimental groups of rats and (B) comparison of the mean bodyweights of rats prior to (open columns) and after treatment (closed columns) either with the HFD alone or with alcohol. Each bar represents the mean ± SEM of 4-6 rats.

Table 2.1 Effect of low, moderate and high alcohol on food intake and feeding efficiency in rats fed with HFD for 15 weeks

Parameters	Normal control	HFD control	Low alcohol + HFD	Moderate alcohol + HFD	High alcohol + HFD
Initial body weight	198.1 ± 3.0	214.4 ± 5.3 ^{ns}	236.1 ± 8.2 ^{ns}	211.0 ± 3.0 ^{ns}	231.375 ± 13.1 ^{ns}
Final body weight	543.4 ± 9.5	582.4± 13.1 ^{ns}	532.0 ± 26.5 ^{ns}	590.2 ± 18.0 ^{ns}	635.1 ± 40.5 ^{ns}
Weight gain(g)	344.8 ± 5.5	370.5 ± 9.2 ^{ns}	282.5 ± 24.0 ^{ns}	363.1± 3.2 ^{ns}	388.5 ± 39.2 ^{ns}
Food consumption (mg/24h/g bw)	62.6 ± 4.6	34.1 ± 2.8$^{###}$	32.6 ± 2.0 ^{ns}	28.2 ± 1.9 ^{ns}	38.2 ± 1.9 ^{ns}
Total energy intake (Kcal/g/day)	194.33 ± 14.42	219.96 ± 18.22 ^{ns}	210.14 ± 12.86 ^{ns}	199.60 ± 13.43 ^{ns}	196.57 ± 11.26 ^{ns}
Feeding efficiency (g/Kcal)	2.52 ± 0.31	2.62 ± 0.34 ^{ns}	2.62 ± 0.34 ^{ns}	2.46 ± 0.28 ^{ns}	2.46 ± 0.24 ^{ns}
Liver weight	19.5 ± 0.34	26.7 ± 1.2$^{##}$	21.90 ± 1.70$^{(0.05)}$	26.3 ± 1.7 ^{ns}	26.3 ± 2.9 ^{ns}
Liver Index	3.41 ± 0.08	4.76 ± 0.14$^{###}$	4.14 ± 0.27$^{(0.08)}$	4.45 ± 0.20 ^{ns}	4.07 ± 0.17 ^{ns}

Values represent the mean ± SEM of 4-6 rats

Significant difference from normal control: $^{##}p<0.01$, $^{###}p<0.0001$

No significant difference from HFD control: ns ($p>0.05$)

2.3.2 Serum triglycerides

The levels of serum triglycerides in different groups are shown in Table 2.2. The HFD-fed control group (n=6) showed a significant ($p<0.05$) 1.6-fold increase in serum triglycerides when compared to the normal control group (n=5) at the end of the treatment. The moderate alcohol-treated group (n=6) showed a non-significant ($p=0.82$) decrease in serum triglycerides when compared to the HFD control group. In contrast, the low alcohol-treated group HFD (n=5) and high alcohol-treated group (n=4) showed non-significant increase in triglycerides levels by 1.1-fold ($p=0.70$) and 1.2-fold ($p=0.35$) respectively when compared to the HFD-treated control group.

2.3.3 Serum total cholesterol

Table 2.2 shows the total serum cholesterol levels among the experimental groups. The rats fed with HFD (n=6) showed significant ($p<0.001$) 2.3-fold increase in total cholesterol level when compared to the normal control group (n=5) at the end 15-week treatment. A 1.2-fold decrease in total cholesterol was noticed in moderate alcohol-treated group (n=6) but failed to achieve significance ($p=0.10$) when compared to the HFD-fed control group. On the other hand, the low alcohol-treated (n=5) and high alcohol-treated (n=4) groups did not show significant change in serum cholesterol ($p=0.96$ to 0.97) when compared to the HFD-fed control group.

2.3.4 Serum HDL cholesterol

The levels of HDL cholesterol observed in different groups are shown in Table 2.2. The serum HDL concentrations of HFD-fed control group (n=6) were

significantly ($p<0.001$) decreased by 3.3-fold when compared to the normal control group (n=5). The low alcohol (n=5) and moderate alcohol (n=6) treated groups showed a mild but significant ($p<0.001$) increase in serum HDL levels by 1.4 and 1.5-fold respectively when compared to the HFD-treated control group. On the other hand, no significant difference ($p=0.99$) in serum HDL levels was observed between high alcohol-treated group and the HFD-treated control groups.

2.3.5 Serum LDL cholesterol

The LDL cholesterol levels were calculated by using Friedewald's equation. The LDL levels of various groups are shown in Table 2.2. Rats fed with HFD (n=6) showed a significant ($p<0.001$) 4.6-fold increase of serum LDL levels when compared to the normal control rats. Moderate alcohol-treated rats (n=6) showed a non-significant ($p=0.28$) 1.3-fold decrease in serum LDL when compared to the HFD-fed rats. In contrast, no significant change in serum LDL was observed with low alcohol-treated (n=5; $p=0.65$) and high alcohol-treated (n=4; $p=0.99$) rats compared to the HFD-fed control rats.

2.3.6 Serum VLDL cholesterol

The VLDL cholesterol levels are shown in Table 2.2. The HFD-fed control group (n=6) showed a significant ($p<0.05$) 1.7-fold increase in serum VLDL when compared to the normal control group (n=5) at the end of the treatment. Moderate alcohol-treated group (n=6) showed a non-significant ($p=0.82$) decrease in serum VLDL when compared to the HFD control group. On the other hand, low alcohol-treated (n=5) and high alcohol-treated (n=4) groups showed a non-significant 1.1-

fold (p=0.70) and 1.2-fold (p=0.35) increase in VLDL respectively when compared to the HFD-fed control at the end of the treatment.

2.3.7 Serum non-esterified fatty acids (NEFA)

Serum NEFA levels are shown in Table 2.2. The HFD-fed control group (n=6) showed a significant ($p<0.001$) 1.4-fold increase in serum NEFA levels when compared to the normal control rats (n=5). While the moderate alcohol-treated group (n=6) showed a significant ($p<0.01$) 1.2-fold decrease in serum NAFA, the low alcohol-treated group did not show any significant change (p=0.99) in serum NEFA when compared to the HFD-fed control group. In contrast, the high alcohol-treated rats (n=4) showed a significant ($p<0.01$) 1.2-fold increase in serum NEFA, when compared to the HFD-fed control group.

Table 2.2 Effects of low, moderate, high alcohol on serum lipid parameters in rats fed with HFD for 15 weeks

Parameter	Normal control	HFD control	Low alcohol + HFD	Moderate alcohol + HFD	High alcohol + HFD
Triglycerides (mg/dL)	48.32 ± 5.39	80.17 ± 5.96$^{\#}$	91.24 ± 13.90^{ns}	71.53 ± 4.55 ^{ns}	98.36 ± 7.40 ^{ns}
Total cholesterol (mg/dL)	147.56 ± 5.89	338.62 ± 24.63$^{\#\#\#}$	352.92 ± 14.84 ^{ns}	276.02 ± 19.73 ^{ns}	325.30 ± 33.74 ^{ns}
HDL (mg/dL)	73.13 ± 2.03	22.11 ± 1.75$^{\#\#\#}$	30.61 ± 0.34***	35.05 ± 0.53***	21.89 ± 0.60 ^{ns}
LDL (mg/dL)	64.77 ± 6.29	300.47± 23.82$^{\#\#\#}$	304.07 ± 14.05 ^{ns}	226.66 ± 20.55 ^{ns}	283.74 ± 33.65
VLDL (mg/dL)	9.66 ± 1.08	16.03 ± 1.19$^{\#}$	18.25 ± 2.78 ^{ns}	14.31 ± 0.91^{ns}	19.67 ± 1.48 ^{ns}
NEFA (µEq/L)	824.7 ± 12.1	1154.54 ± 16.75$^{\#\#\#}$	1151.27 ± 57.79 ^{ns}	986.71 ± 12.61**	1385.28 ± 82.21**

Values represent the mean ± SEM of 4-6 rats.

Significant difference from normal control: $^{\#}p<0.05$, $^{\#\#\#}p<0.001$

Significant difference from HFD control group: **p<0.01, ***p<0.001,

No significant difference from HFD control: ns (p>0.05)

HFD: High-fat diet, HDL: High density lipoprotein, LDL: Low density lipoprotein,

VLDL: Very low-density lipoprotein, NEFA: Non-esterified fatty acids

2.3.8 Serum glucose

The serum glucose levels among the experimental groups are shown in Table 2.3. The HFD-fed control group (n=6) showed a significant ($p<0.001$) 3.2-fold increase in fasting blood glucose compared with the normal control group (n=5). The moderate alcohol-treated group (n=6) showed a mild but significant ($p<0.001$) decrease in serum glucose by 1.8-fold when compared to the HFD-fed control group. On the other hand, the low alcohol-treated rats (n=5) did not show any significant ($p=0.93$) change in serum glucose when compared to the HDF-fed control group. Furthermore, the high alcohol-treated group (n=4) showed a mild non-significant ($p=0.86$) increase in serum glucose when compared to the HFD-fed control group.

2.3.9 Serum insulin

Serum insulin levels of HFD-fed group (n=6) were found to be significantly ($p<0.001$) increased by 2.9-fold when compared to the normal control group (n=5). On the other hand, the low alcohol-treated (n=5) and moderate alcohol-treated (n=6) groups showed a mild non-significant ($p=0.14$ to 0.49) decrease in serum insulin levels when compared to the HFD-fed control group. Furthermore, high alcohol-treated group (n=4) exhibited a mild non-significant ($p=0.94$) decrease in serum insulin levels when compared to HFD-fed control group.

2.3.10 Homeostatic model assessment of insulin resistance (HOMA-IR)

The development of insulin resistance as assessed by the mathematical HOMA-IR model in different groups was shown in Table 2.3. The HFD-fed control

group (n=6) developed a significant ($p<0.001$) 9.4-fold increase in insulin resistance when compared to the normal control rats (n=5). The moderate alcohol-treated group (n=6) showed a significant ($p<0.001$) 1.9-fold increase in serum insulin resistance compared to the HFD-fed control group. On the other hand, low alcohol-treated group (n=5) showed a non-significant ($p=0.11$) decrease in insulin resistance when compared to the HFD-fed control group. Whereas, high alcohol-treated with HFD (n=4) showed a mild non-significant ($p=0.99$) increase insulin resistance compared to the HFD-fed control group.

2.3.11 Serum aspartate aminotransferase (AST)

The serum AST levels among the experimental groups are shown in Table 2.3. The HFD-fed control group (n=6) showed a significant ($p<0.001$) 1.9-fold increase in serum AST levels when compared to the normal control rats (n=5). The moderate alcohol-treated group showed a mild but significant ($p<0.05$) 1.3-fold decrease in serum AST levels when compared to the HFD-fed control group. Similarly, the low alcohol-treated group (n=5) show a non-significant ($p=0.98$) decrease in serum AST when compared with HFD-fed control group. In contrast, the high alcohol-treated group (n=4) showed a significant ($p<0.001$) 1.5-fold-increase in serum AST levels when compared to the HFD-fed control group.

2.3.12 Serum alanine aminotransferase (ALT)

The serum ALT levels among the experimental groups are shown in Table 2.3. A significant ($p<0.001$) 2.7-fold increase of serum ALT was observed in HFD-fed group (n=6) rats when compared to the normal control group (n=5). However,

the moderate alcohol-treated rats (n=6) showed a significant ($p<0.05$) 1.5-fold decrease in serum ALT levels when compared to the HFD-fed control group. Similarly, low alcohol-treated group (n=5) showed a non-significant ($p=0.07$) 1.3-fold decrease in serum ALT levels when compared to the HFD-fed control group. On the other hand, the high alcohol-treated group (n=4) showed a non-significant ($p=0.95$) increase in serum ALT levels when compared to the HFD-fed control group.

Table 2.3 Effects of low, moderate, and high alcohol on serum glucose, serum insulin, insulin index, and serum alanine transaminase (ALT) and aspartate transaminase (AST) in rats fed with HFD for 15 weeks

Parameter	Normal control	HFD control	Low alcohol + HFD	Moderate alcohol + HFD	High alcohol + HFD
Serum glucose (mg/dL)	115.18 ± 3.52	372.90 ± 11.70$^{###}$	359.64 ± 18.15 ^{ns}	211.22 ± 10.90***	390.43 ±1 2.64 ^{ns}
Serum insulin (ng/mL)	1.49 ± 0.13	4.29 ± 0.13$^{###}$	3.63 ± 0.21 ^{ns}	3.93 ± 0.24 ^{ns}	4.11 ± 0.37 ^{ns}
Insulin index	0.42 ± 0.03	3.96 ± 0.20$^{###}$	3.20 ± 0.28 ^{ns}	2.05 ± 0.17***	3.98 ± 0.44 ^{ns}
AST (IU/L)	33.95 ± 2.05	63.21 ± 2.26$^{###}$	61.26 ± 6.69 ^{ns}	49.06 ± 3.18*	91.94 ± 2.07***
ALT (IU/L)	24.40 ± 3.33	73.37 ± 5.30$^{###}$	55.16 ± 3.58 ^{ns}	47.96 ± 2.76**	77.35 ± 11.24 ^{ns}

Values represent the mean ± SEM of 4 to 6 rats

Significant difference from Normal control: $^{###}$p<0.001,

Significant difference from HFD control group: *p<0.05, **p<0.01 ***p<0.001,

No significant difference from HFD control: ns (p>0.05)

HFD: High fat diet, Alanine transaminase (ALT) & Aspartate transaminase (AST)

2.3.13 Hepatic lipid levels

The levels of hepatic triglycerides in different groups are shown in Table 2.4. The HFD-fed control group (n=6) showed a significant ($p<0.001$) 4.8-fold increase in hepatic triglycerides at the end of 15 weeks treatment when compared with the normal control (n=5) fed with standard diet alone. On the other hand, low alcohol-treated (n=5) and moderate alcohol-treated (n=6) groups showed a non-significant ($p=0.30$ to 0.22) decrease in hepatic triglycerides by 1.2 to 1.2-fold when compared to the HFD-fed control group. In contrast, the high alcohol-treated group (n=4) showed a non-significant ($p=0.71$) 1.1-fold increase in hepatic triglycerides when compared to the HFD-fed control.

Table 2.4 shows the hepatic total cholesterol levels among the experimental groups The hepatic total cholesterol levels of HFD-fed group (n=6) were significantly ($p<0.001$) increased by 9.3-fold when compared with the normal control group (n=5). The moderate alcohol-treated group (n=6) showed a significant ($p<0.05$) 1.3-fold decrease in hepatic total cholesterol when compared to the HFD-fed control group. However, the low alcohol-treated (n=5) and high alcohol-treated (n=4) groups showed a non-significant ($p=0.61$ to 0.85) increase in hepatic total cholesterol levels when compared to the HFD-fed control rats.

Table 2.4 Effect of low, moderate, and high alcohol on hepatic triglycerides and total cholesterol in rats fed with HFD for 15 weeks

Parameter	Normal control	HFD control	Low alcohol + HFD	Moderate alcohol + HFD	High alcohol + HFD
Hepatic triglycerides (mg/g liver)	30.54 ± 2.29	148.07 ± 14.69$^{###}$	119.83 ± 16.00 ^{ns}	118.25 ± 12.54 ^{ns}	166.13± 7.71 ^{ns}
Hepatic total cholesterol (mg/g liver)	10.73 ± 0.90	100 ± 7.14$^{###}$	112.78 ± 12.62 ^{ns}	71.95 ± 5.35*	109.39 ± 8.83 ^{ns}

Values represent the mean ± SEM of 4 to 6 rats

Significant difference from Normal control: $^{###}$p<0.001
Significant difference from HFD control group: *p<0.05

No significant difference from HFD control: ns (p>0.05)

HFD: High fat diet

2.3.14 Histological analysis of liver

The H&E staining of HFD-fed rat liver showed increased fat deposits as evidenced by distended intracellular fat in comparison to the liver of rats fed with standard diet was shown in Figure 2.2 A & B. The images of H&E stained HFD-fed liver also displayed macro-vesicular steatosis, as many single large droplets displaced the nucleus and ballooning degeneration causing conspicuous swelling of the cell and cytoplasmic vacuolation as shown in Figure 2.2 B. The low and moderate alcohol-treatment groups showed marked reduction of liver fat deposits (Figure 2.2 C & D) whereas the high alcohol-treated group (Figure 2.2 E) showed a more pronounced steatosis and ballooning degeneration compared to HFD-fed control rat liver.

The Oil red O staining of HFD-fed rat liver showed increased accumulation lipid droplets in liver sections, comparison to the liver of rats fed with standard diet was shown in Figure 2.2 F & G. The histology of low alcohol-treated and moderate alcohol- treated group's showed a marked reduction of lipid droplet accumulation when compared to the HFD control group (Figure 2.2 H & I). In contrast, the high alcohol-treated liver (Figure 2.2 j) showed a more pronounced lipid droplets compared to the HFD-fed control group.

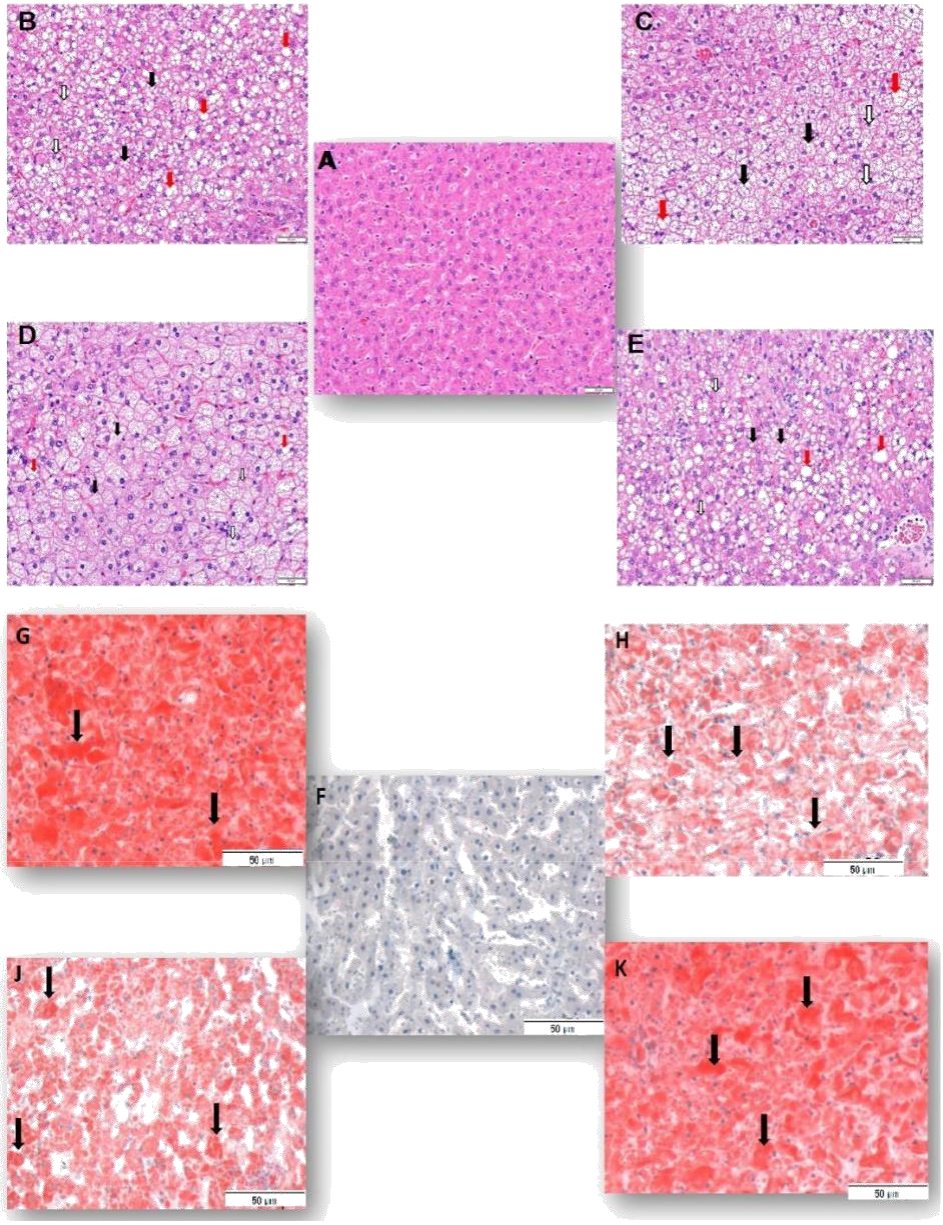

Figure 2.2 Effect of low, moderate, and high alcohol on histological changes produced by HFD-induced NAFLD in rats Representative photomicrographs of H& E staining of the liver of experimental groups at 20x. A: Normal control, B: HFD conrol, C: Low alcohol with HFD, D: Moderate alcohol with HFD, E: High alcohol with HFD. The colour arrows represent histological changes in the liver. Red arrow: Macrovesicular steatosis, Black arrow: Microvesicular steatosis, Open arrow: cytoplasmic vacuolation. The Oil red O staining of the experimental groups at 20x were shown in F: Normal control, G: HFD control, H: Low alcohol with HFD, I: Moderate alcohol with HFD, J: High alcohol with HFD. The black arrows in Oil red O staining represent the lipid droplets.

2.4 Discussion

In the present study, we examined the protective effects of low and moderate alcohol in HFD-fed rat model of NAFLD which is commonly associated with metabolic co-morbidities like obesity, metabolic syndrome and type 2 diabetes [237]. Since rats possess a similar metabolic pattern as that of human beings, it is rational to use this disease model to examine the prophylactic effects of chronic treatment of low and moderate alcohol. Metabolic syndrome is a complex polygenic disorder including obesity, impaired fasting glucose and /or impaired glucose tolerance, reduced insulin sensitivity, hyperinsulinemia, dyslipidaemia, and hypertension has gained importance because of its association with the subsequent development of type 2 diabetes and its complications [238]. Fats become the major source of energy, during a low supply of carbohydrates or when their breakdown is incomplete [239]. As a result, fatty acids are mobilized into the general circulation leading to secondary triglyceridemia in which the total serum lipids, including triglycerides, and cholesterol increase, leading to life-threatening lipid disorder [240]. The development of metabolic syndrome is influenced by a combination of genetic and environmental factors. Among the environmental factors, long-term intake of high-fat is most intensively studied because of its contribution to the development of metabolic syndrome [241]. Apparently, high fat diet/western diet characterised by high-calorie and low-fibre content is believed to be associated with the current day lifestyle in most societies of the world. Long-term intake of HFD leads to abnormal blood lipid profiles which is highly prevalence in metabolic disorders [242]. In addition to hepatic steatosis, HFD-feeding in rats induces insulin resistance, deranged lipid metabolism and other metabolic

syndrome components with pathophysiology that is more similar to that observed in human NAFLD than that generated by other animal models of NAFLD [140, 243].

The main mechanisms involved in diet-induced models in the pathogenesis of NAFLD, include increased *de novo* lipogenesis, increased dietary FFA levels, increased adipose tissue, impaired β-oxidation and impaired VLDL synthesis. These all lead to hepatic triglycerides accumulation and ultimately NAFLD [244]. Feeding rats with HFD, a rodent model of NAFLD, which show the characteristics of metabolic disorders such as insulin resistance, hyperlipidaemia and obesity were widely used due to its similar pathogenesis in humans [133].

Several epidemiological studies suggest that moderate alcohol may possess beneficial effects on the development and the progression of liver damage in patients with NAFLD. Daily consumption of less than 20 g of alcohol lowers the odds of NASH as well as fibrosis in a large biopsy-proven NAFLD population [196]. However, from the results of these epidemiological studies, it cannot be ruled out that compounds are taken in along with alcoholic's beverage (e.g. compounds derived from hops or grapes or lifestyle modification associated with moderate alcohol consumption rather than the alcohol itself. Indeed, it was shown that chronic intake of high amounts of beer and wine in comparison with spirits may have less harmful effects on the liver [218, 245]. Results of animal studies assessing the effect of plain alcohol on the liver in settings of NAFLD are inconsistent as doses and models used varied considerably between studies [213, 214, 217]. We studied that HFD (60% fat with 2% cholesterol) fed rats did not show a significant increase in body weights when compared to control group but

increased liver weights, liver index and metabolic changes were observed. This could be explained by the fact that the caloric ingestion was equivalent in the two groups. Similar results were previously reported by other groups [246-250]. Even though the HFD-fed model used in our study did not show any effect on increase in body weight, but it managed to alter the metabolic changes in rats.

Dyslipidaemia is the most important modifiable risk factor contributing to the development of atherosclerosis in type 2 diabetes [251]. Thus, the importance of blood levels of triglycerides, free fatty acids, cholesterol in the pathogenesis of lipid disorders were extensively reviewed [252]. Hypertriglyceridemia is one of the most common quantitative lipid abnormalities in NAFLD patients [253]. The excess supply of FFAs, directly via intake and via increased lipolysis, brings about triglycerides accumulation in the liver [244]. In present study, HFD-fed control rats exhibited increased concentrations of triglyceride (TG) and TG rich lipoprotein VLDL (very low-density lipoprotein). The *de novo* lipogenesis plays a substantial role in the pathogenesis of NAFLD accounting for 26% hepatic triglycerides in human subjects [24]. Nammi et al [183] demonstrated that light and moderate alcohol on normal rats did not show any significant changes in serum and liver triglycerides. However, in our present study low alcohol-treated group did not show any trend in controlling the serum and liver triglycerides, but the moderate alcohol-treated group managed to reduce the serum and liver triglycerides levels when compared to HFD-fed control group. Furthermore, similar results to our data were reported by others stating that low and moderate alcohol did not show any effect on serum triglycerides [254, 255]..

VLDL receptor belongs to the low-density lipoprotein (LDL) receptor family and is widely expressed in the brain, heart, skeletal muscle, and adipose tissue, whereas its expression is very low in the liver under normal conditions [256, 257]. In normal conditions, insulin downregulates VLDL secretion. Over production of large TG-rich VLDL in hypertriglyceridemia condition is observed in NAFLD [252, 258]. NAFLD was found to be associated with the overproduction of large VLDL in humans [259-261]. In addition, Adiels et al (2006) [259] had demonstrated that HFD-fed rats appear to show the hepatic steatosis and increased secretion of VLDL associated with NAFLD and insulin resistance. HDF fed group showed an increase in serum VLDL levels similar to that of previous results [258, 262], whereas low and moderate alcohol-treated groups did not show any significant trend in regulating VLDL levels in treatment groups.

Accumulation of neutral lipids is one of the hallmarks of NAFLD, which results from an imbalance between lipid availability and lipid removal. Recent data suggest that disturbed hepatic cholesterol homeostasis and liver free cholesterol accumulation are relevant to the pathogenesis of NAFLD/NASH [263]. Cholesterol homeostasis is extensively dysregulated at different levels in NAFLD and thus promotes free cholesterol accumulation. Multi-level changes occur in the liver, including the activation of cholesterol biosynthetic pathways, increased cholesterol de-esterification, and attenuated pathways in cholesterol export and bile acids synthesis. These changes may contribute to free cholesterol accumulation in the setting of NAFLD [263]. Excess free cholesterol in the liver leads to ER stress triggers the release of transcription factors, such as sterol regulatory element

binding protein-1c (SREBP-1c) and SREBP-2 in insulin-resistance, playing a relevant role in the synthesis of fatty acids and cholesterol [264, 265]. Several animal studies showed an increased total cholesterol levels in rats fed with HFD [188, 266]. Our observations from light to moderate alcohol with HFD did not show any significant change in the serum cholesterol, whereas moderate alcohol with HFD managed a significant reduction of total cholesterol in liver tissue.

In our study, a marked increase in LDL cholesterol of serum was found in HFD-fed rats. The increase in LDL-cholesterol may be due to reduced expression or activity of LDL- receptor sites in response to HFD feeding [267]. Therefore, lowering the LDL-cholesterol level may be important in lowering the serum total cholesterol level in rats fed with HFD. Fan et al (2014) demonstrated that low and moderate alcohol increased LDL cholesterol levels in wild-type and as well in as ALDH2 Knock out mice [268]. Likewise, low and moderate alcohol with HFD did not show any significant trend in reducing the serum LDL levels when compared to the HFD group.

Decreased serum HDL-cholesterol is one of the typical characteristics in NAFLD [269]. In the present study, HFD-fed control rats demonstrated decreased concentrations of HDL-cholesterol compared to the normal control rats. However, chronic low and moderate alcohol with HFD feeding markedly elevated the serum HDL levels. Apo-I and Apo-II are the primary protein constituents of HDL-cholesterol. Serum concentration of Apo-I apolipoprotein is significantly reduced in animals with metabolic syndrome [217, 270]. Impaired Lecithin–cholesterol acyltransferase LCAT activity might be responsible for altered cholesterol

homeostasis of acute-phase HDL compared with native HDL. The suggested alcohol protective mechanism takes into consideration its influence on lipoprotein synthesis, mainly through the increase of the HDL-cholesterol levels and a rise in the hepatic Apo A1 production [127]. One additional mechanism that may explain some of the beneficial effects of lower doses of alcohol could be its effects on the liver. The liver is the organ primarily responsible for detoxifying a wide variety of metabolic and environmental toxins so consumption of low doses of alcohol could enhance its ability to remove toxic compounds from the body by potentiation of key liver enzyme systems such as cytochrome P450 [271].

The first event in the development of NAFLD is the accumulation of circulating FFA in the liver. Fatty acids enter into liver cells by simple diffusion and accumulate in high amounts (macro-vesicular steatosis) [272]. Whenever large amount of fatty acids is present in the liver environment, liver cells increase lipid degradation pathways (lipolysis) but also suppress other pathways including insulin receptor activation [273]. Insulin resistance caused by hepatic *de novo* lipogenesis leads to fat accumulation in the liver from different sources including excessive intake of dietary free fatty acids, increased influx of FFA [274, 275]. In NAFLD patients, about 26% of FFAs are accumulated from *de novo* lipogenesis [24]. An earlier study demonstrated that HFD increases the serum FFA in rodents [185, 242, 276]. Likewise, the HFD-fed control rats showed an increase in serum FFA when compared to the normal control group. The hypothetical mechanism behind the salutary effect of moderate alcohol on controlling the serum FFA is alcohol converts to acetate after two successive dehydrogenase activities. The liver releases acetate

into the plasma [277], from where it associates with adipocyte-GPCR43 [278], which initiates a series of signal transduction steps that inhibit lipolysis [279]. With a decrease in lipolysis, there is a subsequent reduction in fatty acid release into the plasma. Thus, the decrease in serum FFA in moderate alcohol-treated group could be due to the acetate, the end product of alcohol metabolism.

Insulin resistance is one of the key components of metabolic syndrome which leads to the overt onset of type 2 diabetes. High content of lipid in the diet is believed to contribute greatly to the occurrence of insulin resistance [280]. Aberration in *de novo* lipogenesis may lead to NAFLD and this is also marked by increased rate of gluconeogenesis, impaired insulin response to suppress gluconeogenesis. Chronic hyperinsulinemia promotes hepatic *de novo* lipogenesis through up-regulation of lipogenic transcription factors [281, 282]. In the present study, HFD-fed rats showed a significant increase in serum glucose and insulin concentrations when compared to the normal control group. Whereas moderate alcohol with HFD treated group showed a significant decrease in serum glucose and HOMA-IR. Contrary to serum glucose levels, low and moderate alcohol with HFD showed a non-significant trend in decreasing but managed to decrease the serum concentration. Insulin resistance is most commonly evaluated by the homeostatic model assessment of insulin resistance (HOMA-IR) which takes consideration of both fasting glucose and insulin concentration. In the present study, the calculated HOMA-IR values of HFD group (3.96 ± 0.20) is significantly higher than the propose cut-off point (=2.29), indicating severe insulin resistance [283]. Fromenty et al (2009) [213] demonstrated that moderate alcohol reduced the

serum glucose and insulin levels in ob/ob mice over a period of six months. Moreover, a large body of compelling evidence from various experimental animal and epidemiological studies had shown significant decrease in blood glucose and insulin resistance [284-286]. The suggested biological mechanisms underlying the association between alcohol consumption and alteration in insulin resistance and hepatic glucose metabolism is by increasing the plasma adiponectin levels, which is an attractive hypothesis because this adipokine is known to improve insulin sensitivity and fatty acid oxidation in different tissues muscle and liver), at least in part through an AMPK-dependent pathway [287, 288].

HFD-induced obesity and abnormal lipid metabolism are associated with NAFLD, causing an hepatocellular injury, increasing the serum concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) which are the major markers for hepatocellular injury [289]. The increased liver enzyme levels and the formation of hepatic steatosis in the HDF-fed group are associated with a significant increase in liver weight [290]. The HFD-fed rats exhibited increased total liver weight. Liver injury was also confirmed by significant increases of serum AST and ALT in the HFD-fed group. An elevated serum activity of ALT was suggested as the fatty liver disease in the general human population [291]. Furthermore, patients with high ALT among those with the metabolic syndrome may leads to systemic inflammation that may, in turn increase the risk of atherosclerosis, leading to coronary artery or cerebrovascular disease [292]. Whereas serum AST levels are characteristically higher than ALT levels which contrasts with the pattern usually seen in NAFLD [293-295]. Among the low, moderate and high alcohol-treated

groups, moderate alcohol with HFD effectively reduce the serum AST and ALT levels in concordance with earlier report [255]. Furthermore, Duly et al.(2015) demonstrated in his study that moderate alcohol reduces serum AST and ALT levels when compared to HFD diet and chow diet groups [296].

Liver tissue for histopathology was removed from each rat, and the same part of each liver was dissected, fixed in 10 % formalin, and then embedded in paraffin wax for staining with haematoxylin and eosin (H&E). In the present study, H&E and Oil red O staining on the liver samples revealed distended hepatocytes with increased lipid droplets, macro- and micro-vesicular steatosis, indicating simple steatohepatitis [297]. The histological abnormalities in the HFD-fed rats of this study were consistent with the findings of previous studies [298, 299]. Previous studies demonstrated that chronic alcohol administration substantially aggravated the development of fatty liver in in ob/ob mice with standard diet and ob/ob mice with HFD [213, 300]. Similar to these results, a developed fatty liver was observed in high alcohol-treated with HFD.

In conclusion, NAFLD induced by chronic feeding of HFD to rats leads to augmented metabolic dyslipidaemia. This is associated with altered serum lipid profile with increased serum insulin and glucose and elevated liver enzymes ALT and AST. Thus, long-term feeding with moderate alcohol reduced the serum glucose, HDL cholesterol and liver ALT and AST enzymes in animals with HFD-induced NAFLD. Whereas as low alcohol with HFD showed its effects on increasing serum HDL levels. Further studies are being undertaken to explain the mechanism behind the lipid regulation by low and moderate alcohol.

CHAPTER–3

Evaluating the mechanistic action of low and moderate alcohol on protecting the liver from HFD-induced NAFLD in rats

3.1 Introduction

Non-alcoholic fatty liver (NAFLD), a condition caused by the pathological accumulation of fat in the liver affecting billions of people in the world. It is a spectrum of disorders, beginning as simple steatosis, which can evolve into non -alcoholic steatohepatitis (NASH) and fibrosis, often resulting in cirrhosis and even hepatocellular carcinoma [301]. NAFLD pathogenesis is mainly characterized by the multiple-hit hypothesis, manifestation of both genetic and environmental factors, dysfunction of various organs, and organelles, as well as the complex interaction between hepatocytes and other cells (*e.g.*, Kupffer and stellate cells) in the liver [185]. Moreover, as liver is a hub for many metabolic pathways making NAFLD a multistage, progressive disease with systemic consequences. It is commonly associated with obesity, insulin resistance, and enhanced risk of cardiovascular disease and mortality. All the above-mentioned cluster of physiological abnormalities are also common in NAFLD which is a component of metabolic syndrome [302].

The diagnosis of NAFLD is confirmed by hepatic steatosis, which is a prerequisite to making a histological diagnosis of NAFLD [14]. Several mechanisms may lead to steatosis, including increased fat supply such as high-fat diet and excess adipose lipolysis; decreased fat export in the form of very low density lipoprotein-triglyceride decreased free fatty -oxidation; and an increased DNL [14, 303-305]. The enhancement of hepatic *DNL* is deemed to be a unique feature in steatosis, increased *DNL* synthesis of fatty acids in hepatocytes, the retention of lipids due to impaired hepatocyte apolipoprotein secretion or β-oxidation [306]. More importantly, insulin resistance appears to be at center stage for the massive

metabolic dysregulations of NAFLD that initiate and aggravate hepatic steatosis [262]. Several prior studies have reported that diets with saturated fat and simple sugars enhances the DNL, increased DNL leads to development of hepatic steatosis with metabolic syndrome and high content of liver fat [23, 307, 308]. Apart from increasing the DNL in liver, decrease in fatty acid oxidation caused by HFD substantially decreased the activities of both isoforms of AMPK in white adipose tissue, heart, and liver [309]. The HFD increase inflammation, obesity, and diabetes, all these physiological changes also leads decrease in AMPK activity [310]. The increase in systemic inflammation, hyperglycaemia or elevated levels of free fatty acids leads to decreases AMPK levels in liver [309].

AMP-activated protein kinase (AMPK) is a master regulator of energy balance, it regulates a large array of intracellular processes including the cellular uptake of glucose and free fatty acids, cell cycling, mRNA stability, and apoptosis [311, 312]. Furthermore, AMPK plays a more global role, regulating multiple aspects of whole-body energy balance including appetite, insulin sensitivity, and the actions of adipokines/cytokines [39, 69, 313]. Apart from AMPK, HFD decreases LKB1 (liver kinase B1) expression which is an up regulator of AMPK which directly phosphorylates AMPK at Thr-172 [53]. Besides AMPK, SIRT1 also plays an important role in regulating metabolic activities related to NAFLD pathophysiology [314]. On the other hand, AMPK activation by LKB1 leads to increase cellular NAD^+ levels which subsequently activates SIRT1 signalling [104]. Moreover, HFD reduces the SIRT1 expression in liver, which is also a novel up-stream regulator for LKB1/AMPK signalling and plays an important role in regulating hepatic lipid metabolism [192]. The effects of alcohol on liver is

controversial. Excessive alcoholic consumption may leads to obesity, accumulation of hepatic fat, altering the physiological and pathological changes in the liver [214, 315]. In contrast to the excessive high alcohol, consumption, the low and moderate alcohol consumption increases AMPK protein expression in animal models [192, 213]. Interestingly, however, an epidemiological review of health status data for a general population has demonstrated that the prevalence of metabolic syndrome is lower in individuals with low to moderate alcohol consumption than in those who do not consume alcohol [316]. Thus, in the present study we investigate the role of low, moderate and high alcohol on the hepatic AMPK-α1, LKB1 and SIRT1 proteins and their downstream targets such as ACC and SREBP1, which play a key role in the pathogenesis of NAFLD.

3.2 Materials and methods

3.2.1 Chemicals used

Electrophoresis and electro-blotting consumables were purchased from Bio-Rad (Hercules, CA, USA). Primary antibodies of AMPK-α1, p-AMPK-α1, p-ACC, LKB1, p-LKB1, SIRT1 and SREBP1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) while ACC was obtained from (Abcam, Cambridge, United Kingdom). Enhanced chemiluminescence kit was obtained from Bio-Rad (Hercules, CA, USA). All other chemicals used were of analytical or molecular biology grade.

3.2.2 Reagents used

(1) Radio immunoprecipitation assay (RIPA) buffer (pH 8.0)

Sodium chloride (150 mM), sodium deoxycholate (0.5%), sodium dodecyl sulfate (0.1%), Tris base (50 mM) and 1 mL of triton X-100 (1%) were added into a volumetric flask and made up to 100 mL with distilled water. The solution was transferred into a beaker and the pH was adjusted to 8.0 and filtered before use through a 0.2 µM membrane.

(2) Running buffer

Glycine (14.42 g), tris base (3.03 g), sodium dodecyl sulfate (1 g) were added into a volumetric flask and made up to 1000 mL with distilled water. The solution was transferred into a beaker and the pH was adjusted to 8.3 and filtered before use through a 0.2 µM membrane.

(3) Transfer buffer

Tris base (3.03 g), glycine (14.41 g) and methanol (200 mL) were added into a volumetric flask and made up to 1000 mL with distilled water. The solution was filtered before use through a 0.2 µM membrane.

(4) Tris-buffered saline (TBS)

Tris-base (2.4 g) and NaCl (8 g) were added into a volumetric flask and made up to 1000 mL with distilled water. The solution was transferred into a beaker and the pH was adjusted to 7.6 and filtered before use through a 0.2 µM membrane.

(5) Phosphate buffered saline (PBS)

Sodium chloride (8 g), potassium chloride (0.2 g), disodium hydrogen phosphate (1.44 g), and potassium dihydrogen phosphate (0.24 g) were added into a volumetric flask and made up to 1000 mL with distilled water. The solution was transferred into a beaker and the pH was adjusted to 7.6 and filtered before use through a 0.2 µM membrane.

3.2.3 Liver protein extraction

Frozen liver samples were homogenized on ice for 30 seconds using a Tissue Master homogenizer (Omni International, GA, USA) with five volumes of radio immunoprecipitation assay (RIPA) buffer (pH 8.0) containing 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 10 l/mL protease and phosphatase inhibitors cocktail. The

homogenates were centrifuged at 4 °C at 10,000 g for 15 minutes, and the supernatants were collected. Protein concentrations were measured by the Bradford assay using bovine serum albumin as standard.

3.2.4 Western blot analysis

The samples were mixed with loading buffer, proteins were denatured by heating at 95 ^{o}C for 5 minutes, and 25 g of total protein was electrophoretically resolved on 10% Mini-PROTEAN® TGX™ Poured gels (Bio-Rad, Australia) at 135 V for 90 minutes and then transferred onto a nitrocellulose membrane (Bio-Rad, Australia) using a wet transfer (100 V for 135 minutes). After blotting, the membranes were blocked with 5% non-fat dry milk for 1 hour at room temperature. The membranes were then washed three times for 5 minutes each with tris-buffered saline-0.1% Tween-20 (TBST, pH 7.6) and incubated for overnight at 4 C with mouse anti-p-ACC reductase (1:1000) or mouse anti-AMPK (1:1000) or mouse anti-LKB1 (1:1000) or mouse anti- p-LKB1(1:1000) or mouse anti- SIRT1 (1:1000) or mouse anti- SREBP1(1:1000) antibody (Santa Cruz, Biotechnology, CA, USA) ,rabbit anti-p-AMPK(1:500) or rabbit anti-ACC(1:1000) (Abcam, Cambridge, UK) diluted with TBST. Blots were then again washed three times for 5 minutes each with TBST and incubated for 1 hour at room temperature with an appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz, Biotechnology, CA, USA) diluted at 1:10,000 with phosphate-buffered saline (PBS, pH 7.4). The membranes were again washed three times for 5 minutes each with TBST and incubated with enhanced chemiluminescence reagent (Clarity™ Western ECL, Bio-Rad, Australia) for 1 minute at room temperature. Immune complexes

were detected after exposing the blots to ChemiDoc™ XRS system (Bio-Rad, Australia) for various time point. Quantitative image analysis was performed using NIH Image software (Image J) to determine the intensity of the protein signal, which was expressed relative to the amount of -actin used as an internal control.

3.2.5 Data and statistical analysis

The results are expressed as mean ± SEM. To analyse the quantitative differences among the experimental groups before or after treatments, the data were subjected to analysis of variance (ANOVA) using the GraphPad 7.03 (GraphPad Software Inc., California, CA, USA) statistical software. Post-hoc comparisons were made using Dunnett's multiple comparisons test.

3.3 Results

3.3.1 Effect of low, moderate, and high alcohol on AMPK-α1 and its phosphorylation in the liver of HFD-fed rats

The effect of low, moderate and high alcohol on the protein expression of AMPK-α1 in rat liver was shown in Figure 3.1A. The HFD-fed control group (n=6) showed a significant ($p<0.001$) 1.7-fold decrease in AMPK-α1 protein expression when compared to the normal control rats (n=5). On the other hand, both the low alcohol-treated (n=5) and moderate alcohol-treated (n=6) groups showed a significant increase in AMPK-α1 protein expression by 1.2-fold ($p<0.05$) and 1.3-fold ($p<0.01$) respectively when compared to the HFD-fed control group. In contrast, the high alcohol-treated group (n=4) showed a significant 1.5-fold ($p<0.01$) decrease in AMPK-α1 protein expression when compared to the HFD-fed control group.

Figure 3.1B illustrates the effect of low, moderate and high alcohol on phosphorylation of AMPK-α1 in rat liver**.** The HFD-fed control group (n=6) showed a significant ($p<0.01$) 1.8-fold decrease in phosphorylation-AMPK-α1 protein when compared to the normal control rats (n=5). On the other hand, both the low alcohol-treated (n=5) and moderate alcohol-treated (n=6) groups showed a significant increase in phosphorylation of AMPK-α1 protein by 1.5-fold ($p<0.05$) and 1.5-fold ($p<0.05$) respectively when compared to the HFD-fed control group. In contrast, the high alcohol-treated group (n=4) showed a non-significant ($p=0.30$) decrease in phosphorylation-AMPK-α1 protein when compared to the HFD-fed control group.

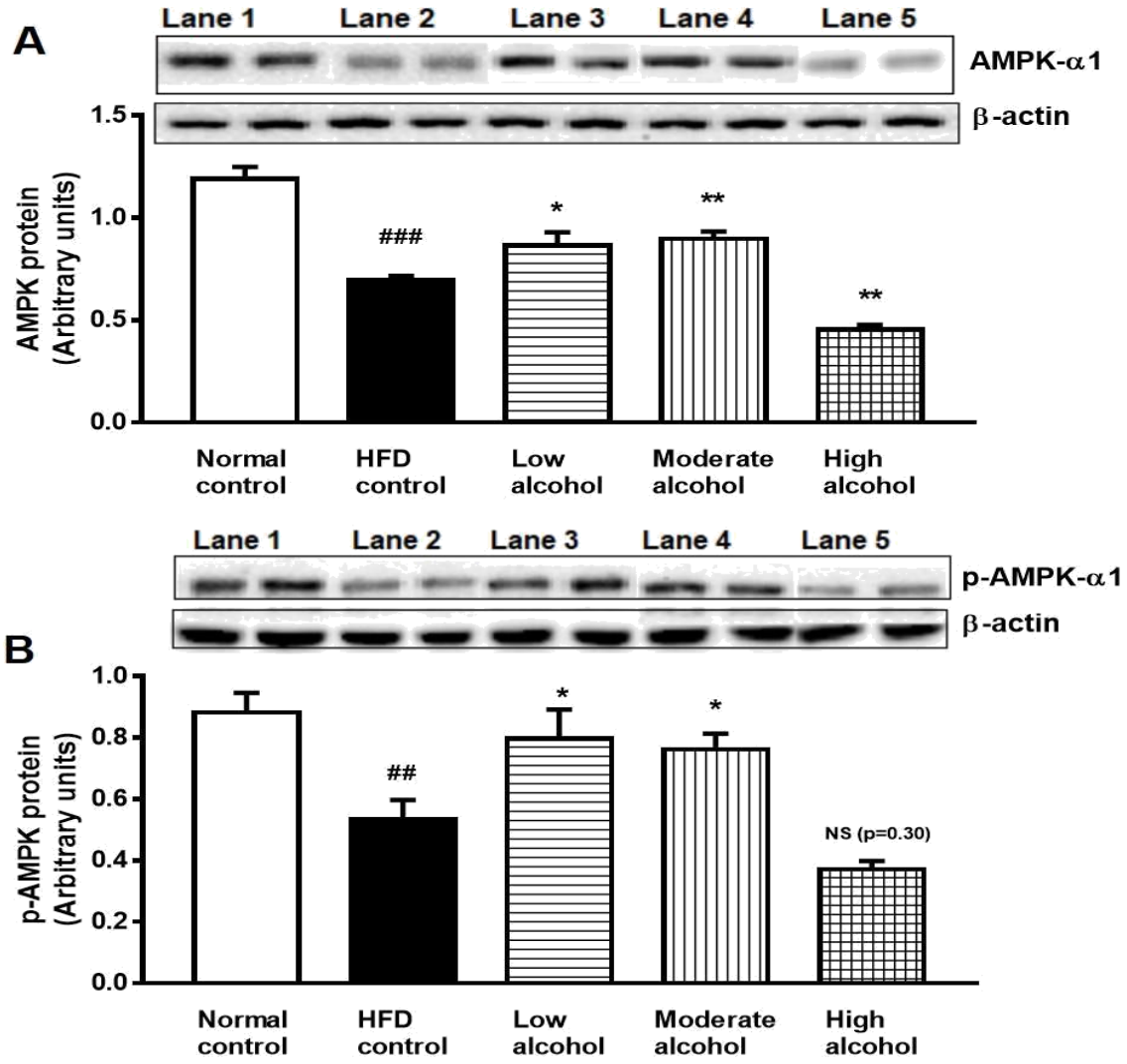

Figure 3.1 Protein expression of AMPK-α1 and p-AMPK-α1 in the liver of HFD-fed rats treated with chronic low, moderate and high alcohol for 15 weeks. (A) AMPK-α1 (B) p-AMPK-α1 protein expression as determined by western blot analysis in the liver of HFD-fed rats for 15 weeks either with low or moderate or high alcohol.

Each bar represents the mean ± SEM of n=4-6 rats calculated relative β-actin used as internal control.

Significant difference from normal control: $^{###}p<0.001$, $^{##}p<0.01$
Significant difference from FFA control: $^{**}p<0.01$, $^{*}p<0.05$
No significant difference from HFD control: ns ($p>0.05$)

3.3.2 Effect of low, moderate, and high alcohol on LKB1 and its phosphorylation in the liver of HFD-fed rats

The effect of low, moderate, and high alcohol on the protein expression of LKB1in rat liver was shown in Figure 3.2A. The HFD-fed control group (n=6) showed significant ($p<0.05$) 1.2-fold decrease in LKB1 protein expression when compared to the normal control rats (n=5). On the other hand, the low alcohol-treated group (n=5) showed a non-significant 1.2-fold ($p=0.09$) increase in LKB1 protein expression when compared to the HFD-fed control group. However, the moderate alcohol-treated group (n=6) showed a mild non-significant 1.1-fold ($p=0.96$) increase in LKB1 expression when compared to the HFD-fed control group. In contrast, the high alcohol-treated group (n=4) showed a non-significant ($p=0.63$) decrease in LKB1 protein expression when compared to the HFD-fed control group.

Figure 3.2B illustrates the effect of low, moderate and high alcohol on phosphorylation of LKB1 in rat liver. The HFD-fed control group (n=6) showed a significant ($p<0.01$) 1.8-fold decrease in *phospho*rylation of LKB1 protein when compared to the normal control rats (n=5). On the other hand, the moderate alcohol-treated rats (n=6) showed a significant 1.2-fold ($p<0.05$) increase in phosphorylation of LKB1 protein expression when compared to the HFD-fed control group. However, the low alcohol-treated group (n=5) showed a non-significant 1.2-fold ($p=0.12$) increase in phosphorylation of LKB1 expression when compared to the HFD-fed control group. In contrast, the high alcohol-treated group (n=4) showed

a non-significant 1.5-fold ($p=0.30$) decrease in phosphorylation of LKB1 protein expression when compared to the HFD-fed control group.

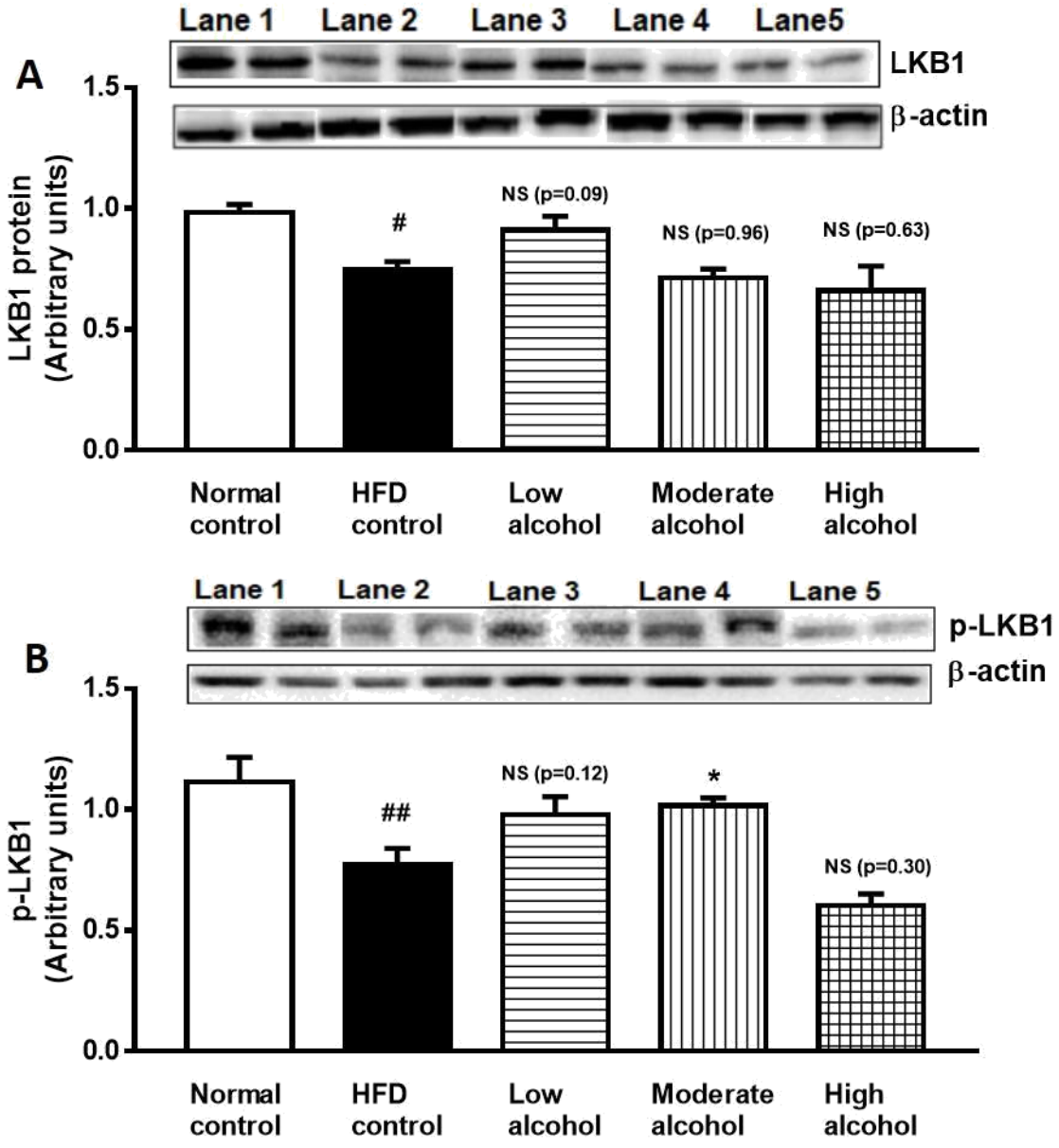

Figure 3.2 Protein expression of LKB1 and p-LKB1 in the liver of HFD-fed rats treated with chronic low, moderate and high alcohol for 15 weeks. (A) LKB1 (B) p-LKB1 protein expression as determined by western blot analysis in the liver of HFD-fed rats for 15 weeks either with low or moderate or high alcohol.

Each bar represents the mean ± SEM of n=4-6 rats calculated relative β-actin used as internal control.

Significant difference from normal control: $^{\#\#}p<0.01$, $^{\#}p<0.05$
Significant difference from FFA control: $^{*}p<0.05$
No significant difference from HFD control: ns ($p>0.05$)

3.3.3 Effect of low, moderate, and high alcohol on SIRT1 protein expression in the liver of HFD-fed rats

The effect of low, moderate, and high alcohol on the protein expression of SIRT1 in rat liver was shown in Figure 3.3. The HFD-fed control group (n=6) showed a significant ($p<0.01$) 1.4-fold decrease in SIRT1 protein expression when compared to the normal control rats (n=5). On the other hand, the moderate alcohol-treated rats (n=6) showed a non-significant 1.2-fold ($p=0.08$) increased trend in SIRT1 protein expression when compared to the HFD-fed control group. Furthermore, the low alcohol-treated group (n=5) showed a non-significant 1.2-fold ($p=0.15$) increase in SIRT1 expression when compared to the HFD-fed control group. In contrast, the high alcohol-treated group (n=4) showed a non-significant 1.2-fold ($p=0.47$) decrease in SIRT1 protein expression when compared to the HFD-fed control group.

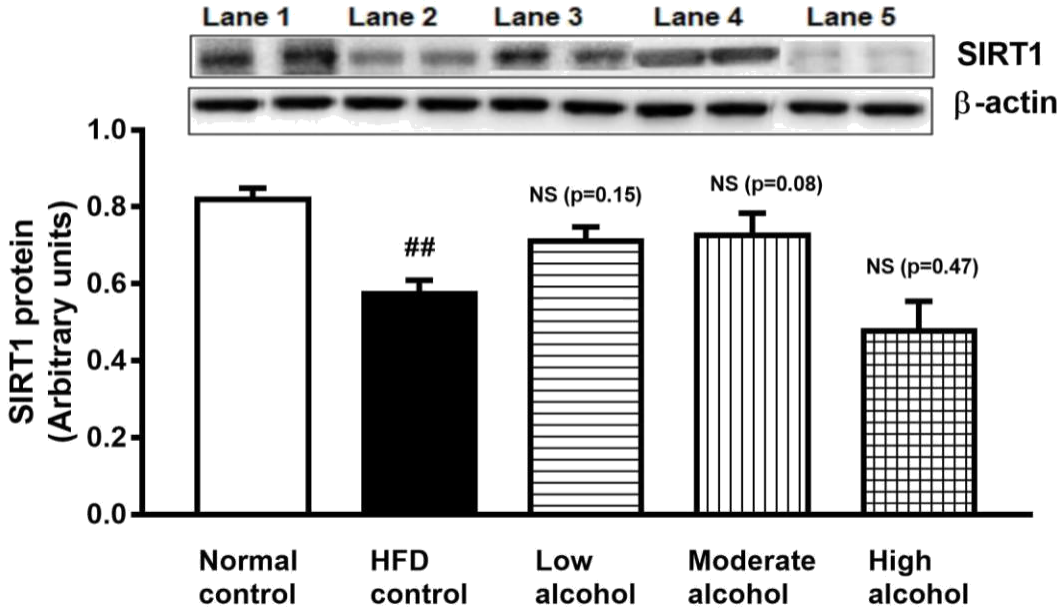

Figure 3.3 Protein expression of SIRT1 in the liver of HFD-fed rats treated with chronic low, moderate and high alcohol for 15 weeks. SIRT1 protein expression as determined by western blot analysis in the liver of HFD-fed rats treated for 15 weeks either with normal diet (normal control) or HFD (disease control) alone or with low, moderate and high alcohol with HFD

Each bar represents the mean ± SEM of 4-6 rats calculated relative β-actin used as internal control.

Significant difference from normal control: $^{\#\#}p<0.01$

No significant difference from HFD control: ns ($p>0.05$)

3.3.4 Effect of low, moderate, and high alcohol on ACC and its phosphorylation in the liver of HFD-fed rats

The expression of ACC protein in rat liver was shown in Figure 3.4A. The HFD-fed control group (n=6) showed a significant ($p<0.05$) 1.5-fold increase in ACC protein expression when compared to the normal control rats (n=5). On the other hand, the moderate alcohol-treated group (n=6) showed a non-significant 1.4-fold ($p=0.07$) decrease in ACC protein expression when compared to the HFD-fed control group. Furthermore, the low alcohol-treated group (n=5) showed a non-significant 1.2-fold ($p=0.44$) decrease in ACC expression when compared to the HFD-fed control group. In contrast, the high alcohol-treated group (n=4) showed a non-significant 1.3-fold ($p=0.38$) increase in ACC protein expression when compared to the HFD-fed control group.

The effect of low, moderate and high alcohol on phosphorylation of ACC in rat liver was shown in Figure 3.4B. The HFD-fed control group (n=6) showed a significant ($p<0.05$) 2.6-fold decrease in phosphorylation of ACC protein when compared to the normal control rats (n=5). On the other hand, both the low alcohol-treated (n=5) and moderate alcohol-treated (n=6) groups showed a significant increase in phosphorylation of ACC protein by 2.8-fold ($p<0.01$) and 2.4-fold ($p<0.05$) respectively when compared to the HFD-fed control group. In contrast, the high alcohol-treated group (n=4) showed a non-significant 1.9-fold ($p=0.77$) decrease in phosphorylation of ACC protein when compared to the HFD-fed control group.

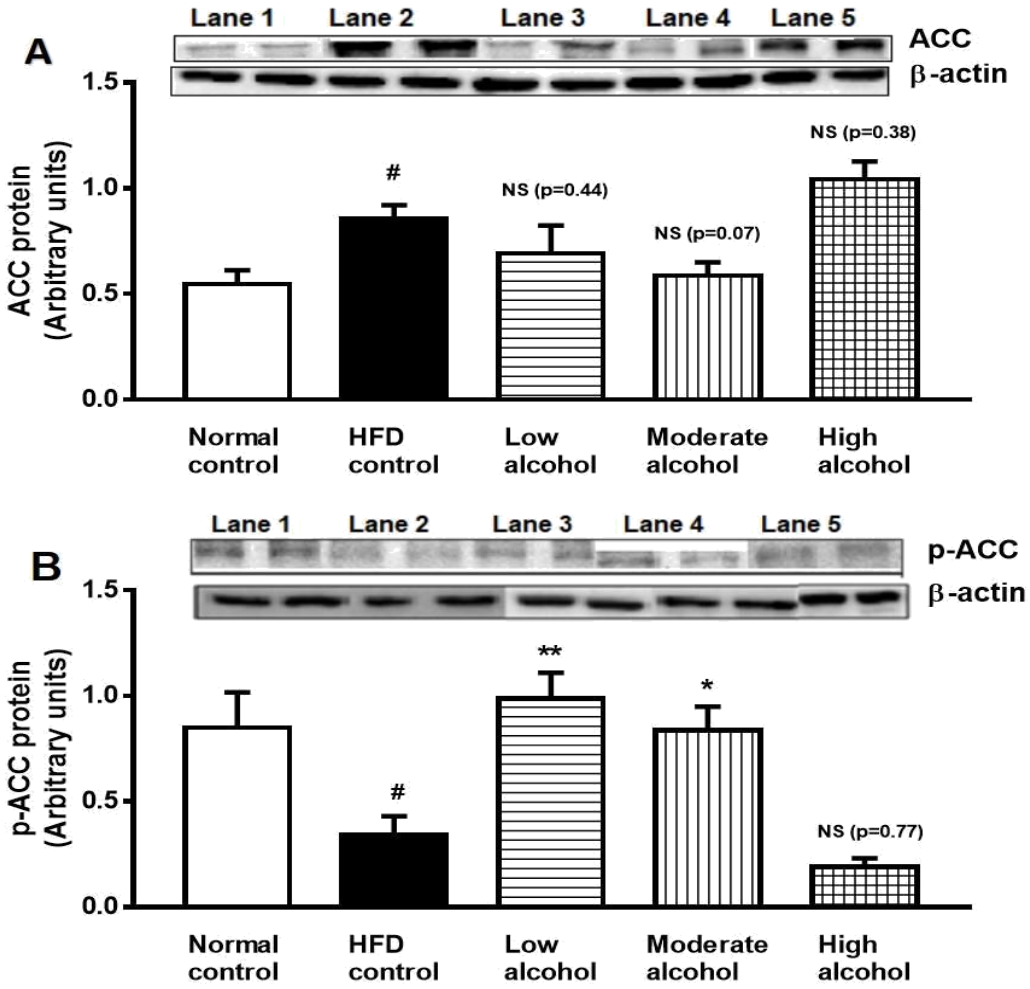

Figure 3.4 Protein expression of ACC and p-ACC in the liver of HFD-fed rats treated with chronic low, moderate and high alcohol for 15 weeks. (A) ACC (B) p-ACC protein expression as determined by western blot analysis in the liver of HFD-fed rats for 15 weeks either with low or moderate or high alcohol.

Each bar represents the mean ± SEM of n=4-6 rats calculated relative β-actin used as internal control.

Significant difference from normal control: $^{\#}p<0.05$
Significant difference from FFA control: $^{**}p<0.01$, $^{*}p<0.05$
No significant difference from HFD control: ns ($p>0.05$)

3.3.5 Effect of low, moderate, and high alcohol on SREBP1 protein expression in the liver of HFD-fed rats

The effect of low, moderate, and high alcohol on the protein expression of SREBP1 in rat liver was shown in Figure 3.5. The HFD-fed control group (n=6) showed significant ($p<0.01$) 1.7-fold increase in SREBP1 protein expression when compared to the normal control rats (n=5). On the other hand, the low alcohol-treated rats (n=5) showed a significant 1.3-fold ($p<0.05$) decrease in SREBP1 protein expression when compared to the HFD-fed control group. In contrast, both the moderate alcohol-treated (n=6) and high alcohol-treated (n=4) groups showed a mild non-significant increase in SREBP1 protein expression by 1.0-fold ($p=0.81$) and 1.3-fold ($p=0.97$) respectively when compared to the HFD-fed control group.

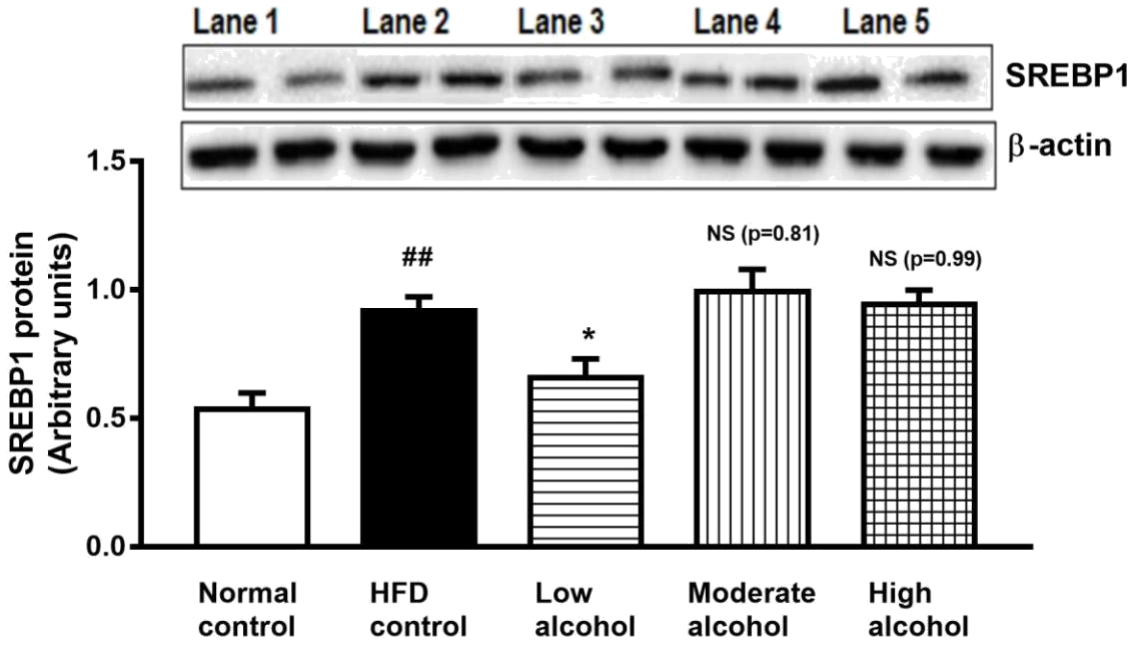

Figure 3.5 Protein expression of SREBP1 in the liver of HFD-fed rats treated with chronic low, moderate and high alcohol for 15 weeks. SREBP1 protein expression as determined by western blot analysis in the liver of HFD-fed rats treated for 15 weeks either with normal diet (normal control) or HFD (disease control) alone or with low, moderate and high alcohol with HFD

Each bar represents the mean ± SEM of 4-6 rats calculated relative β-actin used as internal control.

Significant difference from normal control: $^{\#\#}p<0.05$
Significant difference from FFA control: $^{*}p<0.05$
No significant difference from HFD control: ns ($p>0.05$)

3.4 Discussion

NAFLD, which is a multi-factorial disorder associated with a variety of genetic and environmental factors, is the most common cause of liver disease [317]. It is proven that HFD initiates a serious of molecular events in humans as well as in experimental animal models, leading to elements of metabolic syndrome, including insulin resistance and obesity [249, 312, 318]. In the present chapter, we examined the molecular mechanism(s) behind low, moderate and high doses of alcohol on liver proteins such as AMPK-α1, LKB1 and SIRT1 which play a major role in regulating hepatic lipid metabolism in rats fed with HFD. The results from chapter-2 confirmed that our HFD model showed an increased serum lipids along with serum insulin, glucose, ALT, and AST levels, which are common indicators of liver injury in the majority of liver diseases [319-321]. Thus, long-term feeding with moderate alcohol with HFD fed rats reduced the serum glucose, HDL cholesterol and liver ALT and AST enzymes in animals with HFD induced NAFLD. Whereas, low alcohol group with HFD showed its effects on increasing serum HDL levels. The HFD-induced increased inflammation, obesity, and diabetes, all these physiological changes lead to decrease in AMPK activity [310]. AMPK is a multi-subunit protein kinase found in all eukaryotes. It acts as a metabolic “master switch” regulating cellular processes which are controlled by the body’s energy state [322, 323]. The activity of AMPK is stimulated by stresses which increase the AMP/ATP ratio, and once activated AMPK acts to inhibit pathways that consume ATP and to stimulate multiple events that enhance ATP generation. Thus, AMPK plays a central role in the regulation of lipid metabolism by inhibiting regulatory enzymes involved in biosynthetic pathways, such as ACC, SREBP and 3-hydroxy-3-

methoxyglutaryl-CoA reductase (HMG-CoA) [324]. ACC is a key regulatory enzyme of fatty acid synthesis, and phosphorylation of hepatic ACC *in vivo* is mostly achieved by AMPK, rendering the enzyme inactive [325]. The malonyl-CoA, is a precursor for the biosynthesis of fatty acids and a potent inhibitor of mitochondrial fatty acid oxidation at the carnitine palmitoyl transferase-I (CPT-I) step [326]. Thus, when ACC is inhibited, cellular concentration of malonyl-CoA decreases, and the inhibition of CPT-I is relieved, resulting in increased fatty acid oxidation [327]. Malonyl-CoA is also degraded by malonyl-CoA decarboxylase (MCD), which is activated by AMPK. Several studies have demonstrated that AMPK is the major protein kinase responsible for the inactivation of ACC and the activation of MCD, resulting in reduced malonyl-CoA levels and increased fatty acid oxidation [328]. Our results with HFD and high alcohol group with HFD diet are similar with previous results showing a decrease in AMPK and phosphorylation of AMPK expression in rat liver [214, 329]. The mechanisms behind the decrease in AMPK, are increasing circulating metabolites such as glucose and FFA are decreasing AMPK activity [330]. Moreover, in the model we studied, circulating glucose and the FFA levels were elevated. Thus, elevated circulating levels of metabolic substrates seem likely to be the cause of the HFD-induced reduction in AMPK activity. The mechanism behind the decrease of AMPK and p-AMPK are due to increase in ROS caused by alcohol or increase in fatty acid synthesis, dephosphorylation of AMPK by protein phosphate 2A (PP2A) causing AMPK inactivation, increase in alcohol administration leads to increased ceramide levels, which activates the PP2A, which in turn inhibits the AMPK pathway activity and increases the fatty acid synthesis [331-333]. In contrast to the decreased APMK-α1 in high alcohol with HFD group,

we have observed an increased phosphorylation of AMPK-α1 in the low and moderate alcohol with HFD groups. Our results are similar with the published data demonstrating that moderate alcohol consumption increases AMPK action in rats [183, 192, 213]. The possible mechanism behind the paradoxical dual effects of alcohol could be due to appropriate alcohol metabolic pathway at the threshold of alcohol consumption. It is well known that acetate is produced when alcohol metabolism takes place in the liver [334]. Most of the evolved acetate is oxidized in peripheral tissues. The conversion of acetate to acetyl-CoA, a reaction which generates AMP that triggers AMPK activation and thus support our results. Moreover, binding of AMP to AMPKγ is thought to promote phosphorylation of the critical activation loop threonine (Thr172) in AMPKα, which is required for AMPK activity, largely through suppression of phosphatase activity towards Thr172 [333, 335]. Apart from AMP, LKB1, CaMKKβ (Ca2+/calmodulin-dependent protein kinase kinase β) have identified as physiological kinases upstream of AMPK [53, 336, 337]. LKB1 was first identified in hereditary Peutz-Jeghers syndrome [338]. LKB1 is a serine/threonine protein kinase that can directly activate AMPK and 12 kinds of related kinases [312, 338]. Under normal physiological conditions, LKB1 is located in nucleus and constitutively active [339-341], which makes it necessary to have a counter-mechanism available to prevent persistent or exaggerated activation of AMPK signalling. LKB1 forms a heterotrimeric complex like AMPK with regulating proteins termed STRAD and MO25, which are required for its activation and cytosolic localization [342]. Our results were consistent with the previous results showing that HFD diminishes the expression of LKB1 protein and its phosphorylation in the liver [263, 343]. The effects of chronic low and moderate

alcohol administration on p-LKB1 has been limitedly reported. Moderate alcohol with HFD group showed a significant increase in p-LKB1 expression. The possible mechanisms phosphorylation of LKB1 at Ser-428/431, by activating protein kinase C (PKC)-zeta(ζ) which exports the LKB1 from nucleus to cytosolic for activation and subsequent AMPKα phosphorylation at Thr^{172} [344]. According to recent studies, deacetylation of SIRT1 regulates LKB1 and induces translocation of LKB1 to the cytosol from the nucleus [46, 192]. Translocated LKB1 associates with STRAD and MO25 and exhibits kinase activity leading to the phosphorylation of AMPK [345]. In this study, low alcohol with HFD groups showed an increased trend in p-LKB1 expression. Whereas, high alcohol with HFD group showed a decrease in p-LKB1 levels which are consistent with the previous results [216]. Apart from AMPK and LKB1, SIRT1 also plays a major role in regulating lipid metabolism in the liver. A decrease in SIRT1 is associated with a diminished LKB1 and AMPK activity [46, 192, 346]. SIRT1 is widely expressed in mammalian cells and has been studied in many tissues including brain, skeletal muscle, adipose tissue, pancreas (β-cells), and liver [347-349]. SIRT1 is an NAD1-dependent deacetylase and its enzymatic activity may be regulated by cellular energy and enhanced by an increase in the NAD^+/NADH ratio

[350]. SIRT1 plays a key role in many cellular processes of mammalian cells *in vivo* [351-353]. Apart from LKB1 deacetylation by SIRT1 protein, it deacetylates P53, Tata box-binding protein-associated factor I (TAFI68), p300 (CREB-binding protein homologue), p300/CREB-binding protein-associated factor (PCAF), myoblast determination protein (MyoD), Forkhead transcription factors (FOXO), p65 subunit of nuclear factor k B (NF-kB), Ku70 telomeric protein (a DNA repair factor),

peroxisome proliferators activated receptor γ co-activator 1α (PGC-1α) [167]. SIRT1 and its activators play a key role in lipid and glucose homeostasis and insulin sensitivity via regulating mitochondrial biogenesis and β-oxidation and improving anti-inflammatory activities [354]. A lot of published evidence indicates that HFD and high alcohol reduces SIRT1 expression in the liver [214, 355-357]. In experimental condition, alcohol is firstly metabolized by either alcohol dehydrogenase (ADH) in the cytosol or cytochrome P450 2E1 (CYP2E1) in the endoplasmic reticulum to produce acetaldehyde, which is further rapidly metabolized by mitochondrial aldehyde dehydrogenase (ALDH2) to form acetate and convert NAD+ to NADH [93, 94]. Moreover, reduced NAD+ level and SIRT1 activity were concomitant with dietary energy/nutrition overload status such as high-fat diet and/or high-calorie diet feeding conditions [358, 359]. The effects of moderate alcohol on SIRT1 protein are not consistent. Kanuri et al (2016) [255] demonstrated that moderate alcohol has increased SIRT1 mRNA expression in visceral adipose tissue in mice. In our study, low and moderate alcohol with HFD groups showed an increased trend in SIRT1 protein expression in the liver. The possible mechanism behind the increase in trend in SIRT1 expression is due to AMPK which enhances SIRT1 activity by increasing cellular NAD+ levels, resulting in the deacetylation and modulation of the activity of downstream SIRT1 targets [48].

SREBP-1 and -2 are important transcription factors in the liver, SREBP-1 controls the expression of genes involved in fatty acid and triglyceride synthesis, and SREBP-2 controls the expression of genes involved in cholesterol synthesis and uptake [360, 361]. SREBP-1 and SREBP-2 are structurally similar, but their

regulation in the liver by nutrients and hormones is different. SREBP-1 and SREBP-2 regulate the fatty acid and cholesterol synthesis, respectively, by activating the enzymes involved in the fatty acid and cholesterol biosynthetic pathways including ATP citrate lyase (ACL), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD) and HMG-CoA reductase [360]. SREBPs are expressed as precursor proteins that span the endoplasmic reticulum membrane, are proteolytically cleaved and translocated to the nucleus under the influence of SREBP cleavage-activating protein (SCAP) to activate transcription. Nammi et al (2013) [183] demonstrated that low and moderate alcohol reduced the SREBP1 protein levels in normal rats. Our results were consistent showing that low alcohol with HFD group showed a decreased SREBP1 expression. AMPK activation resulted in a dramatic reduction of SREBP-1 in the liver, suggesting a direct link between AMPK action and SREBP transcriptional activity [362, 363] which support our present observations that low alcohol treatment tended to increase the liver AMPK-α1 phosphorylation and decrease SREBP-1 protein expression leading to increased fatty acid oxidation. Even though moderate alcohol with HFD group showed an increase in p-AMPK levels, but increased p-AMPK protein didn't show any effect in controlling SREBP1 expression. Nevertheless, it is not clear from our studies why increased p-AMPK levels did not regulate the SREBP1. Whereas, high alcohol with HFD group did not show any effect in controlling the SREBP1 expression. Acetyl CoA carboxylase (ACC), a key enzyme in *de novo* lipogenesis, exists around the mitochondria and controls β-oxidation of fatty acids in the mitochondria. ACC is a physiologic inhibitor of CPT, which synthesizes malonyl CoA in the liver [364]. ATP is an energy resource formed by carbohydrate

metabolism and β-oxidation of fatty acids in the liver. Fatty acids are converted into acetyl-CoA through mitochondrial oxidation. Fatty acid oxidation is repressed by dephosphorylated AMPK which conserves serine/threonine protein kinase that acts as a metabolic master switch [365]. Phosphorylated AMPK inactivates ACC by phosphorylation and lessen the intracellular malonyl-CoA level, which is the substrate for synthesis of fatty acid and, at the same time, the inhibitor of CPT-1, the rate-limiting enzyme of mitochondrial fatty acid oxidation. Repression of ACC causes the inhibition of endogenous free fatty acids [366]. In this study, the eminent expressions of ACC were found in HFD fed rats. The increase in p-ACC levels has been observed in low and moderate alcohol groups, possibly due to the increase in p-AMPK which regulated the phosphorylation of ACC.

In the present study we found that HFD increased the expression of SREBP1 and ACC proteins which play a major role in regulating fatty acids synthesis and β-oxidation of fatty acids which leads to increase the lipid deposition in the liver leads to steatosis the first stage of NAFLD. The low and moderate alcohol-treated groups increased AMPK-α1, p-AMPK-α1, LKB, p-LKB1, and SIRT1 proteins which regulate each other and regulates the lipid metabolism pathways. Further studies have been undertaken for understand the role of each protein in protecting the liver from NAFLD.

CHAPTER-4

Evaluating the mechanistic action of low and moderate alcohol on protecting the HepG2 cells from FFA-induced NAFLD

4.1 Introduction

Non-alcoholic fatty liver (NAFLD) has become a significant public health burden effecting millions of people worldwide [186]. NAFLD encompasses a spectrum of liver diseases ranging from a benign fatty liver disorder to the nonalcoholic steatohepatitis (NASH) that may progress to cirrhosis in up to 25% of patients. It is closely associated with metabolic syndrome which is a cluster of complex conditions including central obesity, hypertriglyceridemia, hyperglycemia, hypertension, and low HDL (high density lipoprotein) that are predictive risk factors of cardiovascular disease, stroke, and diabetes [367-369].

From the evidence from Berson et al (1998) Day and James first proposed a two-hit process for the pathogenesis of NAFLD [15, 370]. Accumulation of triglycerides in hepatocytes leads to the development of hepatic steatosis which is the first hit, which increases the vulnerability of the liver to various possible “second hits” that in turn lead to the inflammation, fibrosis, and cellular death characteristics of NASH. The second hit can be a variety of factors, such as oxidative stress, endoplasmic reticulum stress, pro-inflammatory cytokines, and gut-derived bacterial endotoxin [190]. However, increased FFA can lead to excessive hepatic lipid accumulation correlating with disease severity [371]. Numerous studies have suggested that FFA influx and *de novo* lipogenesis are the major pathogenic processes in the development of non-alcoholic fatty liver [372]. This has been supported by animal models of genetic, dietary and combined models characterized by increased hepatic lipid accumulation, where a second insult is necessary to initiate inflammation and fibrosis [373, 374]. Due to ethical

limitations in regards to tissue collection, required long period to study, animal models which depending on the model used may more or less mimic the situation found in humans. More recently, *in vitro* models of steatosis have been used to study the hepatocellular consequences of lipid accumulation in hepatic cells of a human origin [375, 376]. Primary human hepatocytes are the gold standard for *in vito* cellular studies. However, they present issues with availability, inter-donor variability and the short time frame during which they remain differentiated [377, 378]. As a result, proliferating human hepatoma cell models are the most widely used option. A first alternative is the widely used human hepatocellular carcinoma cell line HepG2. These cells are human hepatoblastoma cell line that displays many of the genotypic features, including some liver-specific metabolic functions, have been widely used as a human-derived in vitro model for investigating basic hepatic metabolism and drug hepatotoxicity as well as liver steatosis [379-382]. The main fatty acids in the human body consist of palmitic acid (PA) and oleic acid (OA)

[383]. A combination (OA+PA) or individual usage of FFA are widely used *in vitro* to induce steatosis in cultured human liver cell lines [384-387]. These FFAs are absorbed by liver cells through facilitated transport mechanisms and converted to triacylglycerides [388]. Understanding the intracellular absorption of free fatty acids and their role in cellular cytotoxicity is critical towards elucidating the causes of NASH [389].

Excessive alcoholic consumption leads to induce the oxidative stress and activate the inflammatory pathways, that produce cytokines, tumour necrosis factor-alpha, transforming growth factor-beta (TGF-β) and reactive oxygen species, which are

believed to play a major role in pathogenesis and progression of alcoholic liver diseases [164, 390, 391]. HepG2 cells cannot produce alcohol dehydrogenase activity, however, incubation with alcohol resulted in the appearance of such activity which could explain the alcohol toxicity [392].

In contrast to heavy alcohol that precipitates alcoholic fatty liver disease, low and moderate alcohol showed beneficial effects on rodent model [192]. The results from (chapter 3) prove that low and moderate alcohol protects the liver from NAFLD by increasing the phosphorylation of AMPK-α1 in rats fed with HFD. A numerous *in vito* and *in vivo* studies have demonstrated that AMPK-α1 activation protects the liver from NAFLD by increasing the fatty acid oxidation, inhibition of cholesterol synthesis, glucose production and hepatic lipogenesis [393, 394].

The aim of this chapter was to investigate the role of low to moderate alcohol on protecting the HepG2 cells from FFA-induced hepatic steatosis by increasing the proteins AMPK, and SIRT1 proteins which further suppress the ACC and SREBP1 proteins which play a key role in hepatic steatosis.

4.2 *Materials and methods*

4.2.1 Chemicals used

Electrophoresis and electro-blotting consumables were purchased from Bio-Rad (Hercules, CA, USA). Primary antibodies of AMPK-α1, p-AMPK-α1, p-ACC, LKB1, p-LKB1, SIRT1 and SREBP1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) while ACC was obtained from (Abcam, Cambridge, United Kingdom). Enhanced chemiluminescence kit was obtained from Bio-Rad (Hercules, CA, USA). DMEM (modified basal medium eagle), low glucose, alcohol, Fetal bovine serum (FBS), sodium oleate, sodium palmitate from Sima (Sigma St. Louis, MO, USA). All other chemicals used were of analytical or molecular biology grade.

4.2.2 Reagents used

(1) Radio immunoprecipitation assay (RIPA) buffer (pH 8.0)

Sodium chloride (150 mM), sodium deoxycholate (0.5%), sodium dodecyl sulfate (0.1%), Tris base (50 mM) and 1 mL of triton X-100 (1%) were added into a volumetric flask and made up to 100 mL with distilled water. The solution was transferred into a beaker and the pH was adjusted to 8.0 and filtered before use through a 0.2 µM membrane.

(2) Running buffer

Glycine (14.42 g), tris base (3.03 g), sodium dodecyl sulfate (1 g) were added into a volumetric flask and made up to 1000 mL with distilled water. The solution was transferred into a beaker and the pH was adjusted to 8.3 and filtered before use through a 0.2 µM membrane.

(3) Transfer buffer

Tris base (3.03 g), glycine (14.41 g) and methanol (200 mL) were added into a volumetric flask and made up to 1000 mL with distilled water. The solution was filtered before use through a 0.2 µM membrane.

(4) Tris-buffered saline (TBS)

Tris-base (2.4 g) and NaCl (8 g) were added into a volumetric flask and made up to 1000 mL with distilled water. The solution was transferred into a beaker and the pH was adjusted to 7.6 and filtered before use through a 0.2 µM membrane.

(5) Phosphate buffered saline (PBS)

Sodium chloride (8 g), potassium chloride (0.2 g), disodium hydrogen phosphate (1.44 g), and potassium dihydrogen phosphate (0.24 g) were added into a volumetric flask and made up to 1000 mL with distilled water. The solution was transferred into a beaker and the pH was adjusted to 7.6 and filtered before use through a 0.2 µM membrane.

(6) Stacking gel buffer

Tris base (15.14 g), SDS (1.0 g) were added into volumetric water and made up to 250ml with distilled water. The solution was transferred into a beaker and the pH was adjusted to 6.8 and filtered before use through a 0.2 µM membrane.

(7) Sodium oleate

Sodium oleate (40.58 mg) in 10 mL (13.33 mM) stock of plain DMEM at 37ºC with fatty acid-free BSA and filtered with a 0.2 µM membrane and stored in -20°

(8) Sodium palmitate

Sodium palmitate (18.54 mg) in 10 mL of plain DMEM at 50 ºC with constant shaking with Fatty acid-free BSA and filtered with a 0.2 µM membrane and stored in -20°C.

(9) *400 mM alcohol*

Alcohol 184.28 mg or 235 µL of alcohol in 10 mL of plain DMEM for 400 mM stock and filtered with a 0.2 µM membrane and stored in 4 ºC.

4.2.3 Cell culture

HepG2 cells (passage-7) are obtained from the University of Sydney, which were previously maintained in DMEM with low glucose, 10% FBS, 1% antibiotics (10,000 I.U./mL Penicillin, 10,000 (µg/mL) Streptomycin) as per mentioned in ATCC guidelines for HepG2 cells. They were maintained in same incubator conditions, at 37º C with 5% CO_2 changing the growth medium for every two days. After 70-80% confluence of cells they were washed thrice with PBS and then added 3-5ml of trypsin with EDTA. After the cells completely detach, they were added into DMEM to neutralise the trypsin and the cells were centrifuged at 300 g for 3 minutes. The subculture was done with a ratio of 1:3 or 1:6. The remaining cell were stored with 5% DMSO in growth medium and stored in liquid nitrogen vapour phase.

4.2.4 Experimental design and treatments

HepG2 cells were grown up to 70% confluence and then transferred into 6 well plates at $2.5*10^5$ cells per well and divided into six groups. Control cells were incubated with plain medium and free fatty acid group received 1 mM fatty acids mixture (containing 0.66 mM sodium oleate and 0.33 mM sodium palmitate) whereas the treatment groups received low (10 mM) and moderate (20 mM) concentrations of alcohol with 1 mM fatty acids mixture (0.66 mM sodium oleate and 0.33 mM of sodium palmitate)[395]. The rest of the groups received 10 mM and 20 mM of alcohol without any FFA treatment. Alcohol is a volatile substance in order to maintain a stable alcohol concentration inside the 6 well plate, for the cells, double the concentration of alcohol was placed in a Petri dish during the 24-hour experiment. After 24 hours of treatment, the cells were removed from the incubator and proceeded for lipid extraction and cellular protein extraction.

4.2.5 Extraction of lipids from HepG2 cells

The total lipids from HepG2 cells were extracted by the modified method of Bligh and Dyer [396] as described by Li and Lin [397]. The treated cells were homogenized with chloroform-methanol solution (chloroform-methanol-water,8:4:3). Further the resulting mixture was shaken at 37ºC for 1hour and then centrifuged at 1,100 g for 10 minutes. The bottom layer was collected and again centrifuged, the supernatant was collected and used for analysis of hepatic lipid.

4.2.6 Cell protein extraction

After 24 hours of treatment, 6 well plates with cells were removed from incubator, washed with PBS, and then added Radioimmunoprecipitation assay (RIPA) buffer (pH 8.0) containing 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 10 l/ml protease and phosphatase inhibitors cocktail to each well and cells were scraped on ice over an orbital shaker for 1hr. The homogenates were collected and then sonicated at 60AMP of total time for 30seconds with on time 5 sec and off time 15 seconds. The homogenates were centrifuged at 4 °C at 10,000 *g* for 15 minutes, and the supernatants were collected. Protein concentrations were measured by the Bradford assay using bovine serum albumin as standard.

4.2.7 Western blot analysis

The samples were mixed with loading buffer, proteins were denatured by heating at 95 ^{o}C for 5 minutes, and 25 or 50 g of total protein was electrophoretically resolved on 10% Mini-PROTEAN® TGX™ Poured gels (Bio-Rad, Australia) at 135 V for 90 minutes and then transferred onto a nitrocellulose membrane (Bio-Rad, Australia) using a wet transfer (100 V for 135 minutes). After blotting, the membranes were blocked with 5% non-fat dry milk 1hour at room temperature. The membranes were then washed three times for 5 minutes each with tris-buffered saline-0.1% Tween-20 (TBST, pH 7.6) and incubated for overnight at 4 C with mouse anti-p-ACC reductase (1:1000) or mouse anti-AMPK-α1 (1:1000) or mouse anti- LKB1 (1:1000) or mouse anti- p-LKB1(1:1000) or mouse anti-SIRT1 (1:1000) or mouse anti-SREBP1(1:1000) antibody (Santa Cruz,

Biotechnology, CA, USA) ,rabbit anti-p-AMPK-α1 (1:500) or rabbit anti-ACC(1:1000) (Abcam, Cambridge, UK) diluted with TBST. Blots were then again washed three times for 5 minutes each with TBST and incubated for 1 hour at room temperature with an appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz, Biotechnology, CA, USA) diluted at 1:10,000 with phosphate-buffered saline (PBS, pH 7.4). The membranes were again washed three times for 5 minutes each with TBST and incubated with enhanced chemiluminescence reagent (Clarity™ Western ECL, Bio-Rad, Australia) for 1 min at room temperature. Immune complexes were detected after exposing the blots to ChemiDoc™ XRS system (Bio-Rad, Australia) for various time point. Quantitative image analysis was performed using NIH Image software (Image J) to determine the intensity of the protein signal, which was expressed relative to the amount of -actin used as an internal control.

4.2.8 Data and statistical analysis

The results are expressed as means ± SEM. To analyse the quantitative differences among the experimental groups before or after treatments, the data were subjected to analysis of variance (ANOVA) using the GraphPad 7.03 (GraphPad Software Inc., California, CA, USA) statistical software. Post-hoc comparisons were made using Dunnett's multiple comparisons test.

4.3 Results

4.3.1 Effect of low and moderate alcohol on triglycerides and total cholesterol levels in FFA-treated HepG2 cells

The levels of triglycerides in different experimental groups are shown in Figure 4.1A. The FFA-treated cells (n=6) showed a significant ($p<0.001$) 1.6-fold increase in triglycerides levels when compared to the normal control cells (n=6). On the other hand, both the low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a mild non-significant decrease in triglycerides levels by 1.5-fold ($p=0.19$) and 1.5-fold ($p=0.18$) respectively when compared to the FFA-treated control cells. In contrast, low alcohol treatment in normal cells (n=6) showed a significant 1.1-fold ($p<0.05$) increase in triglycerides levels when compared to the normal control cells, while normal cells treated with moderate alcohol (n=6) showed a non-significant ($p=0.43$) increase in triglycerides when compared to the normal control cells.

The total cholesterol levels in different experimental cellular groups are shown in Figure 4.2B. HepG2 cells (n=6) treated with FFA showed a significant ($p<0.001$) 1.4-fold increase in total cholesterol levels when compared to the normal control cells (n=6). On the other hand, both the low alcohol-treated (n=6) and moderate alcohol-treated (n=6) cellular groups tended to show a non-significant decrease in total cholesterol levels by 1.4-fold ($p=0.44$) and 1.3-fold ($p=0.16$) respectively when compared to the FFA-treated control cells. In contrast, both the low alcohol and moderate alcohol-treatment in normal cells (n=6) showed a

significant increase ($p<0.05$) in total cholesterol levels respectively when compared to the normal cells.

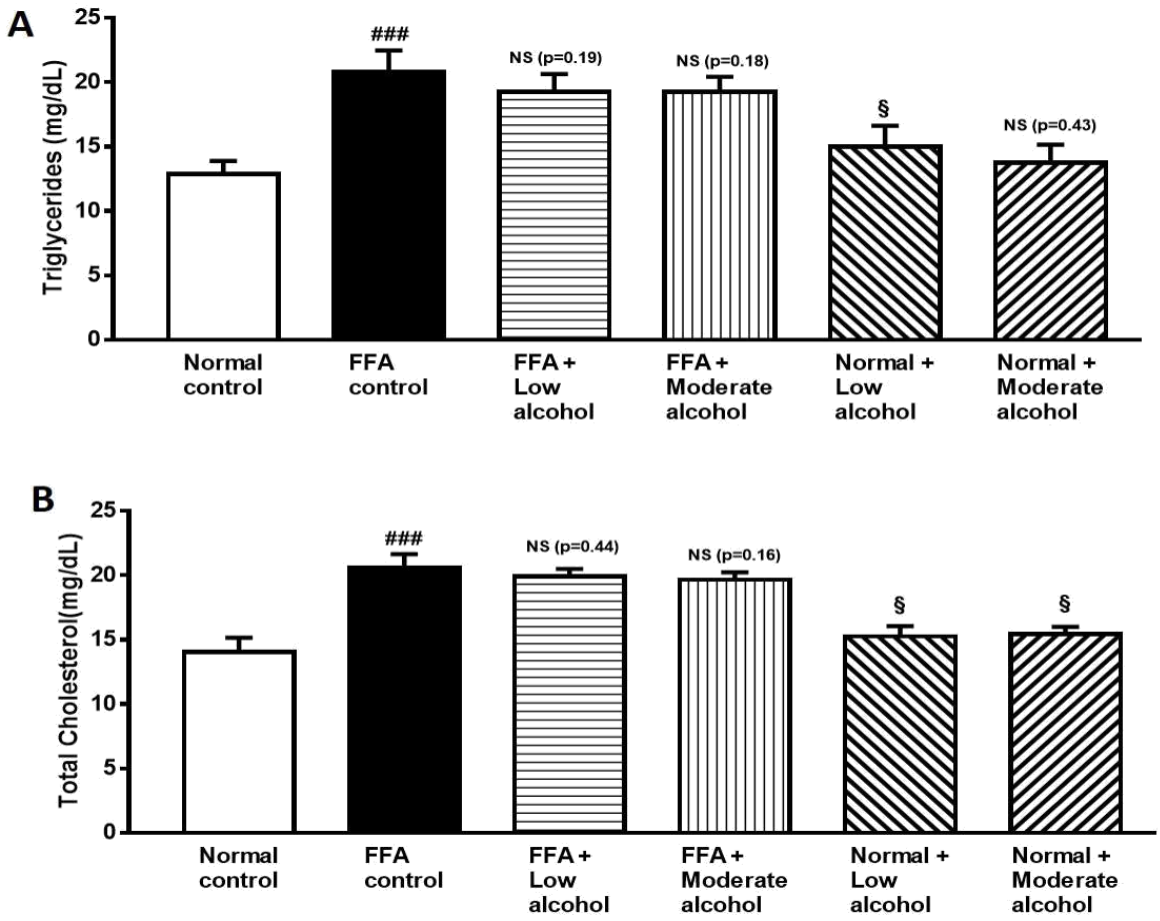

Figure 4.1 Effect of low and moderate alcohol on triglycerides and total cholesterol levels in FFA-treated HepG2 cells. (A) triglycerides (B) total cholesterol accumulation in HepG2 cells incubated with FFA 1mM mixture final concentration for 24 hours either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Values represent the mean ± SEM of n=6

Significant difference from normal control cells: $^{###}p<0.001$,

Significant difference from normal control group vs normal cells treated with alcohol groups: $^{§}p<0.05$,

NS: No significant difference between the groups in comparison

FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

4.3.2 Effect of low and moderate alcohol on AMPK-α1 and its phosphorylation expression in FFA-treated HepG2 cells

The effect of low and moderate alcohol on the protein expression of AMPK-α1 in HepG2 cells is shown in Figure 4.2A. The FFA-treated HepG2 cells (n=6) showed a significant ($p<0.01$) 1.6-fold decrease in AMPK-α1 protein expression when compared to the normal control cells (n=6). On the other hand, cells treated with moderate alcohol (n=6) showed a significant 1.2-fold ($p<0.001$) increase in AMPK-α1 protein expression when compared to the FFA-treated HepG2cells. However, the low alcohol-treated cells (n=6) showed no significant change ($p=0.99$) in AMPK-α1 protein expression when compared to the FFA-treated cells. In contrast, both low alcohol and moderate alcohol treatment in normal cells (n=3) showed a significant increase in AMPK-α1 protein expression by 1.1-fold ($p<0.05$) and 1.5-fold ($p<0.001$) respectively when compared to the normal control cells.

Figure 4.4B illustrates the effect of low and moderate alcohol on phosphorylation of AMPK-α1 in HepG2 cells**.** The FFA-treated control group (n=6) showed a significant ($p<0.01$) 1.2-fold decrease in phosphorylation of AMPK-α1 protein when compared to the normal control cells (n=6). On the other hand, the moderate alcohol-treated group (n=6) showed a significant ($p<0.05$) increase in phosphorylation of AMPK-α1 protein when compared to the FFA-treated control group. The low alcohol-treated group (n=6) tended to show a slight increase in phosphorylation of AMPK-α1 protein but failed to reach significance ($p=0.99$) when compared to the FFA-treated group. In contrast, normal cells treated with moderate alcohol (n=3) showed a significant 1.2-fold ($p<0.05$) increase in phosphorylation of

AMPK-α1 protein when compared to the normal control group while low alcohol treatment in normal cells (n=3) show a non-significant (p=0.88) increase in phosphorylation of AMPK-α1 when compared to the normal control cells.

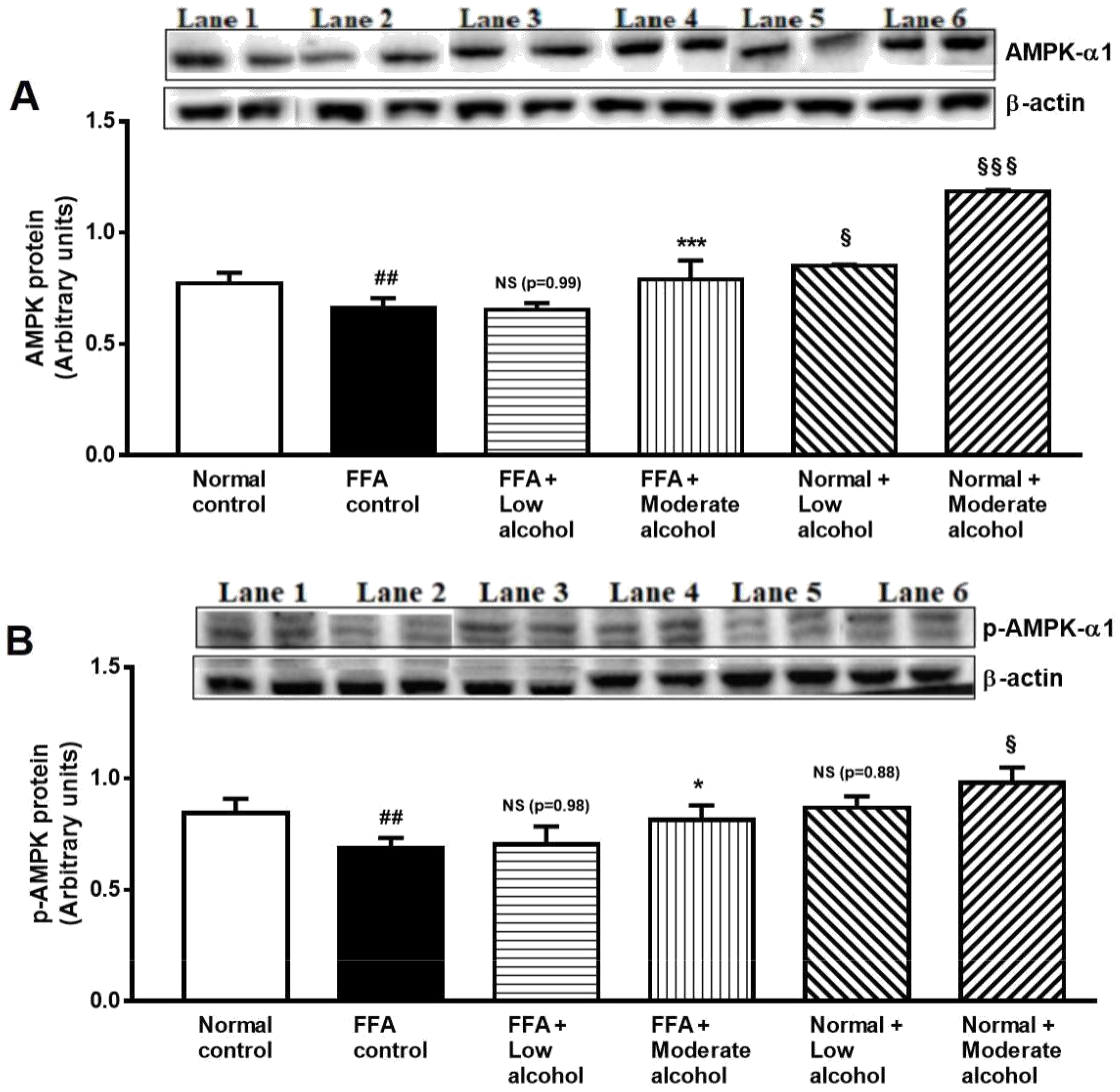

Figure 4.2 Protein expression of AMPK and p-AMPK-α1 in FFA-treated HepG2 cells. (A) AMPK (B) p-AMPK-α1 protein expression as determined by western blot analysis in HepG2 cells treated with 1 mM of FFA for 24 hours either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Each bar represents the mean ± SEM of n=6 & 3 for normal cells treated with low and moderate alcohol groups calculated relative β-actin used as internal control. Significant difference from normal control: $^{\#\#}p<0.01$
Significance difference from FFA control: ***p<0.001, *p<0.05
Significant difference from normal control group vs normal cells treated with alcohol groups: $^{\S}p<0.05$, $^{\S\S\S}p<0.001$

NS: No significant difference between the groups in comparison

FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

4.3.3 Effect of low and moderate alcohol on LKB1 and its phosphorylation in FFA-treated HepG2 cells

The effect of low and moderate alcohol treatment on LKB1 protein expression was investigated by western blot analysis in HepG2 cells Figure 4.3A. The FFA-treated HepG2 cells (n=6) showed a significant ($p<0.001$) 1.4-fold decrease in LKB1 protein expression when compared to the normal control group (n=6). The moderate alcohol-treated group (n=6) showed a significant ($p<0.05$) 1.2-fold increase in LKB1 protein expression when compared to the FFA-treated group. However, the low alcohol treated group (n=6) showed a 1.0-fold non-significant ($p=0.12$) increase in LKB1 protein when compared to FFA-treated cells. Furthermore, normal cells treated with moderate alcohol (n=3) showed a significant ($p<0.01$) 1.3-fold increase in LKB1 protein expression when compared to the normal control group whereas, low alcohol treatment in normal cells (n=3) showed a mild non-significant ($p=0.25$) increase in LKB1 protein expression when compared to the normal control group.

Figure 4.3B, illustrates the effect of low and moderate alcohol on phosphorylation of LKB1 in FFA-treated HepG2 cells**.** The FFA-treated control group (n=6) showed a significant ($p<0.01$) 1.6-fold decrease in phosphorylation of LKB1 protein when compared to the normal control cells (n=6). On the other hand, the moderate alcohol-treated cells (n=6) showed a significant 1.7-fold ($p<0.05$) increase in phosphorylation of LKB1 protein when compared to the FFA-treated control group. Furthermore, the low alcohol-treated group (n=6) showed a mild non-

significant ($p=0.43$) increase in phosphorylation of LKB1 when compared to the FFA-treated control group. In contrast, both the low alcohol-treated ($n=3$) and moderate alcohol-treated ($n=3$) normal cell groups showed a significant ($p<0.001$ and $p<0.05$) decrease in phosphorylation of LKB1 protein respectively when compared to the normal control group.

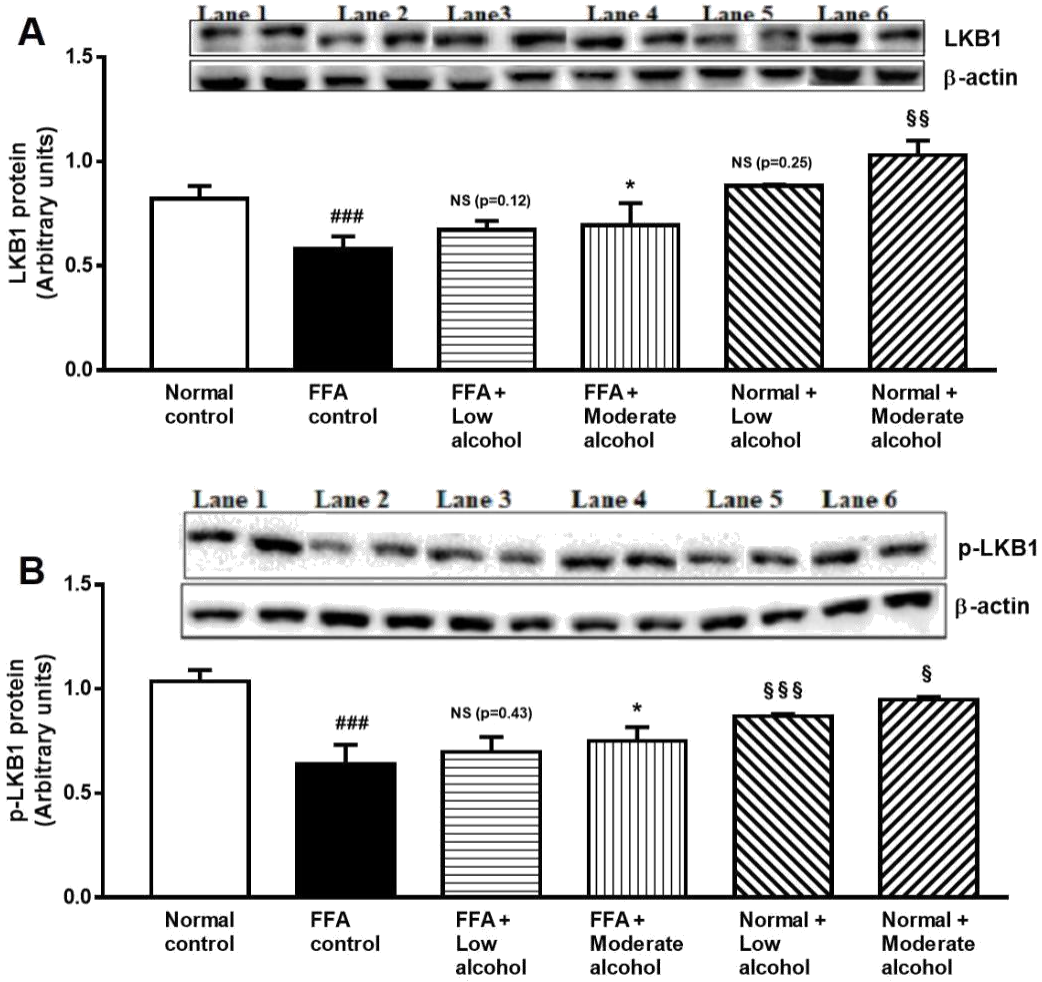

Figure 4.3 Protein expression of LKB1 and p-LKB1 in FFA-treated HepG2 cells. (A) LKB1 (B) p-LKB1 protein expression as determined by western blot analysis in HepG2 cells treated with 1 mM of FFA for 24 hours either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Each bar represents the mean ± SEM of n=6 & 3 for normal cells treated with low and moderate alcohol groups calculated relative β-actin used as internal control. Significant difference from normal control: $^{###}p<0.001$

Significance difference from FFA control: *p<0.05

Significant difference from normal control group vs normal cells treated with alcohol groups: $^{§}p<0.05$, $^{§§}p<0.01$, $^{§§§}p<0.001$

NS: No significant difference between the groups in comparison

FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

4.3.4 Effect of low and moderate alcohol on SIRT1 protein expression in FFA-treated HepG2 cells

The effect of low and moderate alcohol on the protein expression of SIRT1 in FFA-treated HepG2 cells is shown in Figure 4.4. The FFA-treated control group (n=6) showed a significant ($p<0.001$) 1.6-fold decrease in SIRT1 protein expression when compared to the normal control cells (n=6). On the other hand, both the low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a significant increase in SIRT1 protein expression by 1.2-fold ($p<0.01$) and 1.4-fold ($p<0.001$) respectively when compared to the FFA-treated control group. In contrast, both the low alcohol (n=3) and moderate alcohol treatments (n=3) in normal cells showed a non-significant decrease ($p=0.10$ to 0.11) change in SIRT1 protein expression when compared to the normal control group.

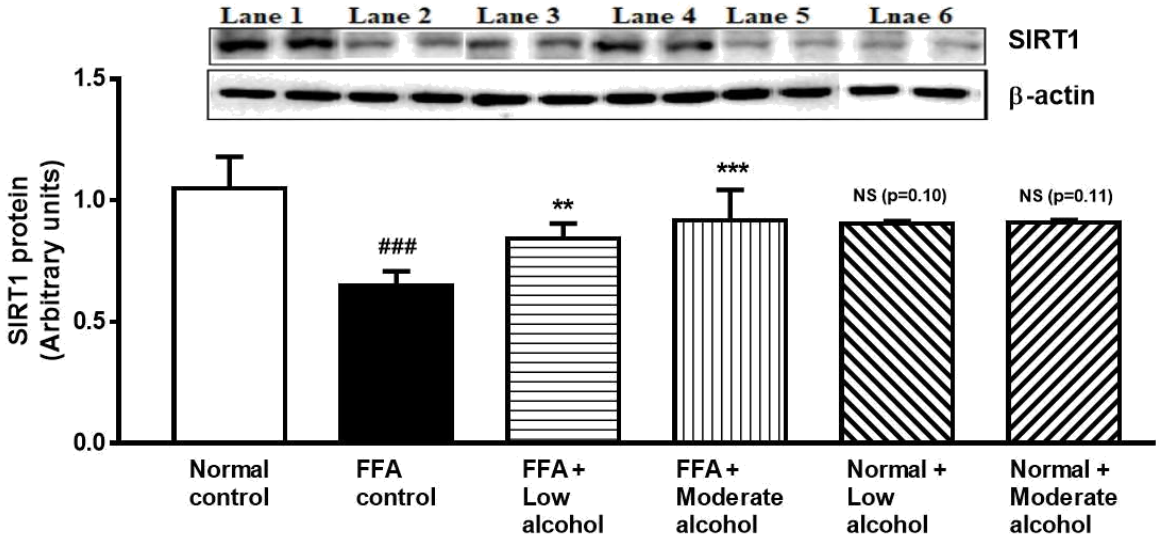

Figure 4.4 Protein expression of SIRT1 in FFA-treated HepG2 cells. SIRT1 protein expression as determined by western blot analysis in HepG2 cells treated with 1 mM of FFA for 24 hours either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Each bar represents the mean ± SEM of n=6 & 3 for normal cells treated with low and moderate alcohol groups calculated relative β-actin used as internal control. Significant difference from normal control: $^{###}p<0.001$
Significance difference from FFA control: **p<0.01, ***p<0.001
NS: No significant difference between the groups in comparison
FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

4.3.5 Effect of low and moderate alcohol on ACC and its phosphorylation in FFA-treated HepG2 cells

The expression of ACC protein in HepG2 cells is shown in Figure 4.5A. The FFA-treated control group (n=6) showed a significant ($p<0.001$) 1.4-fold increase in ACC protein expression when compared to the normal control group (n=6). On the other hand, the moderate alcohol-treated group (n=6) showed a significant 1.1-fold ($p<0.01$) decrease in ACC protein expression when compared to the FFA-treated control group. However, the low alcohol-treated group (n=6) showed a mild non-significant ($p=0.12$) decrease in ACC protein expression when compared to the FFA-treated group. In contrast, normal cells treated with either low alcohol (n=3) moderate alcohol (n=3) treated groups showed a non-significant ($p=0.15$ to 0.69) increase in ACC protein expression when compared to the normal control cells.

Figure 4.5B shows the effect of low and moderate alcohol on phosphorylation of ACC in HepG2 cell**.** The FFA-treated group (n=6) showed a significant ($p<0.001$) 1.9-fold decrease in phosphorylation of ACC protein when compared to the normal control group (n=6). The moderate alcohol-treated group (n=6) showed a significant ($p<0.01$) 1.3-fold increase in phosphorylation of ACC protein when compared to the FFA-treated cells. However, the low alcohol treated group (n=6) showed a non-significant ($p=0.17$) increase in phosphorylation of ACC protein when compared to FFA-treated group. In contrast, both the low alcohol-treated (n=3) and moderate alcohol-treated (n=3) normal cell groups showed a non-significant decrease ($p=0.62$ to 0.77) decrease in phosphorylation of ACC protein when compared to the normal control group.

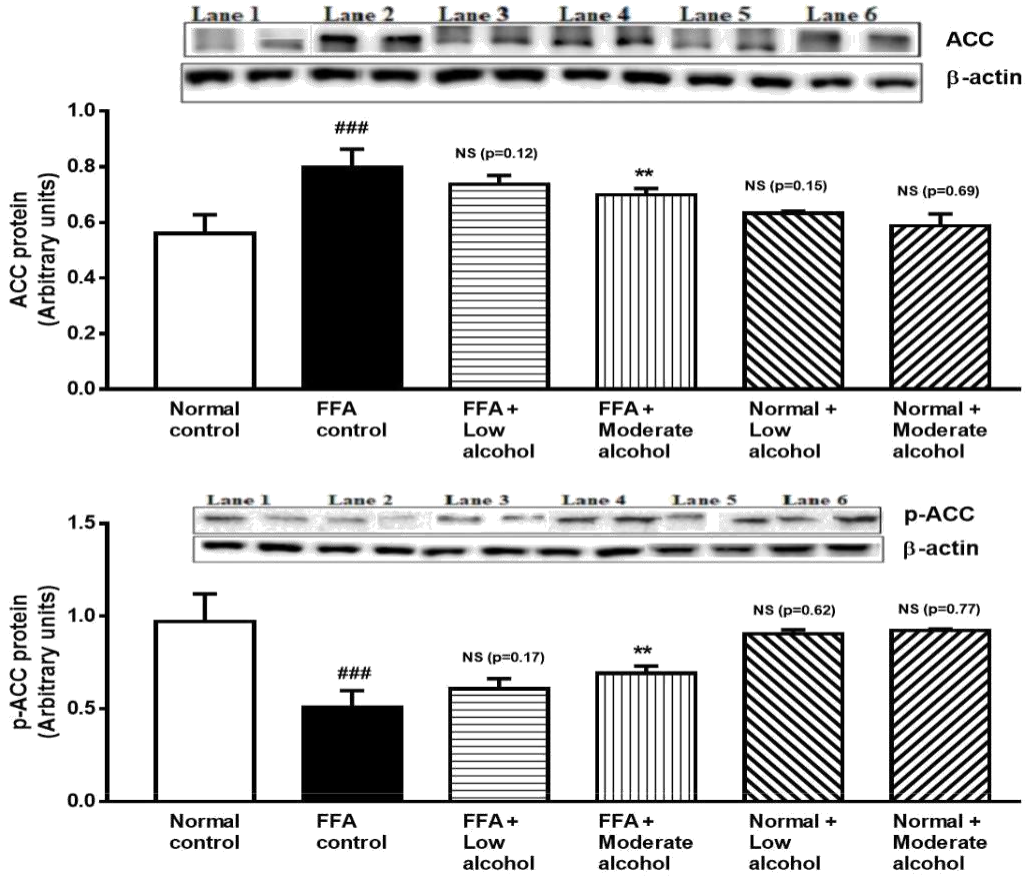

Figure 4.5 Protein expression of ACC and p-ACC in FFA-treated HepG2 cells. (A) ACC (B) p-ACC protein expression as determined by western blot analysis in HepG2 cells treated with 1 mM of FFA for 24 hours either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Each bar represents the mean ± SEM of n=6 & 3 for normal cells treated with low and moderate alcohol groups calculated relative β-actin used as internal control. Significant difference from normal control: $^{###}p<0.001$
Significance difference from FFA control: **p<0.01
NS: No significant difference between the groups in comparison
FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

4.3.6 Effect of low and moderate alcohol on SREBP1 protein expression in FFA-treated HepG2 cells

The SREBP1 protein expression in different experimental groups is shown in Figure 4.6. The FFA-treated cell group (n=6) showed a significant ($p<0.01$) 1.3-fold increase in SREBP1 protein expression when compared to the normal cell group (n=6). Interestingly, the low alcohol-treated cells (n=6) showed a mild increase in SREBP1 protein expression although not significant ($p=0.32$) when compared to the FFA-treated group. On the other hand, the moderate alcohol-treated group (n=6) tended to show a decrease in SREBP1 protein expression although not significant ($p=0.40$) when compared to the FFA-treated control group. In contrast, normal cells treated with either low or moderate alcohol (n=3) showed no significant difference ($p=0.87$ to 0.69) in SREBP1 protein expression when compared to the normal cells.

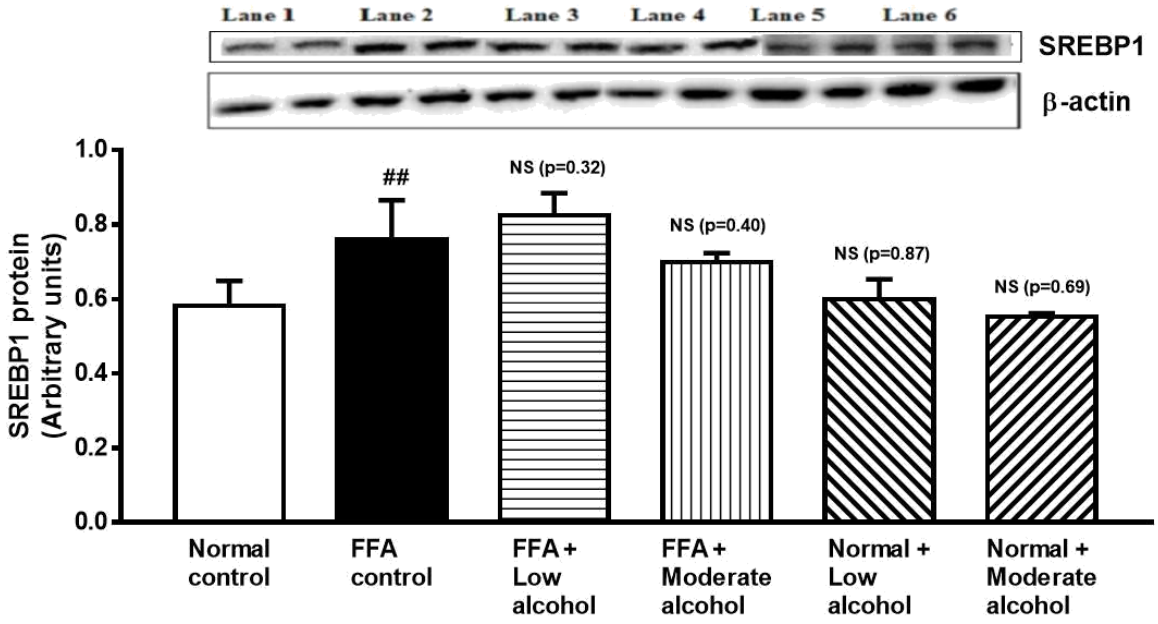

Figure 4.6 Protein expression of SREBP1 in in FFA-treated HepG2 cells. Protein expression as determined by western blot analysis in HepG2 cells treated with 1 mM of FFA for 24 hours either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Each bar represents the mean ± SEM of n=6 & 3 for normal cells treated with low and moderate alcohol groups calculated relative β-actin used as internal control. Significant difference from normal control: $^{\#\#}p<0.01$
NS: No significant difference between the groups in comparison
FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

4.4 Discussion

NAFLD is a leading cause of hepatic dysfunction with intracellular lipid accumulation in hepatocytes in the absence of alcohol. It was first officially reported in 1958, gradually become a widespread and serious disease with an average annual increase of 20% [398]. The combination of genetic factors with external environmental and metabolic stress results in the pathogenesis of NAFLD and the overwhelming theory for the molecular mechanism of NAFLD is the two-hit theory [399, 400]. The first hit is the lipid accumulation in the liver. Fatty degeneration of hepatocytes is more susceptible to 'second' hit (oxidative stress, *etc*.) than normal one, which further promotes the generation of inflammation that is the development of NAFLD. Cellular FFA loading is commonly utilised to develop *in-vitro* models of steatosis, and these models can reliably reproduce key features of hepatic steatosis in humans [401]. Insulin resistance which is commonly associated in NAFLD, along with the resulting hyperinsulinemia leads to increase liver FFA synthesis via *de novo* lipogenesis, along with the increased delivery of FFA from adipose tissue to liver which impairs the FFA β-oxidation. FFA synthesis is essential for the regulation of fat metabolism in the liver. FFA synthesis is mediated by seven enzymes, among which acetyl coenzyme A carboxylase (ACC) is the rate-limiting enzyme. In the present study, HepG2 cells were supplemented with excess FFAs in hepatocytes that results in hepatic steatosis. This was a successful model for hepatic steatosis; the observations are similar with previously published results showing an increased level of intracellular TG and TC [402-404]. Similar to *in vivo* studies (chapter 2), low and moderate alcohol with FFA groups did not show significant ability to control lipid accumulation in HepG2 cells stimulated with FFA.

Jimenez et al.(2002) has demonstrated that HepG2 cells treated with high concentration of alcohol 100 mM for 7 days, which did not show any effect on lipids HepG2 cells, but in contrast our results showed that low and moderate alcohol increases lipid levels in HepG2 cells [405]. Accumulation of triglycerides in liver cells is the first step for hepatic steatosis, which may progress to non-alcoholic steatohepatitis (NASH), which is associated with hepatocyte damage, chronic inflammation, and fibrosis, and may progress to cirrhosis and liver failure. Henriksen et al (2013) has demonstrate that activation of AMPK by AICAR reduces the hepatic triglycerides which protects the liver from hepatic steatosis [394]. Adenosine monophosphate-activated protein kinase (AMPK) is a cellular nutrient sensor and a central nutrient sensor and regulator of energy homeostasis in most tissues and organs including adipose tissue, skeleton muscle and the liver. AMPK regulates hepatic energy metabolism through regulating the genes involved in lipid metabolism or direct phosphorylation of metabolic proteins or enzymes, such as sterol regulatory element binding protein 1(SREBP-1), carbohydrate-responsive element-binding protein (ChREBP), HMG-CoA reductase (HMGCR), acetyl-CoA carboxylase-1(ACC1), and fatty acid synthase (FAS) [406]. In the present study, FFA treated cells managed to decrease AMPK and p-AMPK levels in disease control groups [407]. An increase in AMPK and p-AMPK protein expression was observed in moderate alcohol-treated normal and moderate alcohol with FFA groups. In contrast, an increase in AMPK was observed in normal cells treated with low alcohol.

HepG2 cells, which have a very weak capability of metabolizing alcohol due to the lack of alcohol dehydrogenase (ADH) and cytochrome P450(CYP2E1) [408, 409] resulting in the lack of producing acetate, the end product of alcohol metabolism which enhances AMPK. A very limited studies demonstrated that long-term exposure (12 months) to high concentrations of alcohol can stimulate cultured HepG2 liver cells to significantly increase the expression and enzymatic activity of key alcohol-metabolizing enzymes, i.e., alcohol dehydrogenase and cytochrome P450 2E1 [410, 411]. The possible hypothesized mechanism behind the increase of phosphorylation of AMPK in moderate alcohol group might be either due to activation by LKB1 an upstream kinase for activating AMPK or CaMKK-β which play a physiological role in activating AMPK in mammalian cells [412]. Increase in LKB1 and p-LKB1 was observed in normal cells treated with moderate alcohol, and moderate alcohol with FFA which further confirms that the activation of AMPK is by LKB1 pathway.

In our study, an incresase in SIRT1 was observed in low and moderate alcohol with FFA groups and no change in SIRT1 was observed in alcohol treated normal cells. Sirtuin-1 (SIRT1) is an NAD^+-dependent class III histone deacetylase that acts as a metabolic “sensor,” and is implicated in regulating a wide range of intracellular processes including aging, DNA repair, apoptosis, inflammation, and energy production and storage[413] . SIRT1 activity is regulated by the imbalance of the NAD^+-NADH ratio, and accumulation of the NAD^+ degradation product, nicotinamide (NAM) [414]. Thompson et al.(2015) [415] has proved that no change in SIRT1 was observed in HepG2 cells treated with 0-100

mM of alcohol for 24 and 48 hours. The no change in SIRT1 in HepG2 cells might be due to very low levels of ADH and CYP2E1. The other possible mechanism behind the increase in SIRT1 in low and moderate alcohol treated group might be due to the increase of AMPK. An AMPK activation leads to SIRT1 activation by increasing NAD^+ levels [48].

The hypothesized mechanism behind the increase of LKB1 was, via AMPK, and SIRT1, they regulates each other and increased SIRT1 increases LKB1 and LKB1 regulate each other. Berghein et.al (2008). has demonstrated that SIRT1 lead to deacetylation of one (Lys-48) or more key lysine residues on LKB1. This in turn enhances LKB1 binding to STRAD and MO25, which increases its kinase activity and leads to the phosphorylation and activation of AMPK, MARK1, and possibly other LKB1 targets [46] .

ACC is the enzyme that catalyzes the reaction forming malonyl CoA from acetyl CoA and constitutes the initial step in lipid synthesis [416]. An increase in phosphorylation of ACC was observed in moderate alcohol with FFA. The increased p-AMPK inhibits acetyl-CoA carboxylase (ACC) and results in reduced levels of malonyl-CoA product. Malonyl CoA is a substrate in the *de novo* synthesis of fatty acids and fatty acids elongation. Importantly, it is also an inhibitor of the carnitine palmitoyl transferase-I, required for the transfer of primed cytosolic fatty acids into the mitochondria where they can undergo degradative beta-oxidation [341].

In contrast to previously published results, an increase in p-AMPK and SIRT1 in moderate alcohol with FFA group did not show any effect in reducing

sterol regulatory element binding protein (SREBP1) [407]. SREBP1 is a key lipogenic transcription factor that is nutritionally regulated by glucose and insulin [417]. SREBP-1c preferentially regulates the lipogenic process by activating genes involved in fatty acid and triglyceride synthesis. The dysregulation of SREBP-1c has been implicated in the pathogenesis of hepatic steatosis, dyslipidaemia, and type 2 diabetes [418].

In conclusion, HepG2 cells treated with FFA showed an increase in triglycerides and total cholesterol leads to confirmation of hepatic steatosis, whereas the alcohol-treated groups did not show any effect in controlling the lipids, but in contract the moderate alcohol with FFA group upregulated the expression levels of fatty acid oxidation genes including LKB1, SIRT1 and phosphorylation of AMPK-α1 proteins and down regulated the ACC protein involved in lipid synthesis induced by FFA. LKB1, AMPK, and SIRT1 regulate each other, So a further examination of a possible linkage of each protein among SIRT1, AMPK, and LKB1 proteins by using specific inhibitors studies.

CHAPTER-5

Evaluating the mechanistic action of low and moderate alcohol on FFA-induced NAFLD in HeLa cells: An LKB1 inhibitory study

5.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is a clinic-pathological entity, which is rapidly increasing and one of the most common liver diseases worldwide [419]. It is characterized by hepatic lipid accumulation (> 5%) of liver weight. NAFLD encompasses from steatohepatitis (NASH), cirrhosis, and hepatic cellular carcinoma (HCC) [420]. The pathogenies of NAFLD was proposed by a simplistic two-hit hypothesis [185]. Nowadays, a multiple-hit hypothesis that implicates a myriad of factors acting in a parallel and synergistic manner in individuals with a genetic predisposition is the more accepted view to explain the different phenotypes observed clinically [421]. The accumulation of excessive toxic lipids in the liver, including triglycerides, free fatty acids (FFA), ceramides, and free cholesterol leads to Hepatic steatosis [422]. This can occur from the excessive importation of FFA from adipose tissue, from diminished hepatic export of FFA (secondary to reduced synthesis or secretion of very low-density lipoprotein [VLDL]), or impaired beta-oxidation of FFA. The major sources of triglycerides are from fatty acids stored in adipose tissue and fatty acids newly made within the liver through de novo lipogenesis [423].

Previous studies have demonstrated that impairment of hepatic AMPK levels was observed in both *in vitro* and *in vivo* models of NAFLD [424-426]. Impairment of hepatic AMPK leads to the development of insulin resistance and metabolic disorders associated with metabolic syndrome, including hepatic steatosis [427]. Decreased AMPK may lead to increase lipid synthesis and decreases the β- oxidation. Activation of AMPK, stimulates the ATP producing

catabolic pathways, such as fatty acid oxidation and inhibits ATP consuming process associated with lipogenesis, such as acetyl-CoA carboxylase (ACC) by phosphorylation in the liver [428]. Under fasting conditions, AMPK reduces lipogenesis by suppressing sterol regulatory element binding protein-1c (SREBP-1c) activity in the liver [363]. Phosphorylation of AMPK at Thr172 reflects the degree of AMPK activation. AMPK responds to an increased cellular AMP: ATP ratio and the phosphorylation of AMPK is also regulated by the upstream kinase Liver kinase B1(LKB1) and calcium/calmodulin -dependent protein kinase (CaMKK) in mammalian cells [429, 430]. LKB1 is serine/threonine kinase which was originally found in tumour suppressor function [431]. Apart from phosphorylation and activation of AMPK, LKB1 activates the other members of the AMPK subfamily [432, 433]. A numerous previous studies have proven that AMPK is activated by LKB1 and could be a target for treatment of metabolic disorders [104, 185, 434, 435].

Based on the literature, and our findings from previous chapters 3 and 4 we hypothesize that LKB1, the upstream kinase might be responsible for activating AMPK. This chapter aims to find the role of low and moderate alcohol in HeLa cells (LKB1 deficient cells) on activating AMPK and SIRT1.

5.2 Materials and methods

5.2.1 Chemicals used

Electrophoresis and electro-blotting consumables were purchased from Bio-Rad (Hercules, CA, USA). Primary antibodies of AMPK, p-AMPK, p-ACC, SIRT1 and SREBP1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) while ACC was obtained from (Abcam, Cambridge, United Kingdom). Enhanced chemiluminescence kit was obtained from Bio-Rad (Hercules, CA, USA). DMEM (modified basal medium eagle), low glucose, alcohol, Fetal bovine serum (FBS), sodium oleate, sodium palmitate from Sima (Sigma St. Louis, MO, USA). All other chemicals used were of analytical or molecular biology grade.

5.2.2 Reagents used

(1) Radio immunoprecipitation assay (RIPA) buffer (pH 8.0)

Sodium chloride (150 mM), sodium deoxycholate (0.5%), sodium dodecyl sulfate (0.1%), Tris base (50 mM) and 1 mL of triton X-100 (1%) were added into a volumetric flask and made up to 100 mL with distilled water. The solution was transferred into a beaker and the pH was adjusted to 8.0 and filtered before use through a 0.2 µM membrane.

(2) Running buffer

Glycine (14.42 g), tris base (3.03 g), sodium dodecyl sulfate (1 g) were added into a volumetric flask and made up to 1000 mL with distilled water. The

solution was transferred into a beaker and the pH was adjusted to 8.3 and filtered before use through a 0.2 μM membrane.

(3) Transfer buffer

Tris base (3.03 g), glycine (14.41 g) and methanol (200 mL) were added into a volumetric flask and made up to 1000 mL with distilled water. The solution was filtered before use through a 0.2 μM membrane.

(4) Tris-buffered saline (TBS)

Tris-base (2.4 g) and NaCl (8 g) were added into a volumetric flask and made up to 1000 mL with distilled water. The solution was transferred into a beaker and the pH was adjusted to 7.6 and filtered before use through a 0.2 μM membrane.

(5) Phosphate buffered saline (PBS)

Sodium chloride (8 g), potassium chloride (0.2 g), disodium hydrogen phosphate (1.44 g), and potassium dihydrogen phosphate (0.24 g) were added into a volumetric flask and made up to 1000 mL with distilled water. The solution was transferred into a beaker and the pH was adjusted to 7.6 and filtered before use through a 0.2 μM membrane.

(6) *Stacking gel buffer*

Tris base (15.14 g), SDS (1.0 g) were added into volumetric water and made up to 250 ml with distilled water. The solution was transferred into a beaker and the pH was adjusted to 6.8 and filtered before use through a 0.2 μM membrane.

(7) *Sodium oleate*

Sodium oleate (40.58 mg) in 10 mL (13.33 mM) stock of plain DMEM at 37ºC with fatty acid-free BSA and filtered with a 0.2 µM membrane and stored in - 20°C

(8) *Sodium palmitate*

Sodium palmitate (18.54 mg) in 10 mL of plain DMEM at 50 ºC with constant shaking with Fatty acid-free BSA and filtered with a 0.2 µM membrane and stored in -20°C.

(9) *400 mM alcohol*

Alcohol 184.28 mg or 235 µL of alcohol in 10 mL of plain DMEM for 400 mM stock and filtered with a 0.2 µM membrane and stored in 4ºC.

5.2.3 Cell culture

HeLa cells (passage-9) were obtained from the Western Sydney University, which was previously maintained in DMEM with low glucose, 10% FBS, 1% antibiotics (10,000 I.U./mL Penicillin, 10,000 (µg/mL) Streptomycin). They were maintained in incubator conditions, at 37º C with 5% CO_2 changing the growth medium for every two days. After 70-80% confluence of cells, they were washed thrice with PBS and then added 3-5ml of Trypsin with EDTA. After the cells completely detach, they were added into DMEM to neutralise the Trypsin, and the cells were centrifuged at 300 g for 3 minutes. The subculture was done with a ratio

of 1:3 or 1:6. The remaining cell were stored with 5% DMSO in growth medium and stored in liquid nitrogen vapour phase.

5.2.4 Experimental design and treatments

HeLa cells were grown up to 70% confluence and then transferred into 6 well plates at $2*10^6$ cells per well and divided into six groups. Control cells were incubated with plain medium, and the free fatty acid group received (sodium oleate 0.66 mM and 0.33 mM of Sodium palmitate final fatty acids concentration 1 mM). The treatment groups received low (10 mM) and moderate alcohol (20 mM) with FFA (1 mM of sodium oleate 0.66 mM and 0.33 mM of Sodium palmitate)[395]. The rest of the groups received 10 and 20 mM of alcohol without any FFA treatment. Alcohol is a volatile substance in order to maintain a stable alcohol concentration inside the 6 well plate, for the cells, double the concentration of alcohol was placed in a Petri dish during the 24-hour experiment. After 24-hour of treatment, the cells were removed from the incubator and proceeded for lipid extraction and cell protein extraction.

5.2.5 Extraction of lipids from HeLa cells

The total lipids from Hela cells were extracted by the modified method of Bligh and Dyer [396] as described by Li and Lin [252]. The treated cells were homogenized with chloroform-methanol solution (chloroform-methanol-water,8:4:3). Further, the resulting mixture was shaken at 37ºC for 1-hour and then centrifuged at

1,100 g for 10 minutes. The bottom layer was collected and again centrifuged, the supernatant was collected and used for analysis of hepatic lipid.

5.2.6 Cell protein extraction

After 24-hours of treatment 6 well plates with cells were removed from the incubator and washed with PBS. Radioimmunoprecipitation assay (RIPA) buffer (pH 8.0) containing 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 10 l/ml protease and phosphatase inhibitors cocktail were added to each well and cells were scraped on ice over an orbital shaker for 1hour. The homogenate was then collected and sonicated at 60 amps of total time for 30 seconds with on time of 5 seconds and off time 15 seconds. The homogenates were centrifuged at 4°C at 10,000 g for 15 minutes, and the supernatants were collected. Protein concentrations were measured by the Bradford assay using bovine serum albumin as standard.

5.2.7 Western blot analysis

The samples were mixed with loading buffer, proteins were denatured by heating at 95^{o}C for 5 min, and 25 g of total protein was electrophoretically resolved on 10% lab made gels at 135 V for 90 min and then transferred onto a nitrocellulose membrane (Bio-Rad, Australia) using a wet transfer (100 V for 2-hour 15 minutes). After blotting, the membranes were blocked with 5% non-fat dry milk 1-hour at room temperature. The membranes were then washed three times for 5 minute each with Tris-buffered saline-0.1% Tween (TBST, pH 7.6) and incubated for overnight at 4 C with mouse anti-p-ACC reductase (1:1000) or with rabbit anti-p-

AMPK reductase (1:1000) or with mouse anti-AMPK reductase (1:1000) or mouse anti-SIRT1 (1:1000) or mouse anti- SREBP1(1:1000) antibody (Santa Cruz, Biotechnology, CA, USA) , or rabbit anti-ACC (1:1000) (Abcam, Cambridge, UK) diluted with TBST. Blots were then washed again three times for 5 minutes each with TBST and incubated for 1-hour at room temperature with appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz, Biotechnology, CA, USA) diluted at 1:10,000 with PBS, (pH 7.4). The membranes were again washed three times for 5 minutes each with TBST and incubated with enhanced chemiluminescence reagent (Clarity™ Western ECL, Bio-Rad, Australia) for 1 minute at room temperature. Immune complexes were detected after exposing the blots to ChemiDoc™ XRS system (Bio-Rad, Australia) for various time points. Quantitative image analysis was performed using NIH Image software (Image J) to determine the intensity of the protein signal, which was expressed relative to the amount of -actin used as an internal control.

5.2.8 Data and statistical analysis

The results are expressed as means ± SEM. To analyse the quantitative differences among the experimental groups before or after treatments, the data were subjected to analysis of variance (ANOVA) using the GraphPad 7.03 (GraphPad Software Inc., California, CA, USA) statistical software. Post-hoc comparisons were made using Dunnett's multiple comparisons test.

5.3 Results

5.3.1 Effect of low and moderate alcohol on triglycerides and total cholesterol levels in FFA-treated HeLa cells

The levels of triglycerides in different experimental groups are shown in Figure 5.1A. The FFA-treated group (n=6) showed a significant ($p<0.001$) 1.5-fold increase in triglycerides levels when compared to the normal control cells (n=6). On the other hand, both the low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a non-significant ($p= 0.72$ to 0.29) decrease in triglycerides levels when compared to the FFA-treated control group. In contrast, the low alcohol-treatment in normal cells (n=6) showed a non-significant ($p=0.57$) increase in triglycerides accumulation when compared to the normal control group. Whereas, the normal cells treated with moderate alcohol group (n=6) showed a non-significant ($p=0.36$) decrease in triglyceride accumulation, when compared to the normal control group.

The total cholesterol levels in different experimental groups are shown in Figure 5.2B. The FFA-treated group (n=6) showed a significant ($p<0.001$) 1.5-fold increase in total cholesterol levels when compared to the normal control cells (n=6). On the other hand, both the low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a non-significant ($p= 0.70$ to 0.27) decrease in total cholesterol levels when compared to the FFA-treated control group. In contrast, the normal cells treated with low alcohol-treated group (n=6) showed a non-significant ($p=0.55$) increase in total cholesterol levels when compared to the normal control group. Whereas, the moderate alcohol-treatment in normal cells (n=6) showed a

non-significant (p=0.48) decrease in total cholesterol when compared to the normal control group.

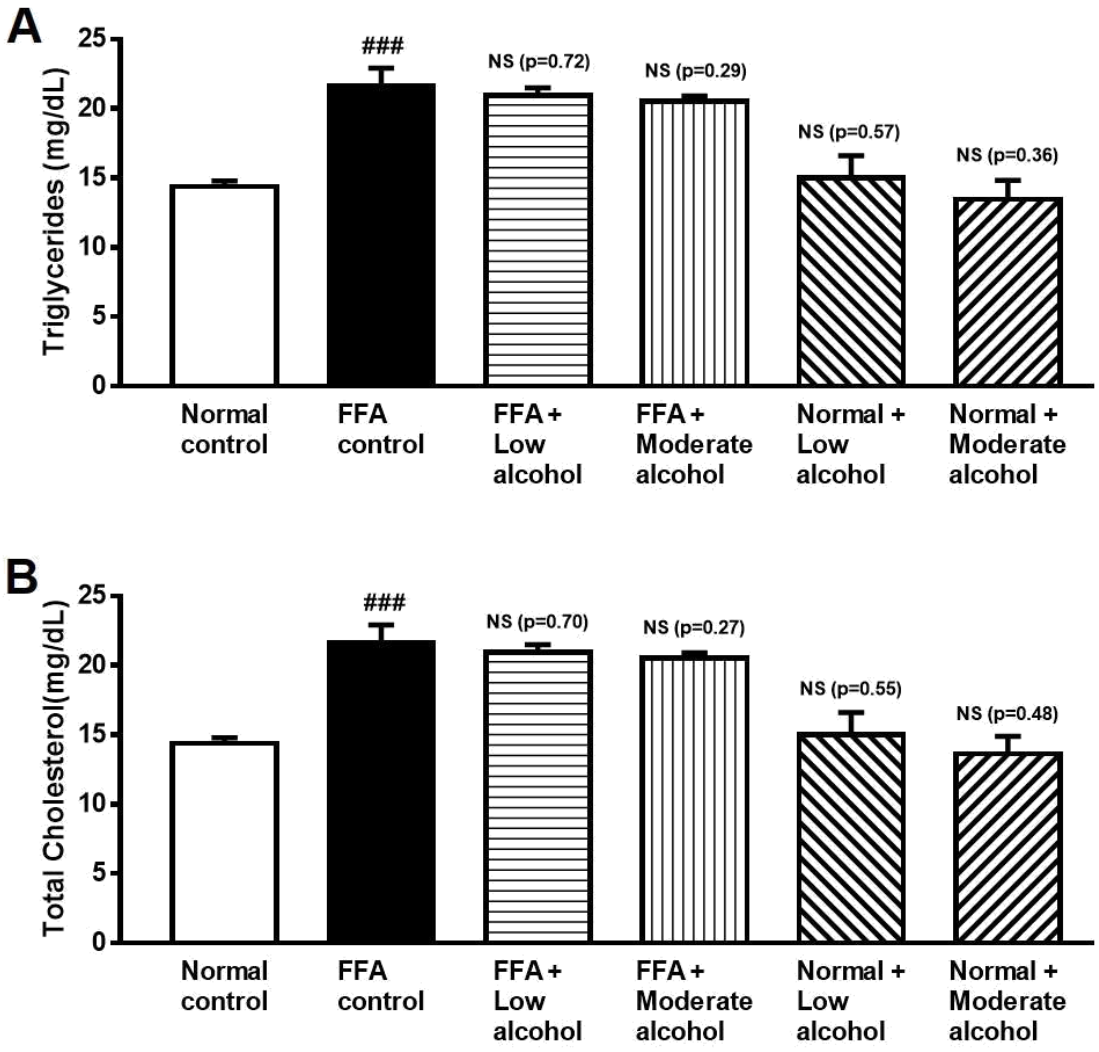

Figure 5.1 Effect of low and moderate alcohol on triglycerides and total cholesterol levels in FFA-treated HeLa cells. (A) triglycerides (B) total cholesterol accumulation in HeLa cells incubated with FFA 1mM mixture final concentration for 24 hours either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Values represent the mean ± SEM of n=6

Significant difference from normal control cells $^{###}p<0.001$,

NS: No significant difference between the groups in comparison

FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

5.3.2 Effect of low and moderate alcohol on AMPK-α1 and its phosphorylation in the FFA-treated HeLa cells

The effect of low and moderate alcohol on the protein expression of AMPK-*α*1 in HeLa cells is shown in Figure 5.3A. The FFA-treated HeLa cells (n=6) showed a significant ($p<0.001$) 1.4-fold decrease in AMPK-*α*1 protein expression when compared to the normal control group (n=6). The low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a mild non-significant increase in AMPK *α*1 protein expression by 1.0-fold ($p=0.97$) and 1.1-fold ($p=0.22$) respectively when compared to the normal control group. In contrast, the low alcohol-treated normal cells (n=3) showed a significant 1.1-fold ($p<0.05$) decrease in AMPK-*α*1 protein expression when compared to the normal control group. Whereas, the normal cells treated with moderate alcohol (n=3) showed a non-significant ($p=0.47$) decrease in AMPK *α*1 levels when compared to the normal control group.

Figure 5.4B illustrates the effect of low, and moderate alcohol on phosphorylation of AMPK-α1 in HeLa cells. The FFA-treated control group (n=6) showed a significant ($p<0.001$) 1.3-fold decrease in phosphorylation of AMPK-α1 protein when compared to the normal control cells (n=6). On the other hand, the low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a mild non-significant ($p=0.17$ to 0.16) increase in phosphorylation of AMPK-α1 protein when compared to the FFA-treated control group. In contrast, the normal cells treated with low alcohol-treated group (n=3) showed a significant 1.1-fold ($p<0.05$) decrease in phosphorylation of AMPK-α1when compared to the normal control group. Whereas, the moderate alcohol-treated normal HeLa cells (n=3)

showed a non-significant ($p=0.05$) decrease in phosphorylation of AMPK-α1 protein when compared to the normal control group.

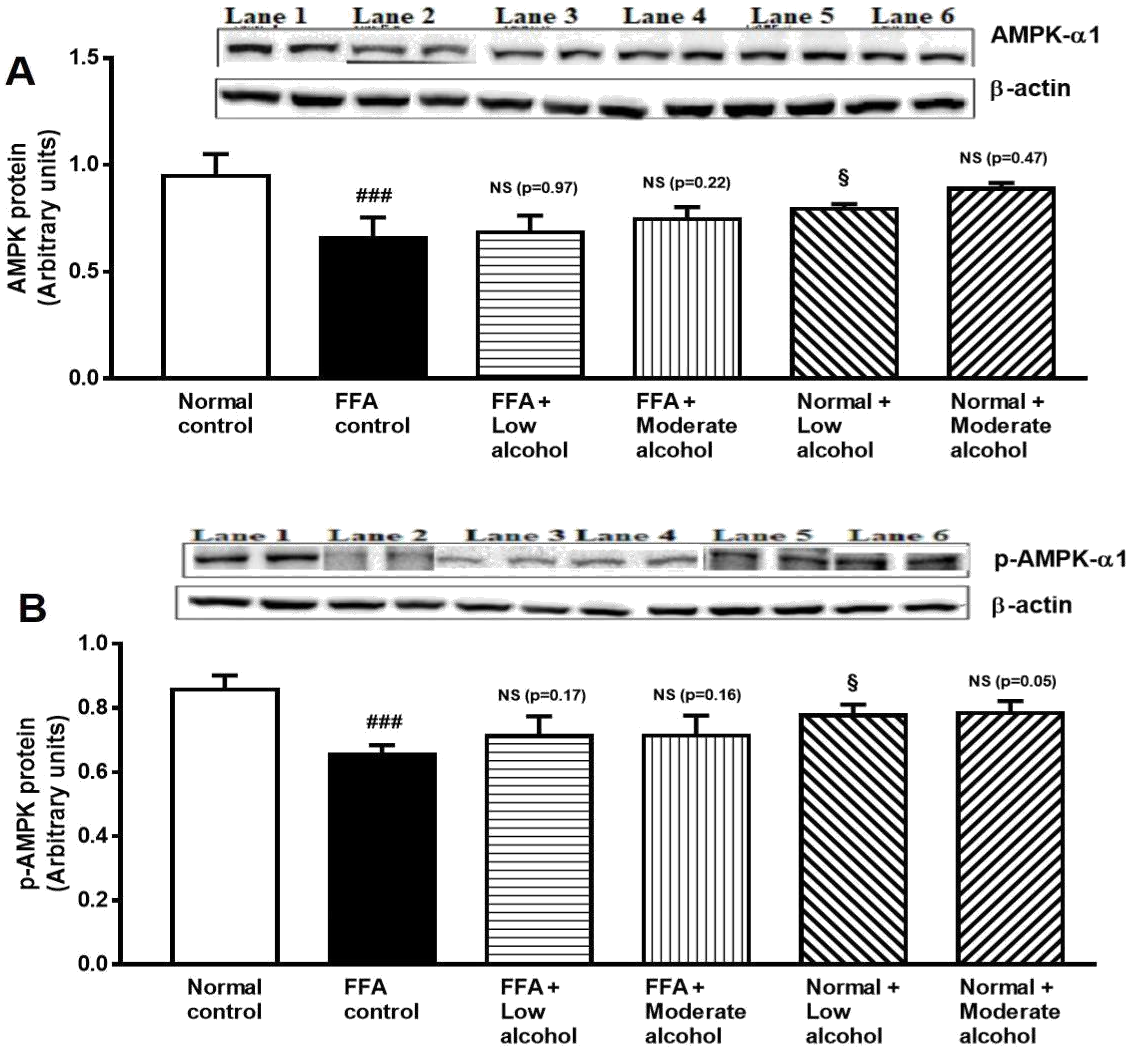

Figure 5.2 Protein expression of AMPK and p-AMPK-α1 in FFA-treated HeLa cells. (A) AMPK (B) p-AMPK-α1 protein expression as determined by western blot analysis in HeLa cells treated with 1 mM of FFA for 24 hours either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Each bar represents the mean ± SEM of n=6 & 3 for normal cells treated with low and moderate alcohol groups calculated relative β-actin used as internal control. Significant difference from normal control: $^{###}p<0.001$
Significant difference from normal control group vs normal cells treated with alcohol groups: $^{§}p<0.05$,
NS: No significant difference between the groups in comparison
FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

5.3.3 Effect of low and moderate alcohol on SIRT1 protein expression in FFA-treated HeLa cells

The effect of low and moderate alcohol on the protein expression of SIRT1 in FFA-treated HeLa cells is shown in Figure 5.3. The FFA-treated control group (n=6) showed a significant ($p<0.05$) 1.3-fold decrease in SIRT1 protein expression when compared to the normal control cells (n=6). On the other hand, both the low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a mild non-significant ($p= 0.95$ to 0.99) increase in SIRT1 protein expression when compared to the FFA-treated control group. In contrast, the low alcohol (n=3) and moderate alcohol (n=3) treated normal cells showed a non-significant decrease in SIRT1 protein expression by 1.2-fold ($p=0.15$) and 1.2-fold ($p=0.10$) respectively when compared to the normal control group.

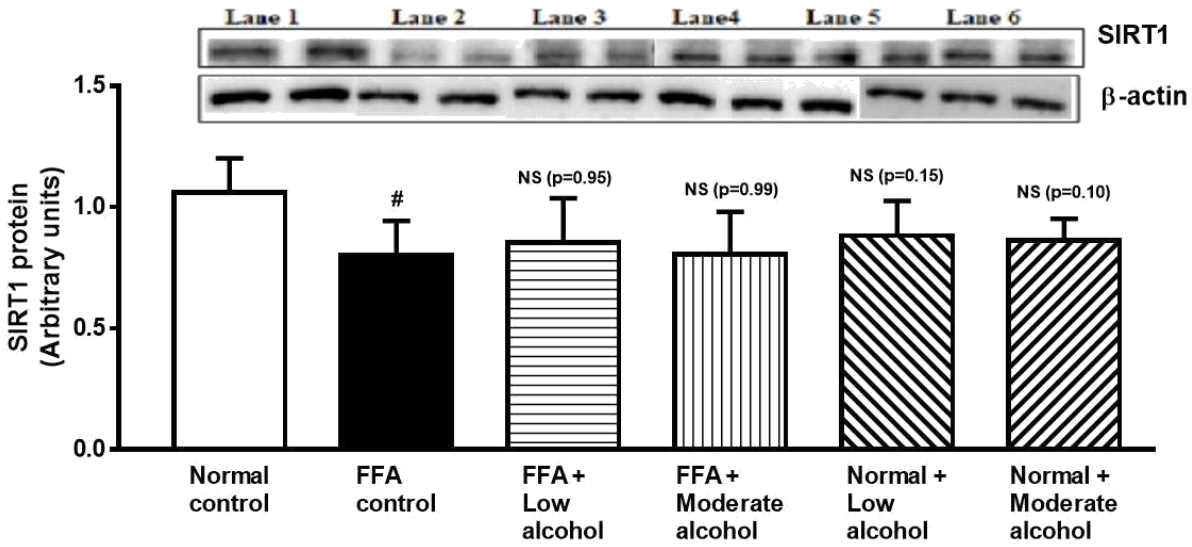

Figure 5.3 Protein expression of SIRT in FFA-treated HeLa cells. SIRT1 protein expression as determined by western blot analysis in HeLa cells treated with 1 mM of FFA for 24 hours either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Each bar represents the mean ± SEM of n=6 & 3 for normal cells treated with low and moderate alcohol groups calculated relative β-actin used as internal control. Significant difference from normal control: $^{\#}p<0.05$

NS: No significant difference between the groups in comparison

FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

5.3.4 Effect of low and moderate alcohol on ACC-α1 and its phosphorylation in the FFA-treated HeLa cells

The expression of ACC protein in HeLa cells was shown is shown in Figure 5.4A. The FFA-treated control group (n=6) showed a significant ($p<0.01$) 1.6-fold increase in ACC protein expression when compared to the normal control group (n=6). On the other hand, the low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a mild non-significant decrease in ACC protein expression by 1.1-fold ($p=0.26$) and 1.0-fold ($p=0.19$) respectively when compared to the FFA-treated control group. In contrast, the low alcohol (n=3) and moderate alcohol (n=3) treated normal cells showed a mild non-significant ($p=0.93$ to 0.99) increase in ACC protein expression when compared to the normal control group.

The below Figure 5.4B explains the effect of low, and moderate alcohol on phosphorylation of ACC in HeLa cells**.** The FFA-treated group (n=6) showed a significant ($p<0.001$) 1.5-fold decrease in phosphorylation of ACC protein when compared to the normal control group (n=6). The low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a non-significant ($p=0.25$ to 0.38) increase in phosphorylation of ACC protein when compared to the FFA-treated group. In contrast, the normal cells treated with low alcohol (n=3) showed a mild 1.1-fold significant ($p<0.05$) decrease in phosphorylation of ACC protein when compared to normal control group. Whereas, the moderate alcohol-treated normal group (n=3) showed a non-significant ($p=0.11$) decrease in phosphorylation of ACC expression when compared to the normal control group.

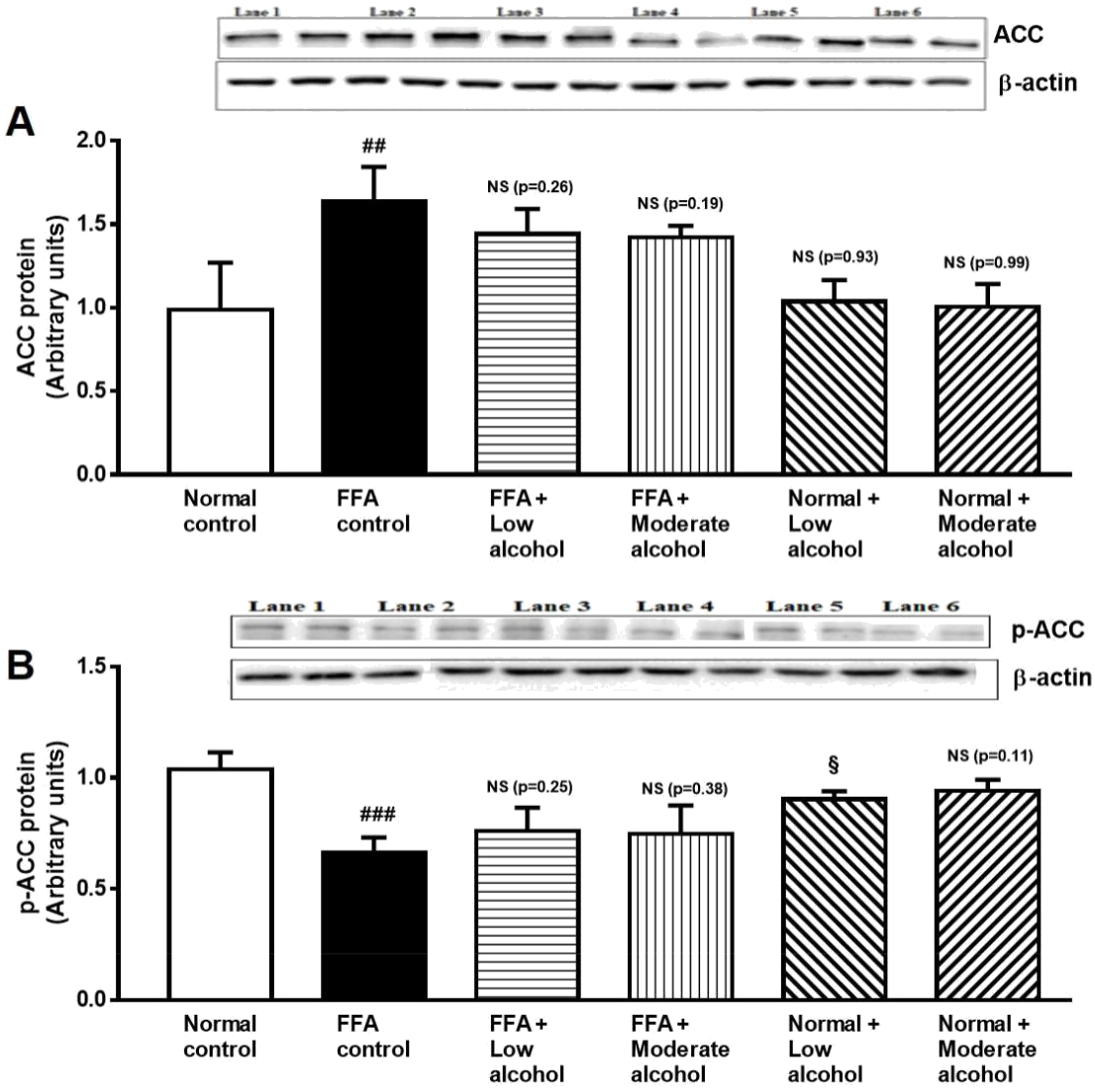

Figure 5.4 Protein expression of ACC and p-ACC in FFA-treated HeLa cells
(A) ACC (B) p- ACC protein expression as determined by western blot analysis in HeLa cells treated with 1 mM of FFA for 24hours either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Each bar represents the mean ± SEM of n=6 & 3 for normal cells treated with low and moderate alcohol groups calculated relative β-actin used as internal control. Significant difference from normal control: $^{###}p<0.001$, $^{##}p<0.01$
Significant difference from normal control group vs normal cells treated with alcohol groups: $^{§}p<0.05$,

NS: No significant difference between the groups in comparison

FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

5.3.5 Effect of low and moderate alcohol on SREBP1 protein expression in FFA-treated HeLa cells

The SREBP1 protein expression of different experimental groups was shown in Figure 5.5. The FFA-treated group (n=6) showed a significant ($p<0.001$) 1.4-fold increase in SREBP1 expression when compared to the normal control group (n=6). On the other hand, the low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a mild non-significant ($p= 0.39$ to 0.23) decrease in SREBP1 protein expression when compared to the normal control group. In contrast, the normal cells treated with low alcohol (n=3) and moderate alcohol (n=3) groups showed a non-significant ($p=0.64$ to 0.72) decrease in SREBP1 protein expression when compared to the normal control group.

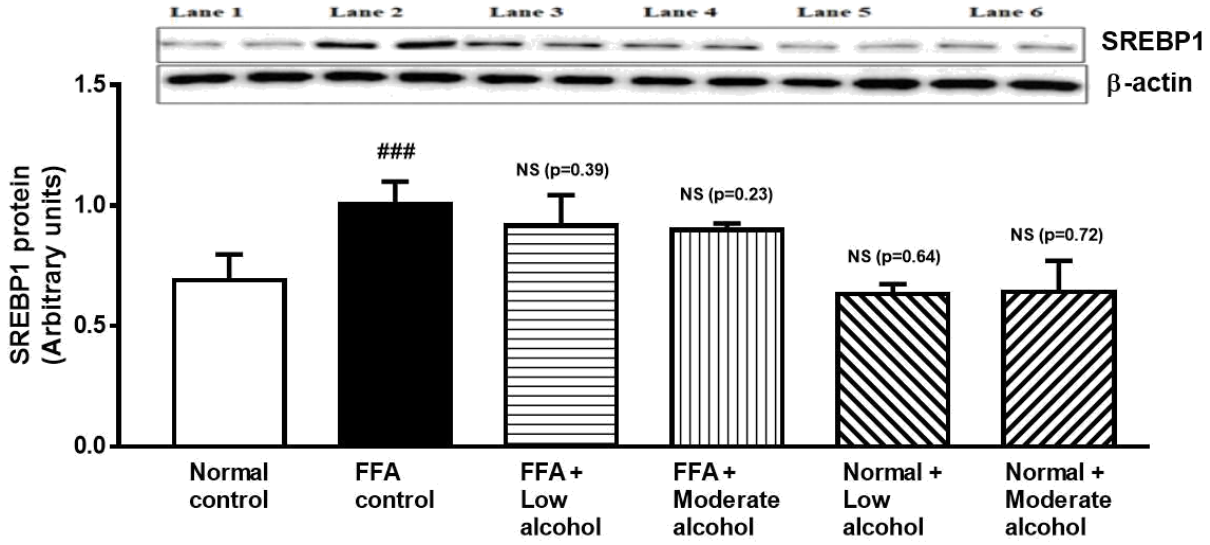

Figure 5.5 Protein expression of SREBP1 in FFA-treated HeLa cells. SREBP1 protein expression as determined by western blot analysis in HeLa cells treated with 1 mM of FFA for 24 hours either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Each bar represents the mean ± SEM of n=6 & 3 for normal cells treated with low and moderate alcohol groups calculated relative β-actin used as internal control. Significant difference from normal control: $^{###}p<0.001$

NS: No significant difference between the groups in comparison

FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

5.4 Discussion

The present study has demonstrated the role of low and moderate alcohol on protecting FFA induced NAFLD in the absence of LKB1 the upstream kinase of AMPK, which subsequently leads to AMPK and ACC phosphorylation. Since HeLa cells lack the functional LKB1, these cells were used for this study [436]. The major findings observed from our study was Hela cells treated with FFA had increased the ACC and SREBP1 proteins which are responsible for the increase in lipid synthesis. The alcohol-treated groups (low and moderate with FFA) had no significant increase in AMPK-α1 and SIRT1 proteins which play a major role in controlling ACC and SREBP1.

The possible explanation for the decrease in phosphorylation of AMPK was may be due to the absence of LKB1. The activation of AMPK is mainly done with an increase in AMP/ATP ratio, LKB1 and Ca^{2+}/calmodulin-dependent protein kinase-β (CaMKKb), the upstream kinases of AMPK. Niu et al., have demonstrated that, bioactive compounds activate AMPK in an LKB1-independent manner by increasing the AMP: ATP ratio [437]. However, alcohol studies showed that H4IIEC3 a (liver cells) treated with alcohol (50 mM) for 24 hour did not show any significant change in AMP: ATP ratio from baseline [332]. Therefore, this confirms that activation of AMPK by alcohol may be due to independent AMP: ATP ratio. Increasing evidence has shown that the phosphorylation of AMPK by natural compounds is dependent on upstream LKB1 activation for the protection of the liver from hepatotoxicity [247]. Furthermore, our previous results from chapter 4 suggests that the activation of AMPK may be dependent on the LKB1-AMPK pathway.

SIRT1 is an NAD+ dependent histone/protein deacetylase whose activity is regulated by nutrients availability. It was proposed that, in fasting conditions an increase in SIRT1 activity can be seen by increasing the abundance of NAD+ and decrease in nicotinamide and NADH, both of which inhibit SIRT1. Apart from AMPK, SIRT1 is also an important regulator of hepatic metabolism[438]. SIRT1 also has considerable interactions with upstream enzymes that regulate the activity of key cellular pathways. Acetylation of LKB1 at specific lysine residues (K48) regulates kinase activity and the activity of its protein substrates. SIRT1 deacetylates LKB1, which leads to activate the LKB1 targets such as AMPK. SIRT1 by virtue of its interaction with the LKB1 blocks the synthesis of lipids via the *de novo* synthesis pathway [439]. Both SIRT1 and AMPK are known to regulate each other and share many common target molecules, and the interaction between SIRT1 and AMPK could be reciprocal [440]. So the hypothetical activation of SIRT1 and AMPK might be due to the decrease in energy state or activation of AMPK by other means that leads to activation of SIRT1, perhaps by increasing NAD or the NAD/NADH ratio [48]. SIRT1 then deacetylates and activates LKB1, which in turn activates AMPK. The joint activation of SIRT1 and AMPK allows for the concurrent deacetylation and phosphorylation of the listed target molecules and presumably others, resulting in a decrease in metabolic syndrome associated disorders. Interestingly, an increase in LKB1 and p-LKB1 was observed in the results from previous chapters 3 and 4. This explains that activation of AMPK was done by LKB1, the lack of LKB1 in HeLa cells explains the decrease in

activation of AMPK and substantially decreases the SIRT1 activity in HeLa cells.

Activated AMPK directly inhibits the downstream enzymatic target ACC, which is involved in the synthesis of malonyl-CoA. AMPK inhibits ACC activity by phosphorylating ACC at Ser77 and Ser79, thereby stimulating fatty acid oxidation and reducing fatty acid synthesis [441]. Apart from ACC, AMPK and SIRT1 also decrease the SREBP-1c. SREBP-1c is a key lipogenic transcription factor, which is abundant in the mammalian liver [442]. Mature SREBP-1c translocate into the nucleus and upregulates SCD-1 and FAS the key enzymes required for *de novo* fatty acid and TG synthesis in hepatocyte [443]. The alcohol treated groups failed to regulate the SREBP1 protein, the possible mechanism behind the regulation of SREBP1 protein was due to decreased AMPK and SIRT1 protein expressions. The present observations showed that low and moderate alcohol treatment has no effect on phosphorylation of AMPK and SIRT1, which increase in the SREBP-1c and ACC protein expression leading to increasing lipid synthesis via *de novo* lipid synthesis leading to increase in lipid profile.

In conclusion, the present study demonstrated that low and moderate alcohol requires LKB1 for activating the AMPK and SIRT1 which regulate each other for inhibiting the SREBP-1C and ACC proteins responsible for lipid synthesis.

CHAPTER-6

Evaluating the mechanistic action of low and moderate alcohol on FFA-induced NAFLD in HepG2 cells: An AMPK inhibitory study

6.1 Introduction

The adenosine monophosphate-activated protein kinase (AMPK) has evolved as an important cellular sensor of reduced energy status that can subsequently phosphorylate its target proteins, slowing the rates of key biosynthetic processes and promoting energy producing pathways. AMPK is a master regulator of glucose and lipid metabolism. For the above reasons, AMPK has been proposed as a therapeutic target for metabolic diseases [444, 445]. It is a serine-threonine kinase comprising a catalytic α subunit and regulatory β and γ subunits. Kinase activity of α subunits is enhanced by energetic stress and posttranslational modification. AMP binds the AMPK γ subunit [147]. AMPKα1-contains complexes localised in the cytoplasm but also in the plasma membrane [446, 447].

AMPK is activated primarily by the increase in AMP/ATP ratio caused by challenges that interfere with ATP production or accelerate ATP consumption. Allosteric activation via the β-subunit and AMP mimetics increases AMPK activity independent of an energetic challenge. Once activated, AMPK inhibits ATP-consuming processes and activates catabolic processes. Apart from AMP/ATP ratio, AMPK is also activated by LKB1 and CaMKK [448]. In liver, AMPK decreases cholesterol and fatty acid synthesis and increases the fatty acid oxidation by inhibiting the enzymes acetyl-CoA carboxylase (ACC), 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase and fatty acid synthase (FAS) and activating the malonyl-CoA carboxylase (M-CoA). Moreover, it downregulates the sterol regulatory element-binding protein-1c (SREBP-1c),

which is a transcription factor for lipogenic genes. In adipose tissue, metformin inhibits lipolysis through attenuation of PKA and ERK1/2 signalling [289].

As it was confirmed by previous publications that AMPK plays a major role in mediating the NAFLD, based on the results from chapter 3 and 4, it is proven that low and moderate alcohol increase AMPK. This chapter aims to investigate the role of low and moderate alcohol against FFA induced hepatocellular lipid accumulation in AMPK inhibited HepG2 cells.

6.2 Materials and methods

6.2.1 Chemicals used

Electrophoresis and electro-blotting consumables were purchased from Bio-Rad (Hercules, CA, USA). Primary antibodies of Phospho-ACC, LKB1, Phospho-LKB1, SIRT1 and SREBP1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) while ACC was obtained from (Abcam, Cambridge, United Kingdom). Enhanced chemiluminescence kit was obtained from Bio-Rad (Hercules, CA, USA). All other chemicals used were of analytical or molecular biology grade.

6.2.2 Reagents used

(1) Radio immunoprecipitation assay (RIPA) buffer (pH 8.0)

Sodium chloride (150 mM), sodium deoxycholate (0.5%), sodium dodecyl sulfate (0.1%), Tris base (50 mM) and 1 mL of triton X-100 (1%) were added into a volumetric flask and made up to 100 mL with distilled water. The solution was transferred into a beaker and the pH was adjusted to 8.0 and filtered before use through a 0.2 µM membrane.

(2) Running buffer

Glycine (14.42 g), tris base (3.03 g), sodium dodecyl sulfate (1 g) were added into a volumetric flask and made up to 1000 mL with distilled water. The solution was

transferred into a beaker and the pH was adjusted to 8.3 and filtered before use through a 0.2 µM membrane.

(3) Transfer buffer

Tris base (3.03 g), glycine (14.41 g) and methanol (200 mL) were added into a volumetric flask and made up to 1000 mL with distilled water. The solution was filtered before use through a 0.2 µM membrane.

(4) *Tris-buffered saline (TBS)*

Tris-base (2.4 g) and NaCl (8 g) were added into a volumetric flask and made up to 1000 mL with distilled water. The solution was transferred into a beaker and the pH was adjusted to 7.6 and filtered before use through a 0.2 µM membrane.

(5) *Phosphate buffered saline (PBS)*

Sodium chloride (8 g), potassium chloride (0.2 g), disodium hydrogen phosphate (1.44 g), and potassium dihydrogen phosphate (0.24 g) were added into a volumetric flask and made up to 1000 mL with distilled water. The solution was transferred into a beaker and the pH was adjusted to 7.6 and filtered before use through a 0.2 µM membrane.

(6) *Stacking gel buffer*

Tris base (15.14 g), SDS (1.0 g) were added into volumetric water and made up to 250 ml with distilled water. The solution was transferred into a beaker and the pH was adjusted to 6.8 and filtered before use through a 0.2 µM membrane.

(7) *Sodium Oleate*

Sodium oleate (40.58 mg) in 10 mL (13.33 mM) stock of plain DMEM at 37ºC with fatty acid-free BSA and filtered with a 0.2 µM membrane and stored in -20°C.

(8) Sodium Palmitate

Sodium palmitate (18.54 mg) in 10 mL of plain DMEM at 50 ºC with constant shaking with Fatty acid-free BSA and filtered with a 0.2 µM membrane and stored in -20°C.

(9) 400 mM Alcohol

Alcohol 184.28 mg or 235 µL of alcohol in 10 mL of plain DMEM for 400 mM stock and filtered with a 0.2 µM membrane and stored in 4ºC.

(10) Dorsomorphin (Compound C) 2HCl

Compound C (10 mg) in 2 ml of plain DMEM for 10 mM stock and stored in 4ºC

6.2.3 Cell culture

HepG2 cells (passage-7) was obtained from the University of Sydney, which were previously maintained in DMEM with low glucose,10% FBS, 1% antibiotics (10,000 I.U./mL Penicillin, 10,000 (µg/mL) Streptomycin) as per mentioned in ATCC guidelines for HepG2 cells. They were maintained in incubator conditions at 37º C with 5% CO_2 changing the growth medium for every two days. After 70-80% confluence of cells they were washed thrice with PBS and then added 3-5 ml of Trypsin with EDTA. After cells completely detach, they were added with DMEM to neutralise the Trypsin and the cells were centrifuged at 300 g for 3 minutes. The

subculture was done with a ratio of 1:3 or 1:6. The remaining cell was stored with 5%DMSO in growth medium and stored in the liquid nitrogen vapour phase.

6.2.4 Experimental designs and treatments

HepG2 cells were grown up to 70% confluence and then transferred into 6 well plates at $2.5*10^5$ cells per well and divided into six groups. Cells were pre-incubated with 40 μM compound C for 30 min, and then washed with PBS and proceeded for alcohol treatment [449]. Control cells were incubated with plain medium and Free fatty acid group received (sodium oleate 0.66 mM and 0.33 mM of Sodium palmitate final fatty acids concentration 1 mM) [395]. The treatment groups received low (10 mM) and moderate alcohol (20 mM) with FFA (1 mM of sodium oleate 0.66 mM and 0.33 mM of Sodium palmitate) the rest of the groups received 10 and 20 mM of alcohol without any FFA treatment. In-order to maintain a stable alcohol concentration for the cells, double the concentration of alcohol was placed in a Petri dish during the 24 hour-experiment. After 24 hour of treatment, the cells were removed from the incubator and proceeded for lipid extraction and cell protein extraction.

6.2.5 Extraction of lipids from HepG2 cells

The total lipids from HepG2 cells were extracted by the modified method of Bligh and Dyer [396] procedure as described in Li Lin [450]. The treated cells were homogenized with chloroform-methanol solution (chloroform-methanol-water,8:4:3). The resulting mixture was shaken at 37ºC for 1 hour and then centrifuged at 1,100 g

for 10 minutes. The bottom layer was collected and again centrifuged, the supernatant was collected and used for analysis of hepatic lipid.

6.2.6 Cell protein extraction

After 24 hour of treatment 6 well plates with cells were removed from the incubator and washed with PBS. Radioimmunoprecipitation assay (RIPA) buffer (pH 8.0) containing 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 10 l/ml protease and phosphatase inhibitors cocktail were added to each well and scrape the cells kept on ice over an orbital shaking for 1hr. The homogenate was then collected and sonicated at 60 amps of total time for 30 seconds with on time of 5 seconds and off time 15 seconds. The homogenates were centrifuged at 4°C at 10,000 g for 15 minutes, and the supernatants were collected. Protein concentrations were measured by the Bradford assay using bovine serum albumin as standard.

6.2.7 Western blot analysis

The samples were mixed with loading buffer, proteins were denatured by heating at 95^{o}C for 5 minutes, and 25 g of total protein was electrophoretically resolved on 10% lab made gels at 135 V for 90 minutes and then transferred onto a nitrocellulose membrane (Bio-Rad, Australia) using a wet transfer (100 V for 2 hour 15 minutes). After blotting, the membranes were blocked with 5% non-fat dry milk 1 hour at room temperature. The membranes were then washed three times for 5 minute each with Tris-buffered saline-0.1% Tween (TBST, pH 7.6) and incubated for overnight at 4 C with mouse anti-PACC reductase (1:1000) or mouse anti-

LKB1 (1:1000) or mouse anti- PLKB1(1:1000) or mouse anti- SIRT1 (1:1000) or mouse anti- SREBP1(1:1000) antibody (Santa Cruz, Biotechnology, CA, USA) , or rabbit anti-ACC(1:1000) (Abcam, Cambridge, UK) diluted with TBST. Blots were then again washed three times for 5 minutes each with TBST and incubated for 1hr at room temperature with appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz, Biotechnology, CA, USA) diluted at 1:10,000 with PBS, pH 7.4. The membranes were again washed three times for 5 minutes each with TBST and incubated with enhanced chemiluminescence reagent (Clarity™ Western ECL, Bio-Rad, Australia) for 1 minute at room temperature. Immune complexes were detected after exposing the blots to ChemiDoc™ XRS system (Bio-Rad, Australia) for various time points. Quantitative image analysis was performed using NIH Image software (Image J) to determine the intensity of the protein signal, which was expressed relative to the amount of -actin used as an internal control.

6.2.8 Data and statistical analysis

The results are expressed as means ± SEM. To analyse the quantitative differences among the experimental groups before or after treatments, the data were subjected to analysis of variance (ANOVA) using the GraphPad 7.03 (GraphPad Software Inc., California, CA, USA) statistical software. Post-hoc comparisons were made using Dunnett's multiple comparisons test.

6.3 Results

6.3.1 Effect of low and moderate alcohol on triglycerides and total cholesterol levels in FFA-treated AMPK inhibited HepG2 cells

The level of triglycerides in different experimental groups are shown in Figure 6.1A. The FFA-treated cells (n=6) showed a significant ($p<0.001$) 1.6-fold increase in triglyceride levels when compared to the normal control cells (n=6). On the other hand, both the low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a mild significant increase in triglyceride levels by 1.1-fold ($p<0.001$) and 1.1-fold ($p<0.001$) respectively when compared to the FFA-treated control cells. In contrast, normal cells treated with moderate alcohol group (n=6) showed a significant ($p<0.05$) increase in triglycerides when compared to the normal control cells. However, the normal cells treated with low alcohol (n=6) showed a non-significant ($p=0.07$) increase in triglyceride levels when compared to the normal control cells.

The total cholesterol levels in different experimental cellular groups in FFA treated HepG2 cells (AMPK inhibited) was shown in Figure 6.2B. HepG2 cells (n=6) treated with FFA showed a significant ($p<0.01$) 1.2-fold increase in total cholesterol levels when compared to the normal control cells (n=6). On the other hand, both the low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a non-significant ($p=0.63$ to 0.52) increase in total cholesterol levels when compared to the FFA-treated control cells. In contrast, both the low alcohol and moderate alcohol-treated treatment in normal cells (n=6) showed a non-significant ($p= 0.26$ to

0.08) increase in total cholesterol levels by when compared to the normal control group.

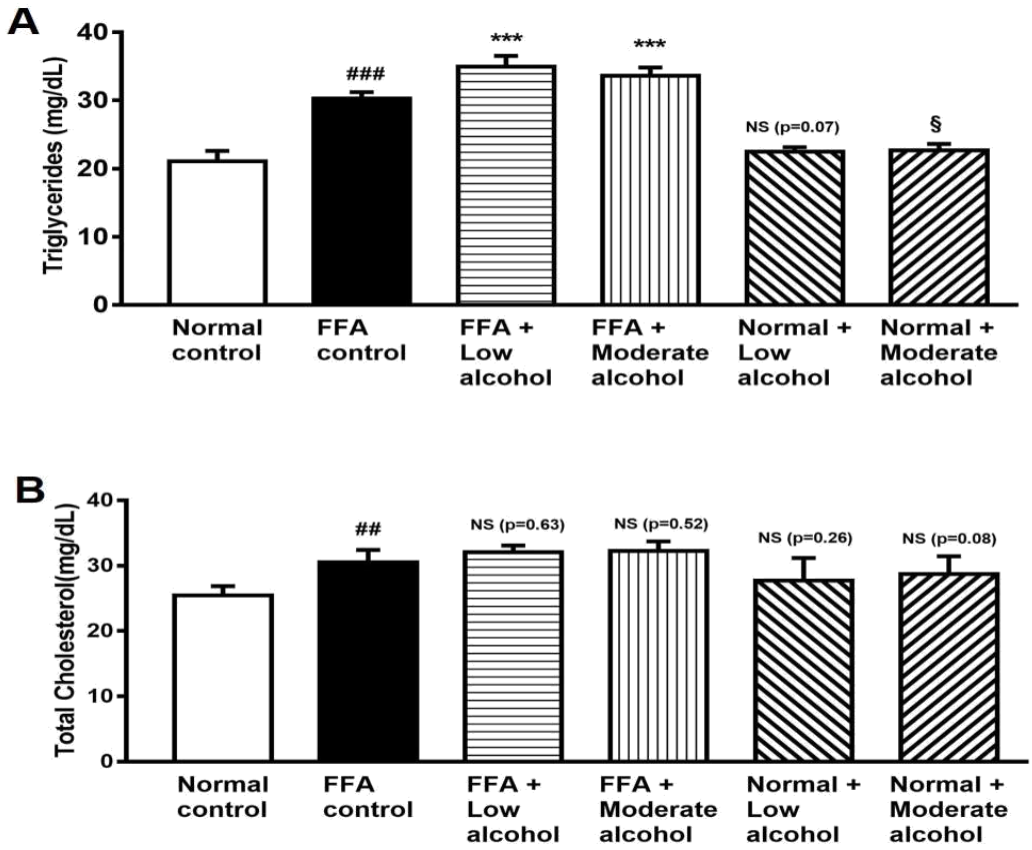

Figure 6.1 Effect of low and moderate alcohol on triglycerides and total cholesterol levels in FFA-treated AMPK inhibited HepG2 cells. (A) triglycerides (B) total cholesterol accumulation in HepG2 cells incubated with FFA 1mM mixture final concentration for 24hrs either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Values represent the mean ± SEM of n=6

Significant difference from normal control cells: $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$
Significant difference from disease control cells: ***p<0.001

Significant difference from normal control group vs normal cells treated with alcohol group: $^{\S}p<0.005$
NS: No significant difference between the groups in comparison

FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

6.3.2 Effect of low and moderate alcohol on LKB1 and its phosphorylation in FFA-treated AMPK inhibited HepG2 cells

The effect of low and moderate alcohol on the LKB1 protein expression was investigated by western blotting in FFA treated HepG3 cells (AMPK-inhibited) Figure 6.2A. The FFA-treated HepG2 cells (n=6) showed a significant ($p<0.001$) 1.5-fold decrease in LKB1 protein expression when compared to the normal control group (n=6). On the other hand, the moderate alcohol-treated group (n=6) showed a significant 1.2-fold ($p<0.05$) increase in LKB1 protein expression when compared to the FFA-treated cells. However, the low alcohol-treated group (n=6) showed a mild non-significant ($p=0.15$) decrease in LKB1protein expression when compared to the FFA-cells. In contrast, normal cells treated with moderate alcohol (n=3) showed a mild non-significant ($p=0.94$) increase in LKB1 protein expression when compared to the normal control group. However, low alcohol treatment in normal cells (n=3) showed a non-significant ($p=0.23$) 1.2-fold decrease in LKB1 protein expression when compared to the normal HepG2 cells.

The below Figure 6.2 (B) illustrates the effect of low and moderate alcohol on phosphorylation of LKB1 in FFA induced NAFLD in AMPK inhibited HepG2 cells. The FFA-treated group (n=6) showed a significant ($p<0.01$) 1.4-fold decrease in Phospho-LKB1 protein when compared to the normal control cells (n=6). On the other hand, both the low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a non-significant increase in phosphorylation of LKB1 protein by 1.0-fold ($p=0.98$) and 1.2-fold ($p=0.12$) respectively when compared to the FFA-treated control cells. In contrast the only low alcohol (n=3) and moderate alcohol

(n=3) showed a non-significant (p=0.06 to 0.83) decrease in phosphorylation of LKB1 when compared to the normal control group.

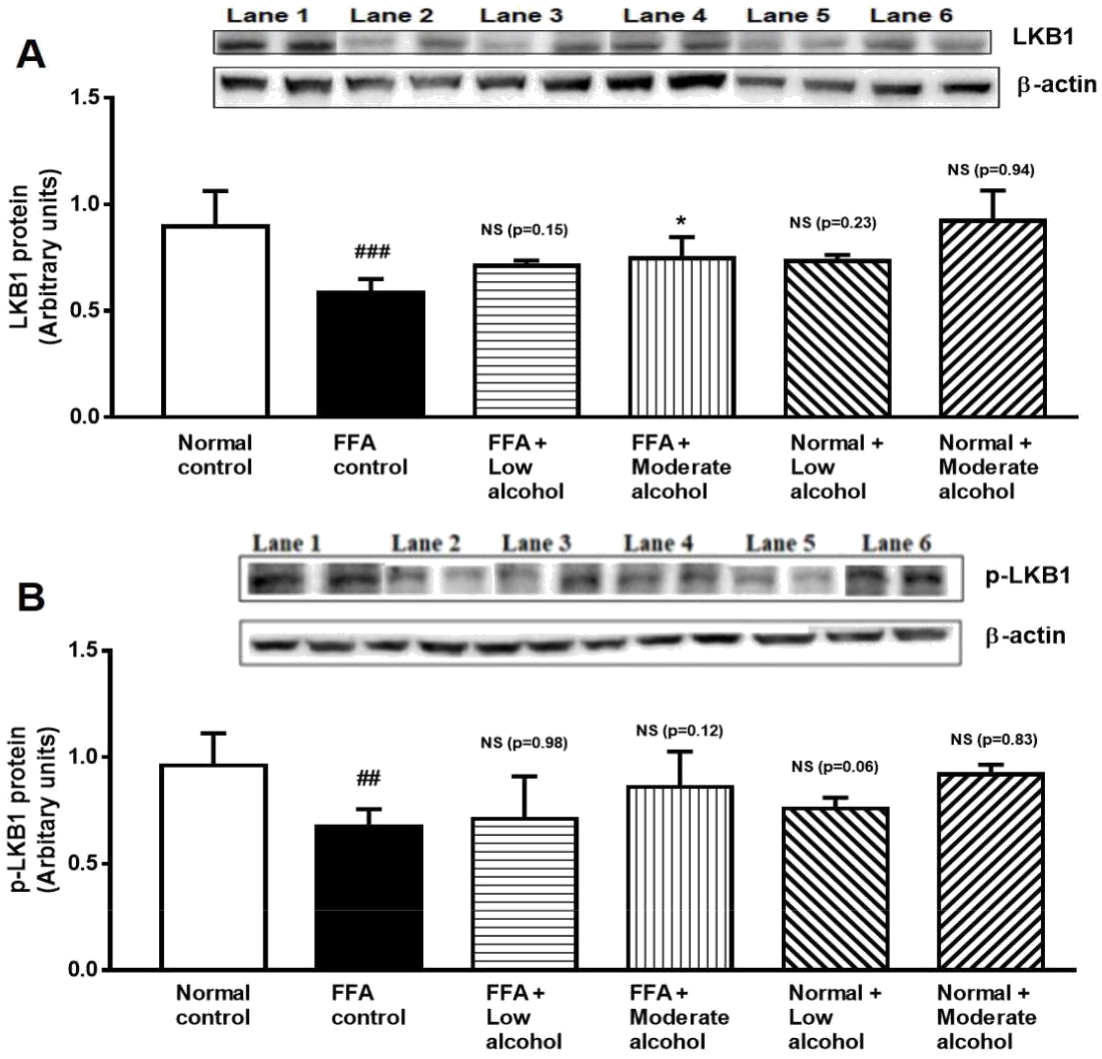

Figure 6.2 Protein expression of LKB1 and p-LKB1 in FFA-treated AMPK inhibited HepG2 cells. (A) LKB1 (B) p-LKB1 protein expression as determined by western blot analysis in AMPK inhibited by Compound C 40 µM in HepG2 cells treated with 1 mM of FFA for 24hrs either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Each bar represents the mean ± SEM of n=6 & 3 for normal cells treated with low and moderate alcohol groups calculated relative β-actin used as internal control. Significant difference from normal control: $^{###}p<0.001$, $^{##}p<0.001$
Significant difference from FFA control: *p<0.05,
NS: No significant difference between the groups in comparison
FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

6.3.3 Effect of low and moderate alcohol on SIRT1 protein expression in FFA-treated AMPK inhibited HepG2 cells

The effect of low and moderate alcohol on the protein expression of SIRT1 in AMPK inhibited HepG2 cells are shown in Figure 6.3. The FFA-treated control cells (n=6) showed a significant ($p<0.001$) 1.9-fold decrease in SIRT1 protein expression when compared to the normal control cells (n=6). On the other hand, both the low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a significant increase in SIRT1 protein expression by 1.5-fold ($p<0.01$) and 1.8-fold ($p<0.001$) respectively when compared to the FFA-treated control cells. In contrast, both the low alcohol (n=3) and moderate alcohol treatments (n=3) in normal cells did not show any change in SIRT1 protein expression when compared to the normal control group.

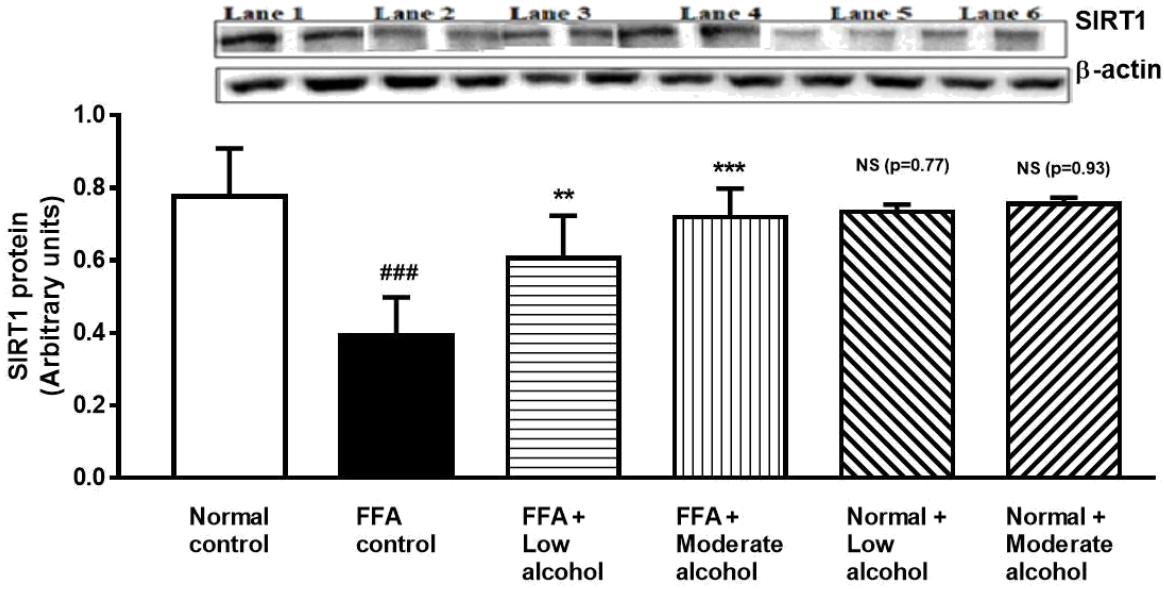

Figure 6.3 Protein expression of SIRT1 in FFA-treated AMPK inhibited HepG2 cells

SIRT1 protein expression as determined by western blot analysis in AMPK inhibited by Compound C 40μM in HepG2 cells treated with 1 mM of FFA for 24hrs either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Each bar represents the mean ± S.E.M. of n=6 & 3 for normal cells treated with low and moderate alcohol groups calculated relative β-actin used as internal control. Significant difference from normal control: $^{###}p<0.001$ Significant difference from FFA control: $^{**}p<0.01$, $^{***}p<0.001$

NS: No significant difference between the groups in comparison

FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

6.3.4 Effect of low and moderate alcohol on ACC and its phosphorylation in FFA-treated AMPK inhibited HepG2 cells

The expression of ACC protein in AMPK inhibited HepG2 cells are shown in Figure 6.4A. The FFA-treated control group (n=6) showed a significant ($p<0.001$) 1.3-fold increase in ACC protein expression when compared to the normal control group (n=6). On the other hand, the moderate alcohol-treated group (n=6) showed a significant 1.1-fold ($p<0.01$) decrease in ACC protein expression when compared to the FFA-treated control group. However, the low alcohol-treated group (n=6) showed a mild non-significant ($p=0.18$) decrease in ACC protein expression when compared to the FFA-treated group. In contrast, normal cells treated with either low alcohol (n=3) and moderate alcohol (n=3) showed a significant increase in ACC protein expression by 1.2-fold ($p<0.01$) and 1.2-fold ($p<0.01$) respectively when compared to the normal control group.

Figure 6.4B shows the effect of low and moderate alcohol treatment on phosphorylation of ACC in AMPK inhibited HepG2 cell**.** The FFA-treated group (n=6) showed a significant ($p<0.01$) 1.4-fold decrease in phosphorylation of ACC protein when compared to the normal control group (n=6). However, the low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a mild non-significant ($p=0.99$ to 0.70) increase in phosphorylation of ACC protein when compared to the FFA-treated control group. Furthermore, normal cells treated with Low alcohol (n=3) showed a significant ($p<0.05$) decrease in phosphorylation of ACC expression when compared to normal control group. Whereas, moderate alcohol treatment in normal cells (n=3) showed a mild non-significant ($p=0.94$)

decrease in phosphorylation of ACC protein when compared to the normal control group.

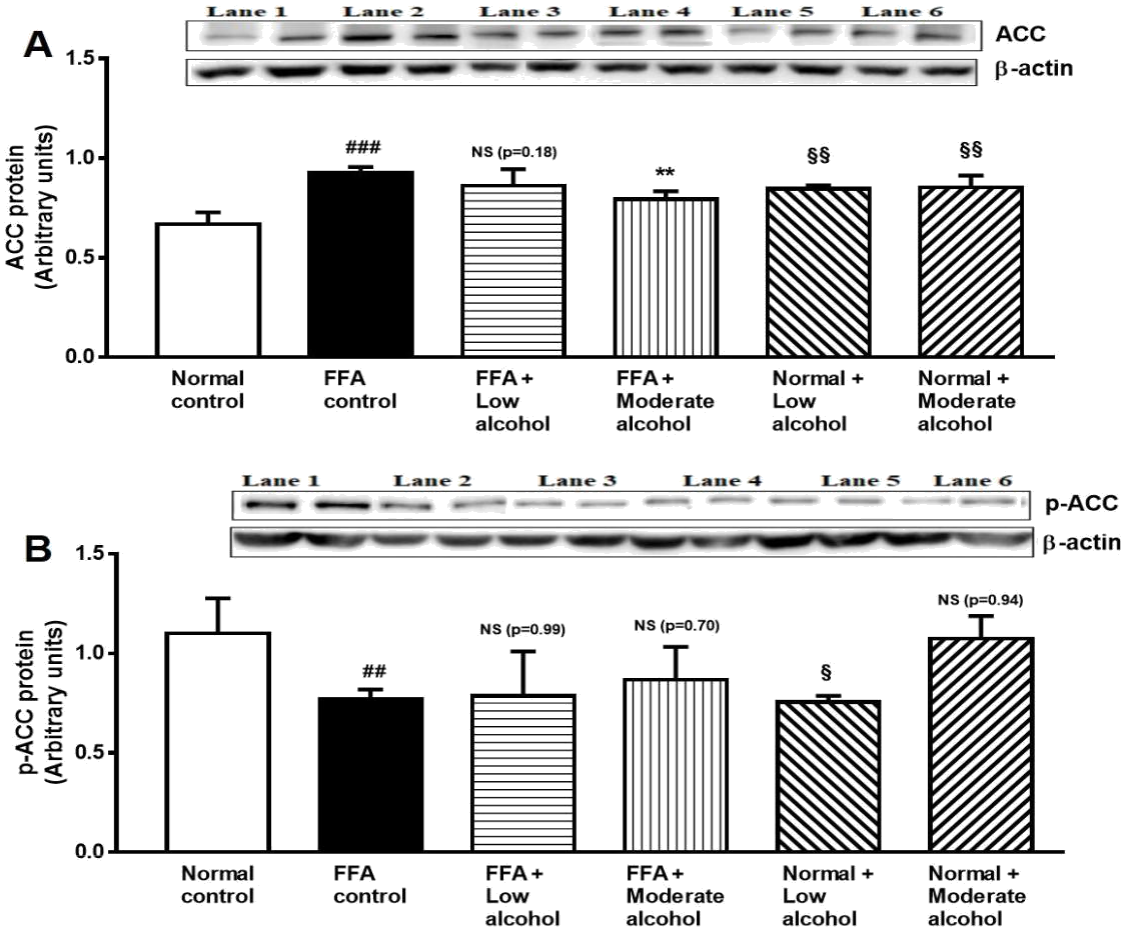

Figure 6.4 Protein expression of ACC and p-ACC in FFA-treated AMPK inhibited HepG2 cells (A) ACC (B) p-ACC protein expression as determined by western blot analysis in AMPK inhibited by Compound C 40 µM in HepG2 cells treated with 1 mM of FFA for 24hrs either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Each bar represents the mean ± SEM of n=6 & 3 for normal cells treated with low and moderate alcohol groups calculated relative β-actin used as internal control. Significant difference from normal control: $^{###}p<0.001$, $^{##}p<0.001$
Significant difference from FFA control: $^{**}p<0.01$,
Significant difference from normal control group vs normal cells treated with alcohol groups: $^{§§}p<0.01$, $^{§}p<0.05$,

NS: No significant difference between the groups in comparison
FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

6.3.5 Effect of low and moderate alcohol on SREBP1 protein expression in FFA-treated AMPK inhibited HepG2 cells

The SREBP1 protein expression in different experimental groups is shown in Figure 6.5. The FFA-treated group (n=6) showed a significant ($p<0.001$) 2.0-fold increase in SREBP1 expression when compared to the normal control group (n=6). The moderate alcohol-treated group (n=6) showed a significant ($P<0.01$) 1.3-fold increase in SREBP1 expression when compared to the FFA- treated control group. However, the low alcohol treated group (n=6) showed a 1.2-fold non-significant ($p=0.11$) decrease in SREBP1 protein expression when compared to the FFA-treated control group. In contrast, the low alcohol treatment in normal cells (n=3) showed a non-significant increase SREBP1 protein expression by 1.5-fold ($p=0.05$) when compared to the normal control group. However, moderate alcohol treatment in normal cells (n=3) showed a mild non-significant ($p=0.82$) increase in SREBP1 protein expression when compared to normal control cells.

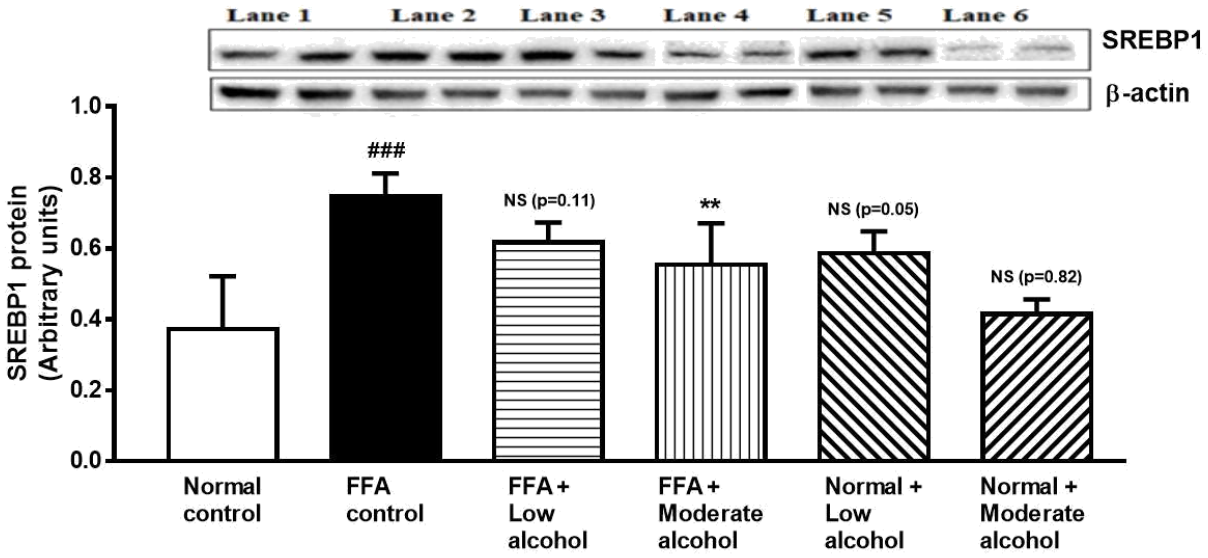

Figure 6.5 Protein expression of SREBP1 in FFA-treated AMPK inhibited HepG2 cells. SREBP1 protein expression as determined by western blot analysis in AMPK inhibited by Compound C 40µM in HepG2 cells treated with 1 mM of FFA for 24hrs either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Each bar represents the mean ± SEM of n=6 & 3 for normal cells treated with low and moderate alcohol groups calculated relative β-actin used as internal control. Significant difference from normal control: $^{###}p<0.001$
Significant difference from FFA control: **p<0.001,
NS: No significant difference between the groups in comparison
FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

6.3 Discussion

The present study demonstrates the role of low and moderate alcohol on protecting FFA induced NAFLD in the absence of AMPK in HepG2 cells. The AMPK itself act individually or by SIRT1/LKB1/AMPK pathway in regulating the lipid metabolism in the liver. To find the role of SIRT1 and LKB1 in regulating lipid metabolism via SIRT1, LKB1 and AMPK pathway, AMPK was inhibited by using compound C for investigating the role of LKB1 and SIRT1in protecting the HepG2 cells. AMPK has multiple effects on lipid metabolism; it has been suggested that the impairment of hepatic AMPK activity is a key pathological event in the development of many metabolic disorders associated with metabolic syndrome including hepatic steatosis[427]. In contrast to the impaired active AMPK, the increased AMPK activates the ATP producing catabolic pathways, such as fatty acid β oxidation, and inhibits ATP consuming processes, such as lipogenesis, directly by phosphorylating regulatory proteins and indirectly by affecting expression levels of genes in this pathway[366]. The first downstream target of AMPK is ACC, which is involved in the synthesis of malonyl-CoA. AMPK inhibits ACC activity by phosphorylating ACC hereby stimulating fatty acid oxidation and reducing fatty acid synthesis[451]. The important finding of the present study was despite the inhibition of AMPK, the low and moderate alcohol increased the SIRT1 and LKB1 protein levels in moderate alcohol group in HepG2 cells treated with FFA which increase ACC and SREBP1 proteins.

In our study, an increase in SIRT1 was observed in low and moderate alcohol with FFA treatment. Although many previous studies have shown

that heavy alcohol decreases the SIRT1 levels in liver [217, 452]. Min et al. have demonstrated that there is no significant change in SIRT1 expression when H4IIEC3 cells treated with alcohol from 0-20 mM whereas, a decrease in SIRT1 expression after cells treated with 50- 100mM alcohol, this confirms that alcohol has no negative effects on SIRT1 at low doses [452]. Furthermore, K.J. Thompson et al. (2015) have demonstrated that alcohol has no effect on SIRT1 expression in HepG2 cells up 100 mM of alcohol up to 48 hours [415]. Based on the results from Min and Thompson it is hypothesized that alcohol has no negative effects on SIRT1 at low concentrations. A further investigation is required for finding out the mechanism behind the increase in SIRT1 levels in alcohol groups. Despite the absence of AMPK, results from previous chapters 3 and 4 suggested that AMPK/LKB1/SIRT1 regulate each other.

An increase in LKB1 was observed in treatment groups but not the phosphorylation of LKB1, the increase in LKB1 may be due to the increase in SIRT1 expression via STRAD and MO25 [453]. Furthermore a decrease in SREBP1 in low and moderate alcohol might be due to SIRT1, as SIRT1 responds to fasting and promotes fatty acid oxidation by activating PPARα and inhibits fatty acid synthesis by targeting SREBP1c for degradation [430, 454]. Increased SIRT1 managed to decrease ACC in moderate alcohol with FFA but failed to phosphorylate the ACC, a further investigation is required to find the role of SIRT1 on phosphorylation of ACC is HepG2 cells.

In conclusion, the present study demonstrated that low and moderate alcohol with FFA increases the SIRT1 despite the inhibition of AMPK which

regulate each other. Further studies are required for investigating the molecular mechanism behind the increase in SIRT1 protein with low and moderate alcohol.

CHAPTER-7

Evaluating the mechanistic action of low and moderate alcohol on FFA-induced NAFLD in HepG2 cells: An SIRT1 inhibitory study

7.1 Introduction

Sirtuins are a group of class III histone/protein deacetylases and the members of the family, silent information regulator 2(SIR2). In mammals 7 different sirtuins (SIRT1-7), have been identified, which have different localisation and expression. SIRT 1, 6, and 7 are localised mainly in the nucleus while SIRT 3, 4 and 5 are localized in the mitochondrial matrix and SIRT2 predominantly cytoplasmic [455]. SIRT1, an NAD+ -dependent protein deacetylase, is an important regulator of energy homeostasis in response to nutrient availability. Currently, the best known and most studied is SIRT1, which is more commonly expressed in metabolic tissues such as liver, skeletal muscle, adipose tissue, pancreas and brain. SIRT1 actions in these tissues include regulation of β-cell and neuron survival, hepatic gluconeogenesis, insulin secretion and adiposity [314, 349].

A decrease in SIRT1 activity would decrease with a decreased level of NAD+ as observed in a NAFLD condition [428, 456, 457]. Impairment of SIRT1 in liver hepatocytes contributes to the pathogenesis of both alcoholic and non-alcoholic fatty liver disease [434, 452, 458]. Previous studies reported that deletion of SIRT1 in liver showed to cause peroxisome proliferator-activated receptor a (PPARα) signal failure and a decrease in fatty acid β-oxidation [430]. At molecular level, SIRT1 plays an important role in regulating various transcriptional networks regulating various critical metabolic processes in the liver [459, 460]. Along those lines, SIRT1 was shown to deacetylate many nonhistone proteins, including p53, nuclear factor kappa B (NF-kB), fork head box class O3 (FOXO3) transcription

factors, PGC-1a, liver X-receptor (LXR), CLOCK, PER2 and TORC2 [314, 353, 461, 462]. Increased expression of SIRT1 decreases the lipogenic genes such as SREBP-1c, ACC and FAS, was central to the pathogenesis of metabolic disorders, including fatty liver [463].

Results from previous chapters 3 and 4 proves that apart from AMPK, SIRT1 also plays a key role in regulating hepatic lipid accumulation. This chapter aims to investigate out the role of low and moderate alcohol against FFA induced hepatocellular lipid accumulation by inhibiting the SIRT1.

7.2 Materials and methods

7.2.1 Chemical used

Electrophoresis and electro-blotting consumables were purchased from Bio-Rad (Hercules, CA, USA). Primary antibodies of AMPK-α1, p-AMPK-α1, p-ACC, LKB1, p-LKB1, and SREBP1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) while ACC was obtained from (Abcam, Cambridge, United Kingdom). Enhanced chemiluminescence kit was obtained from Bio-Rad (Hercules, CA, USA). DMEM (modified basal medium eagle), low glucose, alcohol, Fetal bovine serum (FBS), sodium oleate, sodium palmitate from Sigma (Sigma St. Louis, MO, USA). All other chemicals used were of analytical or molecular biology grade.

7.2.2 Reagents used

(1) Radio immunoprecipitation assay (RIPA) buffer (pH 8.0)

Sodium chloride (150 mM), sodium deoxycholate (0.5%), sodium dodecyl sulfate (0.1%), tris base (50 mM) and 1 mL of triton X-100 (1%) were added into a volumetric flask and made up to 100 mL with distilled water. The solution was transferred into a beaker and the pH was adjusted to 8.0 and filtered before use through a 0.2 µM membrane.

(2) Running buffer

Glycine (14.42 g), tris base (3.03 g) and sodium dodecyl sulfate (1 g) were added into a volumetric flask and made up to 1000 mL with distilled water. The solution

was transferred into a beaker and the pH was adjusted to 8.3 and filtered before use through a 0.2 µM membrane.

(3) Transfer buffer

Tris base (3.03 g), glycine (14.41 g) and methanol (200 mL) were added into a volumetric flask and made up to 1000 mL with distilled water. The solution was filtered before use through a 0.2 µM membrane.

(4) Tris-buffered saline (TBS)

Tris-base (2.4 g) and NaCl (8 g) were added into a volumetric flask and made up to 1000 mL with distilled water. The solution was transferred into a beaker and the pH was adjusted to 7.6 and filtered before use through a 0.2 µM membrane.

(5) Phosphate buffered saline (PBS)

Sodium chloride (8 g), potassium chloride (0.2 g), disodium hydrogen phosphate (1.44 g), and potassium dihydrogen phosphate (0.24 g) were added into a volumetric flask and made up to 1000 mL with distilled water. The solution was transferred into a beaker and the pH was adjusted to 7.6 and filtered before use through a 0.2 µM membrane.

(6) Stacking gel buffer

Tris base (15.14 g), SDS (1.0 g) were added into volumetric water and made up to 250 ml with distilled water. The solution was transferred into a beaker and the pH was adjusted to 6.8 and filtered before use through a 0.2 µM membrane.

(7) *Sodium oleate*

Sodium oleate (40.58 mg) in 10 mL (13.33 mM) stock of plain DMEM at 37ºC with fatty acid-free BSA and filtered with a 0.2 µM membrane and stored in -20°C.

(8) Sodium palmitate

Sodium palmitate (18.54 mg) in 10 mL of plain DMEM at 50ºC with constant shaking with Fatty acid-free BSA and filtered with a 0.2 µM membrane and stored in -20°C.

(9) *400 mM alcohol*

Alcohol 184.28 mg or 235 µL of alcohol in 10 mL of plain DMEM for 400 mM stock and filtered with a 0.2 µM membrane and stored in 4ºC.

(10) Nicotinamide

Nicotinamide (122.12 mg) in 10 ml of plain DMEM for 100 mM stock and filtered with a 0.2 µM membrane and stored in 4 ºC.

7.2.3 Cell culture

HepG2 cells (passage-7) are obtained from the University of Sydney, which were previously maintained as per mentioned in ATCC guidelines in DMEM with low glucose, 10% FBS, 1% antibiotics (10,000 I.U./mL Penicillin, 10,000 (µg/mL) Streptomycin). They were maintained in same incubator conditions, at 37º C with 5% CO_2 changing the growth medium for every two days. After 70-80% confluence of cells they were washed thrice with PBS and then added 3-5 ml of trypsin with

EDTA. After the cells completely detach, they were added into DMEM to neutralise the trypsin and the cells were centrifuged at 300 g for 3 minutes. The subculture was done with a ratio of 1:3 or 1:6. The remaining cells were stored with 5% DMSO in growth medium and stored in liquid nitrogen vapour phase.

7.2.4 Experimental design and treatments

HepG2 cells were grown up to 70% confluence and then transferred into 6 well plates at $2.5*10^5$ cells per well and divided into six groups. Cells were preincubated with 10 mM nicotinamide for 6 hours[346], and then washed with PBS and proceeded for alcohol treatment. Control cells were incubated with plain medium and free fatty acid group received 1 mM fatty acid mixture (containing 0.66 mM sodium oleate and 0.33 mM sodium palmitate)[395] whereas the treatment groups received low (10 mM) and moderate (20 mM) concentrations of alcohol with 1 mM fatty acid mixture (0.66 mM sodium oleate and 0.33 mM of sodium palmitate). The rest of the groups received 10 mM and 20 mM of alcohol without any FFA treatment. Alcohol is a volatile substance in order to maintain a stable alcohol concentration inside the 6 well plate, for the cells, double the concentration of alcohol was placed in a Petri dish during the 24-hour experiment. After 24-hours of treatment, the cells were removed from the incubator and proceeded for lipid extraction and cellular protein extraction.

7.2.5 Extraction of lipids from HepG2 cells

The total lipids from HepG2 cells were extracted by the modified method of Bligh and Dyer [396] as described by Li and Lin [397]. The treated cells were

homogenized with chloroform-methanol solution (chloroform-methanol-water,8:4:3). Further the resulting mixture was shaken at 37ºC for 1hour and then centrifuged at 1,100 g for 10 minutes. The bottom layer was collected and again centrifuged, the supernatant was collected and used for analysis of hepatic lipid.

7.2.6 Cell protein extraction

After 24 hours of treatment, 6 well plates with cells were removed from incubator and washed with PBS. Radioimmunoprecipitation assay (RIPA) buffer (pH 8.0) containing 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 10 l/ml protease and phosphatase inhibitors cocktail was added to each well and cells were scraped on ice over an orbital shaker for 1hour. The homogenates were collected and then sonicated at 60AMP of total time for 30 seconds with on time 5 seconds and off time 15 seconds. The homogenates were centrifuged at 4°C at 10,000 *g* for 15 minutes, and the supernatants were collected. Protein concentrations were measured by the Bradford assay using bovine serum albumin as standard.

7.2.7 Western blot analysis

The samples were mixed with loading buffer, proteins were denatured by heating at 95 ^{o}C for 5 minutes, and 25 g of total protein was electrophoretically resolved on 10% Mini-PROTEAN® TGX™ Poured gels (Bio-Rad, Australia) at 135 V for 90 minutes and then transferred onto a nitrocellulose membrane (Bio-Rad, Australia) using a Mini Trans-Blot® Cell (100 V for 60 minutes) or wet transfer (100 V for 135 minutes). After blotting, the membranes were blocked with 5% non-

fat dry milk 1hour at room temperature. The membranes were then washed three times for 5 minutes each with tris-buffered saline-0.1% Tween-20 (TBST, pH 7.6) and incubated for overnight at 4 C with mouse anti-p-ACC reductase (1:1000) or mouse anti-AMPK-α1 (1:1000) or mouse anti- LKB1 (1:1000) or mouse anti-pLKB1(1:1000) or mouse anti-SREBP1(1:1000) antibody (Santa Cruz, Biotechnology, CA, USA), rabbit anti-p-AMPK-α1 (1:500) or rabbit anti-ACC(1:1000) (Abcam, Cambridge, UK) diluted with TBST. Blots were then again washed three times for 5 minutes each with TBST and incubated for 1 hour at room temperature with an appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz, Biotechnology, CA, USA) diluted at 1:10,000 with phosphate-buffered saline (PBS, pH 7.4). The membranes were again washed three times for 5 minutes each with TBST and incubated with enhanced chemiluminescence reagent (Clarity™ Western ECL, Bio-Rad, Australia) for 1 minute at room temperature. Immune complexes were detected after exposing the blots to ChemiDoc™ XRS system (Bio-Rad, Australia) for various time point. Quantitative image analysis was performed using NIH Image software (Image J) to determine the intensity of the protein signal, which was expressed relative to the amount of -actin used as an internal control.

7.2.8 Data and statistical analysis

The results are expressed as means ± SEM. To analyse the quantitative differences among the experimental groups before or after treatments, the data were subjected to analysis of variance (ANOVA) using the GraphPad 7.03

(GraphPad Software Inc., California, CA, USA) statistical software. Post-hoc comparisons were made using Dunnett's multiple comparisons test.

7.3 Results

7.3.1 Effect of low and moderate alcohol on triglycerides and total cholesterol levels in FFA-treated SIRT1 inhibited HepG2 cells

The levels of triglycerides in different groups are shown in Figure 7.1A. The FFA-treated group (n=6) showed a significant ($p<0.001$) 1.2-fold increase in triglycerides levels when compared to the normal control cells (n=6). On the other hand, both the low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a non-significant ($p=0.15$ to 0.10) decrease in triglycerides levels by when compared to the FFA-treated control group. In contrast, the low alcohol (n=6) and moderate alcohol(n=6) treatment in normal cells showed a non-significant ($p=0.77$ to 0.32) decrease in triglycerides levels when compared to the normal control group.

The total cholesterol levels in different groups of SIRT1 inhibited HepG2 cells are shown in Figure 7.1B. The FFA-treated (n=6) group showed a significant ($p<0.001$) 1.2-fold increase in cholesterol levels when compared to the normal control group (n=6). On the other hand, both the low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a non-significant ($p=0.15$ to 0.05) decrease in total cholesterol levels when compared to the FFA-treated control group. In contrast, the normal cells treated with moderate alcohol (n=6) showed a significant ($p<0.05$) decrease in total cholesterol levels when compared to the normal control group. Whereas, the normal cells treated with low alcohol group (n=6) showed a mild non-significant ($p=0.99$) increase in total cholesterol levels when compared to normal control group.

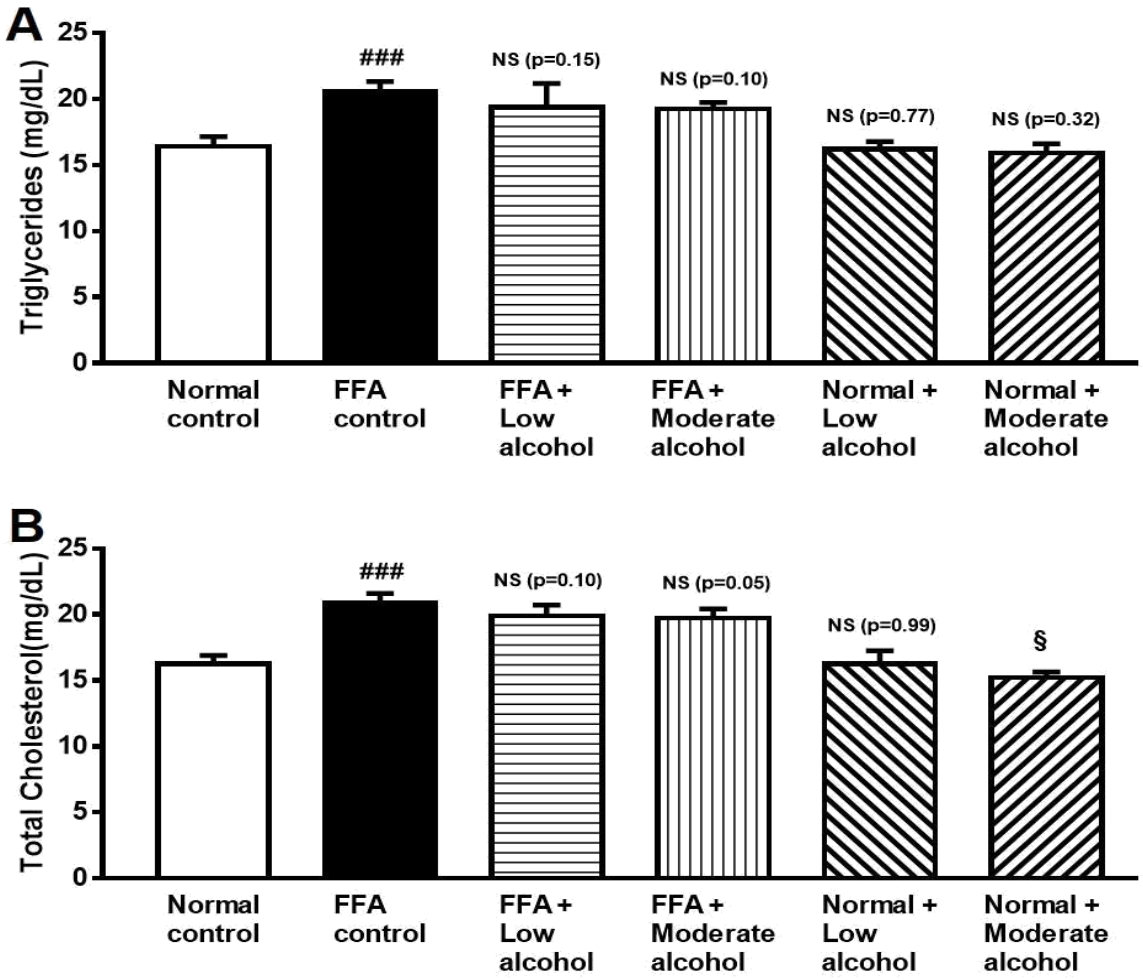

Figure 7.1 Effect of low and moderate alcohol on triglycerides and total cholesterol levels in FFA-treated SIRT1 inhibited HepG2 cells. (A) triglycerides (B) total cholesterol accumulation in HepG2 cells incubated with FFA 1mM mixture final concentration for 24hrs either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Values represent the mean ± SEM of n=6

Significant difference from normal control cells: $^{###}p<0.001$

Significant difference from normal control group vs normal cells treated with alcohol group: $^{§}p<0.005$

NS: No significant difference between the groups in comparison

FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

7.3.2 Effect of low and moderate alcohol on AMPK-α1 and its phosphorylation in FFA treated SIRT1 inhibited HepG2 cells

The effect of low, moderate alcohol on AMPK-*α*1 protein expression in HepG2 cells are shown in Figure 7.2A. The FFA-treated group (n=6) showed a significant ($p<0.01$) 1.4-fold decrease of AMPK-*α*1 levels when compared to the normal control group (n=6). On the other hand, both the low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a significant increase in AMPK-*α*1 protein expression by 1.2-fold ($p<0.05$) and 1.3-fold ($p<0.01$) respectively when compared to the FFA-treated control group. In contrast, both the low alcohol (n=3) and moderate alcohol (n=3) treatment with normal cells showed a non-significant (p=0.80 to 0.99) decrease in AMPK-*α*1 protein expression when compared to the normal control group.

The below Figure 7.2 (B) illustrates the effect of low, moderate alcohol on phosphorylation of AMPK**-***α*1 in FFA treated SIRT1 inhibited HepG2 cells**.** The FFA-treated control group (n=6) showed a significant ($p<0.001$) 1.9-fold decrease in phosphorylation of AMPK-α1 protein when compared to the normal control cells (n=6). On the other hand, both the low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a significant increase in phosphorylation of AMPK-α1 protein by 1.9-fold ($p<0.001$) and 2.0-fold ($p<0.001$) respectively when compared to the FFA-treated control group. In contrast, the moderate alcohol-treated normal cells(n=3) showed a significant 1.6-fold ($p<0.05$) increase in phospho-AMPK-α1 protein when compared to the normal control cells. Whereas, the normal cells

treated with low alcohol (n=3) showed a non-significant 1.2-fold (p=0.06) increase in phosphorylation of AMPK-α1when compared to the normal control group.

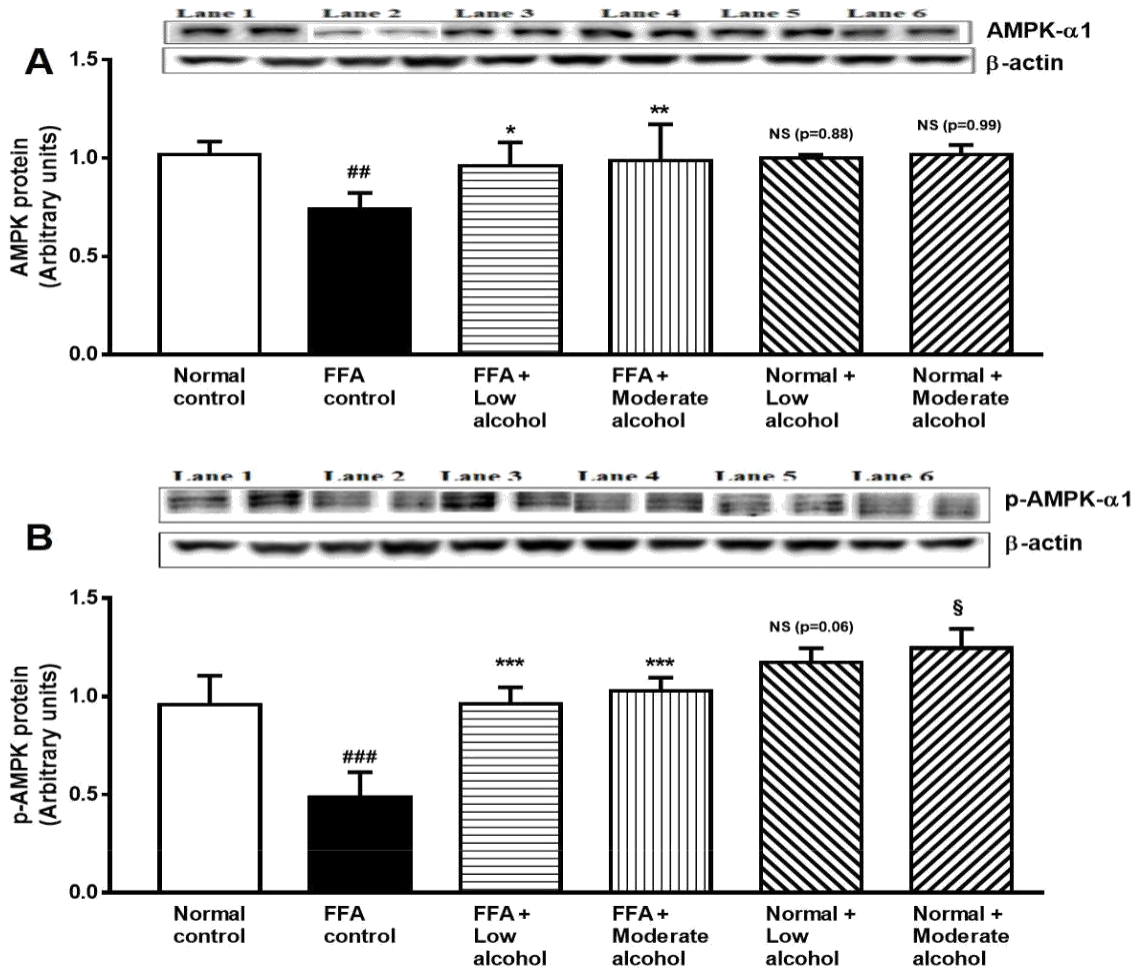

Figure 7.2 Protein expression of AMPK-*α*1 and p-AMPK-*α*1 in FFA-treated SIRT1 inhibited HepG2 cells. (A) AMPK-*α*1 (B) p-AMPK-*α*1 protein expression as determined by western blot analysis in SIRT1 inhibited by Nicotinamide 10 mM in HepG2 cells treated with 1 mM of FFA for 24hrs either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Each bar represents the mean ± SEM of n=6 & 3 for normal cells treated with low and moderate alcohol groups calculated relative β-actin used as internal control.

Significant difference from normal control: $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$

Significant difference from FFA control: *p<0.05, **p<0.01, ***p<0.001

Significant difference from normal control group vs the normal cells treated with alcohol group: $^{\S}p<0.05$

NS: No significant difference between the groups in comparison

FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

7.3.3 Effect of low and moderate alcohol on LKB1 and its phosphorylation in FFA-treated SIRT1 inhibited HepG2 cells

The effect of low and moderate alcohol treatment on LKB1 protein expression was investigated by western blot analysis in SIRT1 inhibited HepG2 cells and was shown in Figure 7.3A. The FFA-treated group (n=6) showed a significant ($p<0.001$) 1.5-fold decrease in LKB1 protein expression when compared to the normal control group (n=6). On the other hand, both the low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a significant increase in LKB1 protein expression by 1.4-fold ($p<0.001$) and 1.5-fold ($p<0.001$) respectively when compared to the FFA-treated control group. In contrast, the normal cells treated with moderate alcohol treated group (n=3) showed a significant ($p<0.05$) 1.1-fold increase in LKB1 expression when compared to normal control group. Whereas, the normal cells treated with low alcohol group (n=3) showed a non-significant ($p=0.99$) decrease in LKB1 expression when compared to the normal control group.

The Figure 7.3B illustrates the effect of low and moderate alcohol on phosphorylation of LKB1 in FFA treated SIRT1 inhibited HepG2 cells. The FFA-treated control group (n=6) showed a significant ($p<0.05$) 1.2-fold decrease in phosphorylation of LKB1 protein when compared to the normal control rats (n=6). On the other hand, both the low alcohol-treated (n=6) and moderate alcohol-treated (n=6) groups showed a significant increase in phosphorylation of LKB1 protein expression by 1.2-fold ($p<0.05$) and 1.2-fold ($p<0.05$) respectively

when compared to the FFA-treated control group. In contrast both the low alcohol (n=3) and moderate alcohol (n=3)- treatment with normal cells showed a non-significant (p=0.69 to 0.18) increase in phosphorylation LKB1 protein when compared to the normal control group.

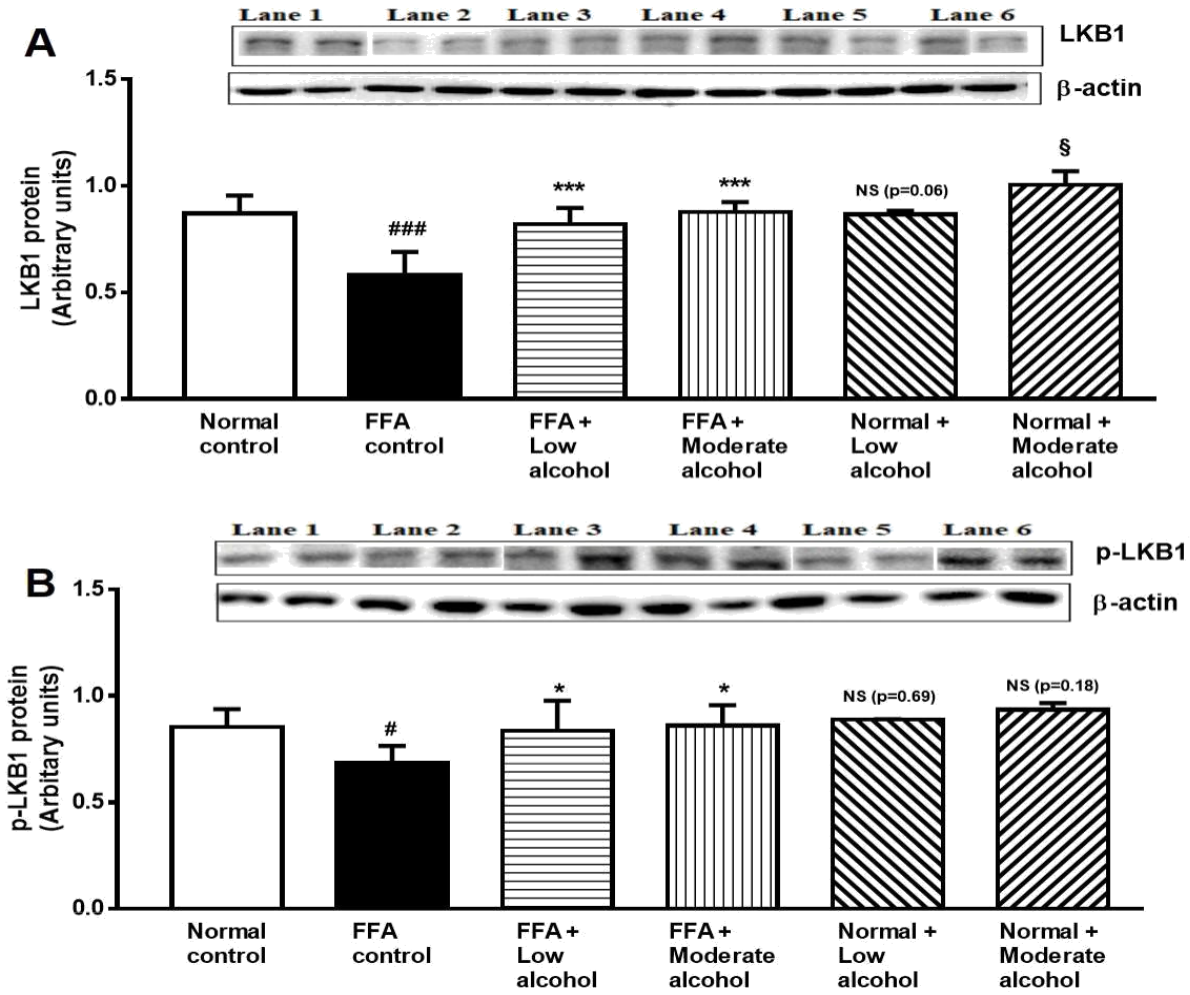

Figure 7.3 Protein expression of LKB1 and p-LKB1 in FFA-treated SIRT1 inhibited HepG2 cells. (A) LKB1 (B) p-LKB1 protein expression as determined by western blot analysis in SIRT1 inhibited by Nicotinamide 10 mM in HepG2 cells treated with 1 mM of FFA for 24hrs either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Each bar represents the mean ± SEM of n=6 & 3 for normal cells treated with low and moderate alcohol groups calculated relative β-actin used as internal control. Significant difference from normal control: $^{\#}p<0.05$, $^{\#\#\#}p<0.001$
Significant difference from FFA control: *p<0.05, ***p<0.001
Significant difference from normal control group vs the normal cells treated with alcohol group: $^{\S}p<0.05$
NS: No significant difference between the groups in comparison
FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

7.3.4 Effect of low and moderate alcohol on ACC and its phosphorylation in FFA-treated SIRT1 inhibited HepG2 cells

The effect of low and moderate alcohol treatment on ACC protein expression was investigated by western blot analysis in FFA treated SIRT1 inhibited HepG2 cells and was shown in Figure 7.4A. The FFA-treated control group (n=6) showed a significant ($p<0.001$) 2.0-fold increase in ACC protein expression when compared to the normal control group (n=6). On the other hand, the moderate alcohol-treated group (n=6) showed a significant 1.2-fold ($p<0.05$) decrease in ACC protein expression when compared to the FFA-treated control group. Whereas, the low alcohol-treated group (n=6) showed a non-significant ($p=0.75$) decrease in ACC protein expression when compared to the FFA-treated group. In contrast, the low alcohol-treated normal cells (n=3) showed a significant 1.5-fold ($p<0.05$) increase in ACC protein expression when compared to the normal control group. Whereas, the moderate alcohol-treated normal cells (n=3) showed a non-significant 1.2-fold ($p=0.25$) increase in ACC when compared to normal control group.

Figure 7.4B explains the effect of low, and moderate alcohol on phosphorylation of ACC in FFA treated SIRT1 inhibited HepG2 cell**.** The FFA-treated group (n=6) showed a significant ($p<0.001$) 1.7-fold decrease in phosphorylation of ACC when compared to the normal control group (n=6). On the other hand, the moderate alcohol-treated group (n=6) showed a mild non-significant ($p=0.99$) increase in phosphorylation ACC when compared to the FFA-treated control group. Whereas, the low alcohol-treated group (n=6) showed a non-

significant 1.2-fold ($p=0.39$) decrease in phosphorylation ACC when compared to the FFA-treated control group. In contrast, both the low alcohol ($n=3$) and moderate alcohol ($n=3$) treatment with normal cells showed a significant decrease in phosphorylation of ACC protein by 2.8-fold ($p<0.001$) and 2.3-fold ($p<0.001$) when compared to the normal control group.

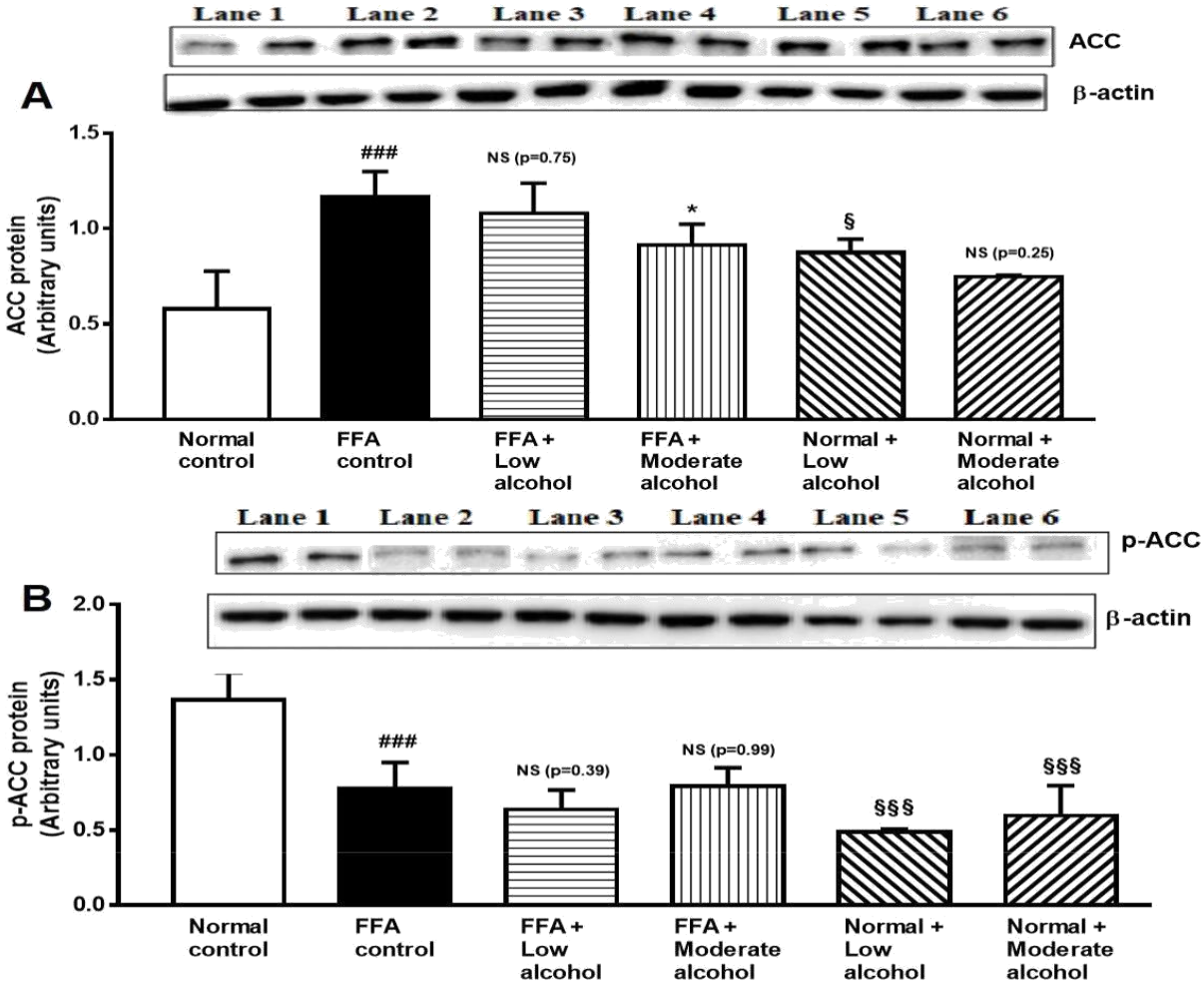

Figure 7.4 Protein expression of ACC and p-ACC in FFA-treated SIRT1 inhibited HepG2 cells. (A) ACC (B) p-ACC protein expression as determined by western blot analysis in SIRT1 inhibited by Nicotinamide 10 mM in HepG2 cells treated with 1 mM of FFA for 24hrs either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Each bar represents the mean ± SEM of n=6 & 3 for normal cells treated with low and moderate alcohol groups calculated relative β-actin used as internal control. Significant difference from normal control: $^{###}p<0.001$
Significant difference from FFA control: *p<0.05,
Significant difference from normal control group vs the normal cells treated with alcohol group: $^{§}p<0.05$, $^{§§§}p<0.001$
NS: No significant difference between the groups in comparison
FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

7.3.5 Effect of low and moderate alcohol on SREBP1 protein expression in FFA treated SIRT1 inhibited HepG2 cells

The SREBP1 expression of different experimental groups has been shown in Figure 7.5. The FFA-treated group (n=6) showed a significant ($p<0.001$) 2.0-fold increase in SREBP1 protein expression when compared to the normal control group (n=6). The moderate alcohol-treated group (n=6) showed a significant ($P<0.05$) 1.2-fold decrease in SREBP1 expression when compared to the FFA-treated control group. Whereas, the low alcohol treated group (n=6) showed a non-significant ($p=0.69$) decrease in SREBP1 protein when compared to FFA-treated control group. In contrast, both the low alcohol (n=3) and moderate alcohol (n=3) treatment with normal cells groups showed a non-significant ($p=0.69$ to 0.52) increase in SREBP1 protein expression when compared to the normal control group.

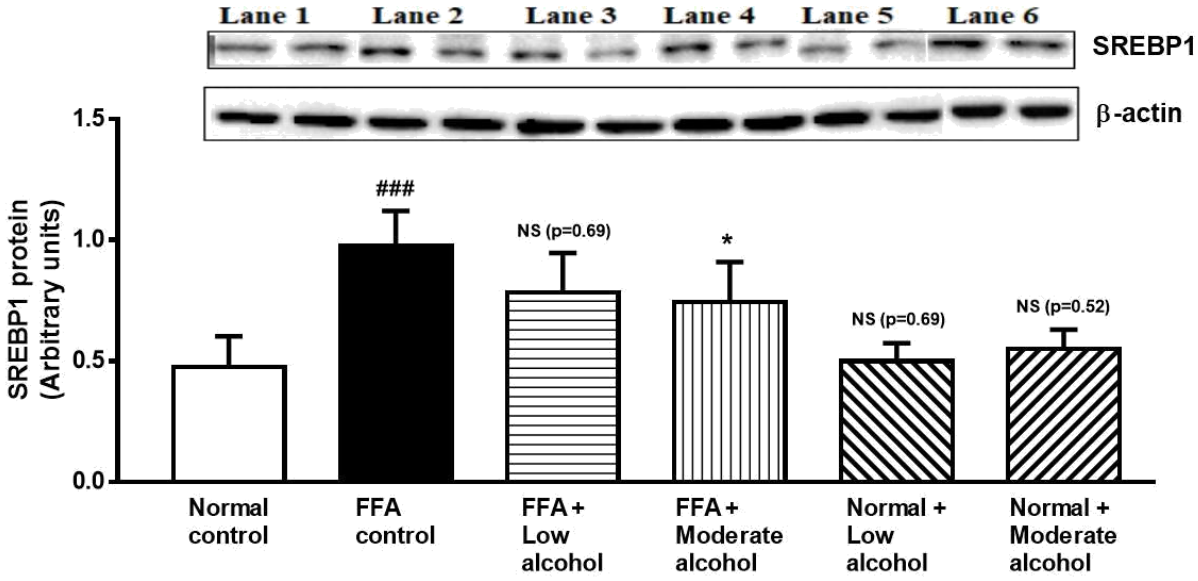

Figure 7.5 Protein expression of SREBP1 in FFA-treated SIRT1 inhibited HepG2 cells. SREBP1 expression as determined by western blot analysis in SIRT1 inhibited by Nicotinamide 10 mM in HepG2 cells treated with 1 mM of FFA for 24hrs either with normal control cells or FFA (disease control) alone or with low, moderate alcohol with FFA and low and moderate alcohol only

Each bar represents the mean ± SEM of n=6 & 3 for normal cells treated with low and moderate alcohol groups calculated relative β-actin used as internal control. Significant difference from normal control: $^{###}p<0.001$
Significant difference from FFA control: *p<0.05,
NS: No significant difference between the groups in comparison
FFA: Free fatty acids 1mM (sodium oleate 0.66mM and 0.33mM of sodium palmitate)

7.4 Discussion

The present study demonstrates the role of low and moderate alcohol on protecting FFA induced NAFLD in the absence of SIRT1 in HepG2 cells. The SIRT1 itself act individually or by SIRT1/AMPK pathway in regulating the lipid metabolism in the liver. To find the role of AMPK/LKB1 in regulating lipid metabolism via SIRT1, LKB1 and AMPK pathway, SIRT1 was inhibited by Nicotinamide for investigating the role of LKB1 and AMPK in protecting the HepG2 cells. The present study results demonstrated that low and moderate alcohol increase the phosphorylation of AMPK and p-LKB1 expression despite the cells treated with FFA which causes an increase in lipid accumulation in HepG2 cells which leads to increase in SREBP1, a lipogenic transcription factor in the liver that regulate the fatty acid and ACC via *de novo* lipogenesis.

LKB1 is a protein kinase that plays a major role in phosphorylates and activates 13 downstream kinases [464]. Based on the results from previous chapters 3 and 4, it is hypothesized that the activation of LKB1 was done by SIRT1. SIRT1 overexpression diminished lysine acetylation of LKB1 and concurrently increased its activity, cytoplasmic/nuclear ratio, and association with the LKB1 activator STRAD. In contrast to the above mechanism, in our study, SIRT1 was inhibited and an increase in LKB1 was observed in the present study. Carling et al (2011) has demonstrated that SIRT1 inhibited showed an HepG2 cells increased total LKB1 levels but decreased p-LKB1 [311]. The mechanism behind the increase of LKB1 with low and moderate alcohol needs further investigation. Apart from LKB1, an increase in p-AMPK was observed in low and moderate

alcohol. The possible explanation behind the increase of p-AMPK was LKB1, the upstream kinase which activates the AMPK by phosphorylating Thr172.

SREBP-1 is an important transcription factor in the liver that regulates the fatty acid respectively, by activating the enzymes involved in the fatty acid pathway including ATP citrate lyase (ACL), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD) [360] (51). SREBPs are expressed as precursor proteins that span the endoplasmic reticulum membrane, are proteolytically cleaved and translocated to the nucleus under the influence of SREBP cleavage-activating protein (SCAP) to activate transcription. Recent studies have further demonstrated that AMPK activation resulted in a decrease in SREBP-1 in the HepG2 cells treated with low and moderate alcohol with FFA, suggesting a direct link between AMPK action and SREBP transcriptional activity [363] which support our present observations that light-to-moderate alcohol treatment tended to increase the liver AMPK-α phosphorylation and decrease SREBP-1 protein expression leading to increasing fatty acid oxidation.

Apart from SREBP1, a decrease in ACC was observed in moderate alcohol with FFA treatment group. AMPK activation and phosphorylation of ACC1 at Ser79 blocks ACC dimerisation, causing a reduction in ACC activity that lowers malonyl-CoA and leads to the inhibition of DNL and increases in mitochondrial fatty acid oxidation [20]

In conclusion, the present investigation demonstrated that low and moderate alcohol increased AMPK activation via LKB1 in the absence of SIRT1, which was associated with decreased expression of SREBP-1 and ACC. So this study proves

that low and moderate alcohol has a beneficial effect on AMPK via LKB1 in HepG2 cells.

CHAPTER-8

SUMMARY AND CONCLUSIONS

8.1 An overview of the principal findings

In this thesis we demonstrated the role of low and moderate alcohol in protecting the liver from non-alcoholic fatty liver disease (NAFLD) via *in vitro* and *in vivo* models for elucidating the mechanism of action.

NAFLD is an inclusive term for describing a broad range of chronic liver pathologies. Several potentially pathogenic mediators are involved during the progression of this chronic liver disease [465, 466]. Initially, it starts with steatosis and may progress to non-alcoholic steatosis (NASH), which involves hepatocellular inflammation and injury which further progresses to hepatic fibrosis [467]. Risk factors for NAFLD include obesity, insulin resistance, and other features of metabolic syndrome. Steatosis is the initial benign stage, characterised by lipid accumulation in hepatocytes due to impaired triglyceride synthesis and export, and/or reduced fatty acid beta-oxidation.

Notably, heavy alcohol consumption leads to alcoholic fatty liver disease. In contrast to the alcoholic fatty liver disease, evidence shows that moderate alcohol consumption has beneficial effects on protection from type II diabetes, protection against the risk of major cardiovascular disease events such as myocardial infarction and coronary artery disease in healthy populations [183]. Henceforward, the purpose of this thesis was to evaluate the beneficial effects of low and moderate alcohol in protecting the liver from high fat diet-fed rat model (HFD). Moreover, the molecular mechanism responsible for protecting the liver from HFD induced dyslipidaemia has been investigated by using *in vitro* model for different inhibitory studies. The principal findings of this thesis were outlined below.

Principal finding 1 (outlined in CHAPTER 2): Chronic long-term feeding of low and moderate alcohol protects the liver from HFD fed rats causing an increase in hepatic lipid accumulation leading to steatosis

Chronic HFD feeding altered the liver functions as evidenced by the biochemical studies and the hepatic tissue as evidenced by the histological studies. The HFD-control rats lead to augment the metabolic dyslipidaemia. This is associated with altered serum lipid profile accompanied by an increase in serum insulin and glucose and elevated liver enzymes ALT and AST which are the key enzymes involved in the hepatocellular injury. The histological architecture of liver tissue in HFD control rats also showed classical features of steatosis. Together with the above mentioned biochemical and histological changes, long-term chronic feeding of HFD to rats leads to augment the metabolic dyslipidaemia. Thus, long-term feeding with moderate alcohol reduced the serum glucose, HDL cholesterol and liver ALT and AST enzymes in animals with HFD induced NAFLD whereas as low alcohol with HFD showed its effects on increasing serum HDL levels. Further studies are being undertaken to explain the mechanism behind the lipid regulation by low and moderate alcohol.

Principal finding 2 (CHAPTER 3): Chronic long-term feeding of low and moderate alcohol increases activation of AMPK-α1 and SIRT1 which plays a key role in controlling the lipid in HFD rats.

Numerous studies demonstrated that non-alcoholic steatosis arises due to the increase in lipid via *de novo* lipogenesis altering the proteins involved in fatty

acid synthesis. The results from chapter-3 revealed that increased hepatic lipid accumulation in HFD control rats is mainly due to increase in SREBP-1 and ACC which also downregulates AMPK-α1, LKB1 and SIRT1. Moreover, the results of chapter 3 also describe that low and moderate alcohol increases the activation of AMPK-α1, SIRT and LKB1. Activation of AMPK-α1 inactivates the metabolic enzymes involved in lipid metabolism such as SREBP-1. It also controls ACC, the key enzyme involved in fatty acid synthesis. Thus, the molecules responsible for hepatic lipid accumulation in the HFD control group was regulated in treatment groups.

Principal finding 3 (CHAPTER 4): The low and moderate alcohol increases activation of AMPK-α1 and SIRT1 which plays a key role in controlling the lipid in HepG2 cells treated with FFA (oleic and palmitic acid) induced NAFLD.

Hyperlipidaemia is a hallmark for NAFLD and is largely due to increasing triglycerides and total cholesterol which also includes the proteins involved in lipid synthesis via *de novo* lipogenesis induced by the treatment of oleic acid and palmitic acid (FFA). The results from chapter- 4 suggest that FFA increases the lipid profile and the proteins involved in lipid synthesis (SREBP-1 and ACC) and decreases the proteins (AMPK-α1, SIRT1 and LKB1) involved in lipid metabolism. On the other hand, low and moderate alcohol failed to decrease the SREBP1 protein despite the increase in AMPK-α1 and SIRT1 proteins which play a major role in regulating the lipids. Thus, the results from chapter-4 indicate that low and moderate alcohol showed a similar result to *in vivo* studies (chapter-3) via the AMPK-α1/LKB1/SIRT1 pathway.

Principal finding 4 (CHAPTER 5): Identifying the role of LKB1 protein on oleic and palmitic acid (FFA) challenged HeLa cells treated with low and moderate alcohol.

Based on the results from chapters-3 and 4, low and moderate alcohol increases the proteins AMPK-α1, LKB1 and SIRT1 which further regulate each other for lipid metabolism regulation. The results from chapter-5 suggest that FFA-treated HeLa cells showed an increase in lipid synthesis proteins along with increase in triglycerides and total cholesterol accumulation. Whereas, the low and moderate alcohol failed to regulate the proteins involved in lipid synthesis such as SREBP1 and ACC via AMPK-α1 and SIRT1. Thus, the results from chapter-5 conclude that LKB1 is required for the activation of AMPK-α1, and SIRT1.

Principal finding 5 (CHAPTER 6): Identifying the role of low and moderate alcohol in FFA (oleic and palmitic acid) induced NAFLD in HepG2 cells (AMPK-inhibited).

The decrease in hepatic AMPK is one of the main protein involved in regulating the lipid synthesis. The aim of chapter-6 is to find the role of SIRT1 and LKB1 in regulating the lipid synthesis in the absence of AMPK in HepG2 cells treated with FFA. The results from this chapter suggest that FFA control group showed a similar result of FFA control group from previous chapters. However, the alcohol-treated groups showed an increase in LKB1 and SIRT1. SIRT1 plays a major role in controlling the post-translation proteins SREBP-1 and ACC. Thus, the result from chapter-6 conclude that alcohol treated HepG2 (AMPK-inhibited) cells, increases the SIRT1 protein in absence of AMPK.

Principal finding 6 (CHAPTER 7): Identifying the role of low and moderate alcohol in FFA (oleic and palmitic acid) induced NAFLD in HepG2 cells (SIRT1-inhibited).

The results from chapter-6 suggest that in the absence of AMPK, SIRT1 alone can regulate the lipid synthesis. To find the role of AMPK-α1, SIRT1 was inhibited in this chapter. In conclusion, the present investigation demonstrated that low and moderate alcohol increased AMPK-α1 activation via LKB1 in the absence of SIRT1, which was associated with decreased expression of SREBP-1 and ACC. So, this study demonstrates that low and moderate alcohol has a beneficial effect on AMPK-α1 via LKB1 in HepG2 cells.

8.2 Conclusions

NAFLD is induced by chronic feeding of HFD to rats leading to augmented metabolic dyslipidaemia. This is associated with elevated serum lipid profile, alongside increased serum insulin and glucose levels, which also includes elevated ALT and AST liver enzymes. Long-term feeding with low to moderate alcohol decreased the serum glucose, HDL cholesterol and liver ALT and AST enzymes in HFD-induced NAFLD.

Long term feeding of HFD leads to up-regulated SREBP-1c protein and contributes to the increase *de novo* fatty acid synthesis. Increased SREBP1c triggers the downstream target proteins such as ACC, which was observed in HFD fed groups. Increased expression of SREBP1c and ACC proteins, plays a major role in regulating the synthesis and β-oxidation of fatty acids . This further leads to increase in lipid deposition in liver, which finally results in steatosis, the first stage of NAFLD. The low and moderate alcohol-treated groups showed an elevated levels of AMPK-α1, p-AMPK-α1, LKB, p-LKB1, and SIRT1 proteins. Apart from AMPK itself, the concurrent regulation of SIRT1 and AMPK controls the SREBP-1 dependent and its downstream proteins by phosphorylating the SREBP1 and ACC proteins which are involved in lipid synthesis.

HepG2 cells treated with FFA showed an increase in triglycerides and total cholesterol levels, which leads to hepatic steatosis. Whereas, the alcohol-treated groups did not show any effect in controlling the hepatic lipids. However, the moderate alcohol with FFAs group showed an increase in phosphorylation of

AMPK-α1, LKB1, and SIRT1, the proteins involved in regulating the lipid synthesis via controlling the SREBP1c and ACC.

From the inhibitory studies, it is concluded that LKB1 is essential for activation of AMPK and SIRT1. In contrast to LKB1 inhibition in HeLa cells, HepG2 cells showed an increased SIRT1 protein levels despite the inhibition of AMPK, SIRT1 deacetylates and inhibits SREBP-1c activity, resulting in down-regulation of lipogenic SREBP-1c gene expression and subsequently decreased fat storage in the liver. Furthermore, the low and moderate alcohol increased AMPK activation via LKB1 in the absence of SIRT1, which was associated with decreased expression of SREBP-1 and ACC.

Taken all together, these findings demonstrated that the low and moderate alcohol protected against the development of HFD induced NAFLD by enhancing the proteins such as AMPK-α1, SIRT1 and LKB1 involved in lipid metabolism. Further, *in vitro* studies demonstrated that the consumption/intake of low and moderate alcohol reduces the lipid synthesis via increasing AMPK-α1, SIRT1 and LKB1 which regulate each other.

8.3 Limitations

Due to time constraints, only protective effects of low to moderate alcohol have been evaluated in this thesis. Curative effects of low to moderate alcohol on *in vivo* studies work have not been performed in this thesis. Furthermore, this thesis includes information about alcohol with HFD and does not include any experimental groups fed with only low to moderate alcohol, which could have delivered information about the beneficial effects of low and moderate alcohol in absence of HFD. This thesis provides information only about the major proteins involve protecting the liver from NAFLD. The enzymatic studies and mRNA expression (RT-PCR) studies work has not been performed in this thesis. This thesis includes only information only about the major outlined proteins involved in lipid pathways. A further detailed proteins involved in lipid pathways need to be quantified, besides those involved in NAFLD. Moreover, this thesis does not provide any information on the role of low to moderate alcohol on the inflammatory and oxidative stress pathways which plays a major role in pathogenesis of NAFLD.

This thesis lacks the information on alcohol metabolism studies in *in vitro* and *in vivo* models. Furthermore, investigations on blood alcoholic concentrations of low to moderate alcohol has not been done. This thesis includes information about the HepG2 cell line, which cannot metabolise alcohol was used for evaluating the role of low to moderate alcohol. This thesis lacks the information about the effects of low to moderate alcohol on the genetically modified HepG2 cell line such as VA-13, which has ethanol metabolism properties. This thesis lack the detailed information about the role of individual protein among AMPK-α1/LKB1/SIRT1 in regulating the lipid metabolism pathways. Furthermore, experiments on *in vitro* histology studies have not been done.

8.4 Future directions

Future studies on the curative effects of low to moderate alcohol on HFD-induced NAFLD are required. Furthermore, extra groups with only alcohol treated rats should be included in the experimental procedure with increased sample size, which will provide a better understating on beneficial effects of low to moderate alcohol on protecting the liver from HFD induced NAFLD. A further detailed evaluation on proteins involved in lipid pathways need to be quantified. The effects of low to moderate alcohol on inflammatory and oxidative stress pathways which plays a major role in pathogenies of NAFLD needs to be investigated. The role of low to moderate alcohol on leptin and adiponectin which are the main metabolic products of adipose tissue have been implicated in the pathogenies of NAFLD and a part of metabolic syndrome needs to be studied.

All the *in vitro* studies need to be done in VA-13 cell lines, a genetically modified HepG2 cell line which has ethanol metabolizing properties and the comparison of results will provide the role of alcohol metabolites in protecting the liver from NAFLD. Furthermore, detailed multiple protein inhibitory studies need to done for evaluating the role of individual proteins in protecting the liver from NAFLD. Moreover, a detailed histopathological studies including H&E staining and Oil Red O staining need to be done for *in vitro* models.

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