EXPOSURE TO ALCOHOL INTERRUPTS ADIPOSE CELL MATURATION, ATTENUATES ADIPONECTIN EXPRESSION, AND CONTRIBUTES TO INFLAMMATORY MARKERS IN 3T3-F442 PRE-ADIPOCYTES

A DISSERTATION

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To the Dean of the Graduate School:

I am submitting herewith a dissertation written by Mahnoush Sophia Shafiei entitled: Exposure to Alcohol Interrupts Adipose Cell Maturation, Attenuates Adiponectin Expression, and Contributes to Inflammatory Markers in 3T3-F442 Pre-adipocytes. I have examined this dissertation for form and content and recommend that it will be accepted in partial fulfillment of the requirement for the degree of Doctor of Philosophy with a major of Nutrition.

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We have read the dissertation and recommend its acceptance

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DEDICATION

This work is dedicated to three people who have taught me the most important lessons in my life; I would not have accomplished what I have without them. I dedicate this work to the memory of my father, who gave me a thirst for science and the knowledge and the potential to seek it, to my mother, whose love is the greatest gift of my life and whose commitment, support, and patience are a true model for all, and to my son, Reza, who inspires me to grow, who will always be a continual source of pride and enlightenment to me, and who brings me the greatest happiness.

I also dedicate the efforts herein to my sisters for their encouragement, patience, and never-ending love. If we are but a collection of those who believe in us, I am lucky to have these people in my life who daily push and inspire me.

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ABSTRACT

MAHNOUSH SOPHIA SHAFIEI

EXPOSURE TO ALCOHOL INTERRUPTS ADIPOSE CELL MATURATION, ATTENUATES ADIPONECTIN EXPRESSION, AND CONTRIBUTES TO INFLAMMATORY MARKERS IN 3T3-F442 PRE-ADIPOCYTES

AUGUST 2018

Drinking alcohol during pregnancy interrupts cellular development, which may have far-reaching health effects on the fetus in its embryonic state and later as a young child. Adipose tissue (AT) is an important target for alcohol action. Alcohol disrupts the synthesis of a wide variety of peptides and adipokines, as well as the endocrine activity of adipose tissue. Adiponectin is an anti-inflammatory and insulin-sensitizing adipokine that is involved in fatty acid breakdown; it is produced in adipose tissue. In this project, it is hypothesized that alcohol disrupts adipose cell development and reduces adiponectin (ApN) expression, concomitant with an elevation of pro-inflammatory cytokines and reduction in anti-inflammatory cytokines. The data suggest that alcohol exposure in 3T3-F442 pre-adipocytes reduces adipocyte proliferation. Pre-adipocytes were exposed to 0.0% (control), 0.25%, 0.5%, 1%, 1.5%, 2%, and 2.5% alcohol solution for 48 hours. Triglycerides and the expression of ApN and several pro- and anti-inflammatory cytokines were measured. Nile Red (NR) staining was used for the detection of adipogenesis and adipocyte differentiation. The results show triglyceride reduction in 3T3-F442 adipocytes and a significant reduction in adipocyte differentiation in

comparison with control (non-alcohol-treated) cells. Furthermore, ApN secretion was reduced in 3T3-F442 cells in response to alcohol. The pro-inflammatory cytokines, IL-6, IL-13, IL-1b, TNF-α, and INF-γ were increased, whereas the anti-inflammatory cytokines, including IL-4 and IL-10, IL-12, were reduced. In conclusion, exposure of alcohol reduced differentiation of 3T3-F442 pre-adipocytes to adipocytes. It was demonstrated in this project that alcohol impairs ApN secretion and increases pro-inflammatory cytokines. The results help to establish the potential role of alcohol in promoting inflammation and reducing adiponectin expression in developing pre-adipocytes, suggesting alcohol may be disruptive in metabolism by disruption of adipose cell differentiation.

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CHAPTER I

INTRODUCTION

Alcohol is a widely used substance with nearly 100 million people in the world suffering from alcoholism (Rehm et al., 2009; Scherer, Williams, Fogliano, Baldini, & Lodish, 1995; Shield, Gmel, Patra, & Rehm, 2012; Smart, 1991). In the United States, alcohol consumption increased significantly between 2002 and 2013 (Dawson, Goldstein, Saha, & Grant, 2015). Women are more prone to develop alcohol-related health issues and alcohol addiction (Qureshi, Couto, Hofvind, Wu, & Ursin, 2012; Tan, Denny, Cheal, Sniezek, & Kanny, 2015; Wu, Vigen, Razavi, Tseng, & Stancyzk, 2012; Zelner & Koren, 2013).

Alcohol consumption during pregnancy causes fetal alcohol spectrum disorders (FASDs) (Bruce, Biousse, Dean, & Newman, 2009; Gemma, Vichi, & Testai, 2007; Jones, 2011; May et al., 2013; Memo, Gnoato, Caminiti, Pichini, & Tarani, 2013; R. A. Mukherjee, Hollins, & Turk, 2006; Werts, Van Calcar, Wargowski, & Smith, 2014). Infants with FASDs are characterized by abnormal appearance, small head size, poor coordination, and behavior problems. These infants also show an increase in feeding and a lack of satiety, followed by accumulation of body fat when affected by fetal alcohol syndrome (FAS) (Fuglestad et al., 2014; Werts et al., 2014). The extent of these symptoms, such as abnormal appearance, depends on the timing, amount, and duration of

alcohol exposure and on genetic susceptibility (Friel, Baer, & Logan, 1995; Gemma et al., 2007).

Alcohol use in pregnant women has serious consequences for the mother and the developing fetus and leads to a range of disorders and health complications (Ridderinkhof et al., 2002; Roussotte et al., 2012; Yang a, Phillips, et al., 2012; Yang b, Roussotte, et al., 2012). Moreover, prenatal alcohol exposure is a risk factor for developing both metabolic and endocrine disorders (Fuglestad et al., 2014; Xia et al., 2014).

Over the past two decades, the discovery of adipokines, adipocyte-derived factors, and their protective action has increased interest in identifying the role these adipocytokines serve in tissue dysmorphogenesis and obesity (Berg, Combs, & Scherer, 2002; Harwood, 2012; Kiess et al., 2008). Adipocytes are recognized as producers of potent hormones, rather than just lipid-storing cells (Ahima, Qi, & Singhal, 2006; Coelho, Oliveira, & Fernandes, 2013; Harwood, 2012). Notably, the pathophysiological impact of AT is now recognized as playing a key role in systemic and metabolic alteration of different tissues (Kershaw & Flier, 2004; Kienesberger et al., 2009). Adipocytes can be identified as early as the sixth week of gestation in humans (Corbetta et al., 2005). The development of differentiated adipose cells (adipocytes) from pluripotential-undifferentiated precursors is called adipogenesis (Billon & Dani, 2012; Chi et al., 2014; Rosen & MacDougald, 2006; Stern & Scherer, 2015). Adipogenesis is regulated by over 100 transcription factors (Rosen & Spiegelman, 2000; Rosen, Walkey,

Puigserver, & Spiegelman, 2000). AT also produces a wide range of proactive cytokines, called adipocytokines (Kang et al., 2016; Tilg & Moschen, 2006).

Adiponectin (ApN), a circulating plasma protein hormone secreted by adipose tissues, is an example of an adipocytokine that has received continued interest in fat metabolism research (Caminos et al., 2005; Chandran, Phillips, Ciaraldi, & Henry, 2003; Corbetta et al., 2005; Yamaguchi et al., 2005). The level of ApN is inversely associated with obesity (Ahl et al., 2015). Thus, low levels of ApN in plasma are recognized as a biological marker for the potential development of obesity and hypertension (Iwashima et al., 2004; Martos-Moreno, Barrios, Chowen, & Argente, 2013). However, ApN may provide protection against various chronic diseases, including obesity, cardiovascular disease, and cancer (Yamamoto et al., 2012; Yamauchi et al., 2003). Animal studies have shown that ApN-overexpressing animals are significantly less susceptible to chemically-induced liver fibrosis compared to controls (Shafiei, Shetty, Scherer, & Rockey, 2011).

Hypertrophy and disruption of AT integrity could induce pro-inflammatory and cell-stress signals (K. Sun, Kusminski, & Scherer, 2011; K. Sun, Tordjman, Clement, & Scherer, 2013). Indeed, this tissue inflammation results from the death of hypertrophic adipocytes and gives rise to distinctive structural differences and an abnormal physical appearance of the cells (Bourlier et al., 2008; Cinti et al., 2005; Giordano et al., 2013; Suganami & Ogawa, 2010).

White AT (WAT) consists of adipocytes, macrophages, and vasculature (Cristancho & Lazar, 2011; Hausman, DiGirolamo, Bartness, Hausman, & Martin, 2001; Tang et al., 2008; Trayhurn, 2007; Q. A. Wang, Tao, Gupta, & Scherer, 2013). During WAT adipogenesis, embryonic pluripotential precursors elicit multipotential mesenchymal cells, which results in the formation of adipoblasts, type I pre-adipocytes, type II pre-adipocytes, and mature adipocytes (Ailhaud, Grimaldi, & Negrel, 1992; Cornelius, MacDougald, & Lane, 1994; Gesta, Tseng, & Kahn, 2007; Gregoire, Smas, & Sul, 1998). In this sequence of development, adipoblasts synthesize proinflammatory cytokines (IL6, TNF-α), but only mature adipocytes are capable of secreting adipocytokines and ApN (Cristancho & Lazar, 2011; Martos-Moreno et al., 2013).

Cytokines are multifunctional proteins that play a critical role in a variety of tissues, performing functions such as regulating inflammation, cell death, cell proliferation, and migration (Hofmann et al., 2002; O'Shea, Ma, & Lipsky, 2002; Turner, Nedjai, Hurst, & Pennington, 2014). Cytokines are categorized as being pro-inflammatory (T helper 1, Th1) or anti-inflammatory (T helper 2, Th2). Interleukins (IL), interferons (IFN), growth factors, and tumor necrosis factors alpha (TNF-α) all represent cytokines (Cousins, Lee, & Staynov, 2002). The main pro-inflammatory cytokines are TNF-α, IL-1, IL-6, and IFN-γ, while the main anti-inflammatory cytokines are IL-4, IL-10, and IL-12 (Dinarello & Pomerantz, 2001; Laso, Vaquero, Almeida, Marcos, & Orfao, 2007). These cytokines activate natural killer and helper T cells, inducing cell-mediated

immunity. IL-8 is a chemokine cytokine that attracts neutrophils to the site of an infection (Dinarello, 2000). In general, activation of Th1 cytokines inhibits Th2 cytokine production and vice versa (Kidd, 2003). The activation of Th1 and inactivation of Th2 create a complicated process that regulates tissue response to inflammation, injury, and healing (Dinarello, 2000; Sandler, Mentink-Kane, Cheever, & Wynn, 2003).

Alcohol is a widely used substance and is known to adversely affect innate immune responses (Crews et al., 2006). Blood levels of the circulating antibodies-immunoglobulin A (IgA) are raised in individual with alcoholism, and immunoglobulin M (IgM) is increased in patients with liver disease (Gonzalez-Quintela et al., 2008). The excessive level of antibodies is associated with increased inflammation, dysregulation, and alteration in cytokine production. The effect of alcohol on the immune response depends on the blood alcohol level as well as the individual's history of alcohol consumption (Viitala, Israel, Blake, & Niemela, 1997). Alcohol increases different cytokines, including, TGF-β, TNF-α, IL- β in different organs, such as the lung, liver, and adipose tissue, in a complex manner (Ishikawa et al., 2011; Kema, Mojerla, Khan, & Mandal, 2015).

Rationale and Significance

The molecular mechanisms and processes underlying the teratogenic effects of alcohol exposure have not yet been fully explored. The successful completion of this body of work will contribute to a better understanding of how alcohol exposure in pre-

adipocytes, which are present in fetal tissue, affects the complex physiology of AT and adipose-derived hormone activities. This may contribute to understanding whether there are physiological reasons for an increased risk for obesity in children with FASDs and point to the need for additional research of these mechanisms in humans.

The murine pre-adipocyte cell line 3T3-F442, which works through insulin-like growth factor (IGF-1), is an ideal model for white adipocyte differentiation, which occurs during gestation in fetal tissue (Gregoire et al., 1998; Rosen, 2002). After stimulation with insulin, and confluent growth, pre-adipocytes start the cell cycle and undergo several rounds of mitosis, which is known as clonal expansion (Gregoire et al., 1998). Mitosis is required for transcription factors to interact with the regulatory response element on the gene that is responsible for the adipocyte's phenotype (Green & Kehinde, 1975; Green & Meuth, 1974).

Using the pre-adipocyte 3T3-F442 cell line may help to characterize how alcohol exposure during differentiation may disrupt AT integrity and differentiation. Therefore, pro-inflammatory signaling, hypoadiponectinemia, and reduction in triglyceride were investigated in this study. This research offers an innovative, investigative approach to examine the role of alcohol in adipose cell differentiation and proliferation.

Alcohol induces release of proinflammatory cytokines and contributes to the alcoholic pathology of some tissues. It is not known how alcohol affects adipocyte

maturation, proliferation, and metabolism. The following questions guided the present dissertation:

1. How does alcohol exposure in pre-adipocytes stimulate secretion of proinflammatory cytokines in adipocytes?
2. How does alcohol exposure in pre-adipocytes affect adipose cell development and ApN production?

Hypothesis and Aims

The hypothesis guiding this research is that early alcohol exposure in 3T3-F442 pre-adipocytes can have lasting effects on physical abnormalities and obesity in offspring.

Hypothesis: Alcohol exposure during early development interrupts adipogenesis by reducing the transdifferentiation of adipocytes, which ultimately decreases triglyceride accumulation in adipocytes. Consequently, arrested growth in adipoblasts is followed by up-regulation of pro-inflammatory cytokines and a subsequent reduction in ApN expression (Figure 1.1).

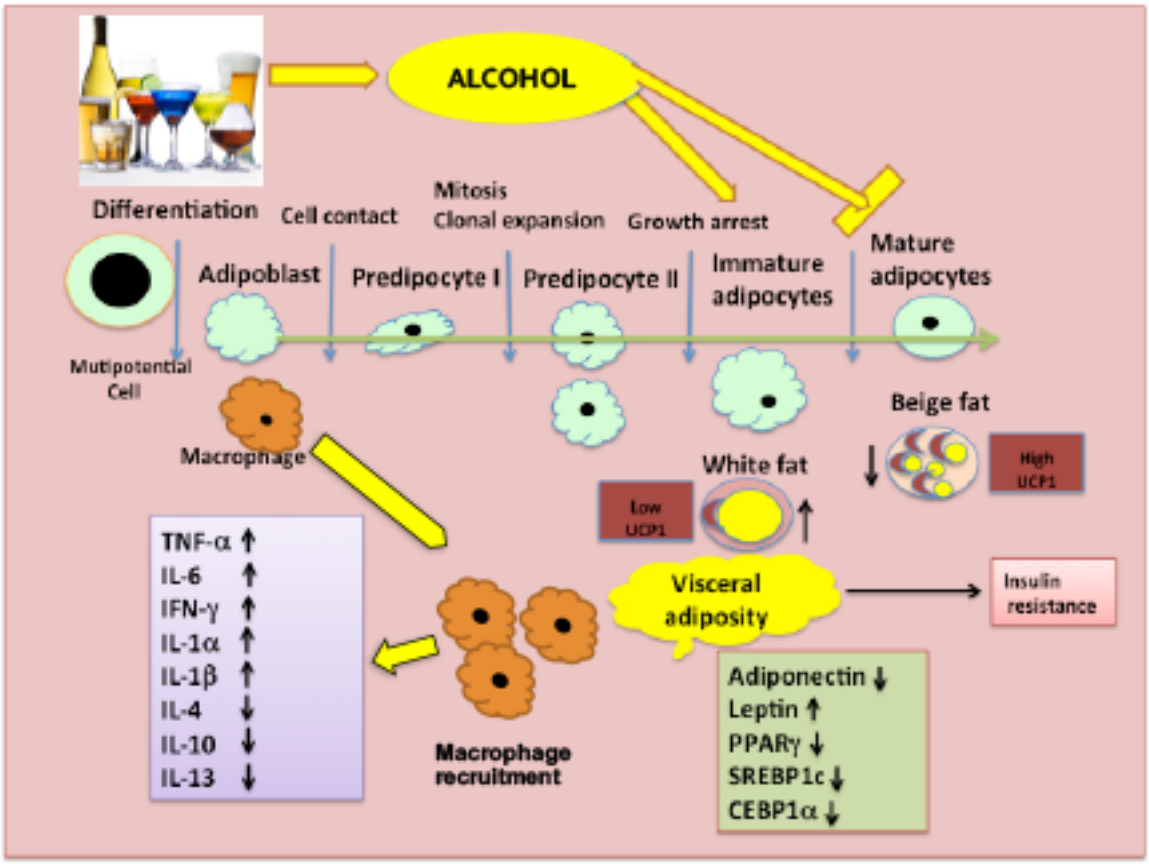

Figure 1.1. Adipogenesis Model of Alcohol-Induced Obesity. Normal adipogenic differentiation of multipotential cells and their pattern of adipokine secretion. The ability to secrete adiponectin is exclusively restricted to the mature adipocytes at the end of this process. Interrupted adipocyte development increases pro-inflammatory cytokines production.

This project has five distinct aims that contribute to the hypothesis:

Specific Aim 1: To determine the effect of alcohol on intracellular lipid accumulation in 3T3-F442 pre-adipocytes.

Specific Aim 2: To delineate the impact of alcohol on adipocyte adiponectin production by performing the above comparison with cells that are not treated with alcohol (control).

Specific Aim 3: To evaluate the effect of alcohol on pro-inflammatory cytokines, chemokines mRNA, and protein expression in 3T3-F442 adipocytes including IL-6, IL1β, TNF-α, IFNγ, and IL17.

Specific Aim 4: To determine the effect of alcohol on anti-inflammatory cytokines including mRNA and protein expression in 3T3-F442 adipocytes including IL-13, IL-10, and IL-4.

Specific Aim 5: To establish whether alcohol exposure on adipocytes will alter lactic acid production in 3T3-F442 cell lines by determining the extracellular acidification rate (ECAR).

Limitations

The research presented here was limited by using pre-adipocyte cell lines rather than AT of animal models. This study should be followed by animal studies and primary AT from mice. In addition, transgenic mice and tissue-specific Cre-lox knockouts (KO) of adiponectin should be attempted.

CHAPTER II

REVIEW OF LITERATURE

Alcohol

Ethanol is a widely used and abused substance in most societies (Barnes, 1984; Murray, Barnes, & Patton, 1994). Around the world, approximately 100 million people have an alcohol-use disorder (Rehm et al., 2009; Shield et al., 2012). In the US, according to the Center for Disease Control and Prevention, there are 88,000 alcohol-related deaths each year (Hingson, Zha, White, & Simons-Morton, 2015; White et al., 2015). Alcohol is estimated to be the third leading preventable cause of death in the US (Polednak, 2007, 2012, 2016; Pongor et al., 2015). Alcohol is a contributing factor for 60 or more diseases in human beings, including obesity, cardiovascular diseases, and type 2 diabetes (Baliunas et al., 2009; Klatsky et al., 2014; Mantena, King, Andringa, Eccleston, & Bailey, 2008). These diseases create a notable burden on the economy in the form of healthcare costs, which were estimated at $223 billion in 2006 in the United States alone (Bouchery, Harwood, Sacks, Simon, & Brewer, 2011; Polednak, 2005, 2007). Alcohol use during pregnancy disrupts cell and tissue development, which can lead to health complications known as fetal alcohol spectrum disorder (FASDs) (Gemma et al., 2007; Memo et al., 2013; R. Mukherjee, Eastman, Turk, & Hollins, 2007; R. A. Mukherjee, Hollins, & Curfs, 2012; R. A. Mukherjee & Turk, 2004). When consumed during pregnancy, alcohol crosses the placenta, which causes fetal blood alcohol levels to be

higher than the maternal levels. Since a fetus cannot break down alcohol at the same rate as an adult, the consumption of alcohol during pregnancy results in high concentrations of alcohol within the fetus (Burd, Roberts, Olson, & Odendaal, 2007). Alcohol-related teratogenic effects and molecular mechanisms remain poorly understood because of differences in genetic susceptibility and differences in the amount and the duration of exposure to alcohol.

Alcohol Metabolism

Alcohol metabolism and elimination are regulated by different pathways (Cederbaum, 2012). The common pathways are associated with two enzymes, including alcohol dehydrogenase (ADH), through which ethanol is broken down to acetaldehyde (CH_3CHO), a highly toxic and carcinogenic compound (Cederbaum, 2012; Edenberg, 2007). These are generally short-lived substances and are further metabolized by aldehyde dehydrogenase (ALDH) into a less toxic byproduct called acetate (Zakhari, 2006). The acetate releases water and carbon dioxide (Figure 2.1) (Zakhari, 2006).

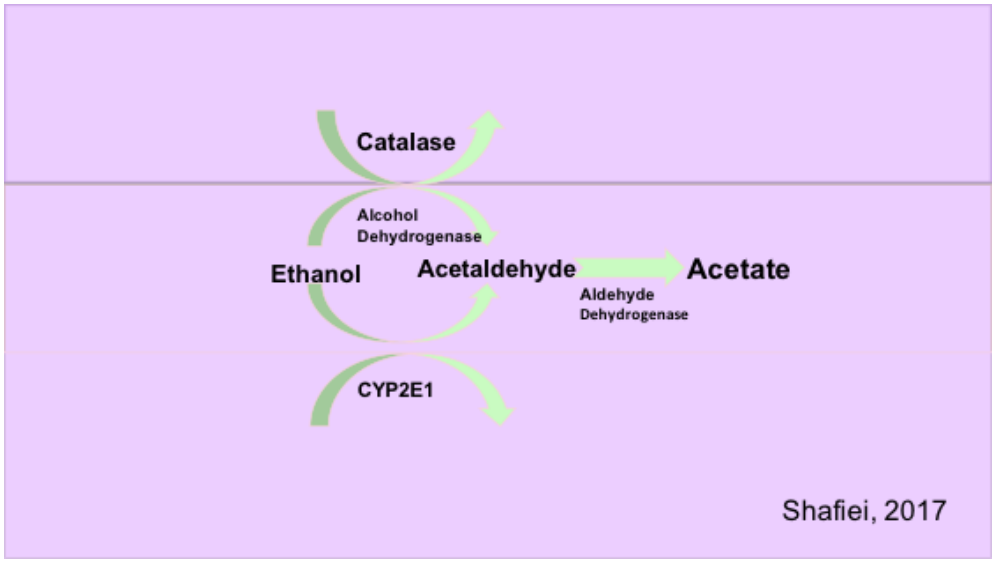

Figure 2.1. Oxidative Pathways of Alcohol Metabolism. Alcohol metabolized by (ADH) to produce acetaldehyde. At high levels of alcohol consumption, cytochrome P450 2E1 metabolizes alcohol into acetaldehyde. Acetaldehyde is further metabolized into acetate in the mitochondria.

Alcohol and Cytokines

The immune system has a distinct and extremely complicated impact on cells and different tissues (J. Wang & Arase, 2014). Cytokines are groups of heterogeneous polypeptides with a molecular weight between 8-45 KD that regulate the immune system as well as inflammatory responses (Ohno, Natsume, & Wakabayashi, 2012). Cytokines play a vital part in the complex network of the immune system that includes interleukins, interferons, tissue necrosis factor a (TNF-α), and growth factors (Arai et al., 1990; Hofmann et al., 2002). Chemokines have a significant role in regulating cell death, proliferation, migration, and inflammation (Luster, 1998). Within immune systems, most cells respond to cytokines by increasing the pro-inflammatory cascade and increasing cytokine synthesis (O'Shea et al., 2002). Other types of cytokines are capable of modulating and inhibiting pro-inflammatory cytokines (Wojdasiewicz, Poniatowski, & Szukiewicz, 2014). T cells are classified as helper T (Th cells, CD4) or cytotoxic T cells (Tc cells, CD8) that are the components of acquired (adaptive) immunity. The helper T cells are separated into two different groups, the Th1 and Th2 subtypes), each of which produce a different type of immune-regulating cytokine (Cherwinski, Schumacher, Brown, & Mosmann, 1987; O'Shea et al., 2002; Romagnani, 1991, 1992, 1994; Sultani, Stringer, Bowen, & Gibson, 2012). Th1 cells produce the key pro-inflammatory cytokines, including IL-6, TNF-α, IL-1, and IFN (Dinarello, 1991). The Th2 subtypes produce the key anti-inflammatory cytokines, IL-4, and IL-10 (Sultani et al., 2012). The

chemokine cytokine IL-8 is a neutrophil-attracting cytokine (Chatterjee, Chiasson, Bounds, & Mitchell, 2014; Dinarello, 1997). The T helper cells and natural killer cells are activated by IL-12 and induce cell-mediated immunity (Becker, 2004; Trott & Harrison, 2014).

The immune system is known to be altered by alcohol (ethanol), which increases blood levels of circulating antibodies (Cook, 1996; Crews et al., 2006). Elevation of IgA occurs in the blood of all alcoholics with elevation of IgM also occurring in people with liver dysfunction (Jakab, 2015). The high level of antibodies is possibly caused by autoimmunity dysregulation and cytokine production alteration. The influence of alcohol on the immune system is affected by the level of alcohol consumption, the duration of alcohol exposure, and the history of consumption of alcohol (Crews et al., 2006).

Wheeler and others have shown in their studies that alcohol-induced liver disease increases macrophages in the liver (Polednak, 2012; Wheeler, Luscinskas, Bevilacqua, & Gimbrone, 1988). These findings reveal that alcohol-induced alcoholic liver diseases are associated with an augmented inflammation of the liver that induces TNF-α regulated oxidant radicals as well as other cytokines (H. J. Wang, Gao, Zakhari, & Nagy, 2012). Finally, the decrease of Th2 anti-inflammatory cytokines further supports the cytokines' role in the inflammation of the liver of alcoholics and progression of hepatic fibrosis (Isayama et al., 2004).

Crews et al. (2004, 2006) reported on the interaction between liver, plasma, and brain cytokines. TNF-α enters the brain upon systemic increase and leads to prolonged neuroinflammation, which shifts transcription factors by increasing the nuclear regulatory factor (Crews et al., 2006; Crews et al., 2004).

Bechara et al., 2004 reviewed studies on the lungs and the way cytokine responses altered by alcohol lead to Acute Respiratory Distress Syndrome (ARDS). The lungs' specific expression of Transforming Growth Factor-βb-1 (TGF-β-1), a pluripotent cytokine implicated in acute lung injury, was dramatically increased by alcohol administration (Bechara, Brown, Roman, Joshi, & Guidot, 2004).

Fetal Alcohol Syndrome Effects, Alcohol, and Metabolic Disruption

Infants with prenatal alcohol exposure do not always show the complete physical characteristics of FAS but may instead have a neurocognitive impairment and abnormalities in the structure of the brain (FASD) (Roussotte et al., 2012). FASD leads to intellectual and neurodevelopmental disabilities and covers several adverse effects associated with developmental disabilities (Bruce et al., 2009; Yang, Roussotte, et al., 2012). In addition, Gemma (2007) suggest prenatal alcohol exposure causes abnormalities in the metabolic pathways in offspring (Gemma et al., 2007).

A few studies suggest children and adolescents with chronic alcohol exposure are also at significant risk for diabetes mellitus, impaired glucose homeostasis, hypertriglyceridemia, hypertension, and abdominal obesity (Fuglestad et al., 2014; Ting

& Lautt, 2006). Moreover, children with FAS are at risk of developing hyperinsulinemia and hyperglycemia. These children also exhibit insulin resistance (de la Monte & Wands, 2010). However, the mechanisms of prenatal alcohol exposure and metabolic syndrome and its incidence in children are not well-established.

In addition, the consequence of chronic prenatal ethanol exposure (CPEE) on metabolism is not well known or understood. Recently, some studies have demonstrated an alteration in metabolic pathways in early ethanol exposure including impaired insulin signaling, impaired glucose metabolism, and increased gluconeogenesis (Dobson et al., 2014). Another study has shown a detailed understanding of the nature of alcohol and tested the effect of an increase in whole-body adiposity in CPEE guinea pig offspring. The study found prenatal ethanol exposure during pregnancy increased whole body adiposity in CPEE guinea pig offspring. This leads to hypertriglyceridemia in adult guinea pig progeny, suggesting CPEE is a risk factor for altered lipid metabolism (Dobson et al., 2012).

In summary, alcohol exposure leads to birth defects and a range of symptoms connected to FAS and FASD, including abnormal facial features, intellectual disability, impaired memory or attention, immune system disruption, inappropriate feeding behaviors, and obesity (Bruce et al., 2009; Burd et al., 2007; Matson et al., 2013; Mattson et al., 2013; Roussotte et al., 2012). Until recently, FASD research and alcohol research has focused on congenital defects, while the mechanistic aspects of FAS and FASD

remain not fully understood. Alcohol use during pregnancy is associated with growth retardation; however, there has been little focus in terms of the link that is associated with FAS and being overweight or obese. Fuglestad et al. (2014) conducted a large clinical study with children with FASD and studied the pervasiveness of high body mass index (BMI) and obesity. The results have shown that in children with FASD, the rate of overweight/obesity is increased (Bruce et al., 2009; Fuglestad et al., 2014). Moreover, this rate is increased in adolescents for any FASD diagnosis, especially in females (Zelner et al., 2012). The data suggest that prenatal alcohol exposure/FASDs increases risk for metabolic or endocrine disruption as well as the development of obesity (Fuglestad et al., 2014). Prenatal alcohol exposure can also affect eating behavior through an increased level of feeding behavior and a lack of satiety (Werts et al., 2014).

Childhood Obesity

In U.S., there has been a 60% increase in childhood obesity since 1990 (Akinbami & Ogden, 2009; Ogden, Carroll, Kit, & Flegal, 2012, 2014). Obesity is a critical problem, and during the past three decades, a dramatic increase in obesity has become a general health crisis (Olds et al., 2011). Obesity is associated with many different chronic diseases and is characterized by the expansion of unhealthy adipose tissue. In U.S., more than 25% of children are overweight and 11% are obese (Fakhouri, Ogden, Carroll, Kit, & Flegal, 2012). Being overweight or obese during childhood significantly

increases physical and psychological co-morbidities (Dehghan, Akhtar-Danesh, & Merchant, 2005; Werts et al., 2014).

Like non-pregnant adults with obesity, obese, pregnant women often have low adiponectin levels (Ianniello, Quagliozzi, Caruso, & Paradisi, 2013). Low adiponectin level during pregnancy is associated with poor fetal development (Hendler et al., 2005; Lekva et al., 2017; Qiao et al., 2017). Pregnant mothers with low circulating adiponectin have an increased risk for giving birth to an infant that is LGA, and they are at risk for high intrauterine abdominal circumference in the fetus (Lekva et al., 2017).

It is of interest whether alcohol exposure in utero could be a contributing risk for obesity in offspring, even if FASD is not present. The proposed study will explore a possible mechanism for increased obesity in offspring exposed to alcohol in utero.

Extracellular Remodeling of Adipose Tissue

Adipocytes are surrounded by extracellular matrix (ECM), provide mechanical support, and contribute to a variety of different signaling events. Expansion of unhealthy AT during development leads to chronic inflammation (Castoldi, Naffah de Souza, Camara, & Moraes-Vieira, 2015; Dalmas, Clement, & Guerre-Millo, 2011; Divoux et al., 2010). Obesity impacts AT macrophages and immune cells and prompts a switch from anti-inflammatory to pro-inflammatory cytokines (Lee, Wu, & Fried, 2010; Lumeng, Bodzin, & Saltiel, 2007). Obesity is associated with low-grade inflammation and the

production of pro-inflammatory cytokines and is characterized by macrophage infiltration of AT (Neels & Olefsky, 2006; Wellen & Hotamisligil, 2005).

Some investigators have shown that with increasing body weight, macrophages are accumulated in AT (Lumeng et al., 2007). Additionally, inhibiting the accumulation of AT macrophages prevents obesity and inflammation as well as insulin resistance (Weisberg et al., 2006). Obesity induces visceral AT expansion and ECM remodeling. Continuing production and deposition of collagen VI (a major ECM constituent in adipose tissue) and fibronectin leads to AT dysfunction and abnormal architecture of AT (Divoux et al., 2010; K. Sun et al., 2011).

AT as an Endocrine Organ

The discovery of "adipocytokines," and their function as hormones in several tissues, has changed our view of AT to an active endocrine organ (Deng & Scherer, 2010). Previously, AT was recognized as a depository for triglycerides, but now it is regarded as an active player in the interaction between adipocytokines and inflammation (Matsuzawa, 2005, 2006, 2007). Adipogenesis starts from pluripotential-undifferentiated precursors very early in the sixth week of gestation (Feve, 2005; Martos-Moreno et al., 2013). Adipocytes regulate the pro-inflammatory population of monocytes and macrophages by modifying the paracrine and endocrine secretions (Hausman et al., 2001). The adipogenesis from embryonic pluripotential precursors results in the formation of type I pre-adipocytes, type II pre-adipocytes, and mature adipocytes.

Adipoblasts synthesize pro-inflammatory cytokines such as IL-6 or TNF-α, but only mature adipocytes have the ability to secrete and synthesize adipocytokines including ApN (Cristancho & Lazar, 2011; Feve, 2005). ApN is composed of 247 amino acids, and a mutation in a single nucleotide causes hypoadiponectinemia (Weyer et al., 2001). A lower level of ApN is directly connected to the degree of obesity, and hypoadiponectinemia is identified as a marker for potential development of obesity (Iwashima et al., 2004; McManus et al., 2012). Moreover, interruption of the integrity of AT and hypertrophy could increase the pro-inflammatory cytokines and cell stress signals (Greenberg & Obin, 2006). Ultimately, the physiological impact of AT is crucial in the metabolic function of different tissues (Giordano et al., 2013).

ApN Structure and Function

A broad range of cytokines called, "*adipocytokines*" are produced by AT(Matsuzawa, 2007). ApN is an adipocytokine and is circulates as a plasma protein (Corbetta et al., 2005; Maeda et al., 1996; Matsuzawa, 2010; Yamauchi et al., 2001). Different groups have been identified APN. First, Scherer characterized it in differentiating 3T3-L1 adipocytes in 1995 (Scherer et al., 1995). In 1996, the sequence was identified in mice as the mRNA transcript revealed an adipose-specific, collagen-like factor (apM1) (Maeda et al., 1996). From the pre- and post-differentiated adipocyte, along with 3T3-L1 and 3T3-F442A mouse fibroblasts, mRNA was generated. The encoded sequences of adipocyte complement-related protein of 30 kDa called AdipoQ

was eventually identified by. Spiegelman (Hu, Liang, & Spiegelman, 1996).

ApN consists of four different regions (similar to C1q): an N-terminal signal peptide, a C-terminal globular domain, a changeable region containing cysteine (at position 39), and a domain that is similar to collagen (Shapiro & Scherer, 1998). APN, at full-length, exists in plasma and is cleaved by an enzyme secreted by activated monocytes, called leukocyte elastase, in order to form the globular head (gAd) from the tail fragment (Waki et al., 2005). The full-length ApN can trimerize and circulate as a low molecular weight (LMW) molecule and through a di-sulphide bridge, join other trimers to form medium molecular weight (MMW) hexamers, high molecular weight (HMW), and multimers (Simpson & Whitehead, 2010). ApN, an active biological molecule, is secreted from adipocytes and circulates in plasma. ApN concentration is inversely correlated with adiposity and insulin resistance (Arita et al., 1999; E. Hu et al., 1996; Yamamoto et al., 2012).

For persons with a normal body mass index (BMI), ApN levels are usually normal. Western blotting demonstrates that ApN levels are about 0.05% of all plasma proteins (Scherer et al., 1995). ApN circulates at a concentration in the range of 1.9 to 17.0 μg/ml with ApN circulating concentration being dimorphic since males are shown to have lower plasma concentration than females (Combs et al., 2003). Differences in these observed levels may be due to the obstructive action of male testosterone, which suppresses the release of ApN from adipocytes (Xu et al., 2005).

There is a direct association between low ApN levels and increased risk of type 2 diabetes and the onset of obesity and diabetes (Bacci et al., 2004; Iwashima et al., 2005; Iwashima et al., 2004; Jang et al., 2005; McManus et al., 2012; Ohashi et al., 2004; You, Considine, Leone, Kelly, & Crabb, 2005). In adult organisms, ApN regulates the metabolism of glucose and fatty acids. ApN reduces intercellular lipid content by regulating the level of insulin/insulin-like growth factor (IGF) (Berg et al., 2002; Combs, Berg, Obici, Scherer, & Rossetti, 2001; Combs & Marliss, 2014; Kiess et al., 2008). ApN also reduces the synthesis of fatty acid and stimulates direct fatty acid oxidation (Combs & Marliss, 2014).

ApN activates lipid metabolism by phosphorylation and activation of AMP-activated protein kinase (AMPK) (Fang et al., 2010; Yamauchi et al., 2003). AMPK in skeletal muscle increases fatty acid oxidation, regulates acetyl-CoA carboxylase (ACC), and stimulates carnitine palmityl transferase 1 (CPT I) (Lee et al., 2006). A key regulator of fatty acid metabolism, peroxisome proliferator-activated receptor α (PPARα), is stimulated by ApN and by controlling CPT I and hormone-sensitive lipase (HSL) (Anderson et al., 2002; Rakhshandehroo, Knoch, Muller, & Kersten, 2010).

AMPK activation induced by ApN is involved in enhanced lipid oxidation and maintains cardiac output in isolated working-heart models (Fang et al., 2010). As is evident from a large body of literature, ApN has protective effects in several disease

states that include obesity, cardiovascular disease and cancer (Berg et al., 2002; Nanayakkara, Kariharan, Wang, Zhong, & Amin, 2012; Obeid & Hebbard, 2012).

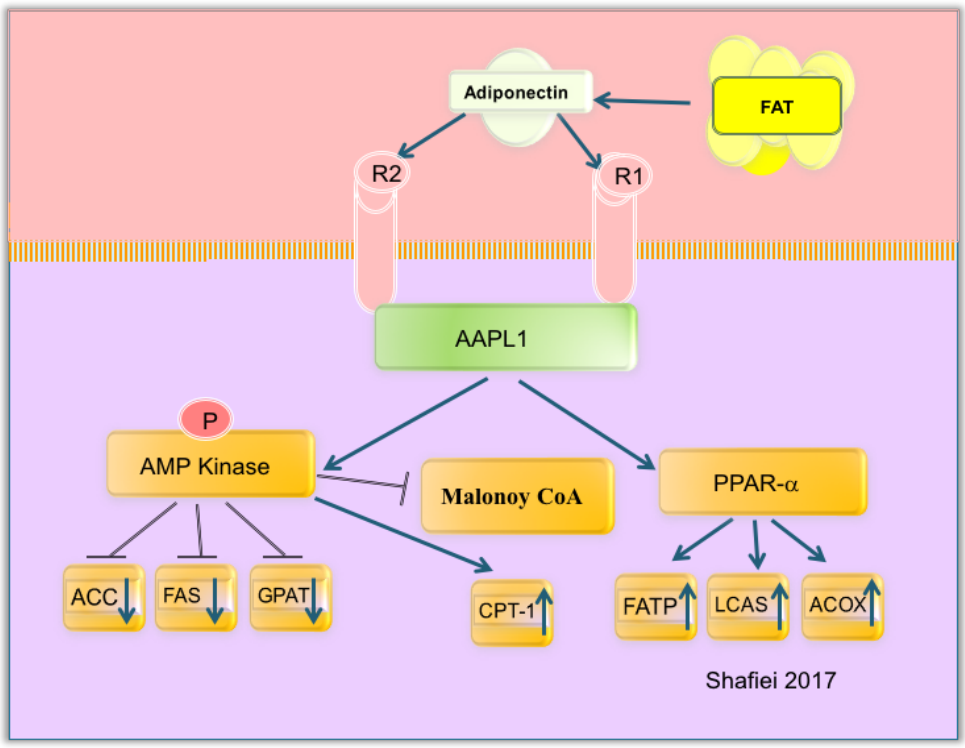

Figure 2.2. Adiponectin signaling. Two ApN receptors (adipoR1 and adipoR2) have been identified and found to be expressed in various tissues including adipose tissue. APPL1, an adaptor protein, appears to be a key-signaling molecule that couples ApN receptors and its downstream Adenosine monophosphate kinase (AMPK). AMPK attenuates acetyl coenzyme A carboxylase (ACC), fatty acid synthase (FAS), and glycerol-3-phosphate acyltransferase (GPAT). PPARα controlling fatty acid oxidation enzyme such as fatty acid transport protein (FATP), long chain acyl-CoA synthesis (LCAS), acyl-CoA oxidase(ACOX).

ApN and Inflammation

ApN contributes to anti-diabetic, anti-obesity effects as it suppresses the inflammatory stimuli that are induced with the activation of NF-κβ (Ohashi, Ouchi, & Matsuzawa, 2011). ApN mediates anti-inflammatory action through downstream mediators of AdipoR1 and AdipoR2, PPAR and AMPK, which increases the anti-inflammatory response by reducing nuclear factor kappa B (NF-κβ) activation (Chandrasekar et al., 2008). ApN blocks fat cell formation in bone marrow by elevating the expression of COX-2 and induced release of prostaglandin E (2) in stromal cell derived prostaglandin (Yokota et al., 2002). ApN receptors in cardiac myocytes and heart tissue decreased in hyperinsulinemia associated with obesity through PI3K/AKT pathway (Cui et al., 2012).

3T3-Pre-Adipocyte Cell Line

The study of adipose differentiation is facilitated by using cell culture models such as the fibroblast 3T3-L1 and the 3T3-F442 cell lines. Both sister cell lines can undergo adipose differentiation after stimulation with insulin, adipogenic serum proteins or growth hormones (Green & Meuth, 1974). 3T3-F442 cells can differentiate into fat pads *in vivo*, whereas 3T3-L1 do not have this feature (Green & Kehinde, 1975, 1979) Therefore, 3T3-F442 cells demonstrate that they can respond to physiological signals in the body, establishing the plasticity of this cell line (Green & Meuth, 1974).

The 3T3-F442A cell line is appropriate for the study of adipose differentiation, adipogenesis-regulating molecules, and the expression of very early genes involved in this pathway (Green & Kehinde, 1975, 1979).Thus, the 3T3-F442 cell line facilitates the study of very early genes involved in adipocyte differentiation and molecules that regulate adipogenesis (Green & Kehinde, 1975, 1979).

It is well known that when pre-adipocytes are induced, the process of differentiation begins with several rounds of mitotic clonal expansion. Then the cells become quiescent when adipogenic gene transcription is initiated (Ntambi & Young-Cheul). Then, pre-adipocytes undergo mitotic clonal expansion during the early stage of adipocyte differentiation through upregulation of C/EBP (MacDougald & Lane, 1995). Little research has been done on the effect of alcohol on differentiation of adipocytes from pre-adipocytes. The current study has the potential to improve our understanding of the way in which alcohol impairs adiponectin secretion in adipocytes and increases pro-inflammatory cytokines. It may also help us to understand adipocyte dysfunction in the context of early alcohol exposure.

CHAPTER III

METHODS

Material

All tissue culture media, Dulbecco's phosphate-buffered saline(PBS), fetal bovine serum (FBS), calf serum, and tissue culture supplies were from Invitrogen/Life Technologies (Carlsbad, CA). The AdipoRed assay reagent kit was from Lonza Inc., (Walkersville, MD), and Oil Red O and ethyl alcohol were from Sigma Aldrich, (St. Louis, MO). The ApN ELISA kit was from Millipore (Bedford, MA) and mouse cytokines were from R&D (Minneapolis, MN).

Methods

Cell Culture

Adipocyte cell culture has been shown to be useful for the study of adipocyte pathology, malfunctioning of AT and adipocyte development (Green & Kehinde, 1975). The cell lines 3T3-F442 and 3T3-L1 have been isolated from 3T3 mouse embryonic fibroblast and can be differentiated to adipocytes (Green & Kehinde, 1975). However, 3T3-F442 cells have a higher rate of spontaneous differentiation than 3T3-L1 cells. In addition, 3T3-L1 requires differentiation inducing agent including dexamethasone and 1-

methyl-3-isobuylxanthine to convert into adipocytes (Green & Kehinde, 1975; Morikawa, Green, & Lewis, 1984).

The murine 3T3-F442 pre-adipocyte cell line is most commonly used to model adipocyte differentiation, which occurs in fetal tissue. Disrupted metabolism can be detected in 3T3-F442 pre-adipocytes by noting undifferentiated fibroblasts or reduction in accumulation of high levels of triglyceride in the cytoplasm (Green & Kehinde, 1975). The degree of differentiation is dependent on hormonal supplements, timing of induction, and passage number (Green & Kehinde, 1975).

3T3-F442A cells (purchased from Dr. Howard Green, Harvard Medical School) were cultured in a $75cm^2$ flask (Thermo Fisher Scientific, Waltham, MA) containing modified Eagle's medium (DMEM) (high glucose) (Life Technologies, Grand Island, NY), 100 U/ml penicillin, and 100ug/ml streptomycin (Life Technologies, Grand Island, NY). Cells were supplemented with 10% donor calf serum (BCS, Life Technologies) at 37°C in a humidified atmosphere of 10% CO_2. After reaching sub-confluence (70-80%), the cells were **seeded at 5×10^4 in six-well plates (6 X six-well plates). The** cells were grown to confluence (90%) using DMEM with 10% donor calf serum (eight days). After reaching confluency (10^5), the differentiation of 3T3-F442 pre-adipocytes into mature adipocytes was stimulated by replacing medium with DMEM containing 10% Fetal Bovine Serum (FBS), Life Technologies) supplemented with 5 μg/ml insulin. The medium was then replaced with DMEM that contained 10% FBS every third day (72

hours). Alcohol at concentrations of 0.25%, 0.5%, 1%, 1.5% and 2% (six wells had same concentration) was added to 10^5cells one hour after induction with insulin and FBS. Same concentration of alcohol was repeated 24 hours after due to evaporation of alcohol. Eight days after induction, with FBS and insulin the fully differentiated adipocytes were used in the experiments.

Cell Viability Assay

After induction of differentiation and two days of incubation with alcohol, a cell viability assay was performed. The supernates were collected and after trypsinization combined with the detached cells. The supernates and trypsinized cell were centrifuged at 1500 RPM for five minutes. After discarding the supernates the cell pellet was suspended in 50 ul of medium and were mixed with 50ul of 4% trypan Blue. Viable and dead cells were counted using a hemocytometer. After 8 days of incubation in differentiation media, in a separate experiment, adipocytes were trypsinized and counted for viable and dead cells as indicated above. Viability of cells was expressed as the percentage of total cells and the trypan blue staining was excluded and presented as dead cells.

Oil Red O Staining of Pre-Adipocytes 3T3-F442

Cell culture was prepared as described previously. Cells were treated with alcohol at the indicated concentration (0.25%, 0.5%, 1%, 1.5%, and 2%) for 48 hours. Oil Red O staining was performed on day 8 of adipocyte differentiation (8 days after FBS and Insulin stimulation) and accumulated lipid droplets in differentiated adipocytes. First,

cells were washed twice with phosphate buffer saline (PBS) for removal of suspended apoptotic cells. Second, cells were fixed with 10% formaldehyde for 40 minutes, and then were washed with PBS and distilled water three times. Cells were stained with ORO dye in 60% isopropanol solution for 30 minutes. Finally, the cells were washed again with distilled water to remove background staining and counted using a phase-contrast microscope.

AdipoRed Assay

The 3T3-F442 cell line accumulates intercellular triglyceride droplets (Green & Kehinde 1975). Thus, intracellular triglyceride is often used as a marker of adipocyte differentiation (Green & Meuth, 1974). The fibroblasts begin to differentiate to adipocytes and accumulate lipid droplets after four to five days of induction, and for an additional one to two weeks the number and the size of the lipid droplets continues to increase. Inhibition of adipocyte differentiation significantly reduces the accumulation of intracellular lipids (Green & Kehinde 1975).

The quantitative measurement of intracellular lipid droplets using Oil Red O requires manual counting of stained cells using a phase contrast microscope. AdipoRed is a reagent that allows for the quantification of intracellular lipid droplets in a high-throughput manner (Memon et al., 2000). AdipoRed reagent is a solution of the hydrophilic stain known as Nile Red (fluorescence-based assay) that allows ones to quantify the accumulation of intracellular triglycerides and provides significant

advantages to detect the effect of alcohol on triglyceride accumulation in adipocytes. It is more accurate than other methods, including Oil Red O assay and is faster and easier than Northern and Western blots.

Intracellular lipid content was quantified by using the AdipoRed assay reagent kit (Lonza Inc., MD). On day 8 of differentiation, cells were treated with AdipoRed reagent and assayed for the quantification of intracellular lipid content following manufacturer's instructions. In brief, differentiated adipocytes were rinsed with PBS and then 140 µL of AdipoRed reagent in 5ml PBS were added to each well (six-well plate) following manufacture's instruction. Cell were incubated for 15 minutes, and a microplate reader, Tecan Infinite M200 (Tecan System Inc., Salzburg, Austria) was used to detect the fluorescence at an excitation wavelength of 485 nm and an emission wavelength of 572nm.

ELISA Assay

Sandwich enzyme-linked immunosorbent assay (ELISA)is based on attachment of antibody specific for a protein of interest coated onto the wells of microplates. Samples containing the protein of interest and standard are added to the wells. After several washing steps to remove unbound substances, a secondary antibody is added to the wells and is allowed to bind to the antibody from the first incubation. After several washing steps, a horseradish peroxidase (HRP) conjugate (detection antibody) is added. After an incubation and washing step, a substrate solution is added and converted to a detected

form by the enzyme (color signal). The intensity of the color signal is directly associated with the concentration of original antibody present and the level of antigen binding to the compound of interest.

Mouse ELISA kits were used to identify specific cytokines involved in inflammation and modulation of cytokine expression after alcohol treatment. 3T3-F442 cell cultures at the density of 5×10^4 were prepared as described previously. The cell culture medium was analyzed using sandwich ELISAs specific for mouse ApN from Millipore (Bedford, MA). For IL-1β, IL-4, IL-6, IL-10, IL-13, IL-17, TNF-α, INF-γ, MCP-1, MIP-α, and MIP-β, ELISA kits specific for mouse (DuoSet@ R&D Systems Inc., Minneapolis, MN) was used. Assays were performed based on the manufacturer's protocol. In brief, the capture antibody was used to coated each well overnight. The next day after several washings steps the sample and detection antibody, as well as streptavidin-HRP, were added to each well. The concentration of captured cytokines in the samples was calculated from a reference standard of known concentrations of cytokine generated in the same assay.

Quantitative Real-Time PCR

The amount of the expressed gene in a cell can be measured by the number of copies of an RNA transcript present in the sample. The first step to utilize PCR is conversion of mRNA into cDNA. RNA was isolated using RNeasy mini kit (Qiagen, Valencia). The isolated RNA sample is first reverse-transcribed using reverse

transcriptase to complementary DNA (cDNA). The cDNA in this reaction can be used directly as a template for PCR with primer with specific gene. The two-steps combination of reverse transcription and RT-PCR facilitates the detection of mRNA. After certain number of cycle, the more abundant transcripts from a highly transcribed gene have higher yield and more product than low transcribed gene (Feckler et al., 2017).

The common methods for the detection of PCR are based on the use of non-specific fluorescent dye that intercalate with any double-stranded DNA, which permits detection of DNA only after the hybridization of fluorescent dye binds to the complementary DNA. Finally, the generated data can be analyzed with computer software connected to the qPCR instrument that calculates the relative gene expression (mRNA copy number) in a sample. This technique quantitatively determines the abundance of a particular gene of interest in the sample.

Cell cultures were grown in 6-well plates as described previously. Total RNA from 6X six-well plate (10^5 each well) was isolated using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA concentration and purity determined spectrophotometrically using NanoDrop™ (Thermo Fisher Scientific) OD 260/280 ratio. The OD at NanoDrop is automatically set up for 230, 260 and 280 calculate the concentration of the RNA and A260/A280 and 260/230 ratio. RNA integrity and ribosomal ratio were determined using Agilent 2100 Bioanalyser System (Agilent Technologies, Santa Clara, CA). After RNA quantified, then only 1μg of RNA was used

in RT Mater Mix reaction. Reverse transcription was performed using RT Master Mix (Thermo Fisher Scientific) containing Oligo (dt) primer and Superscript$^{@}$ III following the manufacturer's directions. After RT reaction, the cDNA was diluted to the same dilution before RT reaction and NanoDrop/Spectrophotometer was used to estimate the quantity of cDNA.

Amplification reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 10μmol/L primer (seeTable 4.1) forward and reverse. One μl of diluted cDNA samples (25 ng) was used for quantitative two-step PCR (Bio-Rad CFX 96). The mRNA expression of IL-1b, IL-6, INF-γ, TNF-α, IL-17, IL-10, IL-4, MCP-1, MIP-1a, MIP-, IL-13, and GAPDH was determined. Each sample was analyzed in triplicate. The mRNA of all gene expression was normalized to GAPDH as an internal control (reference gene). Relative quantification of gene expression was calculated according to the comparative 2 to the power of negative $\Delta\Delta C_T$ (threshold cycle value) method. $\Delta\Delta C_T$ is the differences between the ΔC_T of the treated sample and ΔC_T of untreated sample. The ΔC_T is differences between the C_T gene of interest and the C_T of housekeeping gene. Finally, the fold of gene expression was 2 to the power of negative $\Delta\Delta C_T$. As negative controls, water was used as a template for each reaction (Feckler et al., 2017).

Table 4.1

Primer sequence for quantitative PCR

Primer Name	Forward	Reverse (3′ - 5′)
IL-1β	GACTGACAAGATACCTGTGGCCT	AGACAACAGTGAAGTGCAGCCT
IL-6	CTCTTCACAAGCACCGTCACTCC A	AGCCACTCATTCTGTGAATGCAGC
INF-γ	CTATTACTGCCAGGCCGCGTT	TCCTCTTCCGCTTCCTCAGGTT
TNF-α	AGTAGACAGAAGAGCGTGGT	ATGAGCACAGAAAGCATGA
IL-17	TCTCCACCGCAATGAAGACC	CACACCCACCAGCATCTTCT
IL-10	TGCTATGTTACCTGGTCTTCCTGG	TAGTAGAGTCACCGTCCTGGATGC
IL-4	GCATGTACCAGCTTCATCCAGG	GGCTTCATTCACAGTACAGCAGG
IL-17	AATCTCCACCGCAATGAGGA	ACGTTCCCATCAGCGTTGA
MCP-1	ATGCAGGTCTCTGTCACG	AGTATGACAGAGAACTAG
MIP-A	GCTCAGCCAGATGCAGTTAA	TCTTGAGCTTGGTGACAAAAACT
MIP-B	AGCGCTCTCAGCACCAATG	AGCTTCCGCACGGTGTATG
IL-13	GCAGCATGGTATGGAGTGTG	TGGCGAAACAGTTGCTTTGT
GAPDH	GTC GTG GAT CTG ACG TGC C	TGC CTG CTT CAC CTT CT

Note. IL: Interleukin; INF-γ: Interferon-γ; TNF-α: Tumor necrosis factor-α; MCP-1 Monocyte Chemoattractant Protein-1; MIP: Macrophage inflammatory protein; GAPDH: GlyceIraldehyde-3-phospahate dehydrogenase

Extracellular Acidification Rate (ECAR)

The two major energy production mechanisms in cells are glycolysis and oxidative phosphorylation. Most cells are capable of switching between two pathways and therefore can tolerate changes in their environment. The glycolysis pathway in cells converts glucose to lactate, CO_2, and water. This reaction results in the production and release of protons into the extracellular medium.

The extrusion of protons into the medium results in acidification and reduction of the pH of the medium surrounding the cells. The XF instrument has the ability to measure the acidification rate of the medium, and it reports it as ECAR. The assay workflow has four steps. In the first step, the cells are incubated in the medium in the absence of glucose or pyruvate, and the ECAR is measured. This is followed by injection of saturated concentrated glucose, which is metabolized to pyruvate, ATP, NADH and protons. The protons are released into the medium, increasing the ECAR. This is reported as the rate of glycolysis under basal conditions. The next injection is oligomycin, which blocks the ATP production and shifts energy production to glycolysis. Blocking the mitochondrial ATP production increases the ECAR and reveals the cellular maximum glycolytic capacity. Finally, 2-deoxy-glucose (2-DG), which inhibits the glycolysis pathway, is injected. A decrease in ECAR production confirms that ECAR produced in the medium is based on glycolysis. The difference between glycolytic rate and glycolysis capacity is defined as glycolytic reserve. Prior to injection, the ECAR in the cell is

referred to as the non-glycolytic acidification. Fully differentiated adipocytes were used in the experiments. The glycolytic metabolism of pre-adipocytes 3T3-F442 was measured using XF Cell Mito Stress Tests. The assay medium was supplemented with pyruvate and glutamine. The XF Glycolysis Stress Test kits including glucose, oligomycin and 2-deoxyglucose (2-DG) were purchased from Agilent (Santa Clara, CA). XF24 cell culture plates, sensor cartridges, and XF base medium were obtained from Agilent (Santa Clara, CA). Mouse 3T3-F442 were prepared as described previously and seeded into 24-well XF cell culture microplates (Agilent, Santa Clara, CA) that had previously coated with 0.2% wt:vol solution of gelatin. Eight days later, the fully differentiated adipocytes were used in the experiments. The media then changed to XF assay media (Agilent, Santa Clara, CA). The experiments followed the manufacturer instruction.

Statistical Analysis

All experiments including intracellular lipid droplets, cytokines, chemokines, and ApN expression, were repeated three times (n = 3) and values were presented as means ± SEM. For all assays including Trypan blue, AdipoRed assay, ELISA and Real-Time PCR one-way analysis of variance (ANOVA) and Tukey's post hoc test were performed to determine the difference between groups using GraphPad Prism (GraphPad Software Inc., San Diego, CA) (Figure 4.1-4.-14). Differences between groups in extracellular assays (Figure 4.15) were examined using two-way ANOVA. Tukey's multiple

comparison test was used to assess the differences between means, and a p value of < 0.05 was considered statistically significant.

CHAPTER IV

RESULTS

Alcohol Significantly Reduced Adipogenesis

Adipose tissue has a crucial role in the maintenance of lipid homeostasis with the storage of triglycerides or by the liberation of free fatty acids. As Figure 4-1 shows, a marker of adipogenesis, Oil Red O staining was used to show the effects of alcohol on the formation of fat droplets as a marker of adipogenesis in 3T3-F442 as shown in Figure 4.1. Alcohol treatment significantly reduced ($p < 0.05$) accumulation of lipid in 3T3-F442 adipocytes sequentially in proportion to the alcohol concentration compared to control (without treatment). Adipocytes exposed to the highest level of alcohol had a lower number of mature adipocytes, whereas adipocytes with no alcohol or the lowest level of alcohol exposure, expressed larger quantities.

Effect of Alcohol on Cell Viability

To investigate if alcohol treatment might affect cell viability, trypan blue staining was used. Alcohol toxicity was not apparent as the total viable cells (see Figures 4.2) and the percent of dead cells (Figure 4.3) was not different between differentiated cells treated with different concentrations of alcohol compared with control.

Alcohol Significantly Reduced Adipogenesis and Intercellular Triglycerides

To understand whether alcohol treatment reduced the intracellular lipid content and the differentiation of 3T3-F442, the influence of alcohol on adipocytes was observed.

The AdipoRed reagent is a hydrophobic stain (Nile Red solution) that allows the quantification of intracellular lipid droplets. As shown in (see Figure 4.4), it was found that alcohol reduced intracellular lipid content and triglyceride accumulation. The effect of alcohol on intracellular triglyceride content was dose dependent. The two highest concentrations of alcohol (2% and 1.5%) showed lower intracellular TG compared to the control.

Alcohol Significantly Reduced Adiponectin Secretion and mRNA Expression

To determine whether alcohol exposure and reduction in mature adipocytes would reduce ApN levels, ApN mRNA expression was assessed using qPCR (4.5). The levels of ApN mRNA were significantly reduced (see Figures 4.5). Further, in a separate experiment, the level of ApN secretion was determined using ELISA assay (see Figure 4.6). Similar to the qRT-PCR results, the level of ApN secretion was significantly reduced (see Figure 4.6).

Alcohol Significantly Increased Pro-Inflammatory Cytokines/Chemokines Secretion

To determine the effect of alcohol on the pro-inflammatory cytokine, an ELISA assay was performed. The level of pro-inflammatory cytokines such as IL6, IL-1β (Figure 4.7), TNF-α, IL-17 and IFN-γ (see Figure 4.8) secretion was increased sequentially in proportion to the alcohol concentration. The levels of MCP-1, MIP-1α, and MIP-1β (Figure 4.9) secretion was increased significantly relative to the level of ethanol exposure. Further, ELISA assay was used to determine the level of anti-

inflammatory cytokines. The anti-inflammatory cytokines, including the IL-1 receptor antagonist, IL13 (see Figures 4.10), and IL4, IL-10 (see Figure 4.11) were decreased. Finally, alcohol suppressed the adipocytes differentiation and proliferation whereas adipocytes under no alcohol or the lowest level of alcohol had higher differentiation.

Real-Time qPCR

To determine the effects of alcohol on mRNA levels of pro-inflammatory and anti-inflammatory cytokine and chemokine, Real-Time PCR was performed. The mRNA levels of pro-inflammatory cytokines including IL-6, IL-17, and IL-1β (see Figure 4.12) were increased in alcohol treated cells. There were elevated levels of TNF-α INF-γ (see Figure 4.13). The mRNA levels of chemokines including MCP-1, MIP-1α, and MIP-1β (see Figure 4.14) were elevated. Notably, the mRNA levels for anti-inflammatory cytokines IL-4, IL-10, and IL-13 were significantly reduced (see Figures 4.15).

Measuring Glycolysis Metabolism

To determine whether alcohol exposure in adipocytes altered glycolytic rate and ECAR, the XF glycolysis stress test was used. The sequential injections of glucose, oligomycin, and 2-DG measured glycolysis and glycolytic capacity. The results showed there was an increase in glycolysis, glycolysis capacity, and extracellular acidification rate compared with control or no treatment (see Figure 4.16).

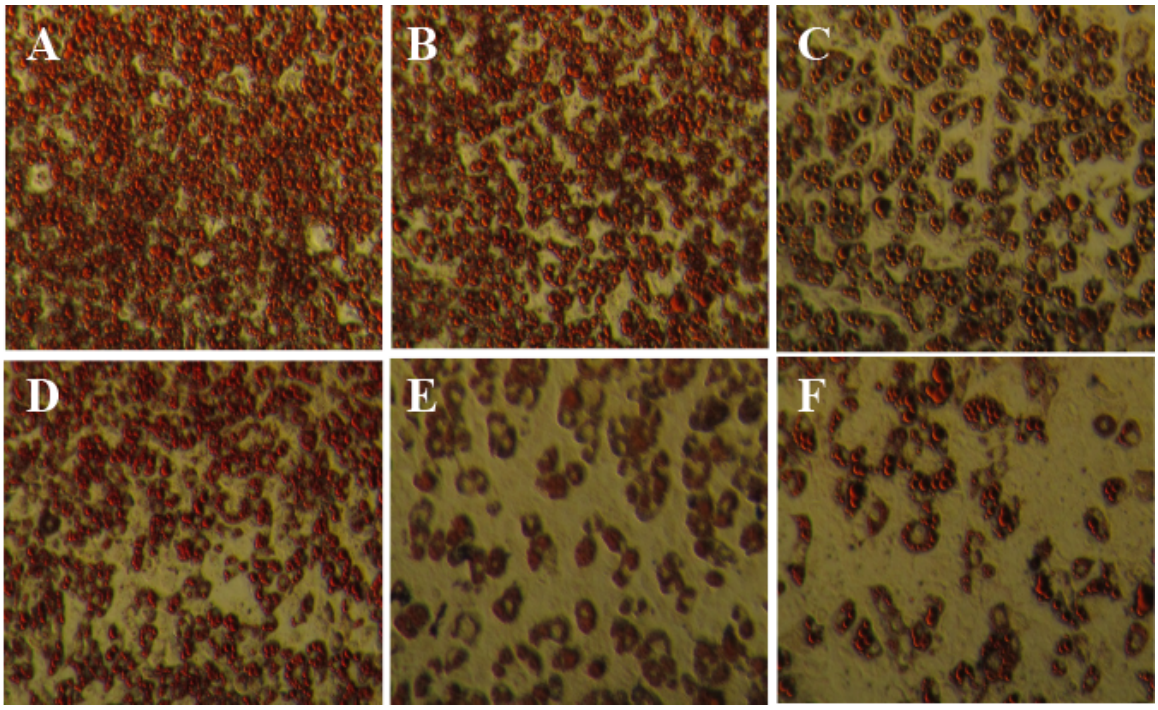

Figure 4.1. Alcohol significantly reduced adipogenesis in 3T3-F442 at early development. As a marker of adipogenesis, Oil Red O staining was used where the diffident effects of alcohol on the formation of fat droplets in 3T3-F442 cells quantified. For the cells culture, 6-well plate was used at the density of 5×10^4 in DMEM at the indicated concentration of alcohol (A) 0%, (B) 0.25%, (C) 5%, (D)1%, (E)1.5%, (F) 2% for 48 hours. Alcohol treatment significantly reduced accumulation of lipid in 3T3-F442 adipocytes sequentially in proportion to the alcohol concentration compared to control (without treatment).

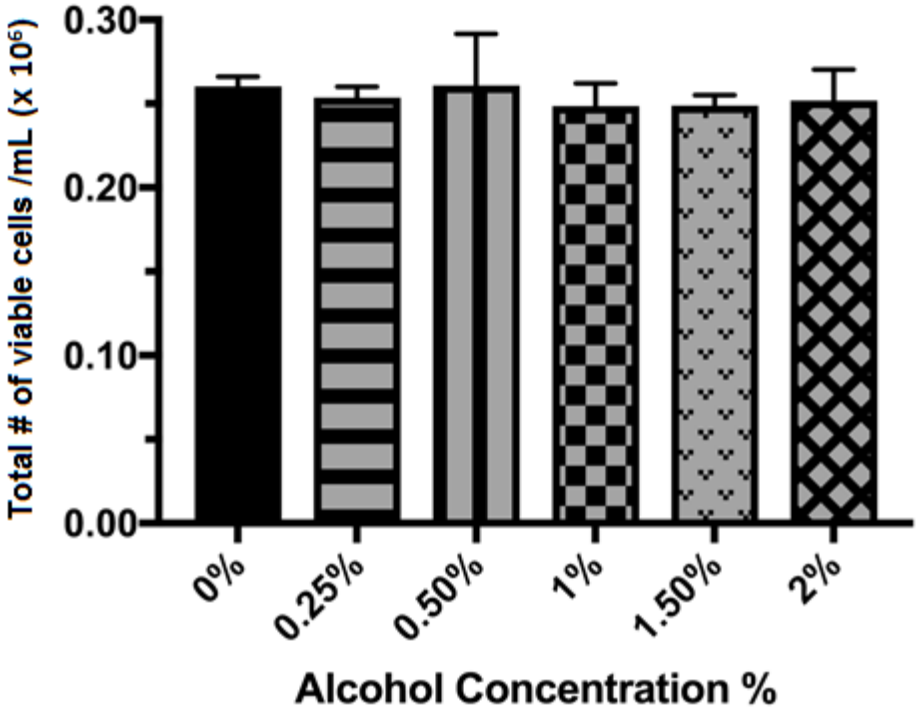

Figure 4.2. The effect of alcohol on the viability of 3T3-F442 adipocytes (viable cells). Cells were cultured in six-well plates at the density of 5×10^4 in DMEM. The confluent cells were treated with ethanol in the presence of insulin and FBS at the indicated concentration. Total viable cell count was determined using Trypan Blue staining. One-way analysis of variance (ANOVA) and Tukey's post hoc test were performed to determine the difference between groups using GraphPad Prism. Data represent the means ±SEM. Data was not statistically significant ($p > 0.05$).

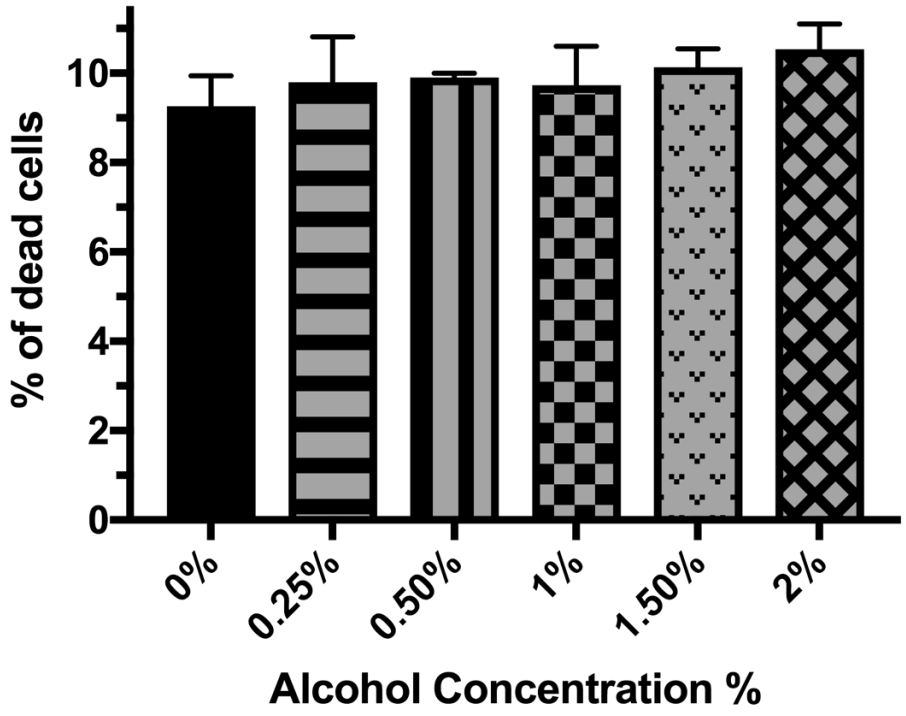

Figure 4.3. The effect of alcohol on the viability of 3T3-F442 adipocytes (dead cells). Cells were cultured in six-well plates at the density of 5×10^4 in DMEM. The confluent cells were treated with ethanol in presence of insulin and FBS at the indicated concentration. Total percent of dead cells was determined using trypan Blue staining and a hemocytometer. One-way analysis of variance (ANOVA) and Tukey's post hoc test were performed to determine the difference between groups using GraphPad Prism. Data represent the means ±SEM. Data was not statistically significant ($p > 0.05$).

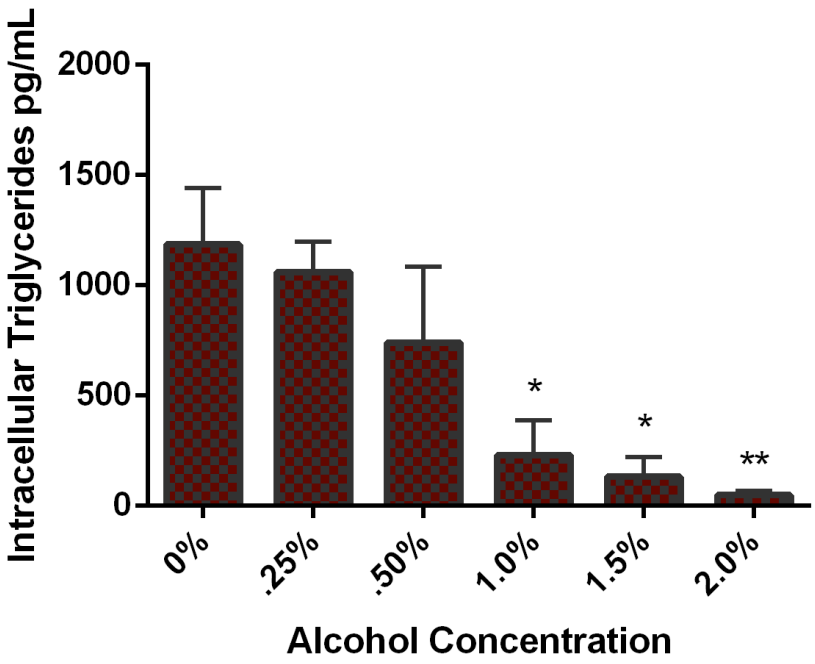

Figure 4.4. Quantification of intracellular triglycerides in 3T3-F442 cells. Cells were cultured in six-well plates at the density of 5×10^4 in DMEM. The confluent cells were treated with ethanol in presence of insulin and FBS at the indicated concentration. The intracellular lipid droplets were quantified by a fluorescent dye (Nile Red) using AdipoRed assay. One-way analysis of variance (ANOVA) and Tukey's post hoc test were performed to determine the difference between groups using GraphPad Prism. Data represent the means ±SEM; (*$p < 0.05$; ** $p < 0.01$).

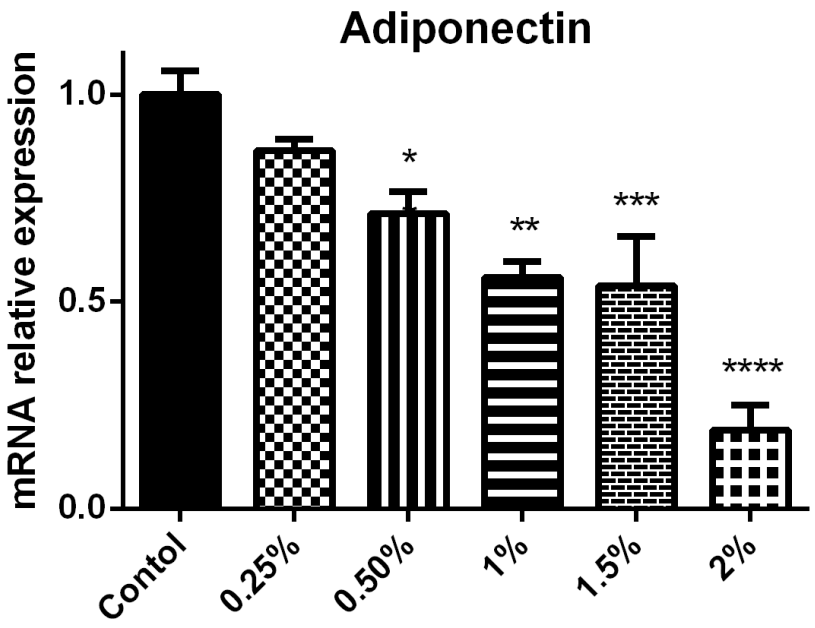

Figure 4.5. Alcohol exposure significantly reduced ApN mRNA expression in 3T3-F442 cell lines. 3T3-F442 were cultured in six-well plates and allowed to undergo adipocyte differentiation for eight days in presence of indicated concentration of alcohol (as described in the Methods section). RNA was extracted and Real-Time PCR was performed as described in the Methods section. One-way analysis of variance (ANOVA) and Tukey's post hoc test were performed to determine the difference between groups using GraphPad Prism. Data represent the means ±SEM; (*$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$; **** $p < 0.0001$).

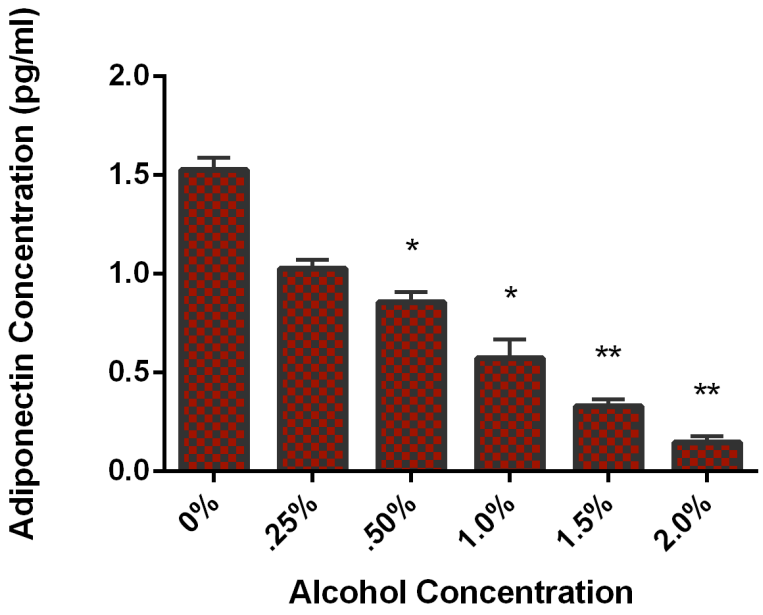

Figure 4.6. Effect of alcohol on adiponectin secretion in 3T3-F442 cells. 3T3-F442 adipocytes were prepared as described in methods. Cells were cultured in six-well plates at the density of 5×10^{6} in DMEM. Mouse ELISA kits were used to detect adiponectin. The level of ApN was significantly reduced at the highest concentrations. One-way analysis of variance (ANOVA) and Tukey's post hoc test were performed to determine the difference between groups using GraphPad Prism. Data represent the means ±SEM; (*$p < 0.05$; ** $p < 0.01$).

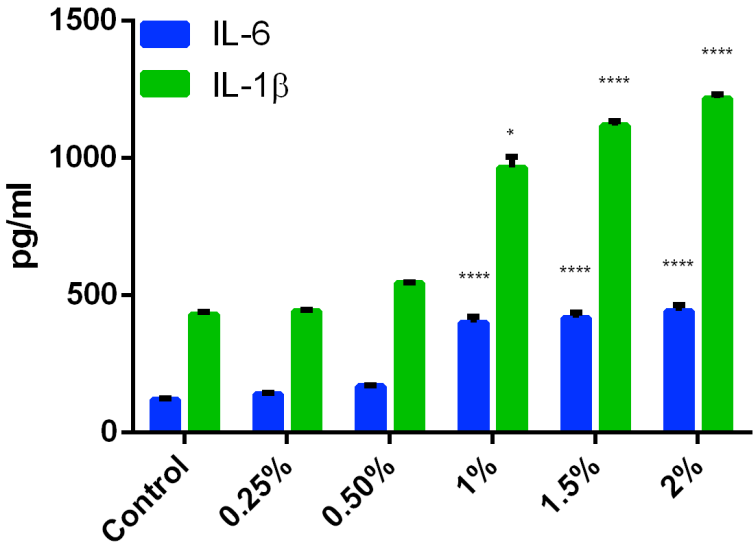

Figure 4.7. Alcohol significantly increased IL-6 and IL-1β secretion. 3T3-F442 were cultured in six-well plates and allowed to undergo adipocyte differentiation for eight days in the presence of the indicated concentration of alcohol (as described in the Methods section). ELISA assay was performed as described previously. All alcohol concentrations were compared with control (or 0%). One-way analysis of variance (ANOVA) and Tukey's post hoc test were performed to determine the difference between groups using GraphPad Prism. The statistical significance levels are *$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$; **** $p < 0.0001$).

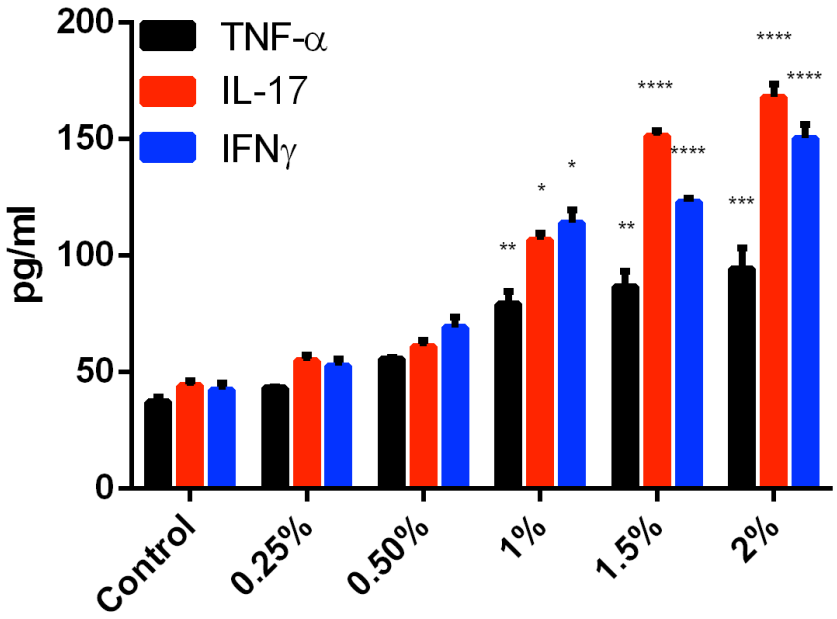

Figure 4.8. Alcohol increased TNF-a, IL-17, and IFN-γ secretion. 3T3-F442 were cultured in six-well plates and allowed to undergo adipocyte differentiation for eight days in the presence of the indicated concentration of alcohol (as described in the Methods section). ELISA assay was performed as described previously. All alcohol concentrations were compared with control (or 0%). One-way analysis of variance (ANOVA) and Tukey's post hoc test were performed to determine the difference between groups using GraphPad Prism. The statistical significance levels are *$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$; **** $p < 0.0001$).

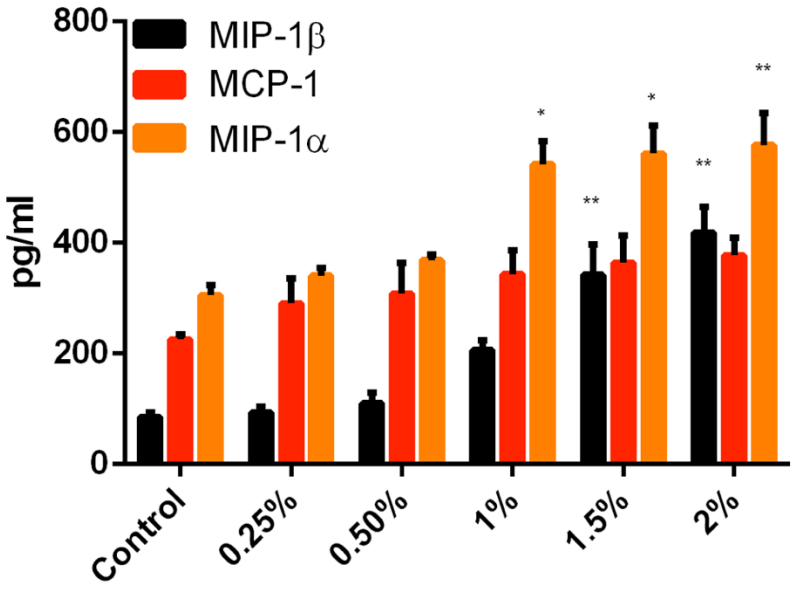

Figure 4.9. Alcohol increased MIP-1β, MCP-1, and MIP-1α secretion. 3T3-F442 were cultured in six-well plates and allowed to undergo adipocyte differentiation for eight days in the presence of the indicated concentration of alcohol (as described in the Methods section). ELISA assay was performed as described previously. All alcohol concentrations were compared with control (or 0%). One-way analysis of variance (ANOVA) and Tukey's post hoc test were performed to determine the difference between groups using GraphPad Prism. The statistical significance levels are (n=3, *$p < 0.05$; ** $p < 0.01$).

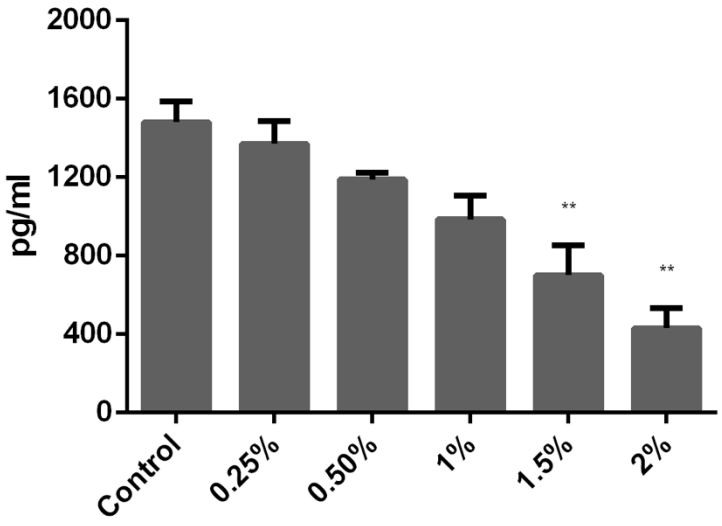

Figure 4.10. Alcohol decreased IL-13 secretion. 3T3-F442 were cultured in six-well plates and allowed to undergo adipocyte differentiation for eight days in the presence of the indicated concentration of alcohol (as described in the Methods section). ELISA assay was performed as described previously. All alcohol concentrations were compared with control (or 0%). One-way analysis of variance (ANOVA) and Tukey's post hoc test were performed to determine the difference between groups using GraphPad Prism. The statistical significance levels are (*$p < 0.05$; ** $p < 0.01$).

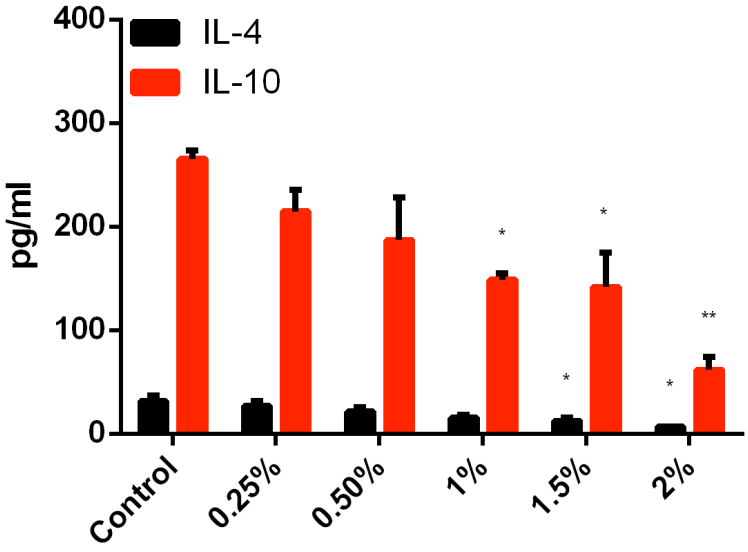

Figure 4.11. Alcohol significantly decreased IL-4 and IL-10 secretion. 3T3-F442 were cultured in six-well plates and allowed to undergo adipocyte differentiation for eight days in the presence of the indicated concentration of alcohol (as described in the Methods section). The assay was performed as described previously. All alcohol concentration was compared with control (or 0%). One-way analysis of variance (ANOVA) and Tukey's post hoc test were performed to determine the difference between groups using GraphPad Prism. The statistical significance levels are (*$p < 0.05$; ** $p < 0.01$).

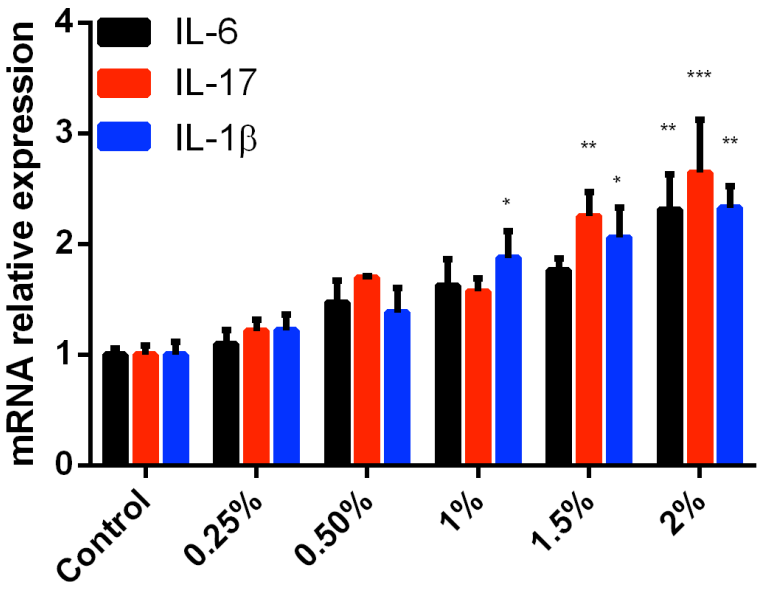

Figure 4.12. Alcohol significantly increased IL-6, IL-17, and IL-1β mRNA expression. 3T3-F442 were cultured in six-well plates and allowed to undergo adipocyte differentiation for eight days in the presence of the indicated concentration of alcohol (as described in the Methods section). RNA was extracted and Real-Time PCR was performed as described in the Methods section. One-way analysis of variance (ANOVA) and Tukey's post hoc test were performed to determine the difference between groups using GraphPad Prism. Data represent the means ±SEM; (*$p < 0.05$; ** $p < 0.01$ vs. 0%)

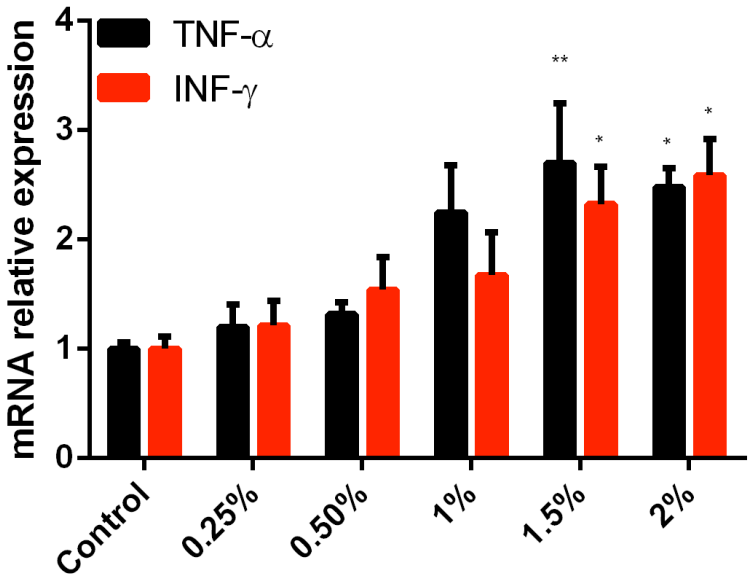

Figure 4.13. Alcohol increased TNF-α and INF-γ mRNA expression. Alcohol exposure increased pro-inflammatory cytokines levels in 3T3-F442 cells. Cells were cultured in six-well plates and allowed to undergo adipocyte differentiation for eight days (as described in the Methods section). RNA was extracted and Real-Time PCR was performed as described in the Methods section. All alcohol concentrations were compared with control (or 0%). One-way analysis of variance (ANOVA) and Tukey's post hoc test were performed to determine the difference between groups using GraphPad Prism. Data represent the means ±SEM; (*$p < 0.05$; ** $p < 0.01$).

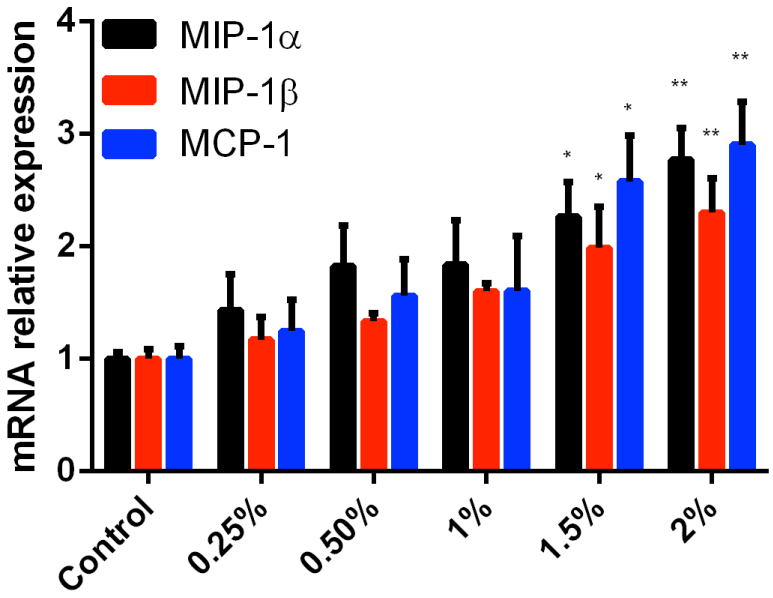

Figure 4.14. Alcohol increased MIP-1α, MIP-1β, and MCP-1 mRNA expression. Alcohol exposure increased chemokines levels in 3T3-F442 cells. Cells were cultured in six-well plates and allowed to undergo adipocyte differentiation for eight days (as described in the Methods section). RNA was extracted and Real-Time PCR was performed as described in the Methods section. All alcohol concentrations were compared with control (or 0%). One-way analysis of variance (ANOVA) and Tukey's post hoc test were performed to determine the difference between groups using GraphPad Prism. Data represent the means ±SEM; (*$p < 0.05$; ** $p < 0.01$).

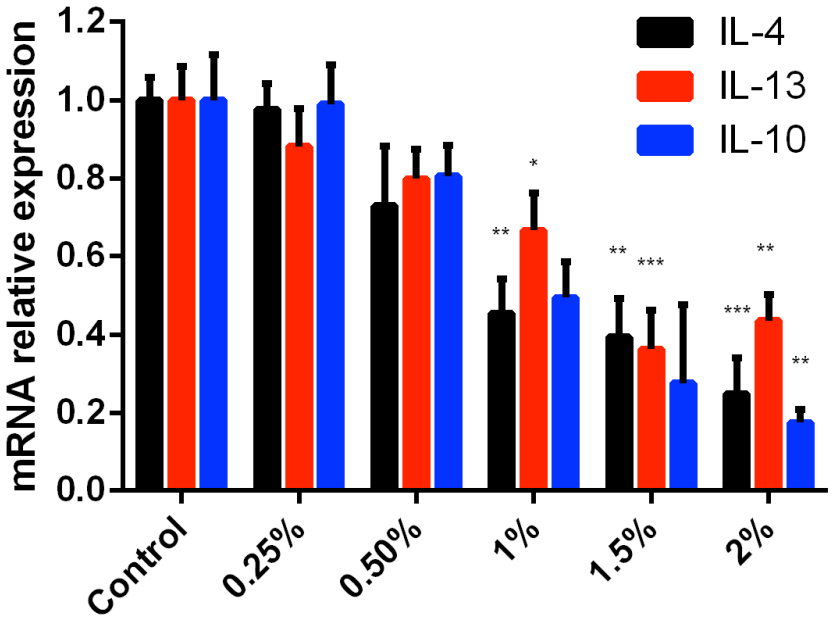

Figure 4.15. Alcohol significantly decreased IL-4, IL13, and IL-10 mRNA expression. 3T3-F442 were cultured in six-well plates as described previously and allowed to undergo adipocyte differentiation for eight days. Total RNA was extracted and Real-Time PCR was performed as described on the experimental procedures. All alcohol concentration was compared with control (or 0%). One-way analysis of variance (ANOVA) and Tukey's post hoc test were performed to determine the difference between groups using GraphPad Prism. The statistical significance levels are (*$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$).

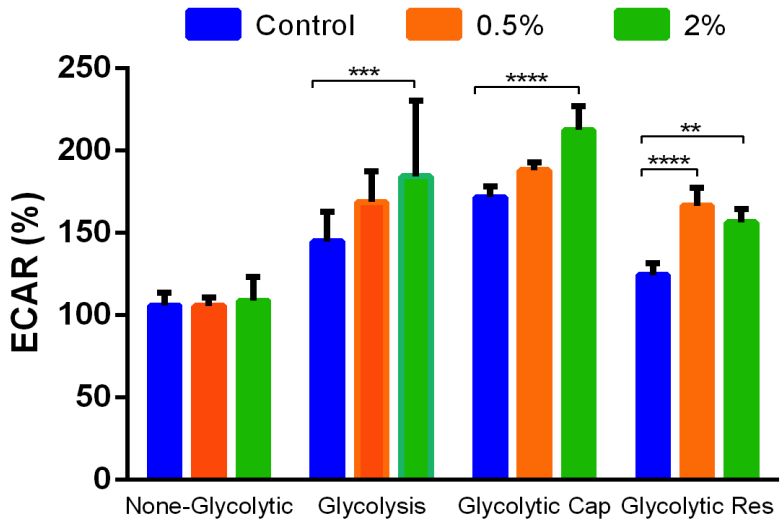

Figure 4.16. The glycolytic metabolism of 3T3-F442. The extracellular acidification rates (ECAR), were measured during a glycolytic stress experiments with XF assay media as described in methods. At baseline (None-Glycolytic) there is no difference between different alcohol concentrations. ECAR was greater in 2% alcohol compare with control in Glycolysis and Glycolytic-Capacity. ECAR was higher in 0.5% alcohol in Glycolytic Reserve compared with control and 2% ethanol. All alcohol concentrations were compared with control (or 0%). Two-way ANOVA and Tukey's multiple comparison test was used to assess the differences between means, and a *p* value

of < 0.05 was considered statistically significant. Data represent the means ±SEM; (per treatment *$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$; **** $p < 0.0001$vs 0.0%).

CHAPTER V

DISCUSSION

AT is a dynamic organ and helps maintain whole-body energy homeostasis (Ahima, 2006). Malfunctioning AT contributes to metabolic disease (Tandon, Wafer, & Minchin, 2018). It has been observed that offspring of mothers using alcohol during pregnancy may be at increased risk for obesity and other metabolic problems. (Fuglestad et al., 2014; Xia et al., 2014). It was hypothesized that disruption in adipocyte formation and abnormal adipocyte differentiation during early development may cause metabolic disorders later in the life. The total amount of mature AT and the amount of adipose tissue is required for proper adipose tissue function. In this study, potential mechanisms of alcohol in modulation of adipose tissue function were examined using 3T3-F442 pre-adipocytes.

Adipocyte cell culture is an important tool for cellular pathophysiology and mechanistic studies (Kershaw & Flier, 2004). Many factors may affect the differentiation of pre-AT in culture. Alcohol alters the cytokine levels in diverse tissues, including adipose tissue, in a complex manner that regulates inflammation, cell proliferation, cell death, and cell migration (Kema, et al., 2015). In the current study, the data have shown that alcohol promotes increases in pro-inflammatory cytokines and reductions in adipose tissue differentiation.

When pre-adipocytes are induced to differentiate, the cells first undergo several rounds of mitotic clonal expansion and then become quiescent while the transcription of adipogenic genes is initiated (MacDougald & Lane, 1995). Expansion in pre-adipocytes is due to upregulation of CCAAT/enhancer binding protein (C/EBP) during the early stage of adipocyte differentiation (Ntambi & Young-Cheul, 2000).

Kerna et al. (2015) demonstrated that alcohol consumption in both rodent and human induced adipose tissue hypertrophy by CYP2E1 mediated oxidative stress and induction of inflammation in adipose tissue. The hyperlipolysis in adipose tissue leads to hepatic steatosis by the excess release of fatty acids from adipose tissue which is transported to the liver (Kema et al., 2015). In this study, it is demonstrated that alcohol induces inflammation and impairs anti-inflammatory function of adipocytes in a model of differentiating adipose cell.

Some studies have revealed that modifications in cytokine production depend on the duration of alcohol exposure as well as alcohol concentration (Ishikawa et al., 2011). The pro-inflammatory effects of alcohol on different organs and tissues depends on the pattern of alcohol exposure and with chronic alcohol exposure accelerating the inflammatory response (Gonzalez-Reimers, Santolaria-Fernandez, Martin-Gonzalez, Fernandez-Rodriguez, & Quintero-Platt, 2014). Data presented in this dissertation,

demonstrated that higher levels of alcohol concentration promoted increases in the level of pro-inflammatory cytokines.

Adipogenesis is a process of the conversion of fibroblast-like cells into round shape adipocytes and occurs in two stages: conversion of mesenchymal stem cells into pre-adipocytes and final or terminal adipocytes differentiation (Lin, Chun, & Kang, 2016). Recently, Scherer's group showed that cell shape and extracellular matrix remodeling regulates pre-adipocytes function (Scherer, 2016). Abnormal adipocytes formation by excess ECM components and their modifiers causes adipose tissue hypertrophy. The integrin receptor and collagen and other ECM receptors are expressed in adipose tissue and regulate adipose tissue expansion (Lin et al., 2016). The total amount of mature AT and the size of AT is important for proper function (Cristancho & Lazar, 2011). In this study, it has been shown that alcohol decreases adipose tissue differentiation and maturation.

Alcohol disruption of cytokines and inflammation contribute to alcohol-related pathologies (Laso et al., 2007). Kerna et al. (2015) demonstrated that alcohol consumption in both rodents and humans reduced adipose tissue mass by CYP2E1 mediated oxidative stress and induction of inflammation in adipose tissue. The hyperlipolysis in adipose tissue leads to hepatic steatosis by the excess release of fatty acids from adipose tissue which is transported to the liver (Kema et al., 2015). In this

study, I have shown that alcohol induced inflammation impaired the anti-inflammatory function of adipocytes.

A previous study in mice demonstrated that chronic alcohol exposure causes a significant reduction in adipocyte tissue (Zhong, et al., 2012). Reduction in the volume of AT was due to alcohol-induced hyper lipolysis, which was likely a major cause of WAT loss. Finally, the data implicated a link between WAT loss and hepatic lipid gain (Zhong et al., 2012).

Adipose tissue, which was recognized as an energy storage organ, also plays an important role in the inducing different metabolic diseases including type 2 diabetes and alcoholic liver diseases (Kema et al., 2015). The three factors including inappropriate ECM remodeling, unresolved inflammation, and insufficient angiogenic potential contributes to the pathogenesis and dysfunction of adipose tissue (Crewe, An, & Scherer, 2017). The interaction between these three factors is important for understanding of the adipose tissue dysfunction. Both inflammation and improper adipogenesis can contribute to inappropriate ECM and unhealthy AT (Crewe et al., 2017).

There is a possibility that alcohol drinking during pregnancy disrupts adipose tissue proliferation which contributes to inflammation, unhealthy AT, and inappropriate ECM production. The data in the present study suggest that alcohol exposure caused subsequent changes in adipogenesis by decreasing mature adipocytes and reducing

adipogenesis in 3T3-F442 cell lines. Fibroblasts did not differentiate into adipocytes in alcohol-exposed cells compared to control.

Several studies exploring the effect of chronic alcohol exposure on the adiponectin production have demonstrated the changes in adiponectin expression and secretion in adipocytes following alcohol treatment (Chen, Sebastian, & Nagy, 2007; Song, Zhou, Deaciuc, Chen, & McClain, 2008). You et al. (2005) showed in differentiated 3T3- L1 adipocytes, incubation of ethanol with unsaturated fatty acids (either oleic or linoleic acid) decreased adiponectin secretion, suggesting ethanol in the cultured adipocytes may interact with specific fatty acids to inhibit adiponectin expression (You et al., 2005). Other groups investigating chronic alcohol feeding to mice and rats found significantly reduced adiponectin mRNA levels in adipose tissue. In addition, protein level of adiponectin was reduced in both AT and plasma in those animals. Finally, all these studies suggested alcohol feeding in animals has inhibitory effects on the adiponectin expression and secretion (X. Chen, Sebastian, & Nagy, 2007; Song, Zhou, Deaciuc, Chen, & McClain, 2008; Thakur, Pritchard, McMullen, & Nagy, 2006; Xu et al., 2003). In the current study, alcohol exposure reduced ApN mRNA expression in the 3T3-F442 cell line (see Figure 4.5). In addition, alcohol reduced adiponectin secretion (see Figure 4.6).

Unresolved inflammation, which causes the formation of inappropriate extracellular matrix and abnormal adipose tissue inflammation may contribute to inappropriate

adipocytokines secretion and expression. The interaction between these factors during AT expansion contributes to the pathogenesis of dysfunction of AT and inflammatory cytokines induction (Crewe et al., 2017). Alcohol accelerates pro-inflammatory responses in different cells, tissues, and organs (Gonzalez-Reimers et al., 2014). Chronic alcohol exposure interferes with the normal functioning of different cell types including AT. All of these effects enhance the susceptibility of liver, numerous organs, and tissue including AT to chronic exposure of alcohol. (Szabo & Saha, 2015). Several factors contribute to the pathogenesis and dysfunction of adipose tissue.

Alcohol treatment also increased pro-inflammatory cytokine secretion, including IL-6, IL1β (see Figure 4.7), TNF-α, IL-17, and INF-γ (see figure 4.8). Additionally, the chemokine levels of MIP-1β, MCP-1 and MIP-1α (see Figure 4.9) were increased in proportion to alcohol concentration. Likewise, alcohol reduced anti-inflammatory cytokines secretion including IL-13 (see Figure 4.10) and IL-4, IL-10 (see figure 4.11). Furthermore, using RT-qPCR revealed the mRNA levels of pro-inflammatory cytokines IL-6, IL-17, IL1-β (see Figure 4.12), TNF-α, INF-γ (see Figure 4.13) and chemokines including MIP-1α, MIP-1β and MCP-1(see Figure 4.14) were increased compared to control.

The idea that the reduction in TG was due to suppression of differentiation instead of cytotoxicity is supported by measuring the total number of adipocytes and the percentage of dead cells. There were no significant changes in the cell viability and cell

death, suggesting that the alcohol effect was independent of cytotoxicity (seecFigure 4.2 & 4.3). From the data herein, it does not appear that alcohol increased apoptosis because there were no significant differences between cell numbers. These findings are consistent with the findings of several other reports indicating that "ethanol" extracts of Alismatis rhizome inhibit adipocytes differentiation through a complex mechanism (Park et al., 2014). The researchers found using "ethanol" (extracted from Alismatis rhizomes) significantly reduced the level of adipogenesis determined using Oil Red O staining in a concentration dependent-manner, without having cell toxicity (Y. J. Park et al., 2014).

In addition, these findings are consistent with the findings of other groups regarding the effect of alcohol on adipocytes. Zhong et al. (2012) demonstrated a significant loss of WAT in a mouse model of alcoholic steatosis. Alcohol reduced adipocyte size in mice, which likely was a major cause of WAT loss and hyper lipolysis. Reduction in adipocytes' masses was due to reduced triglyceride uptake and increased adipocyte lipolysis (Zhong et al., 2012). A member of the nuclear receptor family of transcription factors, peroxisome proliferator-activated receptor-γ (PPAR-γ) is an early (Sente, Van Berendoncks, Fransen, Vrints, & Hoymans, 2016) and essential factor in the differentiation of adipocytes (Chawla, Schwarz, Dimaculangan, & Lazar, 1994). Emerging evidence demonstrates that PPAR-γ agonists including thiazolidinediones promotes differentiation of fibroblast cell lines into adipocytes (Tontonoz, Hu, Graves, Budavari, & Spiegelman, 1994).

Tumor necrosis factor alpha (TNF-α) impairs adiponectin secretion (Sente et al., 2016) and adiponectin antagonize TNF-α gene expression and production (Y. Chen et al., 2017). In ethanol-fed, animal models, circulating adiponectin levels are inversely associated with the serum TNF-α concentration (Thakur et al., 2006; Xu et al., 2003; You et al., 2005). Therefore, in the current study, alcohol suppression of adiponectin might be due to up-regulation of TNF-α expression and secretion. However, it is unclear whether suppression of adiponectin production by alcohol leads to TNF-α induction or increased in TNF-α results in adiponectin reduction.

Importantly, recent studies indicate that alcohol stimulates adipose tissue dysfunction and may induce multiple organ dysfunction (Zhong et al., 2012). Autophagy is a lysosomal degradation that regulates adipogenesis and adipose tissue homeostasis. Emerging evidence demonstrates that alcohol induces autophagy in different cells and tissues (Shibata et al., 2009)

PPARγ agonist, rosiglitazone normalized lipid homeostasis and improved the adipose tissue gene expression (X. Sun et al., 2012). Importantly, recent studies indicated that chronic alcohol consumption in rats' WAT reduced expression of lipogenic enzymes including CCAAT/enhancer binding protein alpha, PPAR-γ suggesting a decrease in lipogenesis, was sufficient to induce adipose tissue atrophy (W. Zhang et al., 2015). Remarkably, aldehyde (a product of alcohol metabolism) treatment in vitro (in culture cell adipocytes cell line) and ex vivo suppressed lipogenic enzymes including

CCAAT/enhancer binding protein alpha, and PPAR-y suggesting aldehyde may inhibit adipogenesis and lipogenesis which contributes to adipose lipodystrophy (W. Zhang et al., 2015). Indeed, in mice and rats, chronic alcohol consumption decreased adiponectin secretion in adipocytes (X. Chen et al., 2007).

Not all concentrations of alcohol were associated with reduced differentiation of adipocytes as no changes in adipogenesis were noticed in low concentrations. This suggests alcohol suppressed adipogenesis in a concentration-dependent manner, meaning there is a minimum level of exposure necessary to induce effects. By maintaining optimum function of adipocytes during early development, metabolic homeostasis may help to be maintained in the body (prevent from metabolic diseases such as type 2 diabetes) and insulin resistance via appropriate fat turnover in adipocytes. By contrast, without adequate adipogenesis as this study seems to indicate with alcohol exposure, adipose cell dysfunction may be promoted.

The present study is limited in its ability to reveal the exact mechanisms of adipogenesis in vivo (mouse, rats) during fetal development because this is purely an in vitro study in murine pre-adipocytes. It was also impossible to show the specific target of alcohol on the adipocyte cell cycle. Using genetically-engineered mice could significantly advance our understanding of individual adipogenic genes and protein during development by exposing them to alcohol. Using conditional knockout mice CRISPER/Cas could allow for deletion of essential genes during adipogenesis. In

addition, extra genetic material has been added to a genome (transgenic mice) for over expression of fundamental adipogenic transcription marker provide us with a better understanding and measure of alcohol toxicity during a particular time in development.

Further studies on humans may elucidate the potential effect of alcohol on adipocyte development and differentiation during pregnancy and fetal development. Elucidation of the levels of adipocytokines including adiponectin during development in alcohol-exposed adipocytes would be needed to affirm these results and further explore the mechanisms and applicability to humans. However, this research suggests that alcohol suppressed AT differentiation and maturation. It is not clear if alcohol increased the lipolysis or suppressed adipogenic transcription factors. This could provide further support to encourage women to abstain from drinking alcohol during pregnancy to avoid alcohol toxicity in the offspring or other metabolic dysfunction.

Finally, alcohol reduced pre-adipocyte differentiation and reduced ApN expression, by blocking several adipogenic transcription factors such as CCAAT/enhancer binding protein alpha, PPAR-y, suggesting decreased in adipogenesis was sufficient to induce adipose tissue atrophy (W. Zhang et al., 2015). This information, combined with knowledge that adiponectin and anti-inflammatory cytokine are sensitive to oxidative stress by alcohol (Furukawa et al., 2004), demonstrated in cell culture an important role for adipocytes and their adipocytokines disruption after alcohol treatment. The current study demonstrated that alcohol impairs anti-inflammatory cytokines and

induced pro-inflammatory cytokines expression and secretion. This information, combined with the knowledge that the adiponectin has many beneficial functions, and inhibition of alcohol toxicity by reducing oxidative stress and alcohol by product including aldehyde may suppresses the pro-inflammatory cytokines during adipogenesis. These data suggest that clinicians could consider the evaluation of pro-inflammatory cytokines and low levels of adiponectin for use as a prognostic marker to detect risk for early incidence of different metabolic disease.

CHAPTER VI

SUMMARY AND CONCLUSION

Over the past two decades, the view of adipocytes has changed due to the discovery of hormones derived from adipocytes, called adipokines, and as a result of studies on the function of these adipokines in various tissues and organs (Scherer, 2006, 2016; Turer & Scherer, 2016). Notably, the pathophysiological impact of AT is recognized as playing key roles in the metabolic alteration of different tissue (Halberg, Wernstedt-Asterholm, & Scherer, 2008).

In the gestation period of humans, adipocytes can be observed during the sixth week of gestation (Symonds, Pope, Sharkey, & Budge, 2012). Adipogenesis is the development of differentiated adipose cells (adipocytes) from pluripotential-undifferentiated precursors (Kiess et al., 2008; Martos-Moreno et al., 2013; Naderi et al., 2014). Some studies have shown that hypertrophy and disruption of the integrity of AT stimulate the pro-inflammatory cytokines and cell stress signals (Z. Zhang & Scherer, 2018; Zhu, Kruglikov, Akgul, & Scherer, 2018). Most of the literature indicates that inflammation of tissue is due to the death of hypertrophic adipocytes that provide a specific structure as well as an unusual physical appearance (Giordano et al., 2013; Henninger, Eliasson, Jenndahl, & Hammarstedt, 2014). In fat metabolism research, very few molecules other than ApN have received much continuous interest. ApN is a circulating plasma protein hormone that is derived from adipocytes and secreted by AT

(Maeda et al., 1996; Scherer et al., 1995; Z. V. Wang & Scherer, 2016). In a previous study (Shafiei et al., 2011), the protective effects of ApN were demonstrated in ApN-overexpressing mice. These mice were notably less susceptible to chemically-induced fibrosis in comparison with controls (Shafiei et al., 2011).

Alcohol increases different cytokines such as TGF-β, TNF-α, and IL-1β in different organs, including the lungs, liver, and brain, in a complex manner. Alcohol-induced pro-inflammatory cytokines activate and contribute to the alcoholic pathology (T. M. Hu et al., 2013). During pregnancy, proper expansion of AT is required for the development of the fetus. In this study, 3T3-F442 pre-adipocytes were used to model characteristics of human pre-adipocytes (T. M. Hu et al., 2013).

In this study, it was hypothesized that alcohol exposure would result in a decrease in adipocyte differentiation in 3T3-F442 cells. Alcohol disrupts the endocrine activity of adipose tissue, which is exerted through synthesis and secretion of a wide variety of peptides and cytokines called adipokines (J. Park, Euhus, & Scherer, 2011; Zhong et al., 2012). Adipokines synthesis in children plays an important role in the control of energy homeostasis, carbohydrate metabolism, and inflammation (Ting & Lautt, 2006). Modifications in adipose secretion and action in early development may induce inflammation (Symonds et al., 2012). Adiponectin provides a crucial link This may lead to increased levels of pro-inflammatory cytokine synthesis and decreased levels of anti-inflammatory cytokines like adiponectin. Adiponectin plays important roles in lipid and

carbohydrate metabolism, and it, therefore, plays equally important roles in metabolic syndrome and obesity (Z. V. Wang & Scherer, 2016).

In individuals with low adiponectin, AT expands beyond the tissue capacity and becomes a major inducer of metabolic disorders, such as type 2 diabetes and obesity (Scherer, 2016). High levels of WAT have a crucial role in obesity-related metabolic diseases, such as several cancers, cardiovascular diseases, and inflammation (Ahima et al., 2006). Therefore, it is important to understand the mechanisms of excess WAT in early childhood and the induction of different metabolic diseases in adulthood. Obesity initiates AT fibrosis, but relatively little is known about obesity and fibrosis (Crewe et al., 2017). Therefore, understanding the mechanisms of AT differentiation and metabolically dysregulated WAT and induction of obesity is critically important.

This work demonstrates the effects of early alcohol exposure on adipose cell differentiation and adipose-derived cytokines and the relationship of this early exposure to inflammation. Further, 3T3-F442 pre-adipocytes were used as an early fat differentiation from fibroblast to identify alcohol effects that cause disruption of AT(adipogenesis) during development. Alcohol significantly reduced adipogenesis and the formation of fat droplets in 3T3-F442 adipocytes. In addition, transdifferentiation of pre-adipocytes to adipocytes was reduced in proportion to alcohol concentration.

The human immune system is regulated by specific cytokines, chemokines, or inducing systemic inflammation. Interleukin (IL)-1 receptor antagonist, IL-4, IL-10, IL-

11, and IL-13 are recognized as major anti-inflammatory cytokines. TNF-α, specific cytokine receptor for IL-1 and IL-18, also functions as a pro-inflammatory cytokine. While this model of adipogenesis did not include immune cells, adipose cells also produce these cytokines (Ahima, 2006). ELISA assay was used to detect pro-inflammatory cytokines and anti-inflammatory cytokines. The results showed the level of expression of pro-inflammatory cytokines such as IL-1β, IL6, IL-17, TNF-α, and INF-γ seemingly increased, sequentially in proportion to alcohol concentration. Further, the levels of anti-inflammatory cytokines, including IL-1 receptor antagonist, IL-4, IL-10, IL-11, IL-13, and IL-18, were reduced.

Data presented in this dissertation have the potential to provide a better understanding of how alcohol toxicity can interrupt adipocyte differentiation. Finally, this work introduces adipocytes dysfunction and adiponectin as a potentially new early biomarker for prediction and potential indicator for multiple metabolic and inflammatory diseases.

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