Studying the Role of Diallyl Sulfide in Ameliorating Ethanol Induced Adipose Tissue Injury

THESIS

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By

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ABSTRACT

Consumption of alcohol has been in existence in the world for many centuries and it has been identified as one of the major causes for death and disability adjusted life years. Several pathways contribute to ethanol mediated tissue injury. Pathogenesis of alcohol causes alcoholic liver disease. Recent studies have identified a major role of adipose tissue in the progression of alcoholic liver disease. Ethanol affects the metabolic and innate immune activities of adipose tissue contributing to alcohol-induced injury of the tissues. Till date there is no effective therapy available to treat alcohol induced tissue injury and alcoholic liver disease. The focus of the current study was to understand the role of diallyl sulphide (DAS) in reducing adipose tissue injury caused due to ethanol consumption.

DAS is an organo sulfur compound derived from allicin obtained from crushed garlic. DAS has hepato-protective, anti-tumorogenic, and anti-mutagenic properties. To understand the effect of ethanol and DAS on adipose tissue function differentiated 3T3L1 cell line, isolated human primary adipocytes, and male Wistar rats were used in the study. Effect of DAS in ameliorating ethanol induced oxidative stress, endoplasmic reticulum (ER) stress, up-regulation in pro-inflammatory cytokines production, down-regulation in anti-inflammatory cytokines expression, and changes in the adipose mass and adipocyte morphology were studied. Further the role of DAS in macrophage switching was also studied in ethanol exposed RAW264.7 macrophage cell line.

Studies have shown that alcohol consumption affects adipose tissue mass and adipokine expression in both humans and animals. Reduction in adipose tissue mass and adipocyte size was observed in rodents fed with alcohol. Further, decreased glucose uptake by adipocytes and increased lipolysis of white adipose tissue was also observed in alcohol fed rodents. Here we

investigated if DAS was effective in restoring adipose tissue mass, adipocyte morphology as well as reduce adipose tissue lipolysis. Treatment with DAS improved adipocyte morphology and adipocyte mass in both ethanol exposed adipocytes and ethanol fed male Wistar rats. DAS was also found to be effective in reducing lipolysis and promoting lipid accumulation in adipocytes as was evident by the decrease in the expression levels of lipolytic enzymes adipose triglyceride lipase and hormone sensitive lipase. Further, estimation of glycerol and free fatty acids released into the medium of ethanol exposed and DAS treated adipocytes also revealed that DAS treatment reduced lipolysis in ethanol exposed cells compared to unexposed and untreated adipocyte cells. Increased lipid accumulation in adipocytes as confirmed by an increase in oil red O absorbance suggested that DAS was effective in ameliorating ethanol induced hyperlipolysis of adipose tissue.

Chronic ethanol consumption causes oxidative stress and ER stress. Here we hypothesized that treatment of ethanol exposed cells or rodents with DAS reduces ethanol induced oxidative and ER stress. DAS treatment improved ethanol induced oxidative stress as was apparent by the decrease observed in the levels of oxidative stress markers ie, reactive oxygen species and malondialdehyde in ethanol exposed adipocytes and ethanol fed male Wistar rats. DAS also reduced the expression levels of ER stress markers like CHOP and Grp78 in ethanol exposed rodents.

Chronic ethanol consumption causes an increase in inflammatory cytokine production and a decrease in anti-inflammatory cytokine production. Here we hypothesized that DAS is effective in regulating the production of cytokines in ethanol exposed in vitro and in vivo systems. DAS was found to be effective in decreasing the levels of inflammatory cytokines and enhancing the levels of anti-inflammatory cytokines in adipocytes and adipose tissue. Alcohol promotes

polarization of macrophages to M1 phenotype which is pro-inflammatory in nature. In the present study, treatment of ethanol exposed RAW 264.7 macrophage cells with DAS was shown to promote polarization of macrophages from inflammatory phenotype (M1 phenotype) to anti-inflammatory phenotype (M2 phenotype). Taken together, the study results prove that DAS is effective in reducing ethanol induced injury of adipocytes and adipose tissue and can be a potential drug target for treating ethanol induced tissue injury.

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Abbreviations

4-HNE	4-hydroxy nonenal
ALD	Alcoholic liver disease
ADH	Alcohol dehydrogenase
AH	Alcoholic steatohepatitis
ALDH	Acetaldehyde dehydrogenase
AMPK	Adenosine monophosphate-activated protein kinase
ATGL	Adipose triglyceride lipase
BAT	Brown adipose tissue
Bid	BH3 interacting-domain death agonist
BMI	Body mass index
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
CHOP	C/EBP homologous protein
Col1α1	Alpha-1 type 1 collagen
COX2	Cyclooxygenase 2
CYP2E1	CytochromeP4502E1
DAS	Diallyl sulphide
DCFDA	2',7'-dichlorofluorescindiacetate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNPH	Dinitrophenylhydrazine

ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FCS	Fetal calf serum
FDA	Food and drug administration
FM	Fat mass
Gro-α	Growth-regulated protein alpha
Grp78	78kDa glucose-regulated protein
Grp94	94kDa glucose-regulated protein
HO-1	Hemeoxygenase-1
HSL	Hormone sensitive lipase
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-12β	Interleukin 12 beta
IL-22	Interleukin-22
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
KCl	Potassium chloride
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein 1
MDA	Malondialdehyde
Mgl2	Macrophage galactose N-acetl-galactosamine specific lectin 2
Mrc1	Mannose receptor C type 1

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
NCCS	National centre for cell sciences
NF-κB	Nuclear factor kappa beta
Nos2	Nitric oxide synthase 2
NOX	NADPH oxidase
OCT	Optimal cutting temperature
ODS	Octadecyl-silica
PBS	Phosphate buffered saline
PNPLA3	Patatin-like phospholipase domain containing protein-3
PPAR-α	Peroxisome proliferator-activated receptor alpha
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PTEN	Phosphatase and tensin homolog
RBP-4	Retinol binding protein-4
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAMe	S-adenosylmethionine
SAT	Subcutaneous adipose tissue
SOCS3	Suppressor of cytokine signalling 3
STAT-3	Signal transducer and activator of transcription 3
TLR-4	Toll like receptor-4
TNF-α	Tumor necrosis factor alpha
UPR	Unfolded protein response
VAT	Visceral adipose tissue

WAT	White adipose tissue
WHO	World health organization

Chapter-1

INTRODUCTION

1.1 Alcoholic Liver Disease

Alcoholic liver disease (ALD) is a complex; multi factorial disease responsible for morbidity and mortality worldwide. Alcohol ranks eighth among World's death risk factors and it is the third major risk factor for disease and disability. Consumption of 6.13 litres of alcohol per capita by each person of the age 15 years or older was documented in World Health Organization (WHO) report 2005. According to WHO report, 4% of the deaths worldwide are attributed to harmful alcohol consumption (approximately 2.5 million deaths). Alcohol is considered as an underlying factor in 60 different kinds of injuries and diseases and is a principal cause in 200 others. A major risk factor for death in males is alcohol, mainly due to injuries, liver damage, cardiovascular failure, and violence. According to WHO report, global distribution of alcohol attributable deaths by disease or injury include 6% neuropsychiatric disorders, 16.6% liver cirrhosis, 29.6% unintentional injuries, 0.1% prematurity and low birth weight, 21.6% cancer, 12% intentional injuries, 14% cardiovascular diseases and diabetes mellitus. Many serious social issues are also related to alcohol consumption including violence, child neglect and abuse, and non-attendance in the work place (WHO report 2014).

Alcohol consumption has been in existence in India for many centuries. The quantity and pattern of alcohol drinking has changed over the past two decades raising apprehension about public health and social consequences of excessive alcohol consumption. India is one of the leading producers of alcohol in the World and contributes to 65% of total production. Nearly 30-35% of adult men and approximately 5% of adult women (male to female ratio being 6:1) consumes alcohol (Gururaj G, Pratima M et al 2011). An alarming concern, say experts, is that people are beginning to drink at younger ages and the average age of initiation has dropped from 19 years to 13 years. In India, most of the alcohol drinkers fall into the group of

hazardous drinking. Alcohol contributes to 15-20% of all deaths and to about 25% of premature mortality in younger age group of 15-44 years, in India. More than a fifth of hospital admissions are alcohol related: 18% of psychiatric emergencies, more than 20% of brain injuries and 60% of all injuries getting admitted to hospital emergency rooms. There has also been a substantial increase in domestic violence due to alcohol consumption. Although the Indian constitution includes alcohol prohibition among its directive principles, the legislative power of individual states dictates the alcohol policy adopted. Since most states derive a major source of their revenue from alcohol taxation, they are ambivalent towards prohibiting alcohol. 483 alcohol detoxification and 90 counselling centres are funded by the Government of India under its National Drug De-addiction Programme, but these programmes have recorded a low success rate and states did not succeed in effectively funding these centres (Prasad R 2009; Das SK, Balakrishnan V et al 2006).

1.2 Alcohol Metabolism

Alcohol is primarily metabolized in the liver hepatocytes at three different sites: cytosol, endoplasmic reticulum (ER) and peroxisome resulting in the formation of acetaldehyde. The acetaldehyde so formed is metabolized in the mitochondria into acetic acid (Thiele GM, Freeman TL et al 2004; Tuma DJ, Casey CA 2003; De Minicis S, Brenner DA 2006). The three major steps in alcohol metabolism include:

1. Reversible oxidation of alcohol to toxic acetaldehyde effectuated by alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1), and catalase enzymes.
2. Irreversible oxidation of acetaldehyde to acetate by acetaldehyde dehydrogenase (ALDH).
3. Breakdown of acetate to water and carbon dioxide (Setshedi M, Wands JR et al 2010).

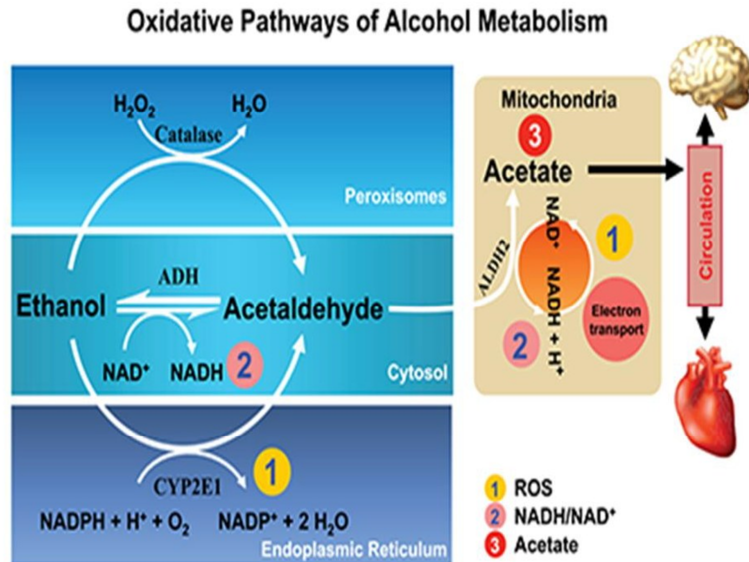

Figure 1.1: Different pathways of alcohol metabolism.

Adopted from: Samir Zakhari, Alcohol Metabolism and Epigenetics Changes, *Alcohol Research: Current Reviews*, Volume 35 (1): 6-16.

1.3 Alcohol disease spectrum and risk factors

The progression of ALD involves different stages: steatosis, steatohepatitis, fibrosis, cirrhosis, and carcinoma of the liver (Hall P 1995). Steatosis is characterized by the accumulation of lipid droplets in the cytoplasmic space of hepatocytes (Rasinen K, Casey CA 2012). 90% of the heavy drinkers develop steatosis which is usually asymptomatic and reversible on abstinence (Orman ES, Odena G et al 2013). Prolonged consumption of alcohol leads to alcoholic steatohepatitis (AH), a condition characterized by hepatic inflammation and injury. Approximately 20% of the heavy drinking population will develop more severe forms of ALD which include fibrosis, cirrhosis, and hepatocellular carcinoma (Altamirano J, Bataller R 2011; Teli MR, Day CP et al 1995). A severe form of acute liver inflammatory disease associated with high short-term mortality rate known as acute alcoholic steatohepatitis can occur at any ALD stage and is characterized by stage IV fibrosis, portal hypertension, bacterial infections, and liver failure leading to death of a person (Gao B, Bataller R 2011; Boetticher NC, Peine CJ et al 2008).

Alcohol intake is considered to be the major risk factor for ALD. Several other risk factors of ALD progression include sex, cigarette smoking, obesity, alcohol drinking patterns, dietary factors and genetic factors (O'Shea RS, Dasarathy S et al 2010; Tsukamoto H, Machida K et al 2009; Wilfred de Alwis NM and Day CP 2007). Women are at higher risk of developing ALD compared to men due to lower gastric alcohol dehydrogenase enzyme levels, estrogen presence and a higher body fat proportion (Becker U, Deis A et al 1996). Obesity and alcohol abuse have synergistic effects resulting in macrophage infiltration and activation, adiponectin and insulin resistance, and ER stress leading to ALD progression (Raynard B, Balian A et al 2002; Naveau S, Cassard-Doulcier AM et al 2010; Xu J, Lai KK et al 2011). Genetic factors like polymorphisms in alcohol metabolizing genes (alcohol dehydrohenase, CYP2E1), cytokines (nuclear factor κB (NF- κB), interleukin-1(IL-1), interleukin-6 (IL-6), Interleukin-10 (IL-10)), genes involved in toll like receptor-4 (TLR-4) signaling mechanism, and patatin-like phospholipase domain-containing protein 3 (PNPLA3) also affect the progression of ALD from steatosis to cirrhosis stage (Bataller R, Brenner DA 2005; Jarvelainen HA, Fang C et al 1999; Stickel F, Buch S et al 2011; Tian C, Stokowski RP et al 2010; Trepo E, Gustot T et al 2011).

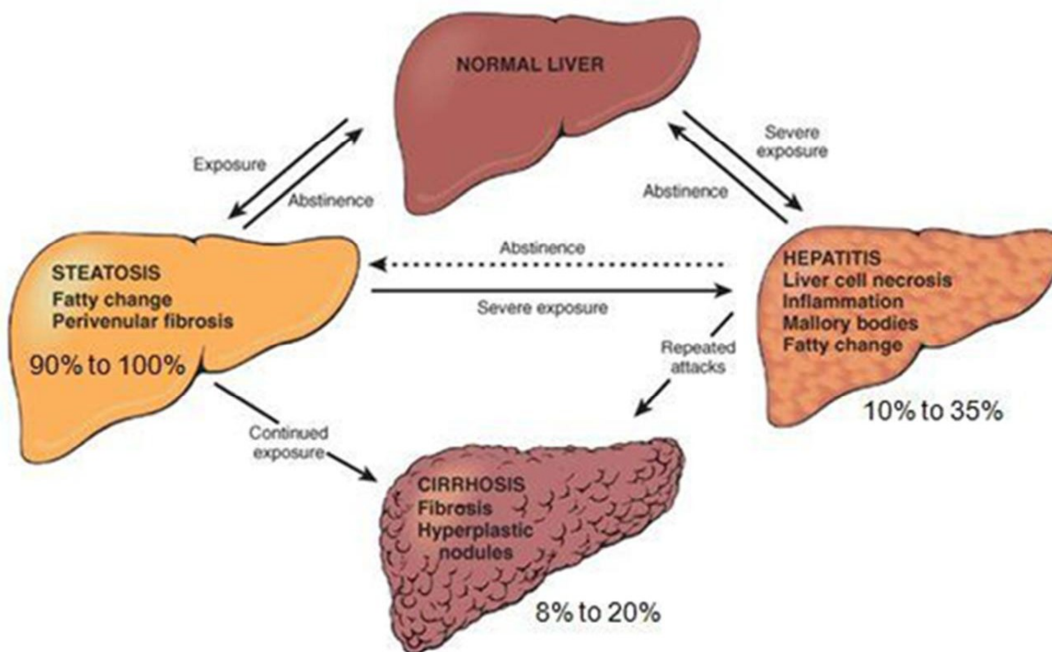

Figure 1.2: Different stages of alcoholic liver disease

1.4 Ethanol and oxidative stress

One of the major factors contributing to ALD is oxidative stress (Lu Y, Cederbaum AI, 2008). Differences in the production of reactive oxygen species/reactive nitrogen species (ROS/RNS) and imbalances in the level and activity of antioxidants results in oxidative stress (Tang H, Sebastian BM et al 2012). A number of studies have shown that ROS generation is the key factor for the progression of liver steatosis to steatohepatitis. Increased ROS production leads to lipid peroxidation resulting in the formation of reactive aldehydes like 4-hydroxy nonenal (4-HNE), which form adducts with proteins and DNA (Galligan JJ, Smathers RL, et al 2012). CYP2E1, induced by chronic alcohol consumption, plays a major role in the generation of ROS. CYP2E1 is mainly found in the ER, but it is also expressed in the mitochondria (Leung TM and Nieto N 2013). Factors like leptin, adiponectin, acetone, insulin, and cytokines regulate the mRNA and protein expression levels of CYP2E1 (Wu D and Cederbaum AI 2005). In order to protect against CYP2E1 induced oxidant stress,

CYP2E1 also induces the expression of anti-oxidant factors like Nrf2. Ethanol induced lipid peroxidation is inhibited by CYP2E1 inhibitors and it is shown that decreased oxidative stress and lipid peroxidation are observed in mice deficient in CYP2E1 when compared to wild type mice on chronic ethanol feeding (Lu Y, Zhuge J et al 2008). Liver is the major site for the expression of CYP2E1 but it is also detected in low levels in the adipose tissue. Activity of CYP2E1 parallels with ethanol induced liver injury and lipid peroxidation (Bell LN, Temm CJ et al 2010; Leung TM and Nieto N 2013). Increase in CYP2E1 levels during chronic ethanol consumption is due to a decrease in the proteasomal degradation which results in increased CYP2E1 stability (Leung TM and Nieto N 2013). Study of the role of CYP2E1 in hepatocyte injury have been carried out using HepG2 cells over expressing CYP2E1, transgenic mice, and CYP2E1 knockout mice. *In vivo* studies on CYP2E1 induction showed an increased oxidative stress that sensitized hepatocytes to lipopolysaccharide (LPS) and TNFα toxicity. Studies on CYP2E1 knock-in mice showed increased hepatic steatosis and liver injury after ethanol feeding. Decreased oxidative stress, increased peroxisome proliferator-activated receptor alpha (PPARα) levels, and protection towards alcohol-induced liver injury were observed in studies done on CYP2E1 knockout mice. Insulin resistance, a feature observed in ALD, increases CYP2E1 expression and activity. This is due to the increased production of ketone bodies from persistent oxidation of mitochondrial fatty acids. The ketone bodies formed will stabilize CYP2E1 protein and prevent its degradation (Ambade A and Mandrekar P 2012; Lu Y and Cederbaum AI 2008).

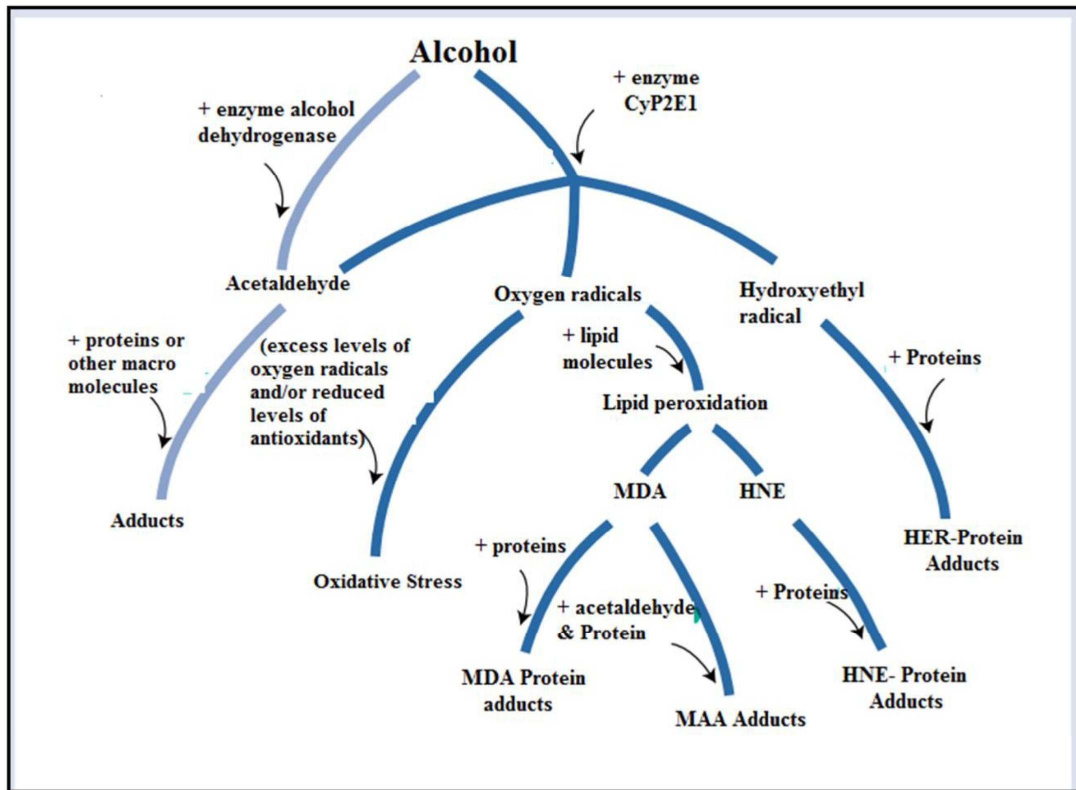

Figure 1.3: Potentially toxic products resulting from the metabolism of ethanol

Adopted from: Brooks PJ, "DNA damage, DNA repair, and alcohol toxicity—a review", Alcohol Clin Exp Res, 1997, 21, 1073-82.

1.5 Ethanol and ER stress: Secretory proteins are trans - located to the endoplasmic reticulum (ER) where they undergo post translational modifications. They are folded in the ER, exported to the Golgi apparatus and then secreted into the extracellular space or are destined for the plasma membrane. Unfolded protein response (UPR), a complex network of 3 interconnected pathways, maintains homeostasis between protein load and the ability of ER to fold the protein (Howarth DL, Vacaru AM et al 2012; Rutkowski DT, Hegde RS 2010). An imbalance in the homeostasis further induces UPR in an attempt to restore the equilibrium, failure of which results in ER stress causing apoptosis (Howarth DL, Vacaru AM 2012; Tabas I, Ron D 2011). Chronic alcohol exposure results in a change in ER morphology and causes ER stress in hepatocytes that leads to ER dysfunction and protein

folding abnormalities that result in impaired protein secretion (Galligan JJ, Smathers RL et al 2012). This effect of alcohol on ER functioning and protein folding is due to the activation of CYP2E1 pathway for alcohol metabolism. Activation of this pathway results in oxidative stress thereby generating reactive oxygen species (ROS) which cause lipid peroxidation and the end products of lipid peroxidation form adducts with proteins which accumulate in the ER causing ER fragmentation (Lu Y, Zhuge J et al 2008; Howarth DL, Vacaru AM et al 2012). Increased ROS is also shown to affect the function of lysosomes and autophagy that leads to mitochondrial injury and hepatocellular death (Tuma DJ, Casey CA 2003; Howarth DL, Vacaru AM et al 2012). Studies with intra-gastric-alcohol treated mice have shown an up-regulation in the ER stress related genes like 78kDa glucose-regulated protein (Grp78), 94kDa glucose-regulated protein (Grp94), and C/EBP homologous protein (CHOP). Knockout of CHOP gene in mice resulted in minimal hepatocyte apoptosis which further supports the role of ER stress in ALD. An elevation in homocysteine levels is one of the reasons for ER stress, which disrupts the disulfide bond formation leading to the accumulation of mis-folded proteins in the ER (Ozcan L, Tabas I 2012).

1.6 Current therapies for treatment and new targets for therapy

Since alcohol consumption is the cause of ALD progression, alcohol abstinence is the only way to prevent development of ALD. Rehabilitation programs together with family support will aid in abstinence. Pharmacological intervention is required for some patients.

1. Use of ADH enzyme inhibitors: Disulfiram, an irreversible ADH enzyme inhibitor, is used to treat alcohol patients but it is not recommended for severe ALD patients as it is hepatotoxic (Soyka M, Rosner S 2010).
2. Use of anti craving drugs: Anti-craving drugs such as acamprosate are recommended for abstinence. Baclofen, a γ-aminobutyric acid B receptor agonist, is recommended for abstinence as it is not hepatotoxic and is safe even for cirrhotic patients.

Naltrexone, an opioid antagonist, is also recommended for reducing alcohol relapse (Soyka M, Rosner S 2010; Mohanty SR, LaBrecque DR et al 2004; Addolorato G, Leggio L 2010).

3. Use of corticosteroids: Corticosteroids are recommended for patients with AH. Studies have shown that use of corticosteroids improved the life expectancy of severe AH patients (Imperiale TF, McCullough AJ 1990; Mathurin P, Deng QG et al 2000).
4. Use of Pentoxifylline: Pentoxifylline, a phosphodiesterase inhibitor, blocks tumor necrosis factor-alpha (TNF-α) transcription. It is recommended for patients with severe AH and for those who donot respond to corticosteroids (Akriviadis E, Botla R et al 2000; De BK, Gangopadhyay S et al 2009).
5. Use of anti-TNF-α agents: Infliximab or etanercept, TNF-α blockers, are used in the treatment of AH. However, studies have shown that use of these drugs increased the mortality rate and risk of infections in AH patients. So these agents are not recommended for AH treatment (Spahr L, Rubbia-Brandt L et al 2002; Naveau S, Cassard-Doulcier AM 2010; Boetticher NC, Peine CJ et al 2008).
6. Use of S-adenosylmethionine (SAMe): SAMe is a methyl donor that has antioxidant functions. It maintains the function of mitochondria, and down regulates TNF-α production. Administration of SAMe has shown a significant decrease in mortality and need for liver transplantation (Lu SC, Martinez-Chantar ML et al 2006; Mato JM, Camara J et al 1999).
7. Supportive and nutrition therapy: It is advised for patients with severe AH to be administered to intensive care unit. Administration of protein and vitamin rich diet is recommended. Antibiotics should be given to prevent infections (Cabre E, Rodriguez-Iglesias P et al 2000; Foody W, Heuman DD et al 2001; Stickel F, Hoehn B et al 2003).

8. Liver transplantation: Liver transplantation is recommended for patients with severe and decompensated ALD who donot respond to medical therapy and who cannot survive the 6 month mandatory abstinence period (Burra P, Senzolo M et al 2010; Dureja P, Lucey MR 2010).

1.7 New therapeutic targets

1. CXC Chemokines: CXC family of chemokines such as interleukin-8 (IL-8), growth-regulated protein-alpha (Gro-α) attracts neutrophils leading to AH. Therefore drugs that target these chemokines need to be developed to treat AH (Colmenero J, Bataller R et al 2007; Dominguez M, Miquel R et al 2009).
2. IL-22: Interleukin-22 (IL-22), a member of IL-10 cytokine family, has anti-apoptotic, anti-oxidant, proliferative, anti-steatotic, and anti-microbial effects. Since the receptors of IL-22 are expressed only on the surface of epithelial cells and its side effects are minimal, IL-22 can be used to treat AH (Ki SH, Park O et al 2010).
3. Gut microbiota and LPS pathway: LPS from gut microbiota causes AH and fibrosis by activating TLR4 signaling pathway. Therefore use of probiotics and TLR4 antagonists that modify LPS signaling pathway can be used to treat ALD (Mencin A, Kluwe J et al 2009).
4. Complement system: Complement syatem activation leads to AH and fibrosis. Therefore drugs that block the activation of complement system or that activate the negative regulators of complement system should be designed to prevent ALD progression (Pritchard MT, McMullen MR et al 2007; Cohen JI, Roychowdhury S et al 2010; Roychowdhury S, McMullen MR et al 2009; Charbel Issa P, Chong NV et al 2011).
5. Endocannabinoids: Endocannabinoids play a role in the progression of ALD via cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2) receptors.

Therefore CB1 antagonists and CB2 agonists need to be developed to limit the progression of ALD (Tam J, Liu J et al 2011; Louvet A, Teixeira-Clerc F et al 2011)

6. Osteopontin: Levels of osteopontin an extracellular matrix protein are markedly increased in ALD. Therefore, developing drugs that target osteopontin need to be developed for ameliorating ALD (Seth D, Gorrell MD et al 2006).
7. Inhibitors of apoptosis: Chronic ethanol consumption causes hepatocyte apoptosis. So drugs that inhibit apoptosis need to be developed to reduce alcohol induced tissue injury (Pockros PJ, Schiff ER et al 2007; Shiffman ML, Pockros P et al 2010; Ratziu V, Chojkiet M et al 2010)

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Chapter-2

REVIEW OF LITERATURE

2.1 Contribution of adipose tissue towards ethanol induced liver injury

Adipose tissue which has been long considered as an energy storage depot is now being identified to play an active role in energy homeostasis and various other processes. It functions as an endocrine organ secreting a variety of cytokines, termed as adipokines or adipocytokines that have important roles at the interface between the metabolic and immune systems. Ethanol consumption effects the metabolic and innate immune activities of adipose tissue and this is a likely factor contributing towards ethanol induced liver injury (Rogers CQ, Ajmo JM et al 2008).

2.2 Adipose tissue histology, location and function

Based on function and histology, adipose tissue is subdivided into white adipose tissue (WAT) and brown adipose tissue (BAT). BAT plays a role in thermogenesis and body temperature regulation while WAT acts as an energy storage and endocrine organ. In mammals WAT is the major energy storage organ and it provides both thermal and mechanical insulation to the body. The location of WAT is not confined but it is distributed as physically unconnected individual pads throughout the organism. Based on the location WAT can be classified as epididymal, mesenteric, subcutaneous, retroperitoneal, and pericardial fat pads.

Adipose tissue is diverse in its cellular composition comprising of mature adipocytes, pre-adipocytes, macrophages, fibroblasts, and endothelial cells. Mature adipocytes can amass excess energy sources with the help of glucose transporters and lipoprotein lipase enzymes in the form of triglycerides inside the cell without compromising their cellular function (Abel ED, Peroni O et al 2001). WAT has three main functions: 1) energy storage 2) triglyceride

hydrolysis to free fatty acids for supporting tissue energy needs and 3) adipokine release (Marra F, Bertolani C 2009).

WAT under positive energy balance conditions stores excess energy in the form of triglycerides whereas under negative energy balance conditions it provides energy for other organs by releasing free fatty acids. Fat storage function disorder of WAT leads to excess influx of fatty acid into the liver leading to steatosis. So, healthy adipose tissue is required to maintain lipid homeostasis throughout the body at the adipose tissue-liver axis (Lafontan M, Girard J 2008; Cusi K 2010; Wree A, Kahraman A et al 2011).

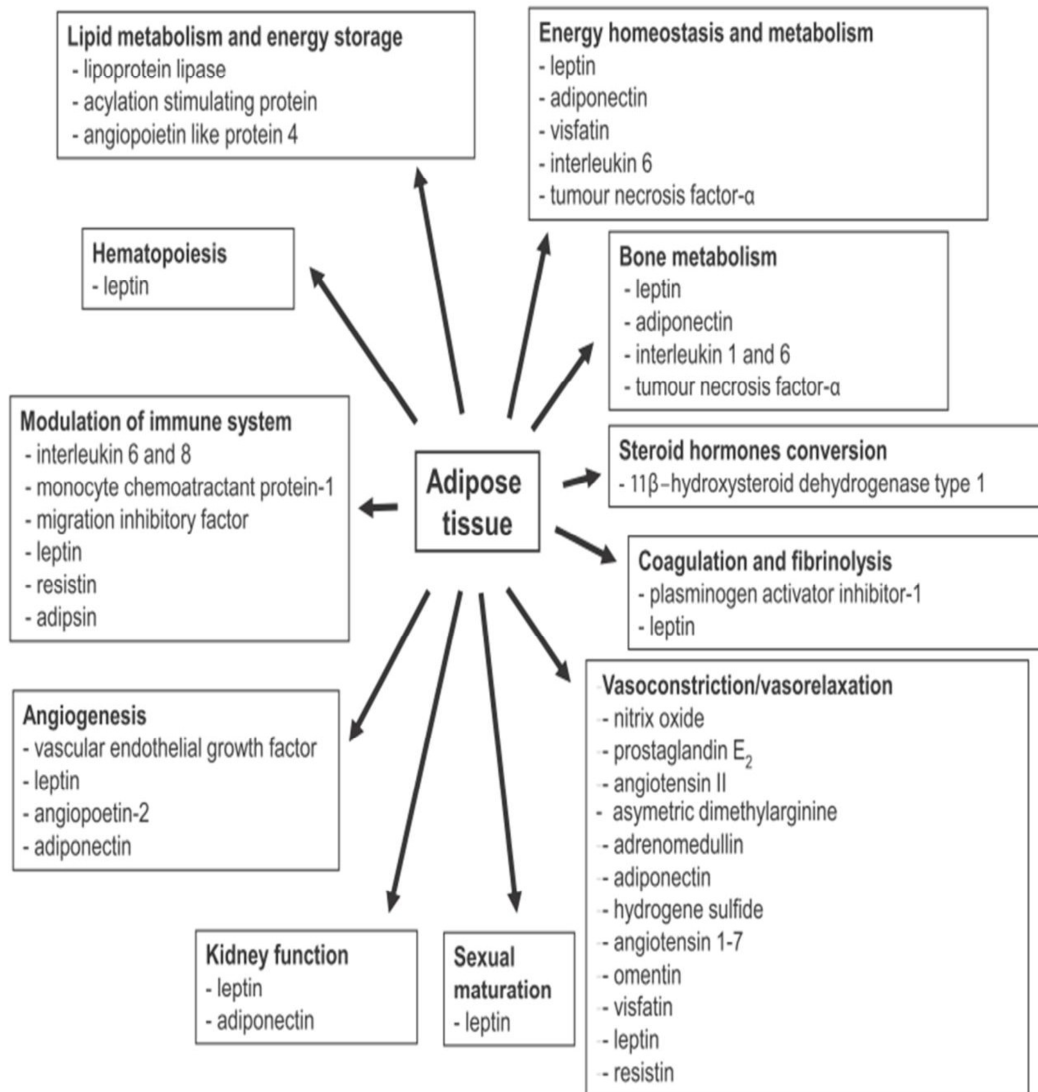


Figure 2.1: Major physiological functions of adipose tissue secretory products

Adopted from: Marcin Adamczak, and Andrzej Wiecek, The Adipose Tissue as an Endocrine Organ, Nephrology, Vol 33, No 1, January 2013, pp 2-13

2.3 Alcohol, adipose tissue and CYP2E1

Ethanol consumption leads to the development of oxidative stress in a number of tissues (Lu Y, Cederbaum AI 2008) including the adipose tissue. Activation of CYP2E1 mediated ethanol breakdown results in oxidative stress and endoplasmic reticulum (ER) stress leading

to adipokine dysregulation and subsequent progression of ALD. Oxidative stress indicators like 4-hydroxynonenal are detected in adipose tissue after chronic ethanol feeding (Kang L, Chen X et al 2007; Chen X, Sebastian BM, Nagy LE 2007). Increased ethanol-induced CYP2E1 expression is found to be critical to the development of adipose tissue inflammation *via* activating redox-sensitive transcription factors that cause increased ROS production. Alternatively, increased CYP2E1 expression and activity also leads to C1q dependent complement system activation and apoptosis mediated by Bid (BH3 interacting-domain death agonist) resulting in an indirect mechanism of CYP2E1 mediated inflammatory responses (Sebastian BM, Roychowdhury S et al 2011). A summary of the effects of alcohol consumption on adipose tissue mass and inflammatory mediators is presented in Figure 2.2 and Table 2.1.

2.4 Endocrine function of adipose tissue

Knowledge about the endocrine function of adipose tissue has emerged after the discovery of leptin expression and production by adipocytes (Zhang Y, Proenca R et al 1994). Studies following leptin discovery have shown that adipose tissue secretes a wide variety of peptide molecules (both hormones and cytokines) known as adipokines or adipocytokines. Adipokines exert their activity on metabolism, immune function, as well as neuroendocrine pathways regulating feeding behaviours. All the cell types contribute to the secretory function of WAT. Both physiological environment and adipose tissue expansion status regulate adipokine production.

The extent of metabolic activity of adipose tissue fat pads varies with the location and certain fat depots secrete specific adipokines more actively than others. Non-adipose tissues also secrete certain adipokines. For example, resistin is found in mouse brain and pituitary (Morash BA, Lia, Murphy PR et al 1999), and in humans resistin mRNA is also observed in

placenta (Yura S, Sagawa N, Itoh H et al 2009) and monocytes (Nagaev I, Smith U 2001; Savage DB, Sewter CP, Klenk ES et al 2001).

2.5 Effect of alcohol on WAT

In human and animal models, alcohol intake causes susceptibility towards non-alcoholic fatty liver disease (Baker SS, Baker RD et al 2010). Studies in both humans and animals have shown that alcohol consumption affects both adipose tissue mass and adipokine secretion. Insulin resistance, increased macrophage infiltration, inflammatory cytokine expression (Kang L, Sebastian B et al 2007), reduced ability of insulin to stimulate glucose uptake and inhibit lipolysis leading to hepatic steatosis are observed after chronic ethanol feeding (Kang L, Chen X et al 2007; Chen X, Sebastian BM et al 2009).

2.5.1 Studies on rodents

Reduction in adipose tissue mass (Kang L, Chen X et al 2007; Kang X, Zhong W et al 2009) and increase in fatty acid uptake by hepatocytes is observed in alcohol exposed rodents (Zhou SL, Gordon RE et al 1998; Berk PD, Zhou S, Bradbury MW 2005). Ethanol consumption in rats apart from decreasing glucose uptake in rat adipocytes (Rachdaoui N, Sebastian BM, Nagy LE 2003), also increases the rate of degradation of triglycerides in adipose tissue thus resulting in increased free fatty acid circulation (Kang L, Chen X et al 2007). Studies on C57BL/6N mice showed that lipid homeostasis at the adipose tissue-liver axis is disturbed on chronic ethanol consumption and this demonstrates that WAT lipolysis is stimulated on alcohol consumption resulting in an excess fatty acid release that get transported to the liver and become deposited as triglycerides (Wei X, Shi X et al 2013). Anatomically, visceral adipose tissue (VAT) is connected to hepatic system *via* the mesenteric and portal veins. Thus chronic alcohol consumption may cause simultaneous accumulation of free fatty acid in liver cells and mesenteric fat tissues. Adipocyte size is also found to be reduced in chronic alcohol exposed mice (Zhong W, Zhao Y et al 2012).

Adipose triglyeride lipase (ATGL) and hormone sensitive lipase (HSL) are found to be activated in association with a significant increase in the release of fatty acid from adipose tissue explants in mice fed with alcohol. Alcohol exposure up regulates fatty acid transport proteins, causes accumulation of lipids in the liver, induces insulin intolerance, inactivates adipose protein phosphatase 1, and also up regulates phosphatase and tensin homolog (PTEN) and suppressor of cytokine signalling 3(SOCS3) proteins in mice. WAT dysfunction can directly impact lipid homeostasis of liver by reverse triglyceride transport. Hyperlipolysis is found to be the major functional defect of WAT after chronic ethanol exposure. Studies suggest that alcohol induced adipose tissue hyperlipolysis and insulin resistance are due to inactivation of adipose protein phosphatase 1 and upregulation of negative regulators of insulin signalling like PTEN and SOCS3 there by dysregulating or inhibiting insulin signal transduction pathway. Study by Zhong et al showed a significant loss in WAT in alcohol fed mouse (Kasztelan-Szczerbinska B, Surdacka A et al 2013).

2.5.2 Studies on alcoholics

Clinical studies in alcoholics have shown an association of lower fat mass with higher liver fat (Addolorato G, Capristo E et al 1997; Addolorato G, Capristo E et al 1998). Lower body mass index (BMI) and fat mass (FM) and higher hepatic fat levels are observed in alcoholic patients (Addolorato G, Capristo E et al 1998; Addolorato G, Capristo E et al 2000; Greco AV, Mingrone G et al 2000). Alcohol intake enhances cortisol secretion that results in change of fat distribution pattern, together with an increase in abdominal and hepatic fat deposition (Leggio L, Malandrino N et al 2009) and subcutaneous adipose tissue (SAT) lipolysis (Krsek M, Ruziicka M et al 2006). Studies show that alcohol consumption is associated with decreased accumulation of SAT and increased accumulation of VAT. Impaired function of adipocytes or surpass in the storage capacity of SAT results in accumulation of fat outside SAT thus resulting in buildup of VAT (Unger RH 2003). VAT accumulation results in

glucose intolerance, onset of diabetes, and an increase in apolipoproteins (Fox CS, Massaro JM et al 2007; Wajchenberg BL 2000; Matsushita Y, Nakagawa T et al 2010). The Framingham Offspring Study reported that intake of large amounts of alcohol is associated with decrease of SAT in women and increase of VAT in men (Molenaar EA, Massaro JM et al 2009).

Consumption of more than 14 standard drinks is shown to be related to an increased risk of metabolic syndrome. No protective effect on adipose tissue accumulation is shown even with low to moderate alcohol intake (Zakhari S and Li TK 2007).

Alcohol energy content of 7.1g/kcal is relatively high (Suter PM, Hasler E et al, 1997) and it increases appetite and promotes energy intake (Westerterp-Plantenga MS, Verwegen CR 1999)A study on the dietary intake of energy from food and alcohol in Koreans showed that with higher consumption of alcohol there was an increase in total energy intake and further it was observed that there was an increase in VAT accumulation with either decrease or no change in SAT accumulation (Kim KH, Oh SW et al 2012).

2.6 Alcohol and adipokines

Alcohol consumption is known to disrupt adipokine release from adipose tissue (Nicolas JM, Fernandez-Sola J et al 2001; Rogers CQ, Ajmo JM, You M 2008), and promote infiltration of macrophages and this leads to adipose tissue phenotype alteration accredited to alcohol metabolism induced oxidative stress (Tang H, Sebastian BM et al 2012). Results from a study reported by Qin and colleagues provided evidence for an increased adipose tissue inflammation due to alcohol binging prior to burn injury (Qin Y, Hamilton JL, Bird MD et al 2013). These observations have important inferences as studies show that increases in pro-inflammatory cytokines not only alter the functional integrity of adipose tissue but also dysregulate metabolism of adipose tissue (Hajer GR, van Haeften TW, Visseren FL 2008).

Studies have shown that consumption of alcohol at moderate levels improves human health, especially cardiovascular related morbidities (Klatsky AL, Udaltsova N 2007). *In vitro* and *in vivo* experimental data demonstrate that alcohol acts as anti-inflammatory agent in presence of stimuli like bacterial lipopolysaccharides that up regulate inflammatory cytokines expression (Syapin PJ, Militante JD, Garrett DK, Ren L 2001). However, in the absence of inflammatory stimuli alcohol acts as a pro-inflammatory agent and up regulates the production of inflammatory cytokines and enzymes like IL-6, TNF-α, inducible nitric oxide synthase (iNOS), and cyclooxygenase 2 (COX-2) (Nanji A, Miao AL et al 1997; Yuan GJ, Zhou XR 2006)

2.6.1 Studies on rodents

Adiponectin and leptin are the key adipokines that modulate hepatic lipid homeostasis towards lowering liver lipid content. Reduced adipose tissue weight, serum leptin and adiponectin concentrations together with the development of fatty liver are observed in chronic alcohol exposed rodents (Kang L, Chen X et al 2007; Zhou SL, Gordon RE et al 1998, Kang X, Zhong W et al 2009; Zhong W, Zhao Y et al 2012; Xu A, Wang Y et al 2003; You M, Considine RV et al 2005; Esfandiari F, You M et al 2007; Song Z, Zhou Z et al 2008; Shen Z, Liang X et al 2010; Otaka M, Konishi N et al 2007; Maddalozzo GF, Turner RT et al 2009). Adiponectin signalling *via* adiponectin receptor activates adenosine monophosphate-activated protein kinase (AMPK) pathway that stimulates fatty acid oxidation and decreases hepatic lipid influx and *de novo* lipogenesis thereby regulating hepatic lipid content (Rogers CQ, Ajmo JM, You M 2008; You M, Rogers CQ 2009; Marra F, Bertolani C 2009; Galic S, Oakhill JS, Steinberg GR 2010). Exogenous adiponectin administration or endogenous adiponectin production stimulation has been shown to attenuate alcohol induced fatty liver in mice (Xu A, Wang Y et al 2003; You M, Considine RV, et al 2005; Shen Z, Liang X et al 2010; Ajmo JM, Liang X et al 2008). Adiponectin has anti-

inflammatory effects that are mediated *via* a heme-oxygenase-1 (HO-1) dependent pathway (Mandal P, Park PH et al 2010; Mandal P, Pritchard MT et al 2010).

Leptin signalling *via* leptin receptor b activates AMPK and signal transducer and activator of transcription 3 (Stat3) pathways (Galic S, Oakhill JS, Steinberg GR 2010; Marra F, Bertolani C 2009). Decreased serum leptin levels are observed in both chronic alcohol or acute alcohol consumption (Greco AV, Mingrone G et al 2000; Santolaria F, Pérez-Cejas A et al 2003; Calissendorff J, Brismar K, Röjdmark S 2004; Kalousová M, Zima T et al 2004). Studies show that leptin deficiency is associated with WAT mass reduction and exogenous leptin administration in leptin deficient mice restored alcohol induced hypoleptinemia and hepatic steatosis. Studies show that during hyperinsulinemic-euglycemic clamp subcutaneous, epididymal, and omental adipose tissue glucose uptake is decreased after chronic ethanol feeding (Kang L, Sebastian BM et al 2007). Studies on male Wistar rats showed macrophage infiltration in epididymal adipose tissue, alteration in mRNA expression levels of adipocytokines like TNFα, IL-6, monocyte chemoattractant protein-1 (MCP-1), adiponectin, and retinol binding protein-4 (RBP4) on chronic ethanol feeding (Kang L, Sebastian BM et al 2007).

Intragastric alcohol feeding of male Wistar rats for 22 weeks showed a decrease in the production of cartonectin and adiponectin in VAT and an increase in the production of leptin, visfatin, chemerin (Ren RZ, Zhang X et al 2012), and resistin in both sera and VAT in a dose dependent manner (Yu HC, Li SY et al 2010).

Concentration dependent up regulation of inflammatory genes IL-6 and TNF-α genes and inflammatory enzymes COX-2 and iNOS is observed in 3T3L1 pre-adipocytes (Kiselova-Kaneva Y, Tasinov O et al 2012) treated with ethanol.

2.6.2 Studies on alcoholic patients

Higher levels of adiponectin (Acrp30) and resistin are found in patients with ALD when compared to control subjects. The increase in levels of resistin is due to inflammation in alcoholic patients while the increase in adiponectin levels suggests a protective and anti-inflammatory role of adiponectin (Kasztelan-Szczerbinska B, Surdacka A et al 2013). This may be explained by Behre's hypothesis which states that increased adiponectin blood levels have a protective role and also help the body to adapt during fasting. In ALD progression malnutrition is frequently observed and this might be the reason for increased plasma levels of Acrp30 in alcoholic patients when compared to controls (Behre CJ 2007).

Studies show that moderate alcohol consumption increased adiponectin and grehlin levels while acylation-stimulating protein concentrations are decreased and these concentrations improve insulin sensitivity (Beulens JWJ, de Zoete EC et al 2008). Recent data show that high levels of TNFα in the SAT are observed in patients with alcoholic hepatitis and a positive correlation is observed between the liver IL6 and liver histological lesions and SAT TNFα levels (Naveau S, Cassard-Doulcier AM et al 2010). Osteopontin expression levels from adipose tissue also correlate with fibrosis of liver in alcoholic patients (Patouraux S, Bonnafous S, Voican CS et al 2012). These data suggest the existence of similar pro-inflammatory mechanisms in adipose tissue and liver. Alcohol consumption also leads to pro inflammatory cytokine expression from SAT. One week after alcohol withdrawal adipose tissue macrophages polarize into a M2 phenotype and an allevation in SAT inflammation is observed in patients with ALD (Voican CS, Njiké-Nakseu M et al 2014).

Serum concentrations of leptin, an adipokine with pro-inflammatory and pro-fibrotic properties, are high in alcoholic patients irrespective of the presence or absence of cirrhosis (Nicolas JM, Fernandez-Sola J, Fatjo F et al 2001).

Studies on patients with ALD established a correlation between TNF-α and IL-10 produced by adipose tissue with histological lesions in the liver and this suggests a relationship between adipose tissue inflammatory process and ALD progression in the liver (Naveau S, Cassard-Doulcier AM, Njike-Nakseu M et al 2010).

Studies on alcoholic patients also established a correlation between the osteopontin expression in adipose tissue and hepatic fibrosis emphasizing the concept of adipose tissue inflammation in ALD (Patouraux S, Bonnafous S et al 2010)

In vitro studies on human adipose tissue fragments showed a significant anti-inflammatory effect of ethanol in a dose and time dependent manner (Wandler A, Bruun JM et al 2008).

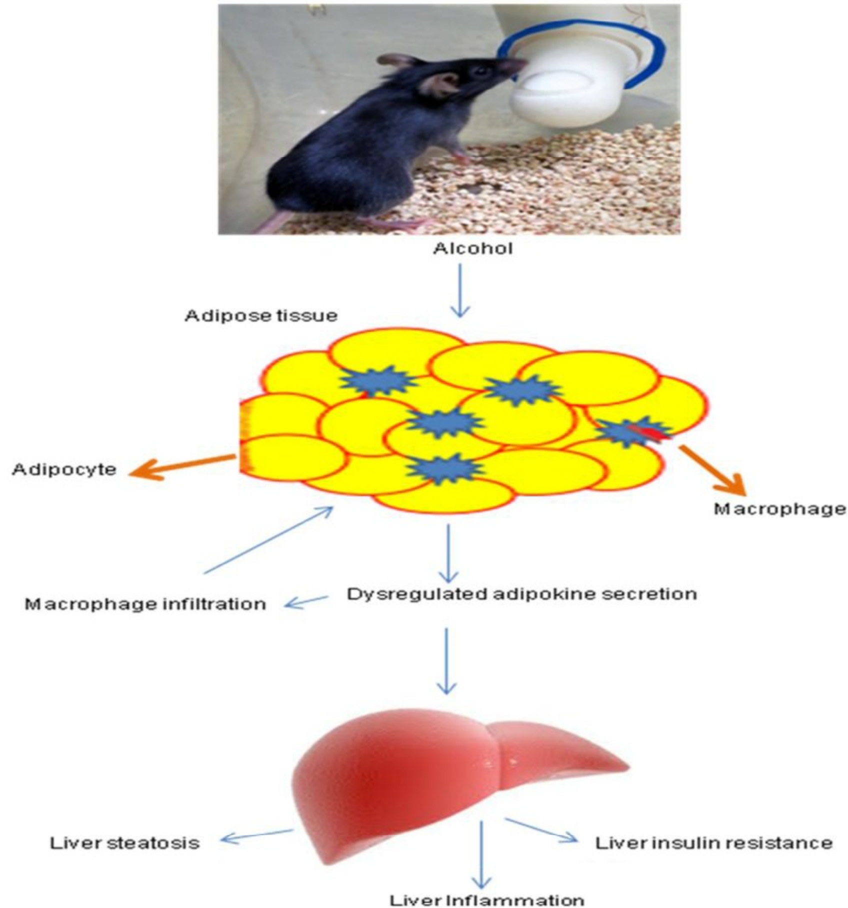
Table 2.1. The effect of alcohol consumption in rodents and humans with respect to adipokine secretion and adipose tissue mass.

Animal Model	Effects caused by alcohol consumption	Reference
Rodent	↓ in adiponectin and cartonectin levels	Song Z, Zhou Z, Deaciuc I et al, 2008.
	↑ in leptin, resistin, IL-6, visfatin, chemerin, TNF-α, MCP-1, RBP-4	Savage DB, Sewter CP, Klenk ES et al, 2001; Kang X, Zhong W, Liu J et al, 2009; Wei X, Shi X, Zhong W et al, 2013.
	↓ in adipose tissue mass and adipocyte size	Rachdaoui N, Sebastian BM, Nagy LE, 2003; Zhong W, Zhao Y, Tang Yet al, 2012.
	↑ in triglyceride degradation	Rachdaoui N, Sebastian BM, Nagy LE, 2003.
	Insulin resistance	Wei X, Shi X, Zhong W et al, 2013.
	↑ in adiponectin, leptin, resistin, grehlin, TNF-α, IL-6	Adamczak M, Wiecek A, 2013; Cusi K, 2010; Morash B, Li A, Murphy PR et al, 1999; Baker SS, Baker RD, Liu W et al, 2010.
	↓ in acylation stimulating protein	Zhou SL, Gordon RE, Bradbury M, 1998.
	↓ in fat mass and ↑ in hepatic fat levels	Shield KD, Parry C, Rehm J, 2013; Addolorato G, Capristo E, Marini M, et al, 2000.

Human	↑ in VAT and ↓ in SAT	Janssens V, Goris J, 2001; Westerterp-Plantenga MS, Verwegen CR, 1999; Santolaria F, Perez-Cejas A, Aleman MR, et al, 2003.
	Glucose intolerance	Molenaar EA, Massaro JM, Jacques PF, et al, 2009; Calissendorff J, Brismar K, R€ojdmark S, 2004.

Adopted from: Venkata Harini Kema, Nishank Reddy, Imran Khan and Palash Mandal (2015). "Effect of alcohol on adipose tissue: A review on ethanol mediated adipose tissue injury", Adipocyte journal, 225-231.

Figure 2.2: Alcohol consumption causes dysregulated adipokine secretion and macrophage infiltration into adipose tissue resulting in liver steatosis and inflammation.

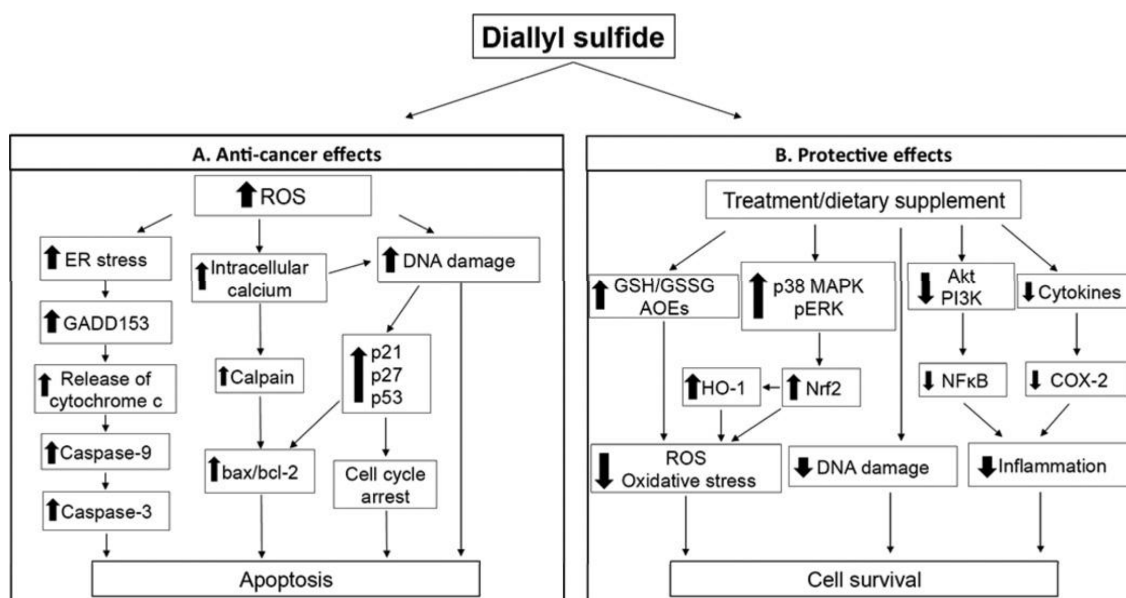


Adopted from: Venkata Harini Kema, Nishank Reddy, Imran Khan and Palash Mandal (2015). "Effect of alcohol on adipose tissue: A review on ethanol mediated adipose tissue injury", Adipocyte journal, 225-231.

2.7 Diallyl Sulfide as a therapeutic agent

Diallyl sulfide is a chief organosulfur condtituent that is produced from the metabolism of allicin present in garlic (Iciek M, Kwiecien I et al 2009). DAS has been extensively studied as an anti-cancer agent and a protective agent.

Figure 2.3: Anti-cancer and protective effects of diallyl sulfide



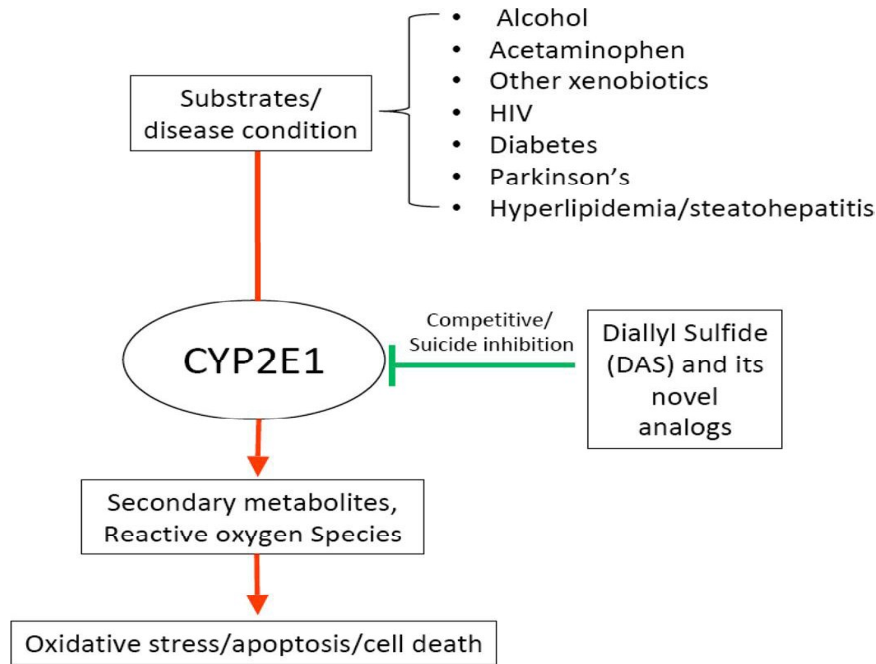
Adopted from: Rao PSS, Midde NM, Miller DD, Chauhan S, Kumar A, and Kumar S (2015) Diallyl sulfide: Potential use in novel therapeutic interventions in alcohol, drugs, and disease mediated cellular toxicity by targeting Cytochrome P4502E1. Curr Drug metab 16(6):486-503.

Anti-cancer effects of DAS are mediated by increased ROS production that results in increased ER stress and DNA damage leading to apoptosis of cells. Protective effects of DAS, on the other hand, are due to decrease in oxidative stress, pro-inflammatory cytokine production, and increase in the anti-inflammatory cytokine production leading to survival of cells.

Because of its inhibitory action on CYP2E1, DAS has attracted interest as a therapeutic agent against ethanol induced liver diseases (Rao PSS, Narasimha M Midde et al 2015). Chronic ethanol consumption activates CYP2E1 mediated ethanol metabolism pathway that generates ROS and acetaldehyde which significantly contribute to clinical and pathological spectrum of ethanol associated tissue injury (Lieber CS 1997). Studies on ethanol exposed and DAS treated hepatic cells (VL-17A) and extra-hepatic cells (U937 monocytic and SVGA astrocytic

cells) have shown a reduction in oxidative stress and cellular toxicity mediated by CYP2E1 pathway of ethanol metabolism (Swaminathan K, Clemens DL et al 2013; Jin M, Ande A et al 2013).

Figure 2.4: Diallyl sulfide as CYP2E1 inhibitor



Adopted from: Rao PSS, Midde NM, Miller DD, Chauhan S, Kumar A, and Kumar S (2015) Diallyl sulfide: Potential use in novel therapeutic interventions in alcohol, drugs, and disease mediated cellular toxicity by targeting Cytochrome P4502E1. Curr Drug metab 16(6):486-503.

2.8 Ongoing Clinical Trials for Alcoholic Liver Disease

Limited success rates have been observed for ALD treatments when drinking continues. Abstinence from alcohol or a significant reduction in alcohol consumption improves patient's survival in any stage of ALD. Currently approved medications for ALD such as disulfiram or naltrexone both increases the hepatotoxicity risk and thereby affect liver function. Therefore

it becomes essential to develop a pharmacotherapy that effectively treats ALD without causing hepatotoxicity.

Study Title	Status	Interventions	Location
Herbal supplements for improvement of liver function in participants with alcoholic liver disease	Clinical trial	Drug – Livitol-70 (3 whole herbs and extracts which has anti-oxidant, hepatoprotective and renoprotective properties)	Mazumdar Shaw Medical Centre, Bangalore, Karnataka, India
A research study to assess the safety, pharmacokinetics and pharmacodynamics of DUR-928 in patients with alcoholic hepatitis	Phase II	DUR 928 (an experimental medication)	DURECT Study Site, United States of America
G-CSF plus NAC in severe alcoholic hepatitis	Phase IV	Drug – Standard medical therapy (Pentoxifylline) Drug – Pentoxifylline + G-CSF Drug – Pentoxifylline + G-CSF + NAC	PGIMER, Chandigarh, India
Pegfilgrastim in patients with alcoholic hepatitis	Phase II	Drug – Standard of care treatment (Prednisolone/Pentoxifylline) + Pegfiltrastim Drug - Standard of care treatment	VA Loma Linda Healthcare System, California, United States of America

		(Prednisolone/Pentoxifylline)	
G-CSF in alcoholic hepatitis	Phase IV	G-CSF	Post Graduate Institute of Medical Education and Research, Chandigarh, India
Comparison of Bovine colostrum versus placebo in treatment of severe alcoholic hepatitis: A randomized double blind controlled trial	Phase III	Drug – Bovine colostrums Drug – Placebo (Pasteurized milk powder)	Dayanand Medical College and Hospital, Ludhiana, Punjab, India

2.9 Gaps in existing research

Studies show that adipose tissue plays a major role in alcohol induced liver disease progression. These effects of ethanol on the adipose tissue innate immunity and metabolic activities lead to ethanol induced tissue injury (Pravdova E, Fickova M 2006) and they also influence behaviours that regulate alcohol consumption (Wurst FM, Rasmussen DD et al 2007; Hillemacher T, Weinland C et al 2009). The above review of literature shows that though much progress has been made in understanding the role of adipose tissue in alcohol related tissue injury, the exact underlying molecular mechanisms linking adipose tissue damage and liver disease progression due to alcohol consumption still needs to be elucidated. As studies show that adipokines play a key role in alcoholic liver disease progression, effective drug leads targeting adipokine signaling pathways need to be developed and tested for amelioration of ethanol induced liver diseases.

A careful introspection reveals that there is a compelling need to look for more effective strategies like

- Developing FDA approved therapies: Although the disease (ALD) progression is well characterized, no food and drug administration (FDA)-approved therapies are currently available to halt or reverse this process in humans.
- Understanding and establishing a correlation between ethanol effect on adipose tissue and ALD progression.
- Developing therapies that specifically target adipose tissue pathways that are altered on ethanol consumption.

2.10 Scope and objective of the work

In an attempt to understand the pathogenesis of ethanol on adipose tissue and study the use of diallyl sulfide (DAS), a CYP2E1 inhibitor, in regulating the effect of ethanol induced adipose tissue injury the following objectives were proposed for the study.

Understanding the role of DAS in regulating:

- The levels of ethanol induced pro-inflammatory and anti-inflammatory markers.
- Ethanol induced oxidative stress.
- Ethanol induced ER stress.
- Ethanol induced macrophage polarization.
- Ethanol induced effects on lipid accumulation, adipocyte morphology and adipose tissue mass and lipogenic gene expression.

In order to achieve these objectives, 3T3L1, a mouse cell line used in biological research on adipose tissue, male Wistar rats, RAW264.7 macrophage cell line and human primary adipocytes were used in the study.

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Chapter-3

MATERIALS AND METHODS

3.1 Chemicals

All chemicals were purchased from Himedia Laboratories, Hyderabad, India unless mentioned otherwise. DAS, DCFDA, isobutylmethylxanthine, Tri reagent were purchased from Sigma-Aldrich, India. FBS, FCS were purchased from Invitrogen, India. cDNA synthesis kit, SYBR green master mix kit were obtained from Stratagene, USA. Primer sequences were commercially synthesized by Sigma-Aldrich, India. All chemicals used were of molecular biology grade and of the highest purity.

3.2 Culturing of 3T3L1 cell line: 3T3L1 cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India and cultured as per the procedure described by Suresh Ambati, Lise Madsen et al (Ambati S, Yang JY et al 2009; Madsen L, Petersen RK et al 2003). In brief, 3T3L1 cells were grown until confluent in Dulbecco's modified Eagle's medium (DMEM) medium containing 10% fetal bovine serum (FBS) and 1% antibiotic - antimycotic solution (10,000 U Penicillin, 10 mg Streptomycin, and 25 μg Amphotericin B per ml in 0.9% normal saline) at 37 ^{0}C in 5% CO_2 atmosphere. Post-confluency (Day 0), the cells were differentiated with DMEM medium containing 10% FBS, 0.5 μM isobutylmethylxanthine, 1 μg/ml insulin and 1 μM dexamethasone for 2 days. The cells were then cultured for the next 2 days in DMEM medium containing 10% FBS and 1 μg/ml insulin. For the next 4 days, the cells were cultured only in DMEM medium supplemented with 10% FBS. Medium was replaced after every 48 hours and more than 90% of the cells differentiated into mature adipocytes characterized by the accumulation of lipid droplets. The mature adipocytes were used for further experiments with ethanol and diallyl sulphide.

3.3 Culturing of RAW264.7 cell line: RAW264.7 cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India and cultured as per the procedure described by

Philip Sawle, Foresti R et al (Sawle P, Foresti R et al 2005). In brief, cells were cultured in DMEM medium supplemented with 10% FBS, 2mM L-glutamine, and 1% antibiotic – antimycotic solution at 37 ^{0}C in 5% CO_2 atmosphere till 80-90% confluency after which they were treated with ethanol and DAS.

3.4 Procurement of human adipose tissue and isolation and culturing of human primary adipocytes: Human adipose tissue samples were collected during inguinal and incisional hernia surgery and processed as described by Majka et al (Majka SM, Miller HL et al 2014). In brief, the isolated adipose tissue was washed with PBS, and minced to produce approximately 1mm fragments. The fragments were then digested with collagenase I. The suspension was incubated for 1 hour at 37 ^{0}C at 100 rpm. Following tissue digestion, the suspension was passed through a 150 µM mesh filter followed by an equal volume of Hanks balanced salt solution. The resulting suspension was centrifuged at 150Xg for 8 minutes. Adipocytes will form a layer at the top of the liquid. The adipocytes were transferred to a 15 ml centrifuge tube and 3-4 volumes of wash buffer were added and the sample was centrifuged at 150Xg for 8 minutes. The adipocyte layer was transferred into a fresh tube. The cells were counted using 0.4% trypan blue solution and cultured on coverslips (NUNC cell culture coverslip) in a 12 well plate for further experiments using DMEM supplemented with 10% fetal calf serum (FCS) and 1% antibiotic-antimycotic solution till confluency. All permissions for working with human adipose tissue protocols were obtained from Owaisi Hospital, Hyderabad, India.

3.5 Cell viability assay: Viability of cells was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay using method described by Terry L Riss et al (Riss TL, Moravec RA et al 2013). In brief, $1X10^4$ cells were seeded in a 96 well plate. Post-confluency and differentiation (3T3L1 cells), cells were exposed to 100 mM ethanol and treated with 50 µM, 100 µM, 200 µM, 300 µM, and 500 µM DAS for 24 and 48 hours.

Control cells were also treated with same DAS concentrations for 24 and 48 hours. After treatment for desired period of exposure, 10 µl of MTT solution at a final concentration of 0.45 mg/ml was added to each well and the plate was incubated in dark at 37 ^{o}C in 5% CO_2 atmosphere for 3 hours. Following incubation, 100 µl of dimethyl sulfoxide (DMSO) (solubilisation solution) was added to each well to dissolve the formazan crystals. The absorbance of the extracted sample was measured at 570 nM in order to determine the percentage of viable cells.

3.6 Oil red O staining of cells and isopropanol extraction of oil red O stain from cells: In order to determine the extent of lipid accumulation in both treated and untreated cells, the cells were first fixed in 10% neutral buffered formalin for 1 hour, followed by washing with water, and staining with oil red O solution for 15 mins. After staining, the cells were extensively rinsed with water. The excess water was evaporated from the stained cells and 1 ml of isopropyl alcohol was added to the culture dish for extracting oil red O from the stained cells. The extracted dye was gently removed and its absorbance was spectrophotometrically measured at 510 nm (Kuri-Harcuch W & Green H 1978; Ramirez-Zacarias JL, Castro-Munozledo et al 1992).

3.7 ROS estimation: Carboxy-H_2-DCFDA (2',7'-dichlorofluorescin diacetate) at a final concentration of 30 µM was added to both treated and untreated cells for measuring ROS production. The cells were then incubated in the culture chamber for 1 hour after which they were harvested by trypsinization. Cells were washed with phosphate buffered saline (PBS), and resuspended in PBS. The cells were then analyzed for ROS production using Flow sight (amnis) using filter with excitation wavelength of 505-560 nm. Before collecting the data, gating was done to remove cell debris and apoptotic cells (Karbowski M, Kurono C et al 1999).

3.8 qRT-PCR: Total RNA was isolated from the cells using Tri reagent extraction method. 1 µg of total RNA was used to synthesize cDNA using affinityscript qPCR cDNA synthesis kit according to the manufacturer's protocol. RT-PCR using brilliant III ultra-fast SYBR green qPCR master mix in Agilent Mx3005P qPCR system was used to study the mRNA expression levels of all genes under investigation. qRT-PCR cycling conditions were 1 cycle of initial denaturation at 95^0C for 10 minutes followed by 40 cycles of denaturation at 95^0C for 30 seconds, annealing at 60 ^{0}C for 1 minute and extension at 72 ^{0}C for 1 minute. The list of primer sets used is tabulated (Tables- 3.1, 3.2, and 3.3). $2^{-\Delta\Delta Ct}$ method was used to calculate gene expression levels in both treated and untreated cells. 18s gene was used as endogenous control.

Table 3.1 List of primers used for qRT-PCR experiments for mouse genes

S.No	Name of the gene	Forward primer sequence	Reverse primer sequence
1	18S	ACGGAAGGGCACCACCAGGA	CACCACCACCCACGGAATCG
2	Leptin	TCTCCGAGACCTCCTCCATCT	CATCCAGGCTCTCTGGCTTCT
3	Resistin	TGAGATGATTCAGTGGGTAAAGATG	TCCACCATGTAGTTTCCAGGAA
4	NADPH Oxidase	CGCTCCCAGCAGAAGGTCGTGATTACCA AGG	GGAGTGACCCCAATCCCTGCCCC AACCA
5	iNOS	CCCTTCCGAAGTTTCTGGCAGCAGC	GGCTGTCAGAGAGCCTCGTGGCT TTGG
6	TNF-α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
7	IL-10	ACTGCTAACCGACTCCTTA	TAAGGAGTCGGTTAGCAGT
8	HO-1	AAGCCGAGAATGCTGAGTTCA	CGGGTGTAGATATGGTACAAGGA
9	IL-6	GACAACTTTGGCATTGTGG	ATGCAGGGATGATGTTCTG
10	Arginase I	GCTGTCTTCCCAAGAGTTGGG	ATGGAAGAGACCTTCAGCTAC
11	Nos2	TGGAGCCAAGGCCAAACACAG	TCCACCAGGAGATGTTGAAC
12	IL 12 β	GAAAGACCCTGACCATCACT	CCTTCTCTGCAGACAGAGAC
13	Mgl2	GATAACTGGCATGGACATATG	TTTCTAATCACCATAACACATTC
14	Mrc1	GCAAATGGAGCCGTCTGTGC	CTCGTGGATCTCCGTGACAC
15	HSL	GCTGGGCTGTCAAGCACTGT	GTAACTGGGTAGGCTGCCAT
16	ATGL	TGTGGCCTCATTCCTCCTAC	TCGTGGATGTTGGTGGAGCT
17	PPAR-γ	GCCCTTTGGTGACTTTATGGA	GCAGCAGGTTGTCTTGGATG

Table 3.2: List of primers used for qRT-PCR experiments for rat genes

S.No	Name of the gene	Forward primer sequence	Reverse primer sequence
1	18S	ACGGACCAGAGCGAAAGCAT	TGTCAATCCTGTCCGTGTCC
2	Leptin	CCTGTGGCTTTGGTCCTATCTG	AGGCAAGCTGGTGAGGATCT
3	Resistin	ACTTCAGCTCCCTACTGCCA	GCTCAGTTCTCAATCAACCGTCC
4	CHOP	GAAAGCAGAAACCGGTCCAAT	GGATGAGATATAGGTGCCCCC
5	Grp78	GAAACTGCCGAGGCGTAT	ATGTTCTTCTCTCCCTCTCTCTTA
6	NADPH Oxidase	GGCATCCCTTTACTCTGACCT	TGCTGCTCGAATATGAATGG
7	iNOS	ACAACAGGAACCTACCAGCTCA	GATGTTGTAGCGCTGTGTGTCA
8	TLR4	GATTGCTCAGACATGGCAGTTTC	CACTCGAGGTAGGTGTTTCTGCTAA
9	TNF-α	CAAGGAGGAGAAGTTCCCAA	CTCTGCTTGGTGGTTTGCTA
10	Col1α1	ATCAGCCCAAACCCCAAGGAGA	CGCAGGAAGGTCAGCTGGATAG

Table 3.3: List of primers used for qRT-PCR experiments for human genes

S.No	Name of the gene	Forward Primer Sequence	Reverse Primer Sequence
1	18S	GATGGTAGTCGCCGTGCC	GCCTGCTGCCTTCCTTGG
2	Adipose triglyceride lipase	ACCAGCATCCAGTTCAACCT	ATCCCTGCTTGCACATCTCT
3	Hormone sensitive lipase	GTGCAAAGACGGAGGACCACTCCA	GACGTCTCGGAGTTTCCCCTCAG
4	PPAR-γ	GGCTTCATGACAAGGGAGTTTC	AACTCAAACTTGGGCTCCATAAAG

3.9 Animal care and chronic ethanol feeding of rats: Adult male Wistar rats weighing 150-160g were used in the study. All permissions for procedures involving animals were approved by the Institutional animal ethical committee at BITS Pilani, Hyderabad campus. Chronic ethanol feeding protocol used in the study has been described previously by Nadia Rachdaoui et al (Rachdaoui N, Sebastian BM et al 2003). In brief, rats were acclimatized with free access to chow food and water for 3 days after arrival. Rats were then allowed free access to Lieber-DeCarli high fat complete liquid diet without alcohol for 2 days following which the rats were randomly assigned into pair-fed (n=6), ethanol-fed (n=6), and ethanol plus DAS fed groups (n=6). Ethanol- fed and ethanol plus DAS fed rats were allowed free access to liquid diet with 17% calories from ethanol for 2 days followed by a diet containing 35% of calories from ethanol. Ethanol plus DAS fed group rats were given 200mg/Kg body weight of DAS through diet during the 4th week of feeding protocol. Control rats were pair-fed with liquid diet that is isocalorically substituted with maltose dextrins instead of ethanol during the entire feeding period. Body weights of the three groups of rats are tabulated in Table- 3.4. All the groups showed an increase in weights during the feeding protocol.

Table -3.4: Body and adipose tissue weights of rats

	Control (pair-fed)	Ethanol fed	Ethanol + DAS treated
Initial body weight (g)	153± 3	158 ± 2	155 ± 2
Final body weight (g)	216 ± 4	222 ± 5	218 ± 4
Weight of adipose tissue (g)	1.64 ± 0.15	0.68 ± 0.02	1.21 ± 0.05

After the feeding period, over-night fasted rats were anaesthetized, visceral and epidydymal adipose tissue samples were removed, preserved in RNA later and stored at -20°C for isolating RNA. Adipose tissue was also flash frozen in liquid nitrogen and stored at -80°C until further analysis. Blood was collected; serum was isolated and stored at -80°C.

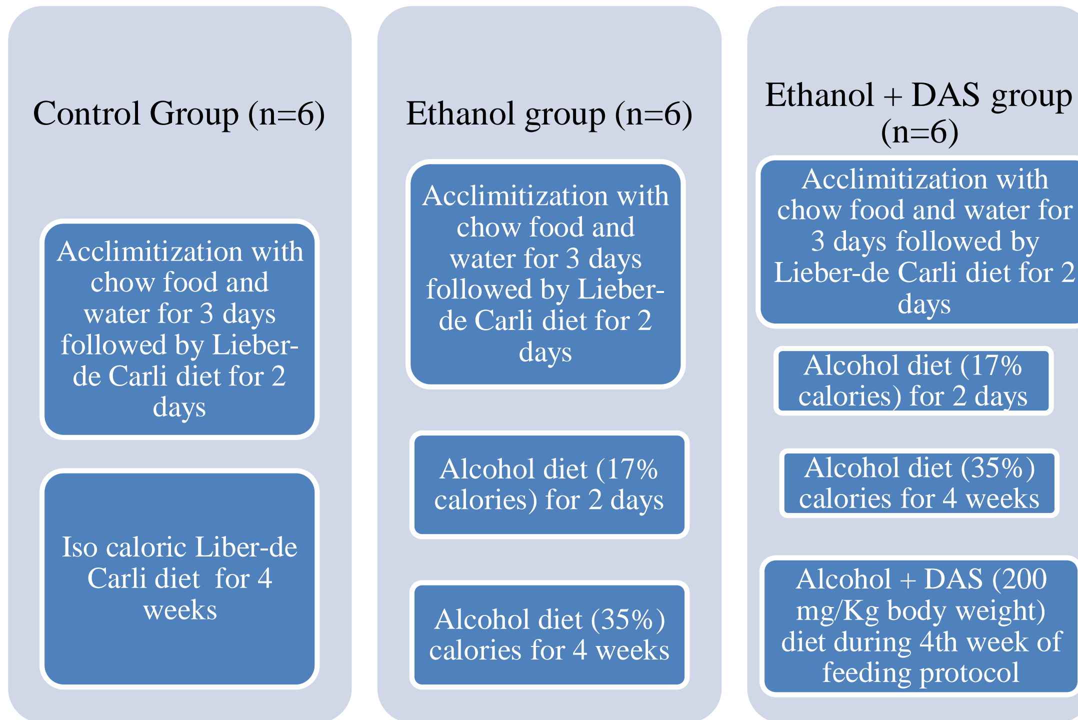

Figure 3.1: Schematic for animal dosing

3.10 H&E staining: Adipose tissue was embedded in optimal cutting temperature (OCT) compound. 7-10 µM sections were cut from the frozen adipose tissue sample using Leica cryostat. The sections were then stained with Harris hematoxylin solution followed by counterstaining with eosin for identifying changes in adipocyte morphology between the differentially treated rat groups.

3.11 Serum analysis: Serum adiponectin, leptin, and TNF-α concentrations were measured using ELISA kits according to the manufacturer's instructions. Rat adiponectin and TNF-α ELISA kits are from Invitrogen while rat leptin ELISA kit was from Sigma-Aldrich. All experiments were performed in duplicate and the concentration of the protein in the serum

was determined from the standard curve constructed using the standards provided in the ELISA kit.

3.12 MDA estimation in serum by HPLC: Total MDA in serum was estimated by method described by Nurten Tukozkan et al (Tukozkan N, Erdamar H, Seven I 2006). In brief, serum sample was hydrolyzed under alkaline conditions, acidified, and derivatized with dinitrophenylhydrazine (DNPH) solution. The derivatized serum sample was then analyzed by Shimadzu HPLC apparatus (Model LC-20AD, Prominence Liquid Chromatograph, Shimadzu Corporation, Kyoto, Japan) using end capped octadecyl-silica 2 (ODS 2) C18 reverse phase analytical column (Spherisorb ODS2-C18, 150mm long, 4.6 mm internal diameter and 5 µM particle size, Phenomenex, Torrance, CA). Acetonitrile-distilled water (38:62) containing 0.2% acetic acid was used as the mobile phase. Isocratic elution of the sample was done using 1 ml/min flow rate. MDA was detected using UV detector set at 310 nm. MDA peak was determined based on the retention time and the concentration of MDA in the serum was determined from the standard curve obtained from MDA derived from 1,1,3,3 tetraethoxypropane.

3.13 Adipose tissue homogenization: Adipose tissue was homogenized using 3 ml/g of homogenate buffer containing 1% Triton-X100, 50mM Tris-HCl, 6.4mM NaCl, 1mM EDTA, 1mM sodium pyrophosphate, 1mM activated sodium vanadate, 10 mM NaF and protease inhibitor cocktail (Sigma-Aldrich) in a Potter-elvehjem tissue homogenizer. Following centrifugation of the homogenate at 15,000g for 5 min, infranatant was removed and used for ELISA and Western blotting experiments.

3.14 MDA estimation in rat adipose tissue by HPLC: 500 mg of the tissue was homogenized in 1.15% KCl using Potter-elvehjem tissue homogenizer. To 1 ml of the homogenate, 200 µL of 6 M NaOH was added and the sample was incubated for 45 min at 60 ^{0}C. Proteins in the sample were then precipitated out by diluting the sample with equal

volumes of acetonitrile. The resulting sample was vortexed for 30 s and centrifuged for 10 min at 15000g. 0.25 ml of the supernatant was then derivatized for 10 min at room temperature with 25 μL DNPH solution (Tukozkan N, Erdamar H, Seven I 2006). The samples were then analyzed by HPLC as mentioned above. Protein levels in the tissue were measured by Lowry method (Lowry OH, Rosebrough NJ 1951) and the MDA data obtained were then normalized to the protein concentration of each sample.

3.15 Western blotting: 10-40μg of the total protein sample was separated on a 12% SDS-PAGE gel. The sample was then transferred to a polyvinylidene difluoride membrane using a semidry transfer technique. Tris-buffered saline (pH 7.6) with 3% BSA and 0.1% Tween-20 was used to block the blots. The blots were then incubated overnight with primary antibody. Dilutions of primary antibodies used were: 1:1000 for ATGL, 1:2000 for p-HSL, 1:5000 for HSL, 1:1000 for PPAR-γ and 1:10,000 for β-Actin. The blots were then incubated with secondary antibody for 1 h and after subsequent washes; the bound antibodies were visualized using chemiluminescence reagent. Signal intensities of the sample were quantified by densitometry using Image J software.

3.16 MDA estimation in human adipocytes by TBA method: Cells were homogenized in 1.15% (w/v) cold potassium chloride (KCl) solution and total MDA in the cell homogenate was estimated by method reported by Hiroshi Ohkawa et al (Ohkawa H, Ohishi N, Yagi K 1979). Flourescence of the resulting supernatant was quantified using SpectraMax M3 microplate reader (Molecular devices, California, USA) at an excitation wavelength of 530 nm and an emission wavelength of 550 nm. Protein levels in the cell homogenate were measured by Lowry method (Lowry OH, Rosebrough NJ 1951).

3.17 Measurement of lipolysis in human adipocytes: Lipolysis was measured as glycerol released per amount of protein present in the samples (mM/mg). The amount of glycerol and free fatty acids released in to the medium were quantified using human adipocyte lipolysis

assay kit (ZenBio, USA) according to the manufacturer's protocol. Following measurements, the data obtained were then normalized to the protein content of each sample.

3.18 Statistical analysis: All experiments were repeated at least thrice. Data are presented as mean ± SEM. Results were analyzed using GraphPad Prism 6 software. Differences between groups were analyzed using one-way analysis of variance with Bonferroni's multiple comparisons test. A p-value less than 0.05 was considered as significant.

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Studying the Role of Diallyl Sulfide in Ameliorating Ethanol Induced Adipose Tissue Injury

THESIS

Submitted in partial fulfillment of
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By

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Chapter-6

SUMMARY AND CONCLUSIONS

6.1 Summary

Chronic consumption of alcohol causes ALD which is the fourth leading cause for death and disability worldwide. The disease progression involves following stages – steatosis, steatohepatitis, fibrosis, and cirrhosis. Adipose tissue apart from functioning as an energy storage organ also secretes several cytokines known as adipokines that are known to play a regulatory role in ALD progression. Although the mechanisms involved in ALD progression are well understood, till date no FDA approved therapies that can effectively cure ALD are available. Hence, the present study was designed to understand the effect of DAS in ameliorating ethanol induced damage of adipose tissue and in restoring the ethanol induced alteration in cytokine release from adipose tissue.

The main objective of the present work was to understand the action of DAS in regulating ethanol induced adipose tissue injury. The objectives of the present work were broadly divided into the following aspects:

- Understanding the effect of DAS in regulating ethanol induced oxidative stress.
- Understanding the effect of DAS in regulating ethanol induced ER stress.
- Understanding the effect of DAS in restoring changes in adipose tissue morphology caused by ethanol.
- Understanding the role of DAS in macrophage polarization.
- Understanding the role of DAS in regulating the levels of cytokines altered due to ethanol consumption.
- Understanding the role of DAS in regulating the levels of lipogenic enzymes

i) Understanding the effect of DAS in regulating ethanol induced oxidative stress:

Differentiated 3T3L1 cells showed a decrease in ROS production when exposed to ethanol and treated with DAS compared to only ethanol exposed cells in a concentration dependent manner. Ethanol exposed isolated human adipocytes showed a reduction in MDA levels when treated with DAS as estimated by TBARS method. The present study showed a reduction in the serum and tissue MDA levels of rats treated with DAS when compared to ethanol fed rats.

The above results showed that DAS ameliorates ethanol induced oxidative stress that was evident by the reduced levels of oxidative stress markers like MDA and ROS in ethanol exposed and DAS treated cells and rats compared to only ethanol exposed cells and rats.

ii) Understanding the effect of DAS in regulating ethanol induced ER stress:

The present study showed that treatment of rats with DAS decreased ER stress proteins like Grp78, and CHOP. The results obtained thus prove that DAS can reduce ethanol induced ER stress damage.

iii) Understanding the effect of DAS in restoring changes in adipose tissue morphology caused by ethanol:

The present study showed that ethanol treatment of male Wistar rats resulted in decrease in adipose tissue mass and change in adipocyte morphology that was improved by feeding rats with diet containing DAS.

Exposure of differentiated 3T3L1 cells and isolated human primary adipocytes with ethanol reduced lipid accumulation in the cells which was improved by DAS addition. The amount of lipid accumulation was found to be greater in cells treated with 100 µM DAS for 24 hours when compared to cells treated with 50 µM DAS for 24 hours. A change in human adipocyte morphology was also observed between ethanol and DAS treated cells.

The results suggested that DAS restored adipocyte morphology that was altered due to ethanol exposure.

iv) Understanding the role of DAS in macrophage polarization

mRNA expression studies showed that DAS promoted up-regulation of macrophage anti-inflammatory genes like arginase-1, IL-10, Mrc-1, Mgl2 that are characteristic of M2 phenotype while expression of pro-inflammatory gene markers like IL-6, NOX, iNOS, TNF-alpha, IL-12β, Nos2 that are characteristic of M1 phenotype were found to be down regulated in ethanol exposed RAW 264.7 cells. The study showed that DAS promotes macrophage polarization to M2 phenotype from M1 phenotype when the cells were exposed to ethanol and treated with DAS thus protecting the cells from ethanol induced injury.

v) Understanding the role of DAS in regulating the levels of cytokines altered due to ethanol consumption:

The present study showed that feeding male Wistar rats with DAS containing diet reduced the levels of pro-inflammatory genes like TNF-α, leptin, resistin, NOX, iNOS in VAT and EAT of rats. Treatment with DAS was found to reduce the levels of TLR4 expression in VAT and EAT of rats.

Expression levels of extracellular matrix protein Col1α was also found to be reduced in rats fed with DAS containing alcohol diet when compared with only alcohol fed rats.

The levels of inflammatory proteins leptin and TNF-α in the serum and adipose tissue were also found to be reduced in DAS treated rat group when compared to ethanol fed rat group while the levels of anti-inflammatory protein adiponectin was found to be increased in the DAS fed rat group.

Differentiated 3T3L1 cells when exposed to ethanol and treated with DAS also showed a decrease in the expression of leptin, resistin, and TNF-α genes and an up-regulation in the

expression of anti-inflammatory genes like HO-1 and IL-10 when compared with only ethanol exposed cells.

vi) Understanding the role of DAS in regulating lipogenic genes:

The present study showed that DAS can reduce ethanol induced levels of lipolytic genes like ATGL and HSL and up regulate the levels of lipid synthesis genes like PPARγ.

6.2 Conclusion

Considering a strong role of adipose tissue in ethanol induced tissue injury, it is critical to test and understand the action of therapeutic agents in ameliorating adipose tissue damage caused due to alcohol consumption. Till date, studies have focused primarily in understanding the impact caused by ethanol on adipose tissue mass, morphology, and immune response. Impairment of anti-inflammatory response and elicitation of pro-inflammatory response is observed in adipose tissue on chronic ethanol consumption. So our research work hypothesized that DAS, a CYP2E1 inhibitor, is capable of improving damage caused by ethanol.

In conclusion the following observations are made about the results obtained in this study.

1. Cell viability was found to be higher at 100 µM concentration of DAS treatment at 24 hours. Viability of cells decreased with time and dose dependent manner of DAS.
2. Ethanol induces oxidative and ER stress that can be reduced by treatment with DAS.
3. Ethanol consumption reduces the production of anti-inflammatory cytokines and enhances the expression of pro-inflammatory cytokines. The condition can be altered by DAS treatment. DAS up-regulated anti-inflammatory gene expression while it down regulated pro-inflammatory gene expression in ethanol exposed cells.
4. Ethanol consumption results in macrophage switching to M1 polarization phenotype resulting in inflammation. DAS treatment changed the macrophages to M2 phenotype

that resulted in the up-regulation of anti-inflammatory gene expression thus protecting the cells from ethanol induced injury.

5. Reduction in adipose tissue mass and change in adipocyte morphology is observed due to alcohol consumption. The amount of triglyceride accumulation in the adipocytes is also found to be decreased due to alcohol treatment. DAS improved adipocyte morphology and enhanced triglyceride accumulation in the adipocytes. Increase in adipose tissue mass was also observed due to DAS treatment.
6. Ethanol consumption reduces the levels of lipid synthesis genes and increases the levels of lipolytic genes. DAS altered this condition and enhanced lipid accumulation in the adipose tissue as was evident by increased adipose tissue mass and oil red O staining.

The results thus prove that DAS can reduce the adipose tissue injury caused by ethanol consumption.

6.3 Major contributions of the work

The present study helps in understanding the role of DAS in reducing the injury caused to adipose tissue on ethanol consumption. The study results infer that DAS can be used in drug formulations to treat ethanol induced tissue injury.

6.4 Future scope of work

The future possibilities arising from the results of the present work are as given below:

- To test effectiveness of DAS with other anti-inflammatory and anti-oxidant agents in in vitro and in in vivo models in minimizing ethanol induced tissue injury.
- To have a better understanding of DAS action in alcohol induced fibrotic animal models.
- The effects of ethanol exposure and DAS treatment at different protein levels require additional investigation.

- To increase the stability of DAS by synthesizing DAS carriers and study the effect of the same in both in vitro and in vivo systems.
- To further extend the study of DAS action in humans with alcohol induced steatohepatitis.