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Role of ethanol as a cofactor in HAART induced hepatic steatosis and injury.

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ROLE OF ETHANOL AS A COFACTOR IN HAART INDUCED
HEPATIC STEATOSIS AND INJURY

By

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ABSTRACT

ROLE OF ETHANOL AS A COFACTOR IN HAART INDUCED
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Highly Active Antiretroviral Therapy (HAART) has led to a significant increase in the life expectancy of HIV patients; however, there are significant side effects including lipodystrophy and hepatotoxicity. Alcohol abuse is highly prevalent in HIV infected individuals and hence may be a significant negative cofactor in HAART induced hepatotoxicity. The present study examines the mechanisms underlying HAART and alcohol induced hepatotoxicity. The effects of HAART drugs (azidothymidine, and Indinavir sulphate) in combination with alcohol were examined in *in vivo* animal model.

Alcohol and HAART drug interactions and hepatotoxicity were also assessed *in-vivo* using an animal model of chronic alcohol feeding. Mice were pair-fed liquid diets (Lieber DeCarli) containing 35% of calories as alcohol (alcohol-fed, AF) or as isocaloric maltose-dextrin (pair-fed, PF) for four weeks. In

addition, HAART treatment groups received AZT (30mg/kg BW) and IDV (50mg/kg BW) by oral gavage for 2 weeks. Animals exposed to both alcohol and HAART developed increased visceral adiposity compared to pair-fed animal suggesting disturbances in lipid metabolism in these mice. Lipodystrophy was also evidenced by macro and microvesicular steatosis in the livers; elevated liver triglycerides and free fatty acids. Additionally, animals receiving combinations of alcohol and HAART exhibited increased inflammation and greater hepatic neutrophil infiltration.

Overall, our data demonstrate that alcohol exacerbates HAART hepatotoxicity, and is a significant cofactor in the development of hepatic steatosis and liver injury.

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INTRODUCTION

HIV/AIDS- An overview

HIV (Human Immunodeficiency Virus) infection is one of the world's leading infectious diseases, claiming more than 25 million lives over the past three decades. HIV infection is considered pandemic by the World Health Organization (WHO). Thirty four million people are affected worldwide. Center for Disease Control (CDC) estimates 1.2 million people in the United States (US) are living with HIV infection. One in five (20%) of those people are unaware of their infection. By race, African Americans face the most severe HIV burden. Men having sex with men (MSM), particularly the young, are most severely affected. HIV leads to AIDS (Acquired Immunodeficiency Syndrome). AIDS was first recognized in the United States in the summer of 1981, when the U.S. (CDC) reported the unexplained occurrence of *Pneumocystis jiroveci* (formerly *P. carinii*) pneumonia in five previously healthy homosexual men in Los Angeles [1].In 1983, human immunodeficiency virus (HIV) was isolated from a patient with lymphadenopathy, and by 1984 it was demonstrated clearly to be the causative agent of AIDS [1]. Since the epidemic began, an estimated 1,108,611 people in the US have been diagnosed with AIDS [1]

HIV & HAART

In order to prevent or delay resistance development, physicians are using a regimen which consists of a combination of several types of antiretroviral drugs that act on different sites on a retro virus and also target different stages in the lifecycle of the virus. Such treatments are called highly active antiretroviral therapy (HAART) [2] . The development of multi-drug combination therapy for treatment of HIV disease is considered one of the great success stories of modern medicine. In a period of approximately ten years, the death rate from HIV disease was reduced by 50 to 80% [3]. Highly Active Antiretroviral Therapy (HAART) as treatment for HIV infection has greatly improved mortality and morbidity for adults and children living with HIV around the world [4, 5]. HAART is the only treatment available to suppress the viral load. Currently used HAART medication include Nucleoside Reverse Transcriptase Inhibitors (NRTIs) (**Zidovudine**, Didanosine, Abacavir), Non-nucleoside Reverse Transcriptase Inhibitors (NNRT) (Nevirapine, Etravirine), Protease inhibitors (PI) (**Indinavir or IDV, Ritonavir**, **Lopinavir**, Atazanavir), Fusion inhibitors (Enfuvirtide), Integrase Inhibitors (Raltegravir), and CCR5 inhibitors all of which act on different sites on HIV facilitating a decrease in viral load. Since the introduction of highly active antiretroviral therapy (HAART) in the mid-1990s, the life expectancy of patients with HIV has increased significantly [6, 7]. Although HAART has been widely used in the treatment of HIV, HIV infected patients have experienced complications associated with HAART, which cannot be ignored. Antiretroviral toxicity, resistance, and adherence rank high on the list of problems that must be

overcome if all, or at least the vast majorities, of HIV-infected patients are to gain the long-term benefits associated with HAART [8]. Among these problems, toxicity, real or perceived, is a major reason for patients either refusing or prematurely discontinuing HAART [9].

The drop in mortality and morbidity due to opportunistic infection has been accompanied by a concomitant increase in HAART-related hepatotoxicity which caused discontinuation of HAART in increasing numbers of patients. HAART hepatotoxicity, including lactic acidosis, hepatic steatosis and lipodystrophy, occurs in approximately 10% of patients and is higher in those with underlying liver disease [10].

Nucleoside Reverse Transcriptase Inhibotors (NRTI)- Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), which inhibit the formation of viral DNA by incorporating into the newly formed DNA molecules, thereby preventing further elongation of those molecules [2].

Zidovudine (AZT) - an important drug for 1st line therapy of HAART:
Zidovudine (AZT) (3'-azido-2'3'-dideoxythymidine) an analogue of thymidine, was first discovered by Mitsuya and associates' to have antiretroviral activity. In 1986, FDA approved this drug for the treatment of advanced HIV infection. Even today it is widely being used in the resource limited settings as one of the important drug for the 1st line therapy of HAART. However, it is now accepted that prolonged use AZT leads to hepatotoxicity [11-13]. Mechanisms of hepatotoxicity

induced AZT involves hepatomegaly or steatosis [14], elevated blood lactate to pyruvate ratios, cytochrome c deficiency [11, 15-17]. Additionally, elevated liver enzymes (AST, ALT) have been reported in patients receiving AZT therapy. These hepatotoxic consequences are related only to AZT administration and are independent of AIDS disease progression. This was confirmed when patients administered with AZT therapy experienced one or more of these hepatotoxic effects but when the therapy was discontinued the adverse effects were resolved [18, 19]. These toxicities are mostly mediated through mitochondrial damage and are associated with mitochondrial DNA depletion [17, 20]. Additional work is required to identify molecular mechanisms to obtain a better understanding of HAART/AZT induced hepatotoxicity and to develop therapeutic strategies. Hence in the present study, we investigate the occurrence and mechanisms involved in Zidovudine (AZT) induced hepatotoxicity in vivo in our well-established animal model.

Protease Inhibitor (PI) - Protease inhibitors block the activity of the protease enzyme, which HIV uses to degrade large polyproteins into the smaller pieces required for assembly of new viral particles. All PIs are metabolized in the liver by CYP3A isoenzymes; consequently their metabolic rates may be altered in the presence of CYP inducers or inhibitors. Hence co-administration of PIs with ritonavir (RTV), a potent CYP3A inhibitor will optimally increase PI exposure. Co-administration of PIs with a potent CYP3A inducer may lead to suboptimal drug concentrations and reduced therapeutic effects of the PI [21]. While HIV can still

replicate in the presence of protease inhibitors, the resulting virions are immature and unable to infect new cells. PI –based regimen has seen demonstrated to have anti-virologic potency. Unlike NRTI resistance, virologic failure rarely selects for PI-resistance, which confers it with increased efficiency in decreasing the viral load.

Indivavir- A commonly used Protease inhibitor in resource limited setting:
In 1996 Indinavir was approved as one of the PIs by the FDA, which dramatically modified the clinical management of HIV-1. IDV is a synthetic peptidomimetic competitive inhibitor of the HIV aspartyl protease, which is involved in the cleavage of the gag and pol gene products into their functional components and, as a consequence, viral particles are unable to undergo final maturation into infectious virions. A number of metabolic abnormalities, including hypertriglyceridemia, hypercholesterolemia, hyperinsulinaemia and lipodystrophy increase in liver enzymes have been associated with PI use [21-23]. Thus along with the efficacious nature of PI based therapy, recent studies have revealed several adverse effects leading to discontinuation of the therapy causing the viral load to increase. Hence this demands a need to investigate the underlying molecular mechanisms that could provide potential therapeutic interventions.

HAART associated hepatotoxicity and liver injury

HAART causes a variety of adverse effects like cardiovascular diseases, neurological disorders, **Liver disease**, lipodystrophy, non-Hodgkin lymphoma to name a few. Liver disease is recognized as an increasingly important problem for the HIV population, and may be due to a variety of factors including co-infection with viral hepatitis, alcohol abuse, and antiretroviral hepatotoxicity. **Liver disease is now a leading cause of death for patients with HIV. In patients co-infected with HIV and hepatitis C, cirrhosis was the underlying cause of death in nearly 50% [9]. In a more general HIV population, liver disease was the second most common non-HIV-related cause of death, trailing only cancer [24].** In a recent American study, which evaluated the causes of death of HIV-infected individuals, discontinuation of ART due to hepatotoxicity increased from 6% in 1996 to 31.8% in 1998-1999 among those mortalities [25]. The severity of liver toxicity ranges from the absence of symptoms to liver decompensation, and the reported incidence of severe liver toxicity after initiating HAART ranges from 2% to 18%. Hepatotoxicity due to HAART is also common and up to 30% of patients on HAART experience World Health Organization grade 3 liver enzyme elevations. Hepatotoxicity induced by these agents has been identified on the basis of circulating liver enzymes (ALT, AST, and GGT) in association with increased lactate levels. In more dramatic situations, liver failure has occurred leading to discontinuation of treatment. The study of hepatotoxicity induced by HAART is complicated by the diverse chemical nature of the agents and by the differences between the effects of agents used singly or in

combination. Hepatotoxicity from antiretroviral drugs has led to adverse patient outcomes either from fulminant hepatic failure, or more commonly, AIDS following discontinuation of HAART. Hence, it is highly relevant to study the underlying mechanisms of HAART-induced hepatotoxicity.

HAAART-induced dyslipidemia and liver lipid metabolism

It has been recently found in long-term and short-term studies that on healthy as well as HIV infected patients, HAART regimen particularly protease inhibitors induces dyslipidemia [23, 26-28]. Often Clinicians supplement these drugs with lipid lowering drugs.

Since liver is the first organ to come in contact with the administered HAART, liver is the key organ in handling multiple downstream processes and their metabolites. Hepatocytes are involved in lipid homeostasis and the metabolism of drugs. Endoplasmic reticulum (ER) plays a key role in regulating hepatic lipid metabolism. The ER is a critical organelle responsible for proper protein folding, cellular calcium levels, lipid synthesis and the secretory pathway. Thus ER stress is induced via depletion of ER calcium stores, changes in ER lipid membrane composition or accumulation of misfolded protein.

Moreover, sterol regulatory element-binding proteins (SREBPs) are transcription factors that are present on ER membrane. The isoforms of SPEBP (SREBP-1c and SREBP-2) play a major role in regulating the expression of key genes such as 3-hydroxy-3-methlgutaryl-CoA (HMG-CoA) synthase, Fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC) involved in lipid metabolism. ER

stress via altering lipid metabolism could be a potential mechanism of PI-induced dyslipidemia and deregulation of hepatic lipid metabolism.

Alcohol as a cofactor in HAART (AZT and IDV) induced Hepatotoxicity

As per National Epidemiologic Survey on Alcohol and Related Conditions (NESARC) an estimated 136 million adults consume alcohol in the United States, and an estimated 14 million Americans are alcoholics[29]. Alcoholic liver disease accounts for 40% of deaths due to cirrhosis and more than 30% of hepatocellular carcinoma. Alcohol abuse and HIV are important health problems. Alcohol use is common in the HIV population. It is estimated that approximately 25% of recently diagnosed HIV patients also developed alcohol addiction, which has been associated with HIV treatment failure [30]. Studies have shown that alcohol abuse is associated with more severe hepatotoxicity in patients on HAART [31]. Importantly, alcoholic liver disease and HAART induced liver injury share many potential mechanisms of injury. These include cytokine dysregulation, mitochondrial dysfunction, and proteasomal dysfunction. Through these and other mechanisms, alcohol and antiretroviral medications converge on the liver in an overlapping fashion to produce hepatotoxicity. Alcohol abuse can cause a variety of metabolic abnormalities including induction of CYP2E1, mitochondrial dysfunction, oxidative stress, depressed hepatic proteasome function, hepatic steatosis, and in some instances more advanced liver disease with chronic abuse [32-34]. Alcohol abuse also can increase susceptibility to certain types of drug-induced liver injury (e.g., acetaminophen) and accelerate the progression of chronic liver disease such as HCV [8, 33, 35]. Additionally, Zidovudine and

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indinavir as mentioned previously has been reported to develop severe hepatic steatosis, elevated liver enzymes and hepatotoxicity. Since alcohol and HAART drugs (AZT, IDV) have similar hepatotoxic effects on liver, we propose that the combination would exacerbate these effects. Hence in this study we are studying the combinatorial effects of alcohol and HAART (AZT, IDV) on liver that would pinpoint some therapeutic interventions. Besides liver, alcohol abuse also has an adverse impact on other organ systems particularly the gut, liver and the immune system. Work done by our research group and others has shown that chronic alcohol exposure leads to intestinal oxidative stress, epithelial barrier disruption and enhancement of gut leakiness and consequent endotoxemia and systemic inflammatory response [36].

Gut-Derived Endotoxemia and Alcohol-induced liver injury:

Due to its anatomical links to the gut, the liver is constantly exposed to gut-derived bacterial products, and functions as a major filter organ and a first line of defense. Gut-derived endotoxin is a crucial mediator of liver injury in alcoholic liver disease. This is demonstrated by the significant reduction of alcoholic liver injury following elimination of the Gram-negative microbiota by antibiotics [37] and the sensitization to LPS-induced liver injury following long-term ethanol exposure [38]. The elevation of endotoxin appears to be predominantly caused by two mechanisms. First, alcohol consumption leads to changes in the intestinal microbiota; and upper gastrointestinal tract bacterial overgrowth is more than six times more frequent in people who are very heavy drinkers than in people who do not drink heavily [39, 40]. However, the effects of alcohol on the composition

of the intestinal microbiota have not been studied in detail. Second, a large body of literature has clearly documented that alcohol ingestion disrupts the intestinal epithelial barrier causing enhanced permeability [41-43] thus allowing increased amounts of LPS to enter the portal circulation. Moreover, it has been suggested that the intestinal microbiota converts ethanol into acetaldehyde, which in turn disrupts tight junctions and increases paracellular permeability [44, 45]. On the other hand, not much is known about the effects of HAART drugs per se on systemic endotoxin or Lipopolysaccharide (LPS). Most of the clinical trial studies focus on HIV-1 infection and the increase systemic endotoxin. Hence more studies are required to tease out the effects of HAART on endotoxin levels and its subsequent hepatic consequences. In order to address this issue we are interested in finding the effects of HAART on systemic endotoxin *in vivo*.

SIGNIFICANCE AND CLINICAL RELEVANCE

In the United States, approximately 2000 cases of acute liver failure occur annually and drugs account for over 50% of them (39% are due to acetaminophen, 13% are idiosyncratic reactions due to other medications). Drugs account for 2-5% of cases of patients hospitalized with jaundice and approximately 10% of all cases of acute hepatitis. HAART has been associated with increase in morbidity and mortality due to HAART-related hepatotoxicity and finally discontinuation of therapy. This is especially true in patients who are co-infected or have other cofactors such as alcohol abuse. The mechanisms for HAART hepatotoxicity in association with alcohol consumption or other cofactors have received very limited attention. There is no well-accepted therapeutic intervention except for abstinence and HAART discontinuation. Recent work indicates that the liver is an important target for alcohol-induced damage and is often associated with gut-generated signals. Findings from this project could provide important mechanisms by which alcohol/HAART-induced pathology in the gut influences the liver inflammatory responses often observed in alcohol abuse. Moreover, the findings from this study would be significant in the development of successful preventive and therapeutic strategies in most-at-risk populations. Finally, the proposed studies will focus on direct hepatotoxicity due to HAART and alcohol as a co-factor by the combinations of Zidovudine and

Indinavir (HAART drugs), defining the disease mechanisms and generate

therapeutic targets.

MATERIALS AND METHODS

Reagents

Zidovudine (AZT) and Indinavir (IDV) were purchased from Sigma-Aldrich (St. Louis, MO). Chloroacetate Esterase Staining reagents were also obtained from Sigma-Aldrich (St. Louis, MO). TRIzol® was obtained from Invitrogen (Carlsbad, CA). Triglycerides and ALT (Alanine Aminotransferase) reagents were obtained from Thermo Fisher Scientific Inc. Reagents for Free Fatty acids were obtained from Wako Chemical USA (Richmond, VA, USA); Endotoxin reagents were obtained from with Limulus Amebocyte Lysate kit (Lonza, Walkersville, MD).

Animal Model

Eight-week-old male C57BL⁄6N mice were obtained from Harlan (Indianapolis, IN). All mice were housed in a pathogen-free, temperature-controlled animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care with 12- hour light ⁄ 12 hour dark cycles. All experiments were carried out according to the criteria outlined in the Guide for Care and Use of Laboratory Animals and with approval of the University of Louisville Animal Care and Use Committee.

Animals were fed a modified Lieber–DeCarli liquid diet containing Ethanol (EtOH) (35% of total calories) and enriched in Unsaturated Fatty Acid (USF) (corn oil); Research Diet, New Brunswick, NJ. Control liquid maltose-dextrin diets provided 40% of energy from fat, 43% from carbohydrate, and 17% from protein. Initially, all mice were given the control liquid maltose dextrin diets (USF, no EtOH) ad libitum for 1 week. Afterward, mice were fed either the liquid EtOH diet or the control liquid maltose-dextrin diet. EtOH was gradually increased every 3 to 4 days from 11.2 to 35% of total calories [(5.0%(v⁄v)]. The mice were fed the Ethanol diet [5% (v⁄v)] ad libitum for 4 weeks. The control mice were pair-fed USF maltose-dextrin diets on an isocaloric basis. For HAART therapy, (AZT 30mg/kg) and (IDV 50mg/kg) were gavaged every day for last 2 weeks to animals administered alcohol or pair-fed (control) diet.

At the end of the feeding experiment, mice were anesthetized with sodium-pentobarbital (nembutal, 80 mg ⁄ kg, intraperitonially), and blood and liver were

collected for assays. The killing sequence was randomized to eliminate any time-dependent variation due to length of fasting. Blood samples were collected from the inferior vena cava using heparinized syringes and were then centrifuged at 7000·g for 7 minutes at 4°C. Whole liver were removed. Part of the liver from left lobe was harvested and fixed in 10% neutral- buffered formalin, while the remaining liver tissue was snap frozen in liquid N2 and stored at 80°C.

Hematoxin and Eosin staining

Staining was performed using Hemo-De or Citrosol. Staining was done in a Copland jar with a stir bar at the bottom of the jar. All slides should be stained with hematoxylin and eosin, respectively. Consequently the slides were kept in Hemo-De for 10mins, 100% EtOH for 10mins, 95% EtOH for 10mins, 85% EtOH for 10mins, 70% EtOH for 10mins and 30% Etoh for 10mins followed by Hematoxylin soln for 1-3mins.

Liver Histological Examination

For histological analysis, liver sections were fixed in 10% buffered formalin for 24 hours and embedded in paraffin. Tissue sections were stained with hematoxylin-eosin and examined under light microscopy at 200· magnification.

Oil Red O staining

Frozen liver sections were washed in phosphate buffered saline twice for 5 minutes. Oil-Red-O and 85% propylene glycol were added with agitation for 15 minutes, followed by washing in tap water.

Chloroacetate Esterase Stain

Neutrophil accumulation was assessed by localizing chloracetate esterase (CAE), a specific marker for neutrophils, in liver tissue by using the naphthol AS-D chloracetate esterase kit (Sigma)

ALT assay

Alanine aminotransferase (ALT) activity was measured as a marker of liver injury using commercially available reagents from Thermo Fisher Scientific Inc. (Middletown, VA).

Liver Triglycerides Assay

For liver TAG assay, hepatic tissue (100 mg) was homogenized in 50 mM NaCl. The homogenate (500 µl) was mixed with chloroform/methanol (2:1, 4 ml) and incubated overnight at room temperature with gentle shaking. Homogenates were vortexed and centrifuged for 5 min at 3000Å~g. The lower lipid phase was collected and concentrated by vacuum. The lipid pellets were dissolved in 1% Triton X100 in phosphate-buffered saline, and hepatic TAG content was determined via enzymatic colorimetric methods.

Hepatic Free Fatty Acids

Liver nonesterified-fatty acid (NEFA) were assayed using a commercially available kit fromWako Chemical USA (Richmond,VA).

Blood Endotoxin Assay

Plasma endotoxin levels were measured with Limulus Amebocyte Lysate kit (Lonza, Walkersville, MD) according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, Inc., La Jolla, CA). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to evaluate significant differences between the 4 compared groups (Pair-Fed Controls+ EtOH, HAART drugs, EtOH+HAART drugs). A p-value of <0.05 was considered statistically significant. Data were expressed as mean ± SEM.

RESULTS

HAART drugs in combination with alcohol increases visceral fat accumulation in mice in comparison with HAART or alcohol-fed groups

Lipodystrophy is one of the common features of long-term HAART therapy commonly seen in HIV-1 infected patients. This is associated with a loss in peripheral fat and an increase in visceral fat accumulation [46, 47]. However, whether alcohol consumption along with HAART drugs would further enhance HAART induced lipodystrophy still remains unclear. Hence, we visually examined the visceral fat accumulation in our experimental groups. Our data suggests that the combination group (Alcohol+HAART) had a significant amount of visceral fat accumulation in comparison with the mice gavaged HAART drugs and/or alcohol-fed groups showing an additive effect of drugs and alcohol. This could be possibly due to decrease in the amount of lipolysis in the adipocytes as well as in the hepatocytes or increased lipogenesis in mice receiving HAART and alcohol (fig. 1).
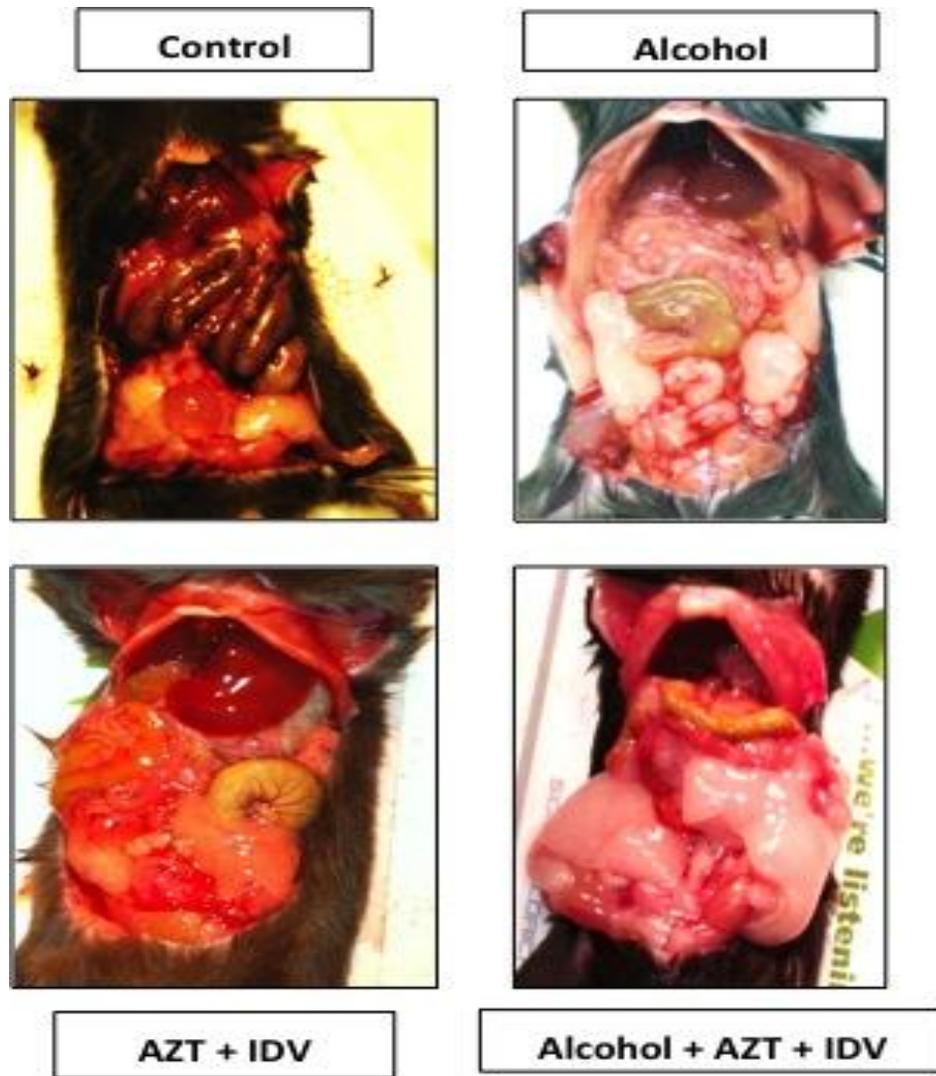
Figure 1: Administration of HAART drugs and alcohol leads to increased visceral fat accumulation

Pictorial representation of mice from different groups such as HAART drugs and alcohol (alcohol+ AZT+IDV), HAART drugs (AZT+IDV), alcohol and pair-fed (controls) showing visceral fat accumulation the most in HAART drugs and alcohol group (alcohol+ AZT+IDV)

Administration of HAART drugs and alcohol enhances alcohol induced Micro- and Macro-Vesicular Steatosis

Since we reported increased visceral fat accumulation in (alcohol+AZT+IDV) group (fig. 1) we were interested in examining the fat deposition (steatosis) in the whole liver. We examined the effect of HAART and alcohol on the whole liver by Hematoxylin and Eosin (H&E) staining and Oil Red O staining. Alcohol further worsened HAART induced steatosis. Indeed, we confirm the development of both micro- and macro vesicular steatosis in (alcohol+AZT+IDV) group (fig. 2). This data suggests that HAART drugs in combination with Alcohol could potentially exacerbate hepatic steatosis in comparison with HAART drugs or alcohol administered mice.
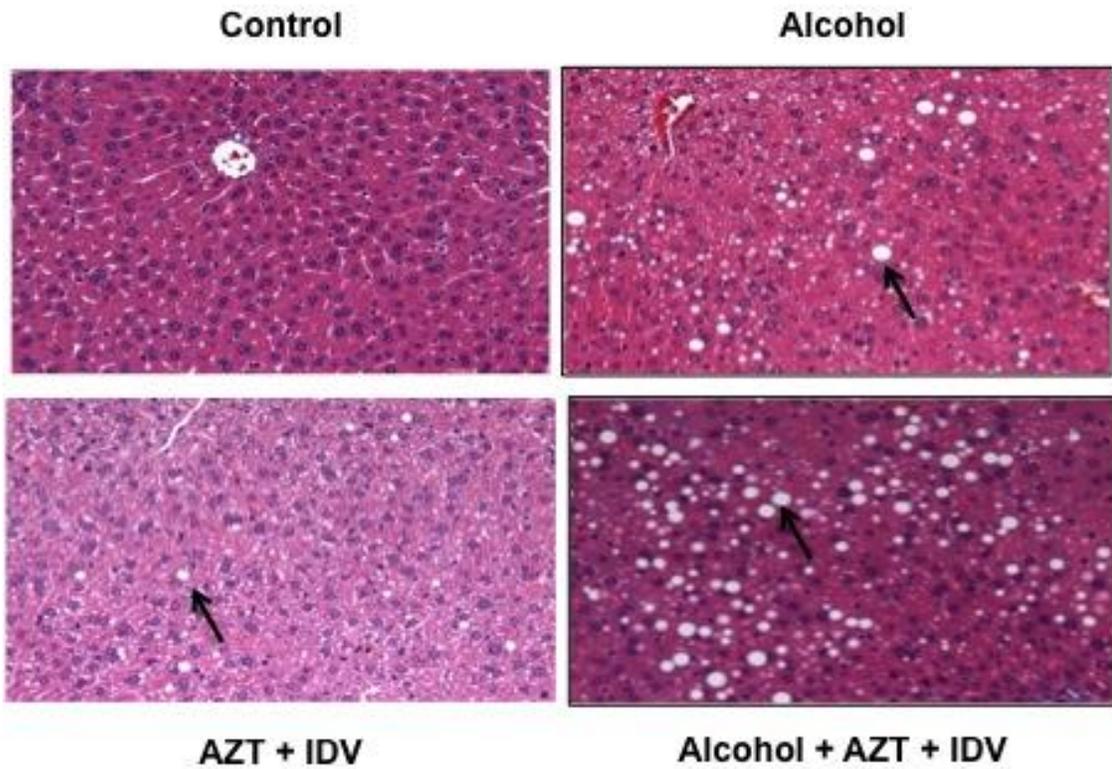
Figure 2: H&E staining of liver tissue

H&E staining of hepatic sections established the presence of micro-vesicular steatosis in alcohol and the (Alcohol+AZT+IDV) fed groups.
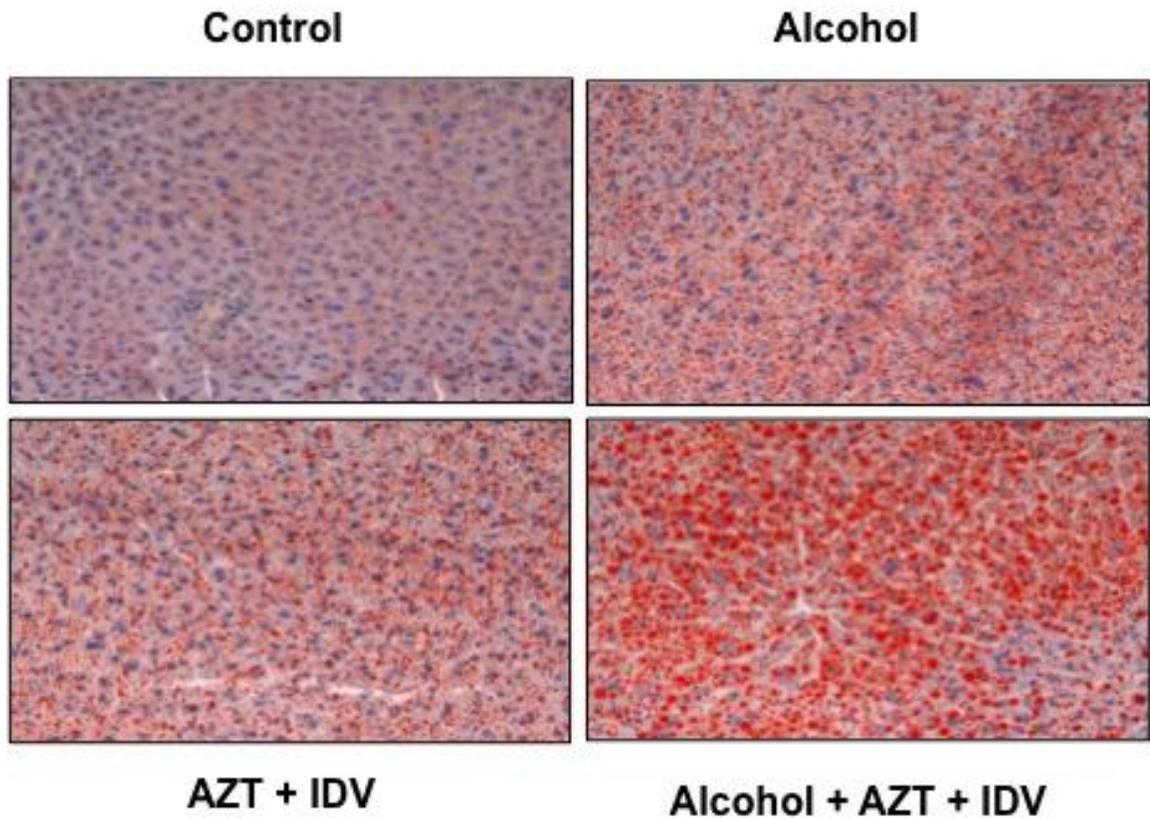
Figure 3: Oil Red O Staining of liver tissue

Oil Red O staining of hepatic sections established the presence of macro-vesicular steatosis in alcohol and the (Alcohol+AZT+IDV) fed groups.

Administration of HAART drugs and alcohol significantly increases hepatic triglycerides whereas hepatic free Fatty acids levels remained unchanged in (alcohol+AZT+IDV) and AZT+IDV groups in comparision with alcohol and controls group

In the liver, glycerol combines with fatty acids to form triglycerides as a byproduct of digestion of fats. Triglycerides play a very important role in digestion and metabolism by acting as transporters of dietary fats. The presence of fatty livers results mainly from the accumulation of triacylglycerols. Moreover free fatty acids are a key mediator of lipotoxicity in the liver. Increase in triglycerides and free fatty acids in the liver could potentially lead to lipodystrophy and dyslipidemia. Hepatic fat accumulation (fig.2, 3) was further confirmed by increase in the hepatic triglycerides. Hepatic triglycerides and free fatty acids were quantified biochemically.

In (AZT+IDV) group hepatic triglycerides were significantly higher than pair-fed (controls). Additionally, hepatic triglycerides in (alcohol+AZT+IDV) were significantly high than (AZT+IDV) or control groups. On the contrary, there was no significant difference in the free fatty acids in the above mentioned groups.
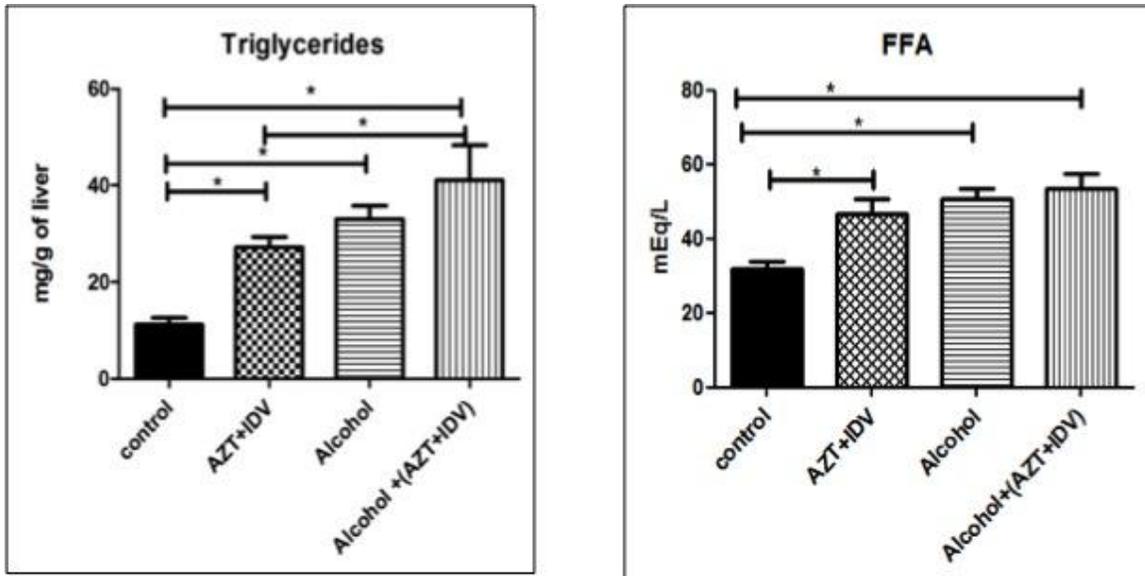
Figure 4: Effects of HAART (AZT+IDV) and alcohol singly and in combination on hepatic triglycerides and free fatty acids (FFA)

Hepatic triglycerides were quantified as (mg/g liver) in mice whereas Free fatty acids (FFA) was measured in (mEq/L) in mice fed with alcohol, (AZT+IDV), (Alcohol+AZT+IDV) and pair-fed (control). (Alcohol+AZT+IDV) significantly increased hepatic triglyceride levels compared to pair-fed (control), (AZT+IDV) whereas FFA levels remained unchanged. Data is presented as mean ± SEM, n=5 animals/group; * p<0.05 when compared to controls. Anova was used to evaluate significant differences between the 4 different groups

Administration of HAART drugs and alcohol causes increased serum endotoxin levels in mice.

Work done by our research group and others has shown that chronic alcohol exposure leads to intestinal oxidative stress, epithelial barrier disruption and enhancement of gut leakiness and consequent endotoxemia and systemic inflammatory response [36]. Acute and chronic ingestion of alcohol lead to a significant elevation of portal and systemic levels of endotoxin in animal models and humans[48-50]. In our experimental groups we reported significantly higher serum endotoxin levels specifically in (alcohol+AZT+IDV) fed mice as compared to (AZT+IDV) and pair-fed (control) groups (fig. 5).

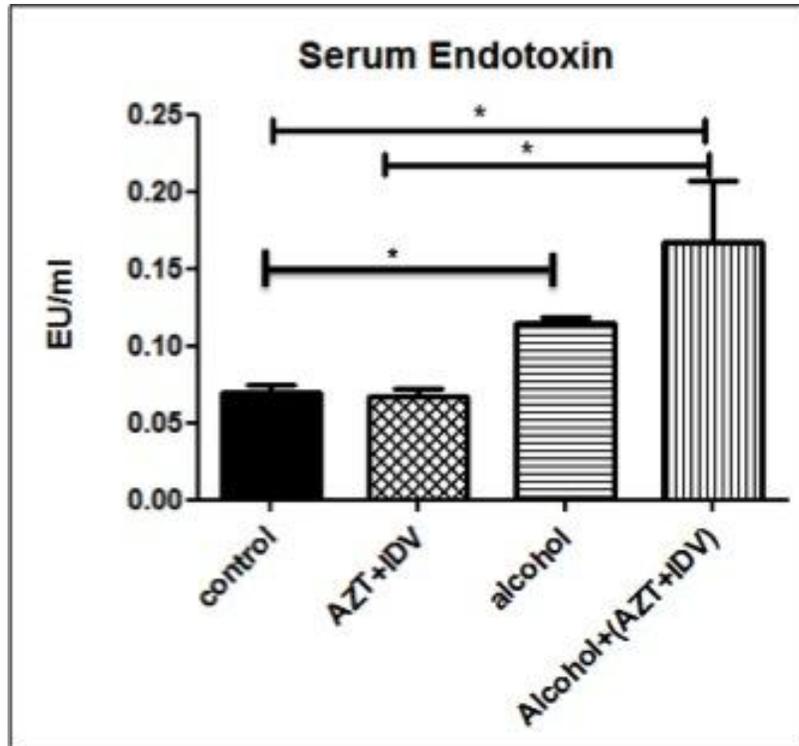**Figure 5: Effects of HAART drugs (AZT+IDV) and alcohol singly and in combination on serum endotoxin**

Serum endotoxin levels were quantified as (EU/ml) in mice fed with alcohol, (AZT+IDV), (Alcohol+AZT+IDV) and pair-fed (control). (Alcohol+AZT+IDV) significantly increased systemic endotoxin levels as compared to (AZT+IDV) and pair-fed(control). Data is presented as mean ± SEM, n=5 animals/group; * $p < 0.05$.

Administration of HAART drugs and alcohol leads to an increase in neutrophil infiltration (Inflammation)

Neutrophils accumulate within the hepatic microvasculature, which includes sinusoids and in postsinusoidal venules. A variety of inflammatory mediators such as TNF-α, IL-1, CXC chemokines [(e.g., IL-8, Macrophage inflammatory protein-2 (MIP-2), keratinocyte-derived chemokine (KC), cytokine-induced neutrophil chemoattractant-1 (CINC-1)], and platelet activating factor (PAF)), can cause neutrophil infiltration within the hepatocytes [51-55]. It is well known that alcohol consumption in humans results in neutrophilic steatohepatitis [56-58]. Neutrophil infiltration in the liver is known to significantly contribute to the pathologic findings noted in alcoholic liver injury (ALI) [56, 57, 59].

In order to evaluate the Neutrophils accumulation we performed Chloroacetate Esterase (C&E) Staining procedure in the whole liver. We confirmed increased levels of neutrophils in HAART and alcohol administered mice livers as compared to individual groups.
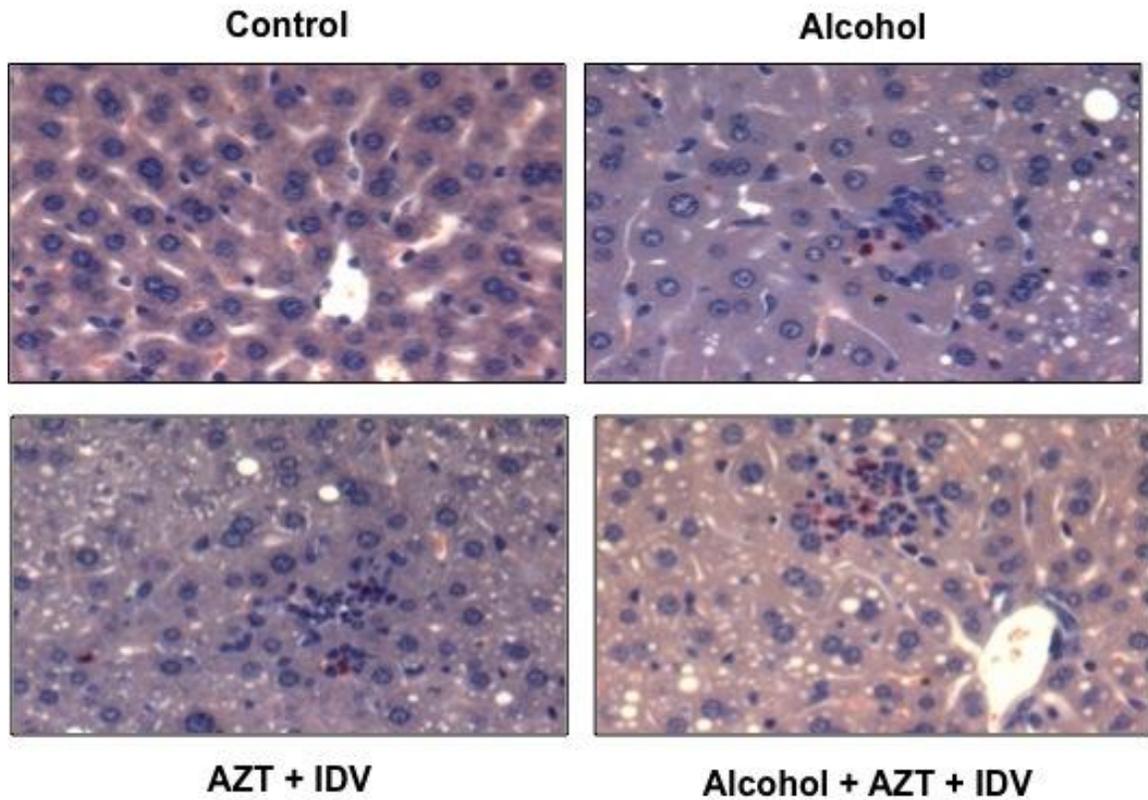
Figure 6: CAE staining of liver tissue

CAE staining of hepatic sections established the presence of neutrophil

recruitment in (Alcohol+AZT+IDV) mice group

Administration of HAART drugs and alcohol lead to an increase in serum ALT levels

Serum alanine aminotransferase (ALT) levels are the hallmark of liver injury. It has been well documented that chronic alcohol consumption leads to elevated ALT levels in the blood indicating liver injury. In the present study, ALT levels were significantly elevated in Alcohol and in (alcohol+AZT+IDV) groups in comparison with pair-fed (Control). Although, ALT levels in (alcohol+AZT+IDV) were not significantly higher than alcohol group.
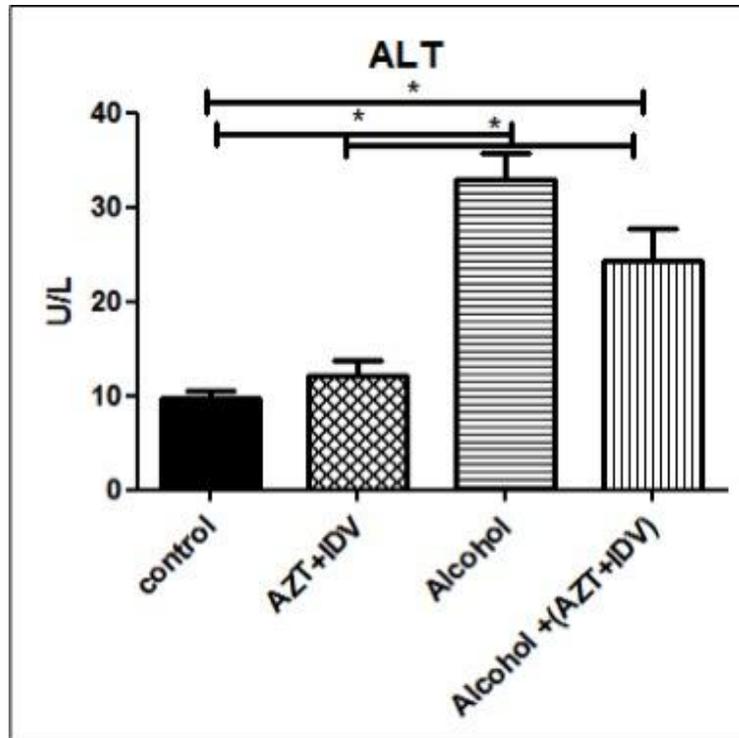
Figure 7: Effects of HAART (AZT+IDV) and alcohol on serum ALT levels

Serum ALT levels (U/L) were measured using ALT reagent from Thermo Scientific as per manufacturer's instructions. Chronic alcohol & HAART (alcohol+AZT+IDV) administration resulted in significant increase in the serum ALT levels in comparison with pair-fed (control) , and AZT+IDV fed mice. Data is presented as mean ± SEM, n=5 animals/group; * $p<0.05$. Anova was used to evaluate significant differences between the above mentioned groups.

DISCUSSION

In this study, we tested the combinatorial the effects of HIV medication HAART specifically a combination of NRTI and PI with chronic alcohol consumption on hepatic injury in animals. Chronic alcohol consumption (4 weeks) lead to a fulminant steatohepatitis and moderate dyslipidemia that is consistent with other well documented studies. Administration of HAART drugs further increased the hepatotoxic consequence of alcohol.

In our results, combination of HAART and alcohol resulted in a synergistic increase in visceral fat accumulation already seen in the group of animals on HAART drugs. It is well-documented that visceral-fat accumulation is associated with the use of PIs like Indinavir [60]. This data clearly suggests that there is a possibility of dysregulation in the body fat redistribution and potential lipodystrophy in animals receiving HAART and alcohol. Consequently, we confirmed that micro- and macro- vesicular steatosis was significantly increased in animals receiving HAART and alcohol as compared animals administered HAART or alcohol alone. This further established the occurrence of dyslipidemia in these animals.

It has been shown in humans as well as animals that chronic alcohol consumption leads to the development of fatty liver [61]. This incidence of fatty liver is due to the accumulation of triacylglycerols. Since we confirmed the

occurrence of macro- and micro- vesicular steatosis in the whole livers of these mice we analyzed the lipid profile specifically hepatic triglycerides and free fatty acids. As expected, we reported marked increase in the hepatic triglycerides in animals receiving alcohol as well as HAART in comparison with HAART or alcohol and pair-fed controls.

Work done by our research group and others has shown that chronic alcohol exposure leads to intestinal oxidative stress, epithelial barrier disruption and enhancement of gut leakiness and consequent endotoxemia and systemic inflammatory response. Moreover, alcohol consumption affects both aerobic and non-aerobic intestinal bacteria resulting in altered gut bacterial composition (dysbiosis). Additionally, alcohol induced endotoxemia is also influenced by depressed hepatic clearance of circulating endotoxin shown in both acute and chronic alcohol consumption causing intestinal barrier dysfunction. Moreover, there are no clinical reports or literature on the effect of HAART drugs per se (without any viral load) on systemic endotoxin. In this regards, we report a significant increase in the circulating systemic endotoxemia in animals on HAART and alcohol administration when compared with HAART or alcohol group animals alone. Serum Aminotransferase activity, which is an indicator of liver injury also followed the same tread of being significantly higher in in animals receiving HAART and alcohol together.

Overall, our data suggests that the combination of alcohol and HAART (AZT & IDV) would have detrimental on liver. Hence the mechanisms underlying the liver toxicity due to HAART and alcohol need further investigation which

would lead to potential therapeutic targets. These pharmacological interventions

could be supplemented with HAART drugs that would help in continuation of the

therapy without any hepatotoxic effects.

SUMMARY

The highly Active Anti-Retroviral therapy has been shown to improve the life expectancy of HIV-1 patients during the last two decades [6, 9]. It is now clear that alcohol abuse can alter/suppress immune function, including CD4 T-lymphocyte depletion, which can accelerate HIV progression. Subjects who consume alcohol may be more likely to develop certain infectious diseases that can complicate HIV, examples being various types of pneumonia and tuberculosis. Patients who consume alcohol also may be less compliant with their HIV therapy, which can affect disease progression. Moreover, Liver disease is now a leading cause of death for patients with HIV. Liver disease may be due to a variety of factors including co-infection with viral hepatitis, alcohol abuse, and antiretroviral hepatotoxicity. Hence in this preliminary animal study we are shedding some light on the mechanisms of alcohol and HAART mediated hepatic steatosis, inflammation and injury.

Our data suggests that HAART medication along with a cofactor like alcohol causes an increase in micro- and macro-vesicular steatosis leading to an increase in hepatic triglycerides possibly leading to dyslipidemia. Neutrophil infiltration (inflammation) was clearly increased in animals receiving HAART and alcohol. Serum endotoxemia was significantly more in animals receiving HAART drugs along with alcohol indicating intestinal barrier dysfunction. We also

reported liver injury in the form of increased serum aminotransferase activity in animals on HAART and alcohol. In conclusion, alcohol/HAART combination causes severe hepatotoxic consequences. Further investigations are required to understand the underlying mechanisms leading to potential targets for therapeutic interventions.

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Acute exposure to acrolein, a ubiquitous environmental pollutant, and anti-HIV Highly Active Antiretroviral Therapy (HAART) medication leads to hepatotoxicity.

Smita Ghare, Hridgandh Donde, Swati Joshi-Barve, Craig McClain and Shirish Barve (Manuscript in preparation)

POSTER PRESENTATION:

Potential Role of FasL Expression and Signaling in the Induction of Hepatotoxicity by AZT.

Hridgandh Donde, Smita Ghare, Wei-Yang Chen, Swati Joshi-Barve, Craig McClain and Shirish Barve. **Research! Louisville, University of Louisville** (2011)

Ethanol is a cofactor in HAART induced hepatotoxicity

Hridgandh Donde, S. Ghare, I Kirpich, L Gobejishvili, W Chen, S Joshi-Barve, C McClain and Shirish Barve. **Research! Louisville, University of Louisville** (2012)

Ethanol is a cofactor in HAART induced hepatotoxicity

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Ethanol is a cofactor in HAART induced hepatotoxicity

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