Synopsis of the thesis entitled

Potential of Carbon monoxide releasing molecule-A1 against hepatotoxicity and in improving Nonalcoholic steatohepatits

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INTRODUCTION

The term non-alcoholic steatohepatitis (NASH) was first introduced by Ludwig et al. (1980) to describe a pathophysiological condition of fatty liver in non-alcoholic individuals. These condition characterized by accumulation of lipids in the liver of non-alcoholic individuals and consequent oxidative stress leading to cirrhosis of liver in the long run (Duvnjak et al., 2007). According to the American Association for the Study of Liver Diseases (AASLD), development of fatty liver in patients with no prior history of chronic high alcohol intake (i.e., alcoholintake is <20 g ethanol/day) is referred to as nonalcoholic fatty liver disease (NAFLD) (Neuschwander and Caldwell, 2003).

Based on the preclinical data available, Day and James were the first to propose a "two-hit" hypothesis for explaining the pathogenesis of NASH. The same was very well accepted and stayed as the only comprehensive explanation for NASH (Day, 1998). The first hit, marked by triglyceride loading within hepatocytes known as steatosis or fatty liver (NAFLD) is due to an overflow of free fatty acids (FFA) into the liver and consequent esterification. The hepatic steatosis marked by high triglyceride accumulation is reflective of an excessive inflow of FFA and this, rather than triglyceride per se, seems to be the factor responsible for the first hit development of NAFLD (Shiota and Tsuchiya, 2006) and the subsequent vulnerability of the liver for second hits leading to NASH and/or fibrosis.

This background of hepatic steatosis sets the stage of vulnerability for a second hit which, as it appears now, may represent a set of factors (multi hits) that may involve complex interactions between hepatocytes, stellate cells, adipose cells, Kupffer cells, inflammatory mediators, and reactive oxygen species driving NAFLD state to NASH. Though, the cause of progression from NAFLD to NASH/fibrosis remains unclear, animal studies tend to suggest that the driving force is mainly oxidative stress and compromised mitochondrial health and function.

Oxidative stress is a major factor in the mechanismunderlying liver diseases. It contributes to the initiation as well as progression of liver injury (Saito *et al.,* 2010). Factors such as alcohol, drugs, heavy metals, and high-fat diet are now identifiedas inducers of hepatic oxidative stress (Knight *et al.,* 2002). Oxidative stress also induces proliferation of stellate cells and collagen synthesis, thus promoting fibrosis and cirrhosis. In response to over whelming oxidative stress, there is a significant use of antioxidant proteins, along with an increase in lipid peroxidation. However, to maintain redox homeostasis, hepatocytes have a sophisticated antioxidant system comprising antioxidant proteins, enzymes, and transcription factors to combat oxidative stress. Hence, regulation of hepatic oxidative stress can play a critical role in thetreatment of various liver diseases.

Nuclear erythroid 2-related factor 2 (Nrf-2), a transcriptional factor, is the major regulator of oxidative stress in hepatocytes. Nrf2 protects against oxidative stress and liver injury by increasing the expression of both metabolizing enzymes and ARE genes [viz. NAD(P)H:quinine oxidoreductase 1 (NQO1), glutamate-cysteine ligase – catalytic and modifier subunits (GCLC and GCLM) and heme oxygenase-1 (HO-1) Mani *et al.,* 2013). Therefore, in models of liver injury, a variety of Nrf-2 activators such as phytochemicals (Jadeja *et al.,* 2016), drugs (Zhang *et al.,* 2017) or gases (Liu *et al.,* 2012) have been investigated for their therapeutic potential. In animal models, nitric oxide donor and hydrogen sulphide reduce APAP-induced liver injury by increasing intracellular GSH, thus providing evidence that gasotransmitters may have therapeutic potential.

Physiological role of carbon monoxide (CO) as a regulator of cellular function is well investigated (Ryter*et al.,* 2006) CO is easily diffusible across cellular and biological membranes and therefore can mediate functional changes rapidly (Motterlini, R. and L.E. Otterbein., 2010). Some known CO targets include soluble guanyl cyclase, heme containing potassium channels, transcriptional factors (BACH1 and NPAS-2), caveolae, nitric oxide

synthase (NOS) and NADPH oxidase (Boczkowski*et al.*, 2006). CO also interacts with cellular components lacking transitional metal (Otterbein, L.E., *et al.*, 2010).

Carbon monoxide releasing molecules (CORMs) with a core of third transitional metal such as manganese or ruthenium are fast releasers of CO (Motterlini, R., *et al.*, 2006). Carbon monoxide releasing molecule A-1 (CORM A-1) has a boron core and releases CO slowly (t1/2 =21 min). CORM A-1 is water soluble and has been investigated for its therapeutic effect in diabetes (Nikolic, I., *et al.*, 2015), myocardial infarction (Varadi, J., *et al.*, 2010), posterior uveitis (Fagone, P., *et al.*, 2015) and neurogenesis. In addition to preclinical studies, clinical trials have been initiated to examine safety and efficacy of CO in patients with idiopathic pulmonary fibrosis, pulmonary arterial hypertension, lung inflammation and acute respiratory distress syndrome. Unlike other gasotransmitters such as NO and H2S, CO is more stable and its action is specific to transitional metals.

DEFINITION OF THE PROBLEM

Incidence of Non-Alcoholic Steatohepatitis (NASH) is increasing in Asian countries and is becoming the cause of death due to its asymptomatic nature, unavailability of the relevant diagnostic tests and its wrong diagnosis as hepatitis. Obese individuals and/or insulin resistant ones are prone to NASH because of the strong positive co-relation existing between these diseases. Decline in mitochondrial functional leads to a mismatch between fatty acid beta-oxidation and oxidative phosphorylation leading to accumulation of partially oxidized intermediates which further exacerbate insulin resistance and leads to the progression of NASH. Various interwoven signalling pathways control the cellular metabolic program and that are subjected to complex alterations of various degrees in NASH. Many of these pathways have been shown to be redox sensitive with ROS playing a key modulatory role. Therefore, many antioxidants have been tested for treating NASH with varying degrees of

therapeutic success. Also, lipid lowering agents have been tried out against NASH with moderate success but great degree of side effects. Hence, search for a single therapeutant with multipronged action against NASH is an important area of research amongst scientific fraternity working in the field of life style disorders.

RELEVANCE OF THE WORK

NASH is a multifactorial disease and hence, a therapeutant with multipronged effects and with minimal side effects is desired. No single pharmacological preparation is available till date for treating NASH. Almost all modes of liver injury including NASH are associated with increased oxidative stress and an overwhelmed antioxidant defence system. Since Nrf2 activation is associated with the enhancement of endogenous antioxidant system, as well as in improving mitochondrial biogenesis and function. Hence, it can be an ideal therapeutic target for reducing pathogenesis of NASH. Carbon monoxide is an endogenous product of heme degredation and lower levels of the same have been associated with progression of several diseases such as diabetes or circadian dysregulation. Carbon monoxide positively affects mitochondrial biogenesis in neurons. These facts are relevant in the present context to use CORM A-1 (as carbon monoxide source) as a possible therapeutant in experimentally induced NASH.

OBJECTIVES:

1. **Role of CORM A-1 in modulating oxidative stress and in improving experimentally induced liver injury.**

   This study was conducted on APAP treated swiss albino mice. Hepatotoxicity and subsequent CORM A-1 mediated protection was assessed. t-BHP induced HepG2 cells were assessed for CORM A-1 induced improvement in cell viability, oxidative

stress, Nrf2, ARE genes. Efficacy of CORM A-1 as a hepatoprotectant and its underlying mechanism of Nrf2 translocation were investigated in detail.

2. CORM A-1 mediated mitochondrial bioenergetics in Non-alcoholic steatohepatitis (NASH).

In this study, HFD induced NASH was developed in C57BL6/J male mice. CORM A-1 was injected daily and effects were observed in liver. Antioxidant, toxicity and related parameters such as staining techniques and expression levels of key genes and proteins for mitochondrial biogenesis were investigated in detail. Also, mitochondrial respiration and ATP generation was assessed using SeaHorse-XF flux analyzer.

1. Role of CORM A-1 in modulating oxidative stress and in improving experimentally induced liver injury.

Depletion of intracellular antioxidants is a key event in oxidatively stressed hepatic tissue. Strategic pathways are targeted using novel test compounds to improve intracellular antioxidants including GSH under diseased conditions. Recent research interest in Nrf2-Keap1 pathway up regulating cytoprotective genes including HO-1 is in focus. *Hmox1* knockout mouse show high percentage lethality, shortened life span and organ anomalies that underlines the functional importance of HO-1. Exogenous carbon monoxide (CO) has beneficial effects in pathophysiological a condition that mimics the role of HO-1. CO carriers containing manganese (CORM-1), ruthenium (CORM-2 and -3) and boron (CORM A-1) are currently being investigated to tailor therapeutic approaches for the prevention of vascular dysfunction, inflammation, tissue ischemia and organ rejection.

We hypothesized that CO modulates oxidative stress in liver injury. To test this hypothesis, we conducted a proof-of-concept investigation to determine the effects of CORM A-1 in two established models of oxidative stress-mediated liver injury viz. t-BHP-induced injury in HepG2 cells and APAP-induced liver injury in mice—a clinically relevant model.

Materials and Methods

Maintenance of HepG2 Cells and Treatment Schedules

Human Hepatoma (HepG2) cellswere procured from National Centre for Cell Science (NCCS, Pune, India) and maintained in CO_2 incubator (Thermo scientific, forma series II 3110, USA) at 37°C with 5% CO_2 in DMEM (10% FBS and 1% antibiotic antimycotic solution). Cells were sub-cultured using 1X TPVG every third day. To assess cytotoxicity, cells were treated with 5, 10, 25 and 50 µM t-BHP alone or with

CORM A-1 (100 µM) for 2 h. Cells were also treated with CORM A-1 alone (10-100 µM).

Cytotoxicity and Intracellular Reactive Oxygen Species Assay

HepG2 cells were seeded in 96-well plates (10^4 cells/well), allowed to grow overnight and were treated with t-BHP (5, 10, 25, 50µM) in the absence or presence of CORM A-1 (100µM) for 2 h. Later, cells were incubated with MTT (5mg/ml) for 4 h and resultant crystals of formazan were dissolved in DMSO (150µl/well). Absorbance measured at 540 nm using Synergy HTX Multimode Reader (Bio-Tek instruments, Inc., Winooski, VT), and cell viability calculated.

HepG2 cells treated with t-BHP and CORM A-1 were also stained with 10 µM 2, 7-dichlorodihydrofluoroscein diacetate (CM-H2-DCFDA) at 37°C for 30 min. Cells were photographed (EvosFloid cell imaging station; Life technologies, USA) and the intracellular fluorescence quantified using Image J software.

Experimental Animals

Experimental protocol was approved by Institutional Animal Ethical Committee (IAEC) (Approval no. MSU-Z/IAEC/04-2017)and all experiments were conducted in CPCSEA-approved *(827/GO/Re/S/04/CPCSEA)* animal house facility at the Department of Zoology, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India.Swiss albino male mice (n=42, 6-8 wks old, 25-30 gm each) were procured (Flair lab, Surat, Gujarat, India) and maintained as per standard guidelines (23±2˚C, LD 12:12, laboratory chow and water ad libitum). All experiments were performed after acclimatization for 1 week.

APAP dose (300 mg/kg), route of administration (*i.p.*) and time of sacrifice were used as described previously by James *et al*, 2003. Overnight fasted mice were divided into seven groups (n=6). Group 1: Untreated; Group 2: Saline treated; Group 3: APAP

(4h); Group 4: APAP + CORM A-1 (4 h); Group 5: APAP (12h); Group 6: APAP + CORM A-1 (12 h); Group 7: iCORM A-1 (12 h).

APAP (300 mg/kg) was injected intraperitoneally and CORM A-1 (20 mg/kg) was administered one hour later. Literature suggests varied doses of CORM A-1 (2 mg/kg every day/twice a day or a single dose of 60 mg/kg) in mice depending on the experimentally induced disease scenario. In a pilot experiment, we had used 2 mg/kg dose of CORM A-1 that had minimal effect on APAP-induced liver injury in mice. Since CORM A-1 has a very short half-life (21 min), to achieve anti-oxidant effect, following administration of APAP one log greater dose of 20 mg/kg was employed to achieve a robust antioxidant effect. Mice were euthanized at 4 h or 12 h. Livers were dissected, stored in 10% formalin, RNAlater and at -80˚C.

Observation

CORM A-1 (100 µM) was effective in inducing ~ 3- 3.5 fold increase in HO-1 expression at 2h and 4h respectively (fig 1). Reactive oxygen species (ROS) generated in t-BHP (25 and 50 µM) treated cells increased intracellular oxidative stress. The same was negated in CORM A-1 co-treated cells wherein, improved cell viability (~10-15%) was observed. Positive regulation of HO-1, Nrf2, GCLC, GCLM and NQO-1 genes (fig3) was recorded in CORM A-1 + t-BHP treated group. Similar observations recorded in Cellular Reduced Glutathione (GSH) levels further corroborate the findings and imply towards CORM A-1 induced modulation of Nrf2-Keap1 pathway in oxidatively stressed hepatocytes.

These data indicate that CO releasing molecules reduce oxidative stress-mediated liver injury. The highlights of our study are

i) In HepG2 cells, CORM A-1 facilitates nuclear translocation of Nrf 2, reduces oxidative stress, upregulates ARE genes, prevents GSH depletion and promotes cell viability;

ii) In mice, CORM A-1 attenuates APAP-induced liver injury by upregulating ARE genes, preventing GSH depletion and reducing hepatocyte necrosis;

iii) In mice, CORM A-1 reduced APAP-induced mortality.

iv) Docking analyses suggest that CORM A-1-mediated results are due to inhibition of interaction between Nrf2 and Keap-1.

Important leads on therapeutic potential of CO releasing ligands in improving liver function have been generated till date and the findings have been accepted for publication.

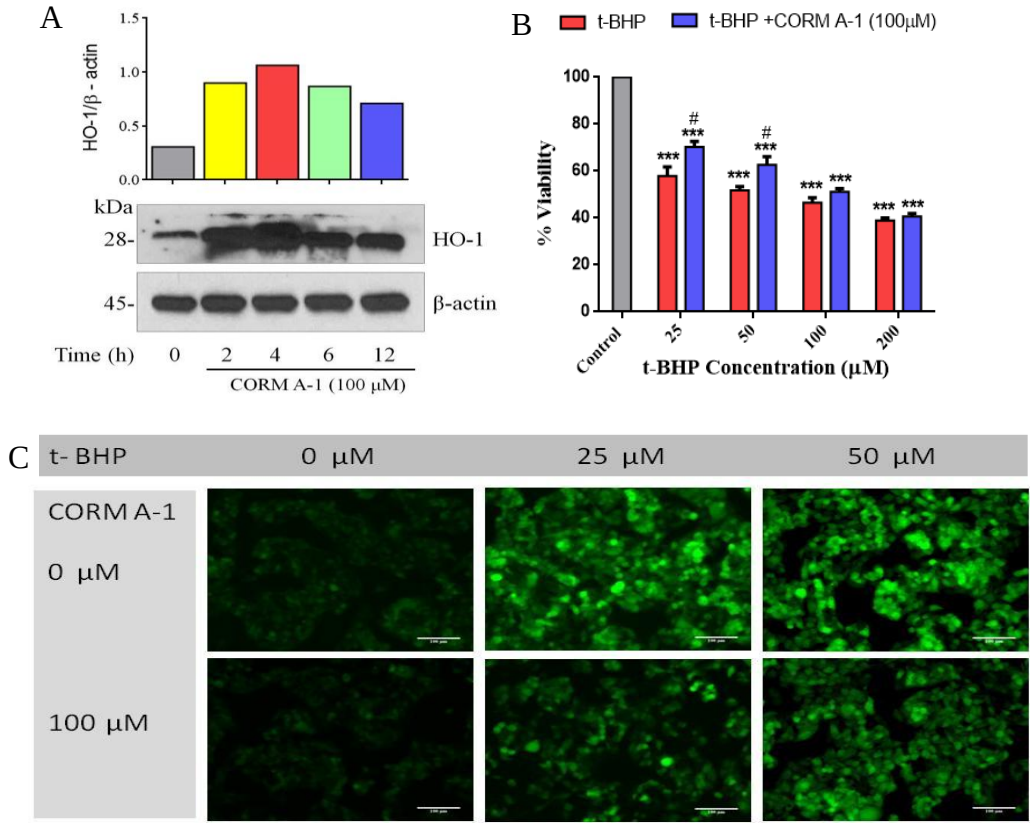


Fig 1.(A) Time-point analysis of HO-1 expression post CORM A-1 treatment. Densitometric analysis was carried out using Image J software (NIH) (B) Determination of cell viability by MTT assay. *** $p<0.001$ for control vs experimental groups; # $p<0.05$ for comparison of t-BHP with respective t-BHP + CORM A-1 group (C) Effect of CORM A-1 on t-BHP induced intracellular oxidative stress by DCFDA staining. Scale bar = 100 μm

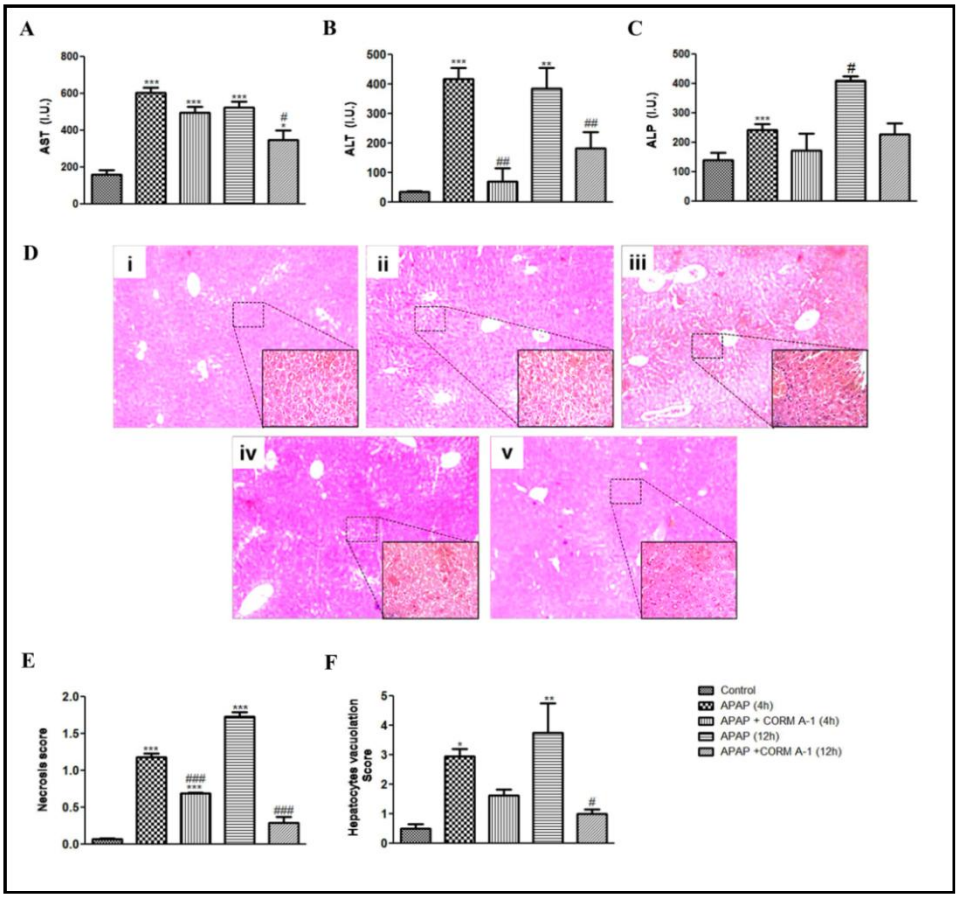


Fig.2. Activity levels of marker enzymes of liver function in serum and microscopic evaluations of liver of control and treated mice. (A) AST, (B) ALT (C) ALP (D) H&E-stained liver sections (2.5X and insert 40X). Livers sections (3 per liver sample) scored for toxicity viz. (E) hepatocyte necrosis (F) hepatocyte vacuolation. CORM A-1 improved liver function and hepatocyte necrosis.

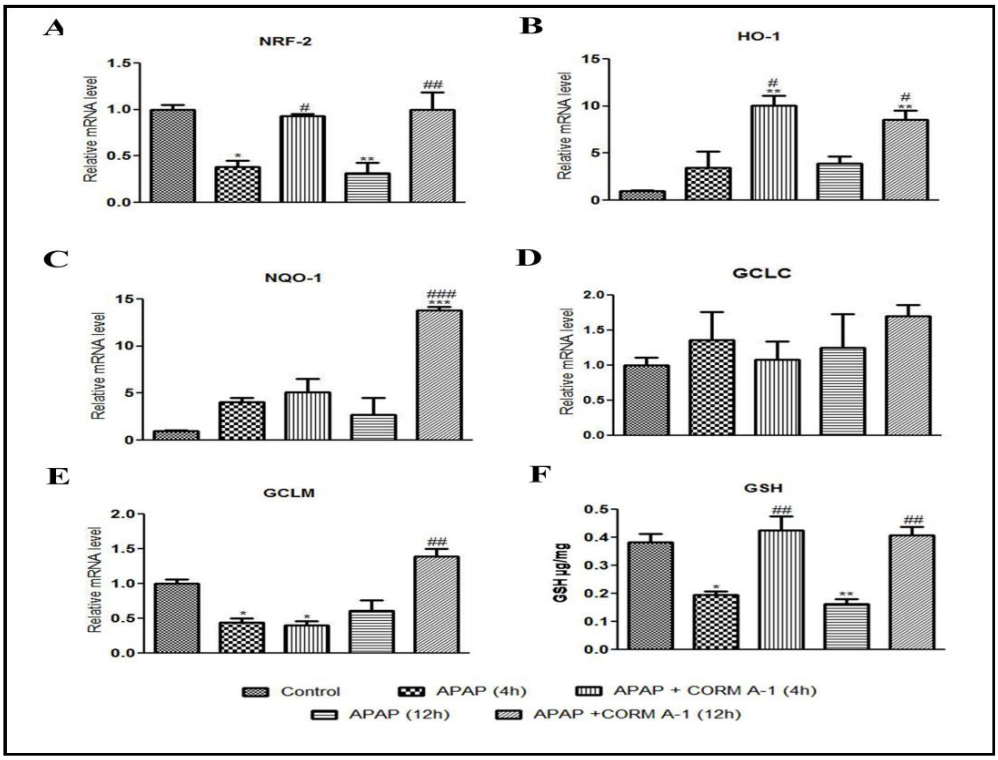


Fig. 3. mRNA levels of Nrf2-ARE and cytoprotective genes viz. (A) Nrf2, (B) HO-1 (C) NQO-1, (D) GCLC (E) GCLM and (F) hepatic GSH content of control and treated mice.Results expressed as Mean ± SEM. *P<0.05, **P<0.01 and *** P<0.001 as compared to control group whereas; #P<0.05, ##P<0.01, and ###P<0.001 is when compared with respective APAP treated group. CORM A-1 induced transcriptional activation of said genes that resulted in improvement in hepatic GSH.

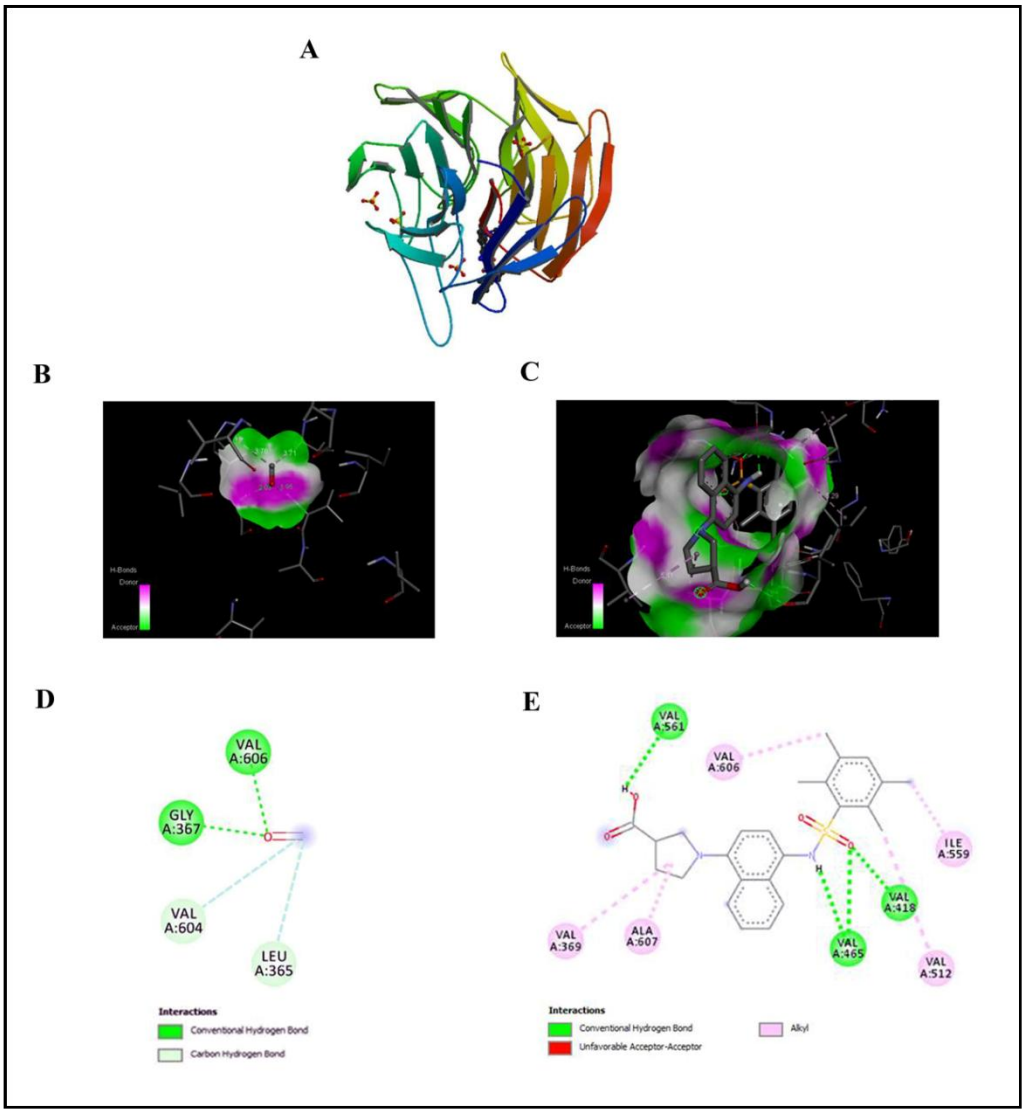


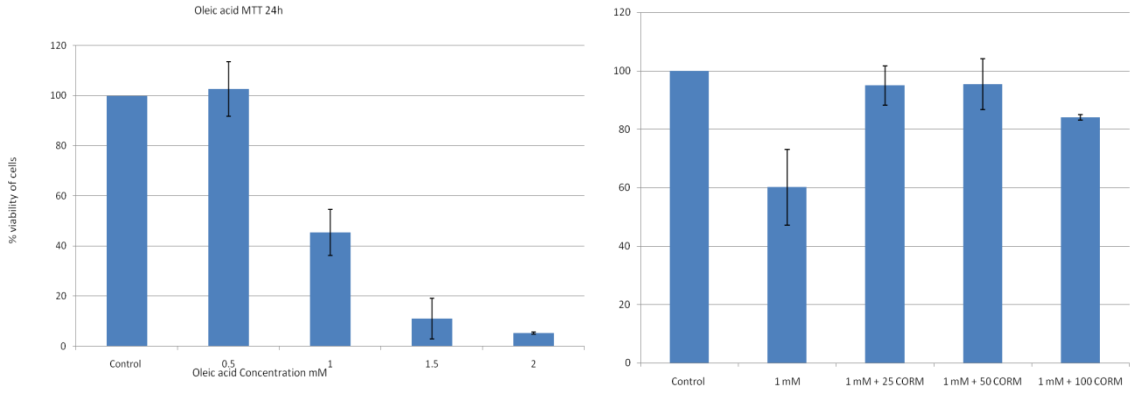
Fig. 4. Molecular docking analysis of CO with Keap1 Protein (A) Keap1 Protein PDB ID:5CGJ Crystal structure of murine Keap1 in complex with RA839, a non-covalent small-molecule binder to Keap1 and selective activator of Nrf2 signalling. (B) Top view of Keap1 in complex with CO (C) Top view of Keap1 in complex with RA839 (D) and (E) 2D interaction map of Keap1 with CO or RA839. The colour codes indicate potential interactions between amino acid residues and CO or RA839.

2. **CORM A-1 mediated mitochondrial bioenergetics in Non-alcoholic steatohepatitis (NASH).**

Mitochondrial loss and poor energetics majorly contribute to liver dysfunction in a disease condition or toxicity. Carbon monoxide releasing molecule A-1 (CORM A-1) is a gasotransmitter that has been reported to improve cellular function in acute liver injury by promoting nuclear factor erythroid 2–related factor 2 (Nrf2) upregulation. But, the mechanism of CORM A-1 in improving mitochondrial bioenergetics and inducing biogenesis in experimental model of Non-alcoholic Steatoheaptitis (NASH) lacks clarity.

HepG2 cells were cultured with saturated fatty acids in presence or absence of CORM A-1 (100 µM) and expression levels of ARE genes (HO-1, GCLC and GCLM) were monitored. Also, changes in mitochondrial parameters viz. mass (mitotracker dye), DNA copy number, membrane potential (JC-1 staining) and function (complex I & II activity) were studied. Marker genes for mitochondrial biogenesis (Nrf2, Nrf1, PGC-1α and Drp1, TFAM) were studied in CORM A-1 treated or siRNA mediated Nrf2 downregulated HepG2 cells. Molecular Docking of CO in kelch domain of Keap-1 protein (an Nrf2 inhibitor) was studied by open source software Auto Dock Vina. Nrf2 translocation was further confirmed by immunostaining.

A



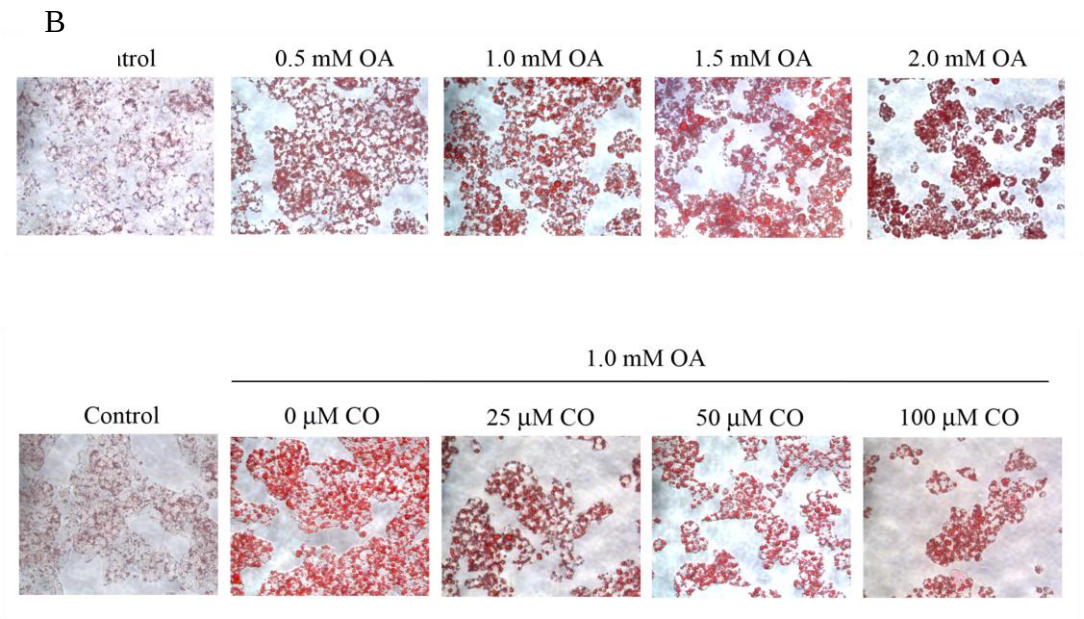


Fig 5.(A) Determination of cell viability by MTT assay (B) Effect of CORM A-1 on Oleic acid induced lipid accumulation by ORO staining.

CORM A-1 treatment accounted for an improved cell viability (MTT, LDH and ATP) and status of mitochondrial membrane potential as compared to lipotoxic cells. mRNA levels of Nrf1, Nrf2 and ARE genes were improved in CORM A-1 treated lipotoxic cells. Also, significant reduction in complex I & II activity, mitochondrial DNA content and mass were observed in lipotoxic cells but CORM A-1 treatment accounted for an improvement in the said parameters. Upregulated expression of PGC-1α, Nrf1, Drp1 and TFAM were recorded in CORM A-1 treated lipotoxic cells, implying towards mitochondrial biogenesis and an improved function. Nrf2 downregulated HepG2 cells revealed lowered expression of Nrf2, Nrf1, PGC-1α and Drp1 irrespective of CORM A-1 treatment. Molecular docking of CO to Keap-1 protein reveled binding of CO to the base of Nrf2 binding site on Keap-1. Immunostaining of Nrf2 in nuclei of CORM A-1 treated HepG2 cells confirmed its nuclear translocation. Hence, it is surmised that mechanism of CORM A-1 mediated

mitochondrial biogenesis is routed via Nrf2 activation in lipotoxic HepG2 cells thus also providing prima facie evidence on its therapeutic potential against NASH.

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CONFERENCE PRESENTATIONS

- Abstract accepted for poster presentation at "Liver meeting 2018", November 09-13, 2018, San Francisco CA, U.S.A.
- Presented "International Conference on Reproductive Biology and Comparative Endocrinology (ICRBCE)" organized by Department of Animal Biology, University of Hyderabad, Hyderabad - 500046, India. 9th - 11th February, 2017.
- Attended One Day National seminar on "Biomedical research in Gujarat:the road ahead" organized by Gujarat State Biotechnology Mission (GSBTM), DST, Government of Gujarat and The civil Hospital Ahmedabad, Gujarat, India. 18th June 2016.
- "International conference on bioactive chemicals for reproduction and human health" organized by Life Science Department, Davangere University, Karnataka, India. 26th-28th February 2015.

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