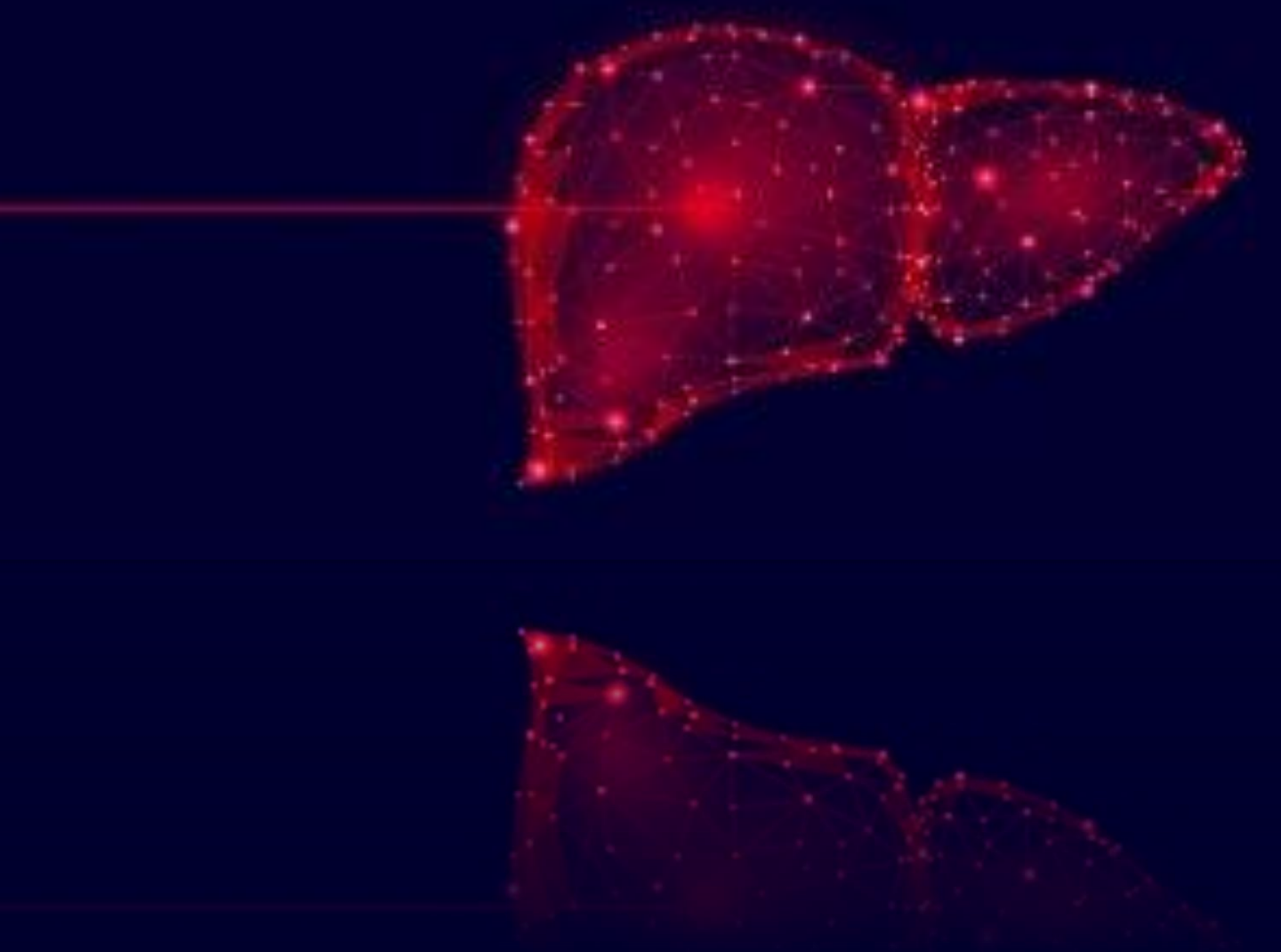
Chapter-4A

Alleviation of experimentally induced Nonalcoholic steatohepatitis (NASH) by CORM-A1: Targeting Nrf2 and antioxidant signaling



Introduction

NASH cover spectrum of diseases which often precedes liver fibrosis, cirrhosis, and hepatocellular carcinoma. It is associated with other type of metabolic diseases including obesity, type 2-diabetes. Based on recent discoveries and detailed understanding scientific fraternity has come up with 'multiple parallel hits' hypothesis, which states occurrence of multiple events parallel on same time, to exacerbate liver injury (Buzzetti *et al.*, 2016). Hence, a potent anti-NASH therapeutants should possess multipronged approach and orchestrate multiple target effect to effectively combat the pathological conditions. The plausible therapeutic targets for the NASH that top the list are insulin resistance, lipotoxicity, oxidative stress, mitochondrial dysfunction and regulation of inflammation (Larter *et al.*, 2006, Suliman *et al.*, 2016).

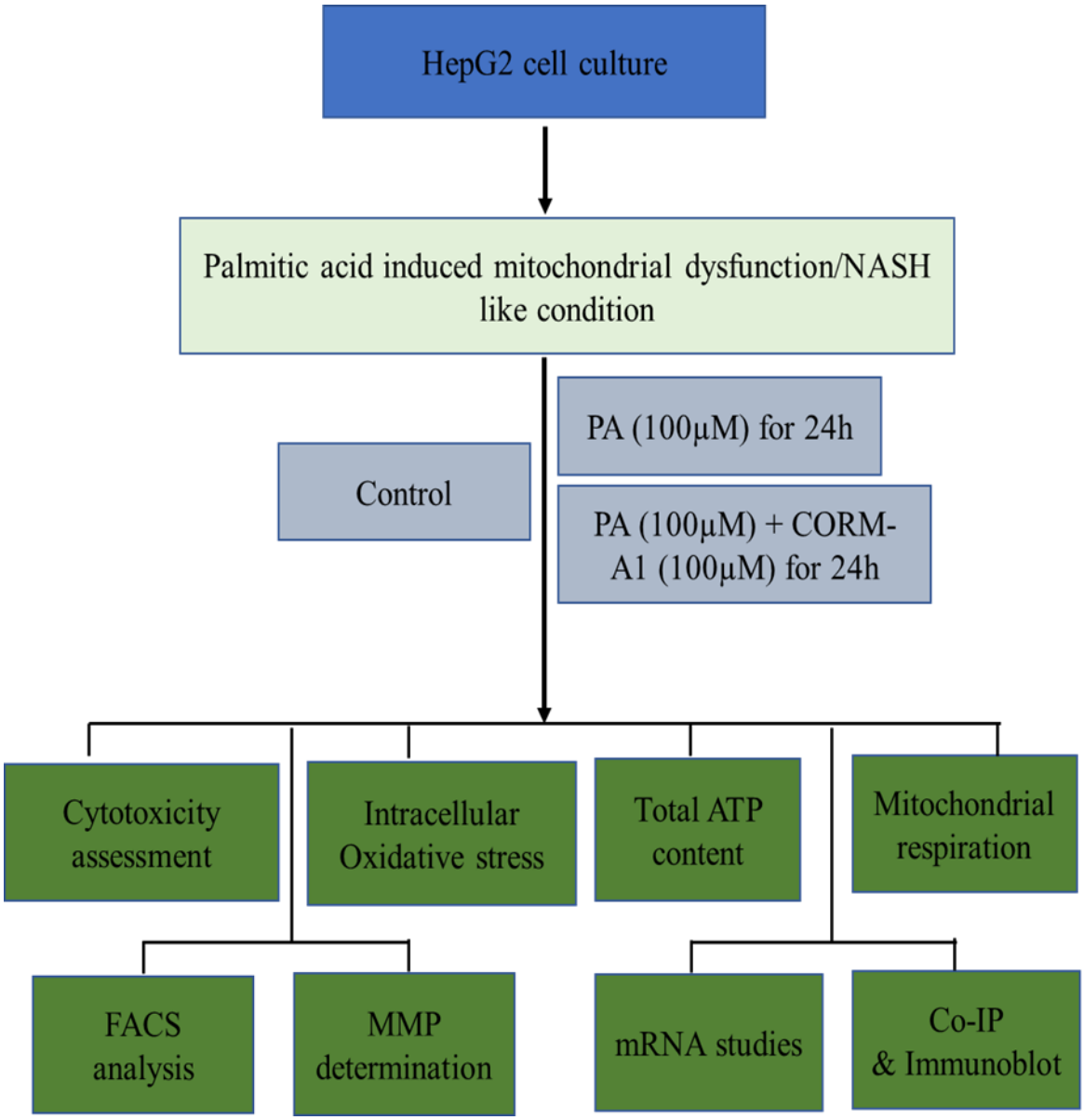
High oxidative stress and impaired antioxidant defense have been extensively documented in the development of NASH. This imbalance appears as a common denominator in various pathological processes in which an oxidative insult causes cell death and tissue damage (Tariq *et al.*, 2014). Physiological low levels of ROS have been indicated as mediators in various vital cellular processes and signaling networks. ROS are formed through oxidative processes within the cell. In the endoplasmic reticulum, monooxygenases, such as cytochrome P450 reductase, contribute to increased formation of hydrogen peroxide and superoxide anion (Zangar *et al.*, 2004), whereas peroxisomes are sources of cytosolic hydrogen peroxide associated with fatty acid oxidation. Under physiological conditions, peroxisomal hydrogen peroxide is removed by catalase, a ROS-metabolizing

enzyme present in these organelles (Fransen *et al.*, 2012). Mitochondria are a principal source of cellular ROS as the result of inefficiencies in the flow of electrons along the electron transport chain (ETC).

Nuclear erythroid 2-related factor 2 (Nrf2) has been extensively reported as principle regulator of cellular redox homeostasis as they are equipped to counterbalance the production of mitochondrial ROS (Dinkova-Kostova *et al.*, 2015, Ma, 2013). Nrf2 enables cell survival and adaptation under conditions of stress by regulating cytoprotective proteins, intracellular antioxidants, anti-inflammatory and detoxifying enzymes (Wu *et al.*, 2012). Nrf2 plays a crucial role in maintaining cellular redox homeostasis by regulating glutathione, thioredoxin, NADPH biosynthesis and controlling the production of ROS and NADPH oxidase (Tanito *et al.*, 2007, Wu *et al.*, 2011). Therefore, in metabolic disorders, a variety of Nrf2 activators such as phytochemicals, drugs or gasotransmitters have been investigated for their therapeutic potential (de Haan, 2011, Jadeja *et al.*, 2016, Liu *et al.*, 2012).

Since the antioxidant defense system is highly compromised in NASH, the present study investigates changes orchestrated by CORM-A1 in pathology of NASH. Our investigation is targeted at deciphering the role of Nrf2 in improving the pathology of NASH with CORM-A1 administration.

Experimental Design



Results

CORM-A1 facilitates Nrf2 translocation, modulates Keap1 expression and activates ARE genes in liver of HFHF fed mice.

Owing to cytoprotective nature of Nrf2 a detailed investigation of Nrf2 was performed in cytosolic and nuclear fractions. CORM-A1 is known to execute anti-oxidative properties by facilitating Nrf2 activation [Chapter 1 &2]. An investigation on Nrf2 expression was performed in HFHF fed and CORM-A1 treated mice. Liver of CORM-A1 treatment accounted for lowered levels of Nrf2 protein in cytosol and significantly higher levels in nucleus as compared to HFHF fed mice (Fig.4A.2-A). Further, the mRNA expression and protein content of HO-1 showed non-significant changes but CORM-A1 treatment resulted in a 2-fold increment. Conversely, Keap1 protein was significantly reduced in CORM-A1 treated group (Fig.4A.2-B). Supportive evidence was obtained in from of increased mRNA expression of ARE genes viz. GCLC, GCLM and NQO-1 in CORM-A1 treated mice (Fig4A.1). These set of findings demonstrate that CORM-A1 induced Nrf2 activation and subsequent upregulation of antioxidant genes in HFHF fed mice.

CORM-A1 mediated Nrf2 translocation and activation of ARE genes in HepG2 cells.

Preceding findings indicate towards anti-NASH potential of CORM-A1 in HFHF fed mice via activation of Nrf2-ARE pathway. Progression of hepatic steatosis comprises of multiple/overlapping molecular events. Hence, PA treated HepG2

cells were used as an in vitro model to affirm the findings in vivo. PA treatment resulted in heightened levels of intracellular ROS as evidenced CellROX (green) (Fig.4A.3) and by DHE (red) (Fig.4A.4) staining but CORM-A1 co-supplementation resulted in significant decrement in red and green florescence respectively. Translocation of Nrf2 protein content (Cytoplasm to nucleus) was further confirmed in HepG2 cells wherein, CORM-A1 treatment accounted for significantly higher Nrf2 protein in nucleus as compared to PA treated cells (Fig.4A.6-A). Keap1 protein (negative regulator of Nrf2) showed a significant decrement in PA and CORM-A1 treated groups. Co-immunoprecipitation (co-IP) results revealed time depended decrement in Keap1 protein content after treatment with CORM-A1 to HepG2 cells (Fig.4A.6-B). Increase in HO-1 mRNA and protein was recorded in PA treated cells whereas, more pronounced effect in mRNA and moderate nonsignificant increment in protein was noted in CORM-A1 co-supplemented group (Fig.4A.6-C). Other ARE genes viz. GCLC, GCLM and NQO-1 were also studied wherein, GCLM mRNA levels was decreased significantly following PA treatment. But, GCLC and NQO-1 mRNA levels in the same group showed non-significant changes (Fig.4A.5). CORM-A1 co-supplementation to PA treated HepG2 cells was marked by significantly elevated mRNA levels of GCLC, GCLM and NQO-1.

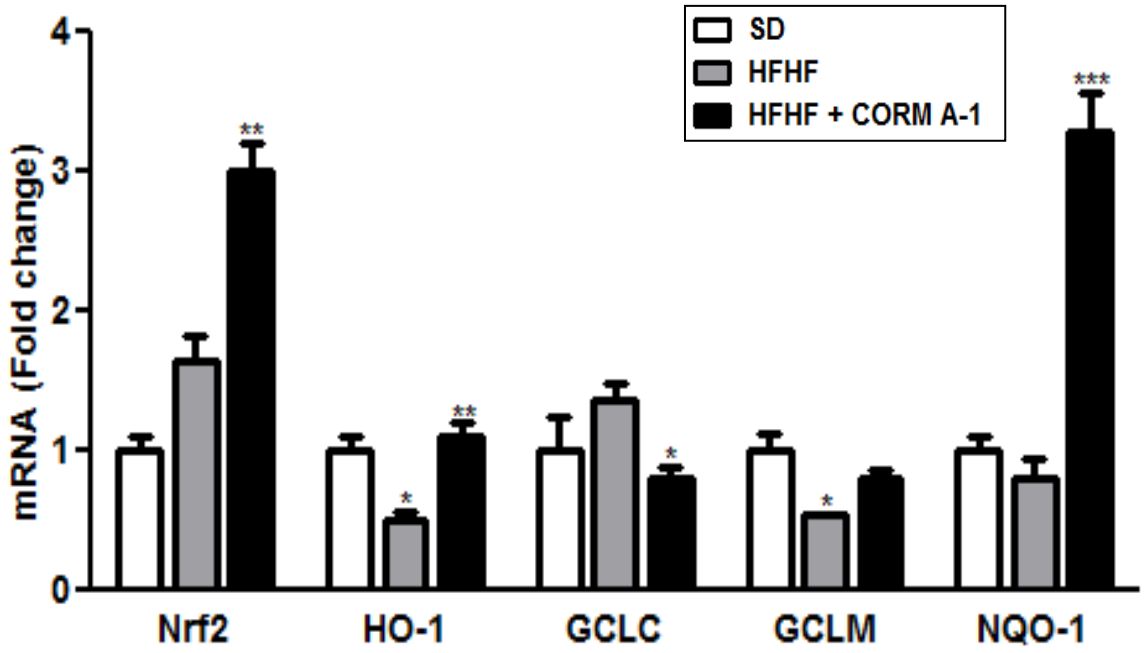


Fig.4A.1. CORM-A1 improves oxidative stress via upregulation of Nrf2-ARE genes in liver of HFHF fed mice. Results are expressed as mean ± S.E.M. *p<0.05, **p<0.01 or ***p<0.001 is when HFHF compared to SD and HFHF + CORM-A1 is compared to HFHF group.

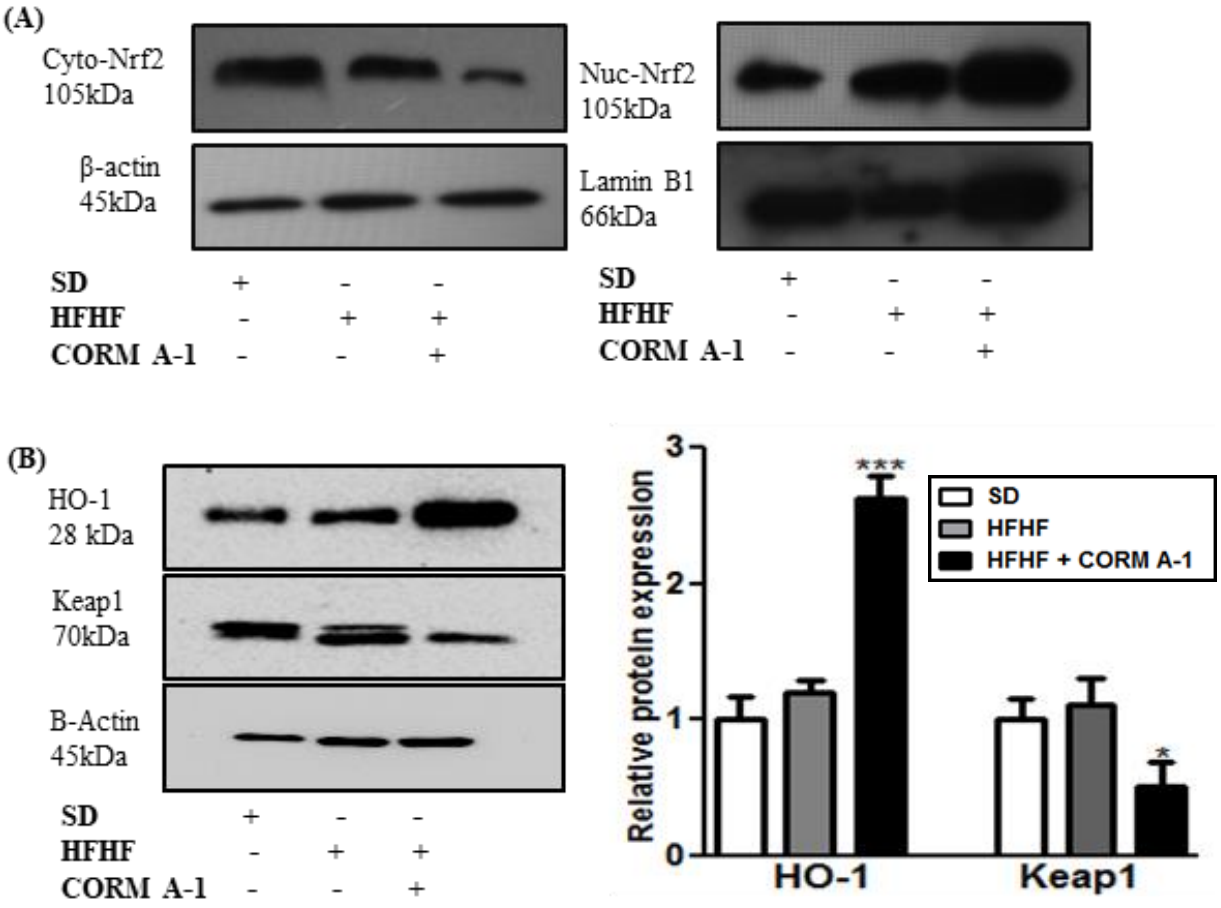


Fig.4A.2. CORM-A1 promotes Nrf2 translocation by modulating Keap1 expression and regulate ARE and lipid metabolic genes in liver of HFHF fed mice. (A) Western blot image of cytoplasmic and nuclear Nrf2 protein. (B) HO-1 and Keap1 protein content and their quantification. Results are expressed as mean ± S.E.M. *p<0.05, **p<0.01 or ***p<0.001 is when HFHF compared to SD and HFHF + CORM-A1 is compared to HFHF group.

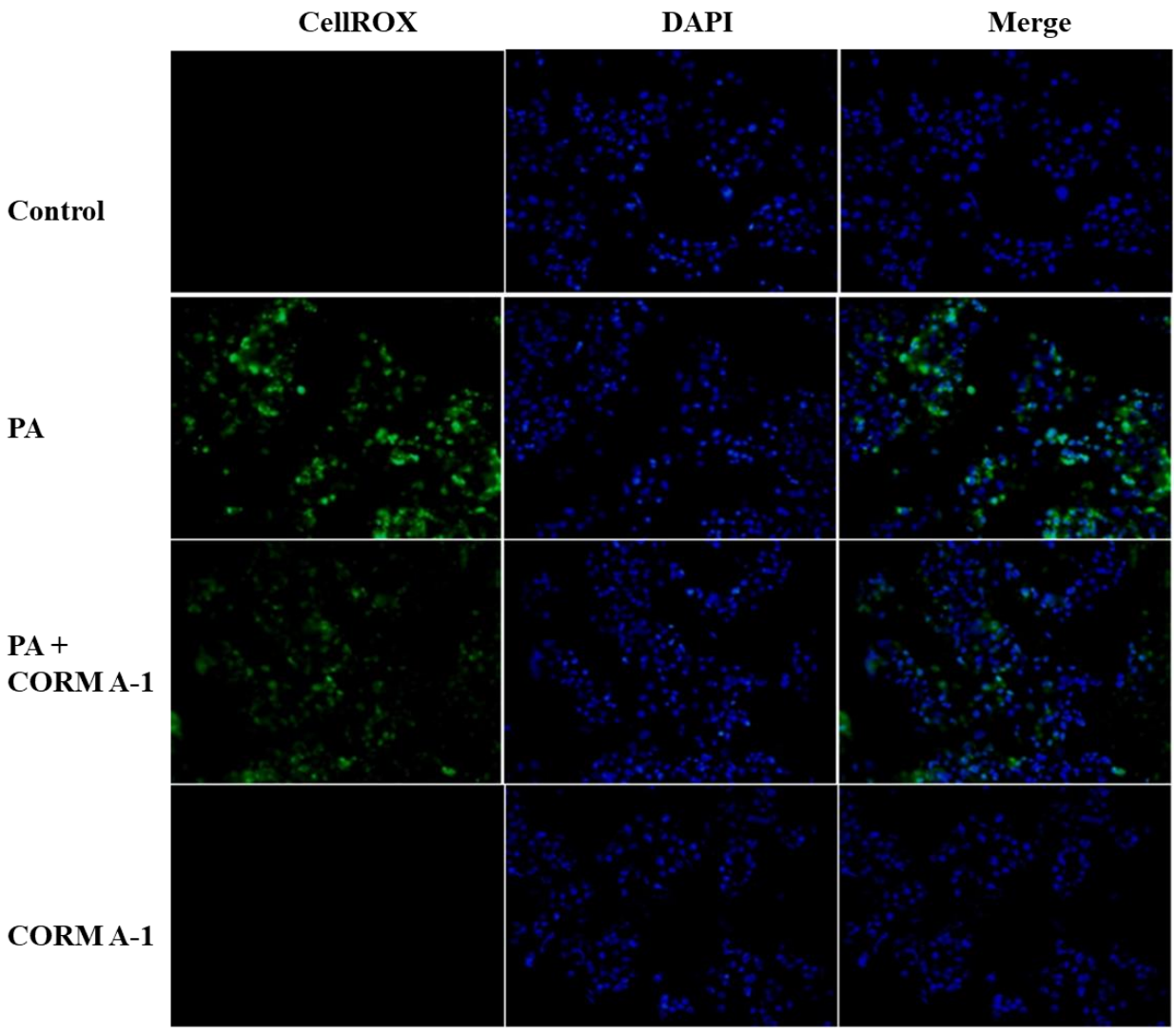


Fig.4A.3. CellROX staining of HepG2 cells showing CORM-A1 mediated improvement in oxidative stress.

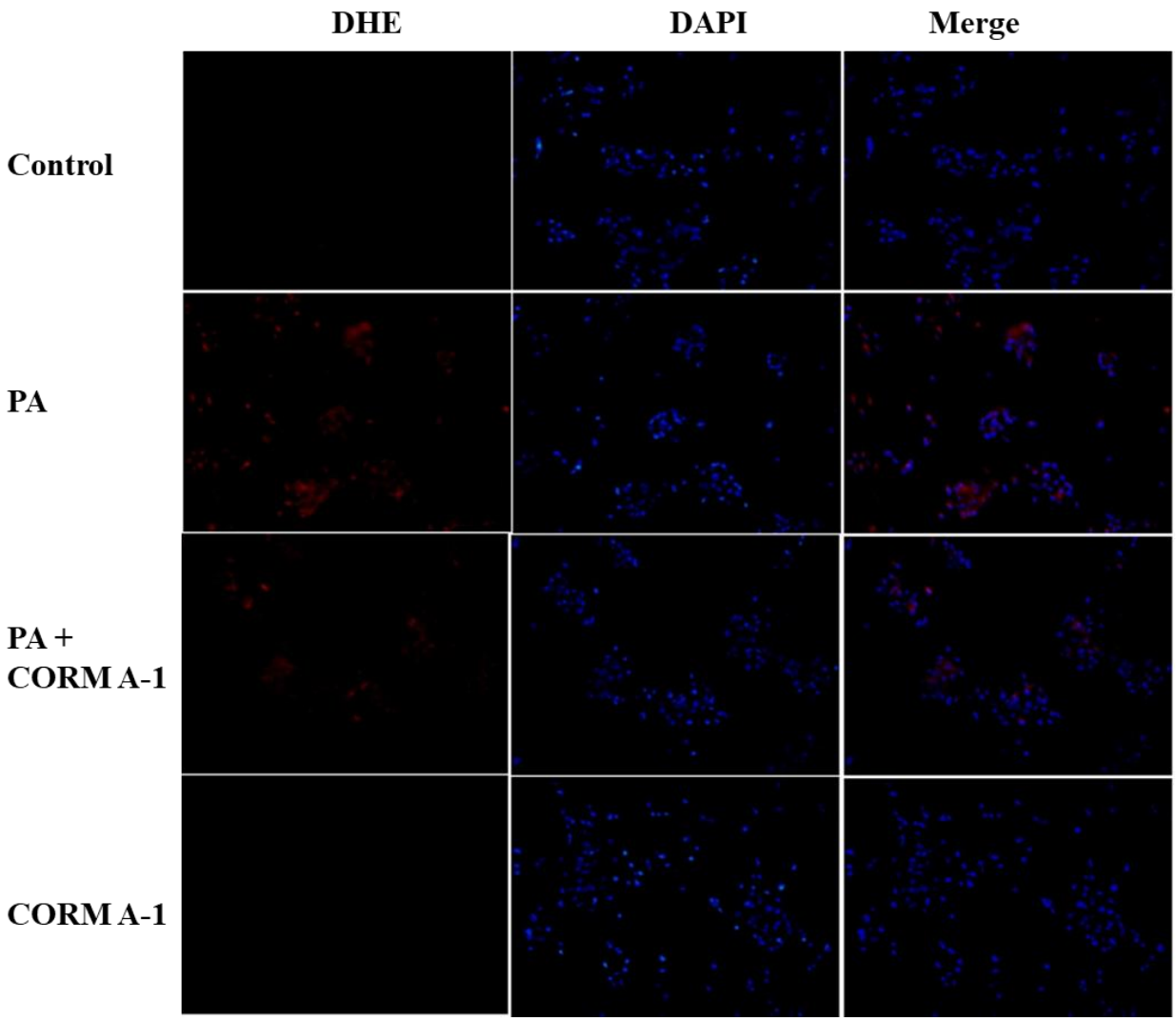


Fig.4A.4. DHE staining of HepG2 cells showing CORM-A1 mediated improvement in oxidative stress.

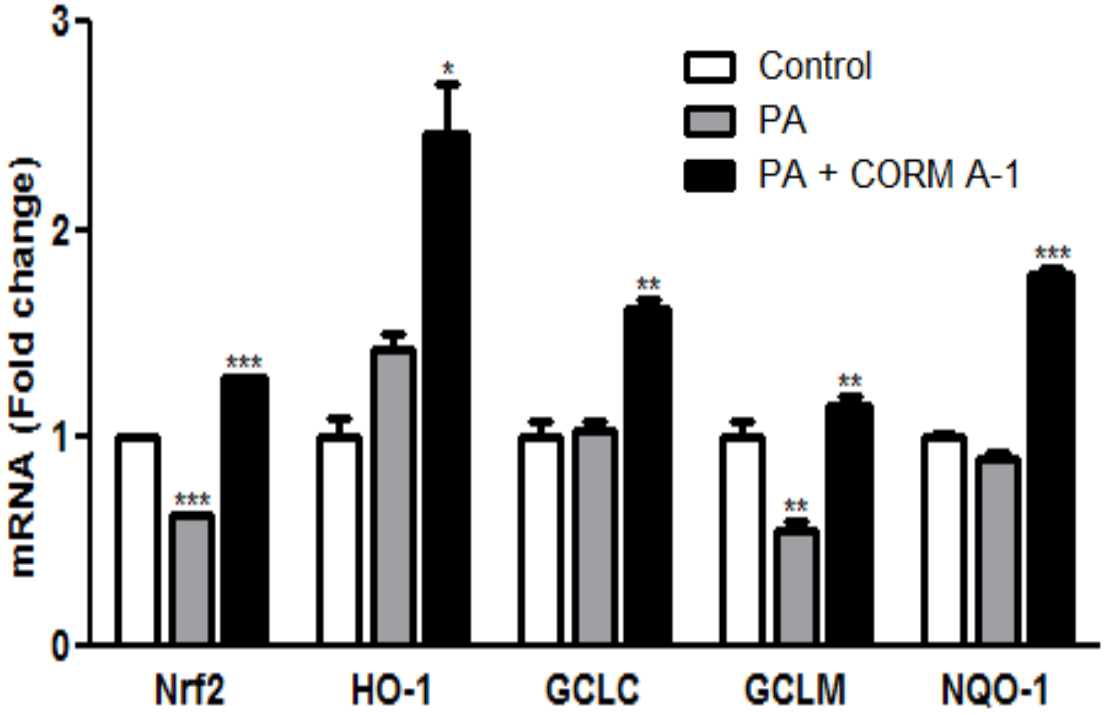


Fig.4A.5. CORM-A1 improves oxidative stress via Nrf2 translocation and activation of ARE genes in hepatocytes. Image showing expression levels of antioxidant gene recorded using qPCR analysis. Results expressed as mean±S.E.M. *p<0.05, **p<0.01 or ***p<0.001 is when PA treatment is compared to control cells and PA + CORM-A1 is compared to PA alone group.

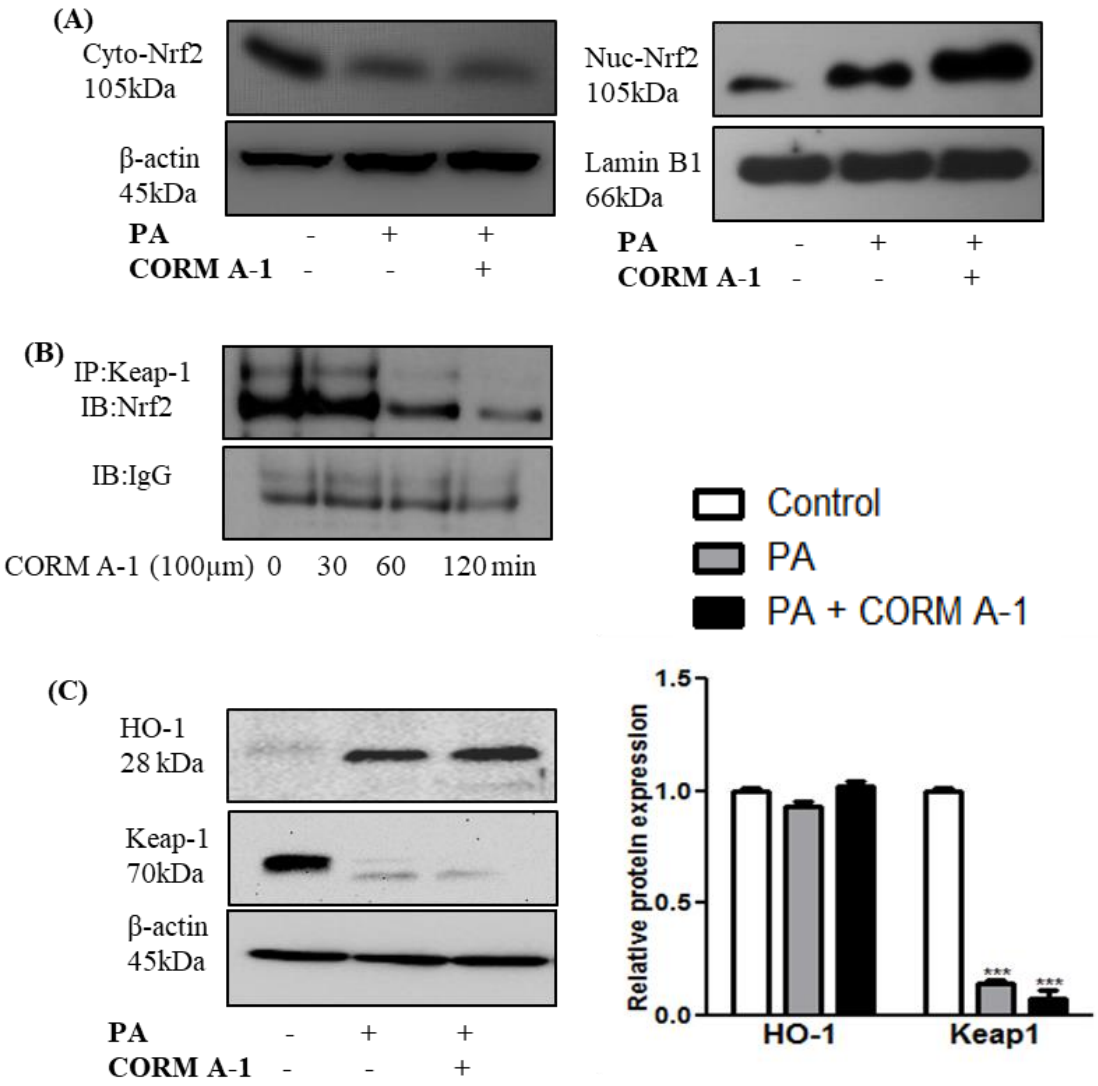


Fig.4A.6. CORM-A1 mediates Nrf2 translocation via downregulation of keap1 and activation of HO-1 protein. (A) representative blot image of Nrf2 protein in cytoplasm and nucleus (B) co-immunoprecipitation results demonstrating time depended decrement in keap1 protein. (C) qualitative and quantitative levels of HO-1 and Keap1 protein. Results expressed as mean ± S.E.M. *p<0.05, **p<0.01 or ***p<0.001 is when PA treatment is compared to control cells and PA + CORM-A1 is compared to PA alone group.

Discussion

High levels of CO in atmosphere (>1500 ppm) and resultant toxicity is well known but its endogenous production in humans (16·4 mmol/h) by HO-1 mediated heme cleavage and its role as gasotransmitter has drawn attention of scientific fraternity (Wu *et al.*, 2005). CO binds reversibly to its molecular targets that make pharmacokinetics simpler and relevant. Also, slow and sustained release of CO from CORM-A1 had been reported in biological systems. Persistently elevated lipid profile and an inflamed liver, over a period of time, makes a transition towards NAFLD from NASH through a complicated cascade of overlapping events that are now extensively reported. CORM-A1 imparts cytoprotection and improves cellular function, via Nrf2 activation in a variety of cells and tissues (Fagone *et al.*, 2011, Mangano *et al.*, 2018). There is no report on the same in steatotic liver. Multiple lines of evidence support an increased production of ROS in cells or tissues due to Nrf2 KO, and such scenario has also been documented in cardiomyocytes and mouse embryonic fibroblasts in culture (Leung *et al.*, 2003, Li *et al.*, 2014). In contrast, Nrf2 activation results in decreased ROS production by mitochondria and cytosolic NADPH oxidase system (Kovac *et al.*, 2015). Previous study from our lab on CORM-A1 induced prevention of APAP mediated hepatotoxicity and related role of Nrf2 forms the basis of our current study. In the present study we report CORM-A1 mediated nuclear translocation of Nrf2 and subsequent activation. Keap1 negatively regulates Nrf2 by protein-protein interaction and polyubiquitination from transcriptional activation of Nrf2-ARE genes (Jadeja *et al.*, 2016). The Nuclear translocation of Nrf2 is imperative and our observation on

CORM-A1 induced decrement in keap1 protein also justifies transcriptional activation of antioxidant genes like HO-1, GCLC, GCLM and NQO-1.

In silico docking analysis of CO [Chapter 1] had indicated that CORM-A1 induce Nrf2 activation via CO-mediated release of Nrf2 from kelch domain of Keap1. In the present study, the observed time dependent dissociation of Keap1 from Nrf2 protein endorses CORM-A1 mediated Nrf2 translocation in HepG2 cells. CORM-A1 mediated Nrf2 activation leads to upregulation of ARE genes that are in agreement with observations of our in vivo study. PA accounted for decrement in Nrf2 mRNA that, as a consequence, had a negative impact on lipid metabolism as indicated by increased level of de novo lipogenesis and lowered titers of lipid metabolizing mRNAs. CORM-A1 co-supplemented group was instrumental in preventing intracellular lipid accumulation (as evidenced by ORO staining) and the mRNA levels of said gene that were comparable to result obtained in CORM-A1 treated HepG2 cell.