# Chapter-3

## CORM-A1 improves Nonalcoholic steatohepatitis (NASH) in HFHF fed C57BL/6J mice

**Introduction**

The main events in NASH pathognomonics are excess lipid accumulation in hepat ocytes (simple hepatic steatosis), elevated oxidative stress and inflammation (Fuchs *et al.*, 2012), that furthers into cirrhosis and culminates in hepatocellular carcinoma (HCC). But subtle variation such as ethnicity, genetics, environment and nutritional practice leads to the reported discrepancy in patients of NASH (Day, 2010).

In present day scenario of developing and developed country a large amount people devour high fat, high fructose diet and live a sedentary lifestyle (Younossi *et al.*, 2018). There is constant increment in calories scale as physical activities have come down to minimum. The summative effect of the said lifestyle culminates into increased lipid titers in plasma as well as hepatocytes (Cheng *et al.*, 2016). Higher titers of lipid shift the metabolism from catabolism to anabolism, the resultant fat accumulation, in hepatocytes, paves way to beginning of onset of NASH, also referred to as first hit. Subsequently due to fat accumulation the cell experiences stress. Mitochondria gets overwhelmed with lipid load in the cell and fatty acid oxidation produces reactive oxygen species (ROS) that develops intracellular oxidative stress. Asymptomatic increment in circulating titers of aspartate transaminase (AST) and alanine transaminase (ALT) are pointers to onset of NASH pathology (Brunt, 2005).

Heightened levels of oxidative stress and ROS have been implicated to be the key players in metabolic disorders such as NASH (Takaki *et al.*, 2013). The reported attributes of CORM-A1 in alleviating oxidative stress and providing cytoprotection forms the basis of our hypothesis Previous study [Chapter 1&2] conducted with CORM-A1 reports Nrf2 activation and cytoprotection in t-BHP induced oxidative stress in HepG2 cells and APAP induced liver injury in Swiss albino mice (Upadhyay *et al.*, 2018). In this study, High fat high fructose (HFHF) fed C57BL/6J mice and palmitic acid treated HepG2 cells have been used as the experimental model of NASH. In this, CORM-A1 mediated corrective changes in regulatory genes governing cellular lipid metabolism and inflammation in NASH has been investigated.

**Experimental Design**

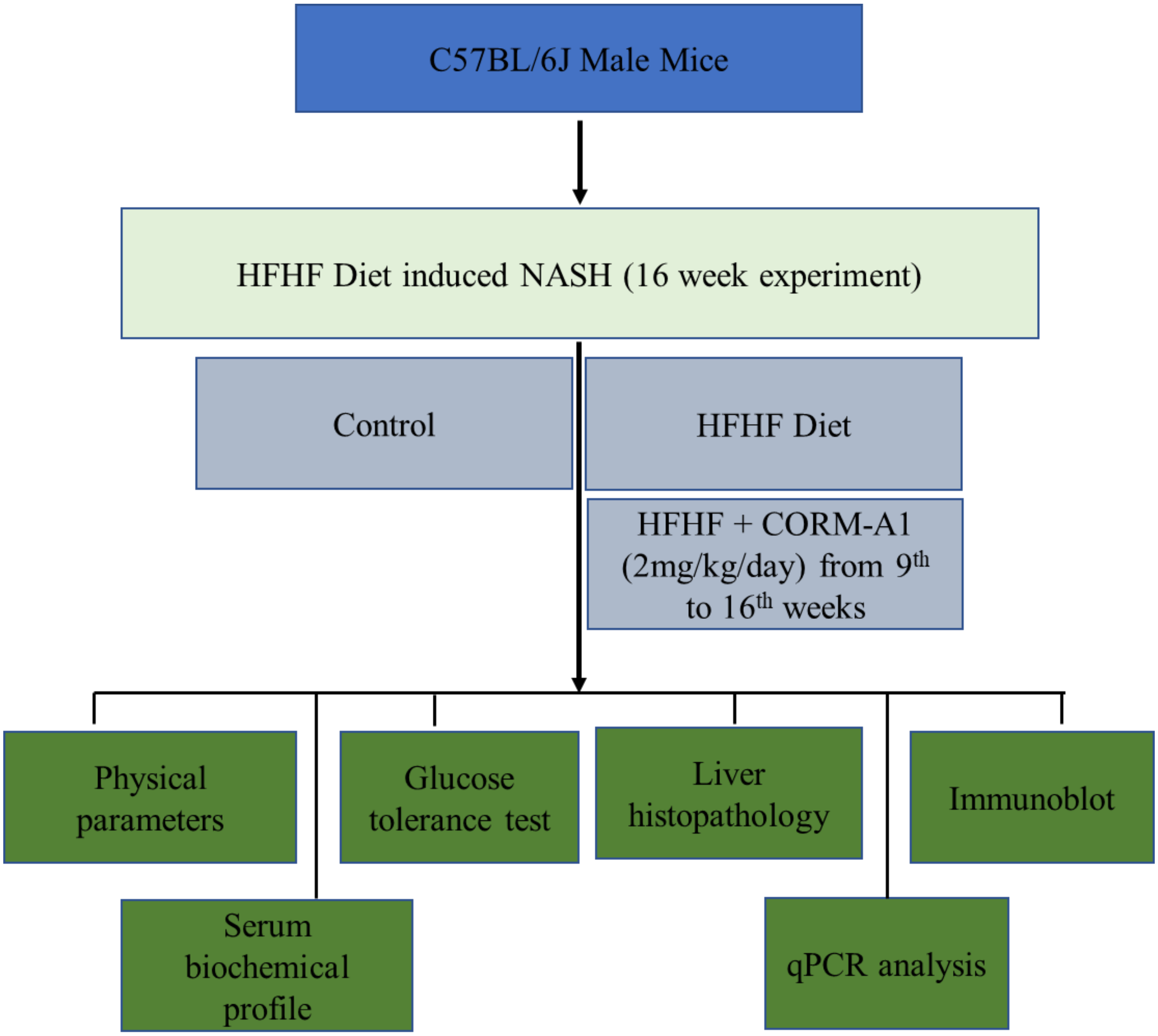
**Animal studies and experimental protocols**

Male C57BL/6J mice (6–8 weeks of age) were purchased from ACTREC Mumbai and maintained as per CPCSEA standard guidelines (23±2˚C, LD 12:12, laboratory chow and water ad libitum) followed by a week-long acclimatization. Protocol was approved by Institutional Animal Ethical Committee (IAEC) (Approval no. MSU-Z/IAEC/02-2017) and experiments were conducted in CPCSEA approved *(827/GO/Re/S/04/CPCSEA)* animal house facility of Department of Zoology, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India.

Mice were randomly divided into three groups with six animals per group. The entire period of experiment was of 16 weeks.

- Group I (SD) was fed with standard diet (SD).
- Group II (HFHF) was fed with high fat diet + 20% Fructose in water (HFHF diet) (Love *et al.*, 2017).
- Group III (HFHF + CORM-A1) was fed with HFHF diet for 16 weeks and CORM-A1 was injected (ip:2mg/Kg B.W.) from 9$^{th}$ to 16$^{th}$ weeks.

Food intake, water intake and body weights were recorded every week throughout the period of study.


C57BL/6J Male Mice
HFHF Diet induced NASH (16 week experiment)
Control
HFHF Diet
HFHF + CORM-A1 (2mg/kg/day) from 9th to 16th weeks
Physical parameters
Glucose tolerance test
Liver histopathology
Immunoblot
Serum biochemical profile
qPCR analysis

## Results

### CORM-A1 treatment attenuates weight gain in HFHF fed mice.

Mice (n=18) were maintained on HFHF diet for the period of 16 weeks for development of NASH. At the end of 8 weeks, mice (n=6) were treated with CORM-A1. CORM-A1 was injected (2mg/kg; ip; Daily injection) from 9$^{th}$ to 16$^{th}$ weeks along with HFHF diet. Body weights of HFHF fed mice were significantly higher than that of control mice at the end of 16 weeks. CORM-A1 treatment to HFHF fed mice resulted in lack of weight gain as compared to SD mice (Fig.3.1-A), without any significant difference in food intake (Fig.3.1-C). Also, HFHF fed mice recorded significant increment in abdominal fat as compared to the control mice but the CORM-A1 treated mice had visibly less fat accumulation (Fig.3.1-B). Section of visceral adipose tissue revealed larger adipocytes diameter in HFHF fed mice as compared to SD mice. CORM-A1 treated HFHF fed mice recorded significant decrement in adipocytes diameter and had a mixed population of larger and medium size adipocytes (Fig.3.2).

### CORM-A1 alleviates histopathology of steatotic liver

Preliminary examination of liver of HFHF fed mice showed difference in color (pale yellow) as compared to SD mice (dark reddish brown) (Fig.3.3-A). Also, HFHF fed mice recorded significant increment in liver to body weight ratio (~35%). Liver of CORM-A1 treated mice was reddish brown in color and its weight was comparable to that of SD mice (Fig.3.4-C).

H&E stained sections of liver showed significantly higher amount of micro vesicular hepatic steatosis in HFHF fed mice as compared to SD mice. Whereas, no such evidence of hepatic steatosis was seen in CORM-A1 treated mice (Fig.3.3-B). Steatotic changes in the liver were further confirmed by detailed microscopic evaluation that revealed a greater number of ballooning hepatocytes and mallory hyaline formation as compared to CORM-A1 treated mice (Fig.3.4-A&B). Taken together, NASH like condition was clearly prevailing at the end of 16 weeks in HFHF fed mice as compared to CORM-A1 treated mice.

**CORM-A1 improves liver functional enzymes in HFHF fed mice**

Functional status of liver was estimated by assaying the activity levels of serum marker enzymes (AST, ALT and ALP) in control and treated mice to corroborate histopathological observation. The results showed a significant increment in the activity levels of the said parameter in HFHF fed mice at the end of 16 weeks, but CORM-A1 treatment resulted in reduced activity levels of these enzymes (Fig.3.5).

**CORM-A1 maintain a healthier metabolic profile in HFHF fed mice**

HFHF fed mice recorded significant increment in serum glucose and decrement in serum protein, but CORM-A1 treatment accounted for reciprocal changes in the said parameters. Also, the serum lipid profile (TL, TC, TG, LDL, VLDL, CHL/HDL, LDL/HDL ratio) was significantly elevated in HFHF fed mice but CORM-A1 treatment recorded significant decrement. A significant decrement in serum HDL was also recorded in steatotic mice but CORM-A1 treatment accounted for moderate non-significant increment in the same (Fig.3.6).

**CORM-A1 regulates mRNA profile of lipid metabolizing genes in liver of HFHF fed mice.**

To determine anti-NASH potential of CORM-A1, we assessed mRNA expression of lipid regulatory genes in liver samples of control and treated mice. A significant increment in mRNA level of FAS and Srebp-1C were recorded in HFHF fed mice but CORM-A1 treatment accounted for significantly lowered mRNA expression. Though the changes in PPAR-α were non-significant in HFHF fed mice, a moderate decrement was recorded in CORM-A1 treated mice. mRNA profiles of CD-36, SIRT-1 and CPT-1 recorded moderate yet non-significant changes in steatotic liver but prominent increment in CORM-A1 treated mice was noteworthy (Fig.3.7.).

**CORM-A1 improves fasting blood glucose and glucose tolerance in liver of HFHF fed mice.**

To investigate effect of CORM-A1 on fasting blood glucose and insulin resistant, we conducted IPGTT test using Accu-Chek Active blood glucose meter in whole blood obtained from tail vein. HFHF fed mice recorded higher blood glucose as compared to CORM-A1 treated mice. Compared to SD mice, HFHF fed mice indicate reduced glucose tolerance which is possibly due impaired clearance of exogenous glucose as indicated by high glucose level. CORM-A1 treatment was effective in clearing extra glucose load hence showed lowered levels at 120 min as compared to HFHF fed mice (Fig.3.8).

**CORM-A1 alleviates hepatic inflammation in HFHF fed mice**

Increased hepatic inflammation is hallmark of diet induced NASH in human. In our study we determine the effect of CORM-A1 on hepatic inflammation. CORM-A1 treatment accounted for significant decrement in mRNA expression of inflammatory genes (IL-1β, IL-6 and TNF-α) in HFHF fed mice (Fig.3.9)

**Qualitative and quantitative assessment of NASH in HepG2 cells**

PA treated HepG2 cells were used as an in vitro model to affirm the findings in vivo. PA supplementation to HepG2 cell resulted in dose dependent (10-1000 µm; PA) decrement in cell viability. A study performed on HepG2 cells revealed that 100 µM PA recorded ~50% cell viability. Also, dosing of CORM-A1 (100 µm) recorded 18% increase in cell viability at the end of 24h (Fig.3.10). Lipid accumulation in HepG2 cell was assessed using ORO staining. Quantitative analysis of ORO staining revealed higher accumulation of lipid in PA supplemented cells than CORM-A1 treated cells (Fig.3.11).

**CORM-A1 improves lipid metabolizing gene in PA treated HepG2 cells**

PA treated HepG2 cells recorded significantly elevated mRNA levels of FAS and Srebp-1c. Whereas, PPAR-α and SIRT-1 mRNA level in the same group recorded nonsignificant decrement. Also, the mRNA levels of CD36 were found to be significantly low in this group. CORM-A1 co-supplementation accounted for non-significant decrement in FAS but significant increment in Srebp-1c, PPAR-α, CD-36 and SIRT-1 mRNA levels (Fig.3.12).

**Table-3.1.** Effect of CORM-A1 on physical parameter of HFHF fed C57BL/6J mice.

| | **Control** | **HFHF** | **HFHF+CORM-A1** |
|---|---|---|---|
| Body Weight (g) | 23.10±0.633 | 31.30±0.144 | 21.60±0.747 |
| Abdominal Circumference (cm) | 6.088±0.878 | 7.67±0.108 | 5.75±0.880 |
| Liver: Bodyweight Ratio | 0.039±0.0005 | 0.045±0.002 | 0.036942±0.001 |

**Table-3.2.** Effect of CORM-A1 on serum liver functional enzymes.

| | **Control** | **HFHF** | **HFHF+CORM-A1** |
|---|---|---|---|
| AST | 90.31±6.629 | 128.30±20.403 | 72.866±5.570 |
| ALT | 36.76±5.826 | 72.07±17.170 | 32.41±1.603 |
| ALP | 117.82±40.875 | 179.48±0.785 | 83.78±11.007 |

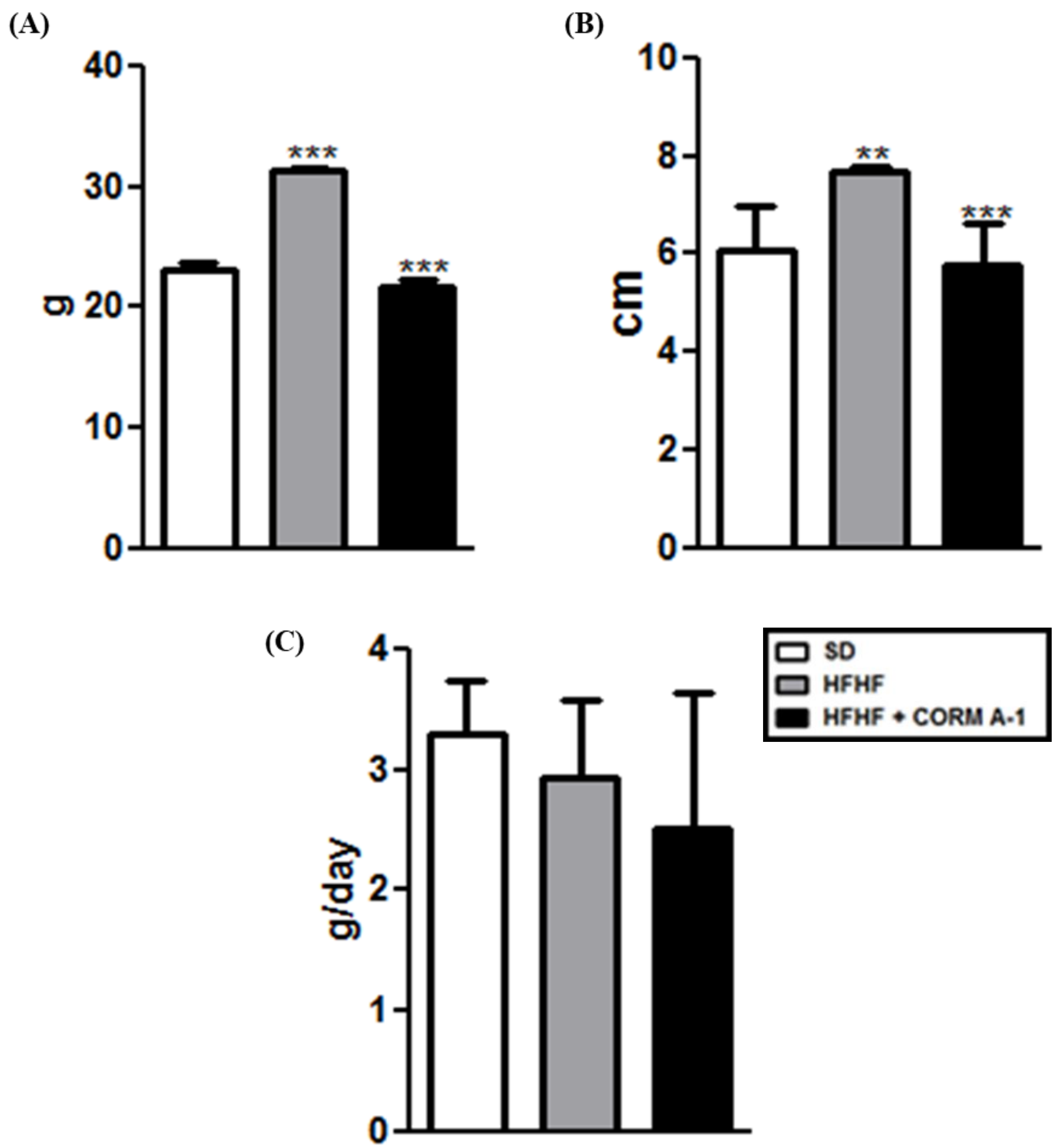



**Fig.3.1.** Effect of CORM-A1 on (A) Body weight gain (B) Abdominal circumference and (C) food intake. Results are expressed as mean±S.E.M. *p<0.05, **p<0.01 or ***p<0.001 is when HFHF compared to SD and HFHF + CORM-A1 is compared to HFHF group.

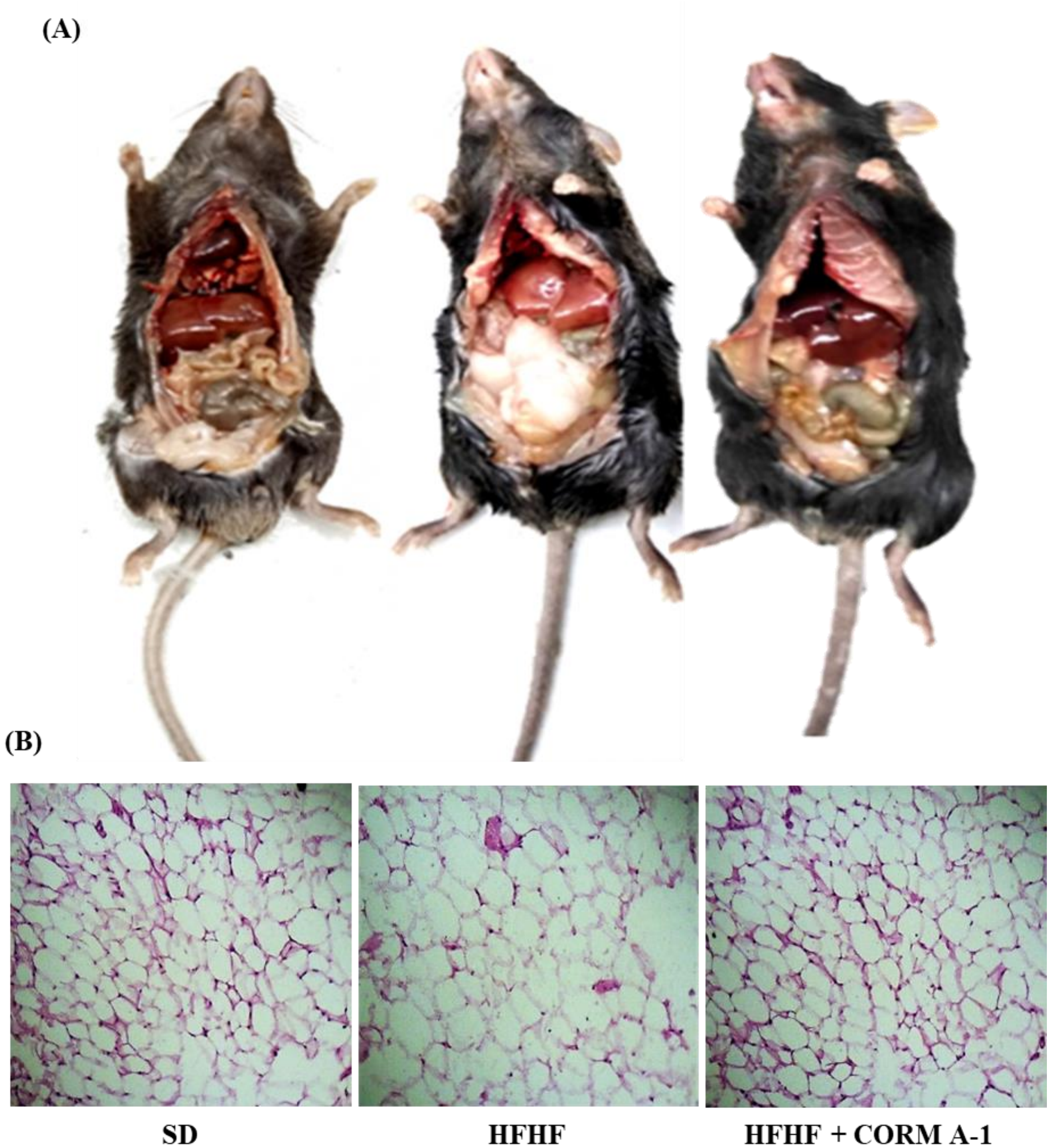



**Fig.3.2.** CORM-A1 treatment attenuates weight gain in HFHF fed mice. C57BL/6J mice viscera showing adipose build-up and (B) cross section area of respective adipocytes. (magnification 200X).

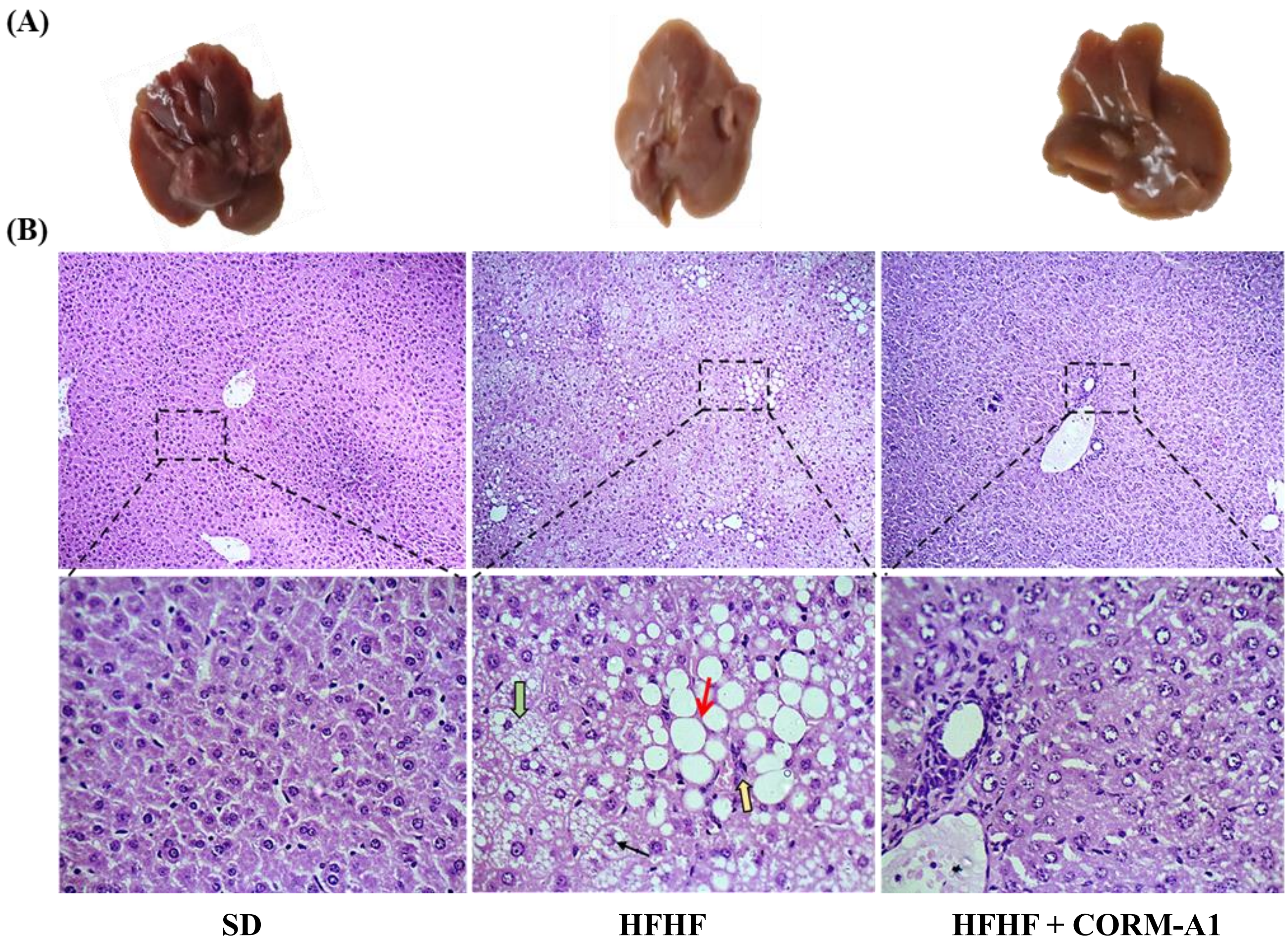



**Fig.3.3.** Mice treated with CORM-A1 showed improvement in histopathology of liver. (A) Autopsy and (B) H&E staining of liver section of C57BL/6J mice fed with SD, HFHF diet or HFHF + CORM-A1 (magnification 100X and 400X), Black arrow indicate Mallory hyaline, Green arrow indicate ballooning hepatocytes, yellow arrow indicates inflammation and red arrow indicate steatosis in liver.

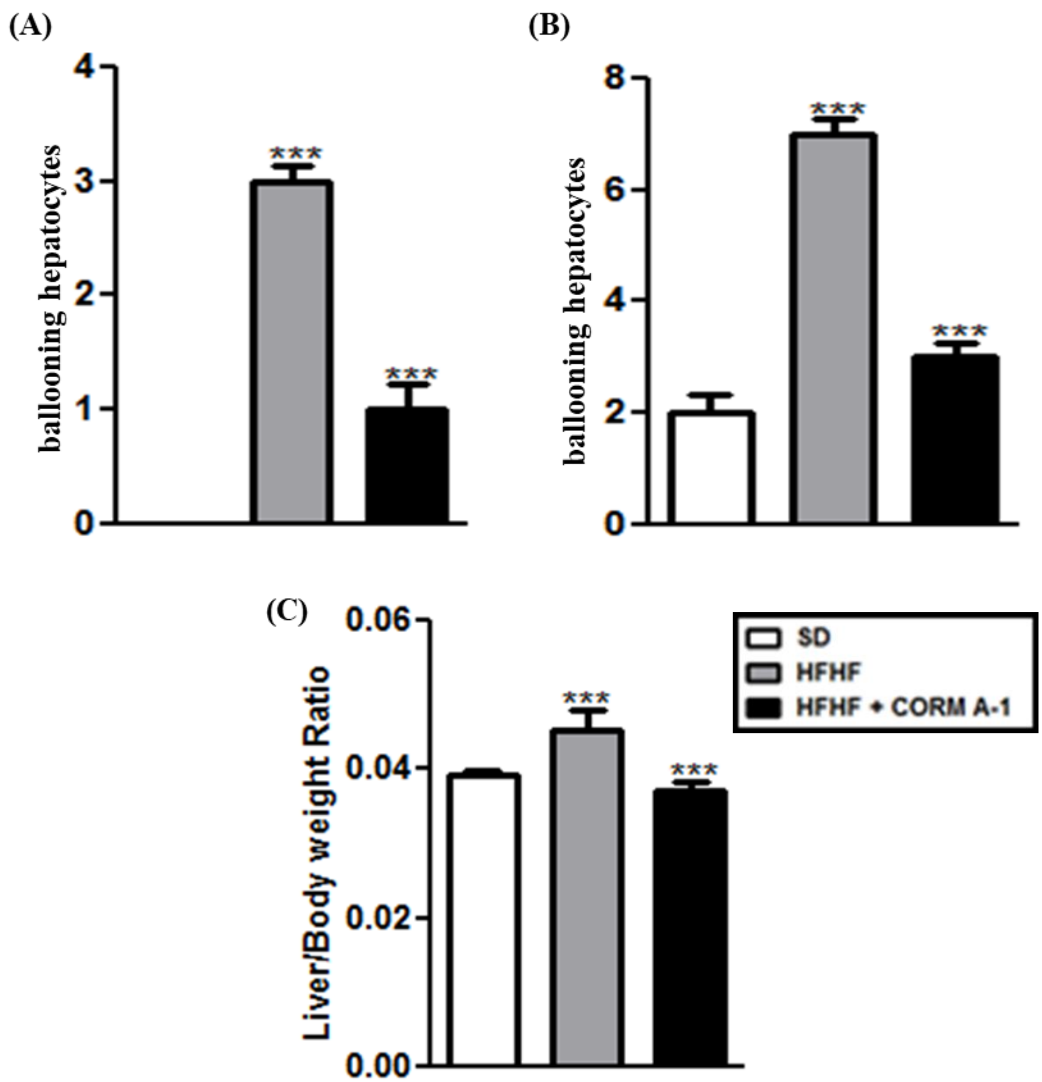



**Fig.3.4.** (A) Scoring of ballooning hepatocytes. (B) Scoring of steatosis and (C) Liver/Body weight ratio. Results are expressed as mean±S.E.M. *p<0.05, **p<0.01 or ***p<0.001 is when HFHF compared to SD and HFHF + CORM-A1 is compared to HFHF group.

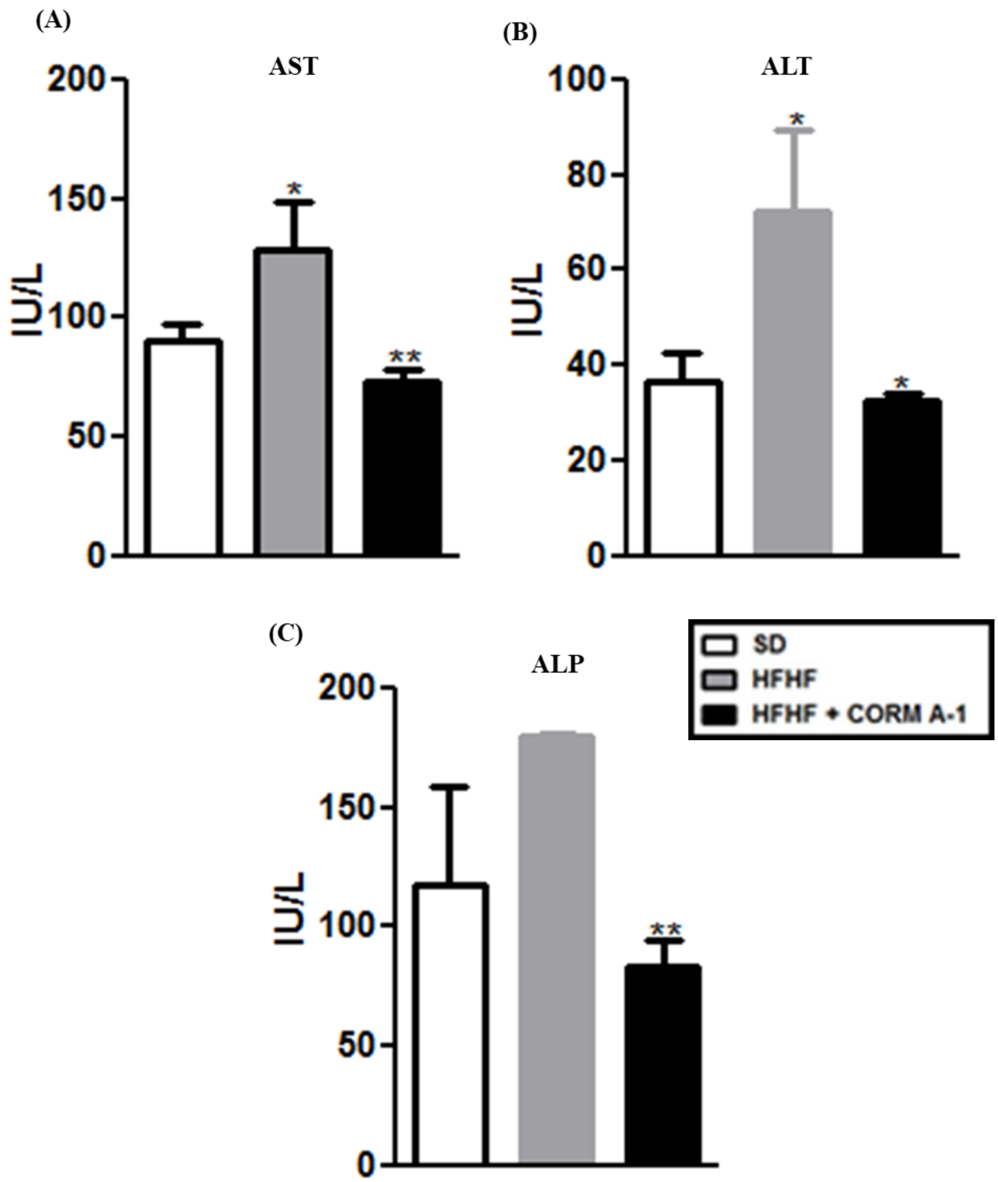
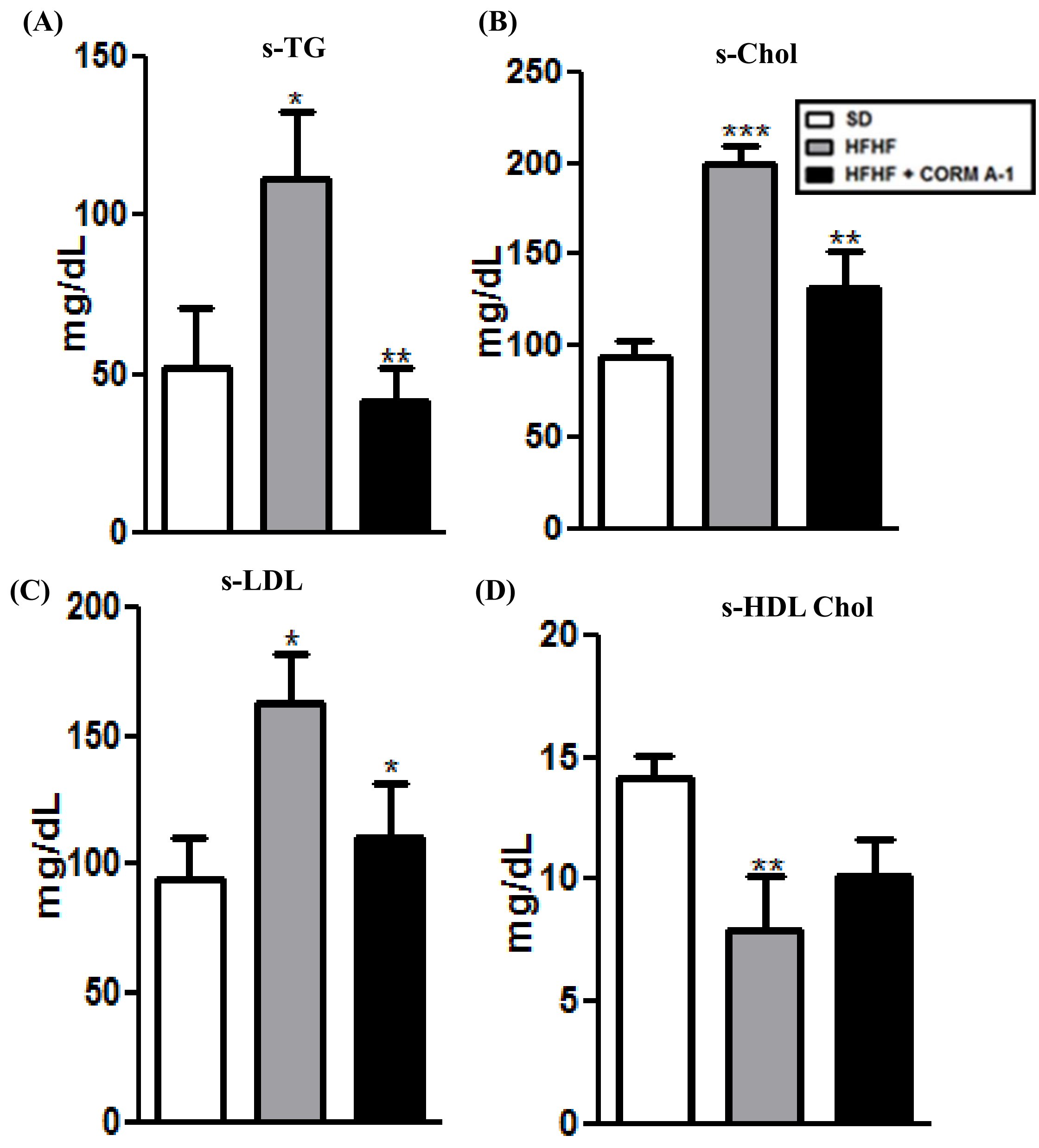



**Fig.3.5.** CORM-A1 improves liver function enzymes (A) AST (B) ALT and (C) ALP. Results are expressed as mean±S.E.M. *p<0.05, **p<0.01 or ***p<0.001 is when HFHF compared to SD and HFHF + CORM-A1 is compared to HFHF group.


(A)
s-TG
150
100
50
0
mg/dL
*
**
(B)
s-Chol
250
200
150
100
50
0
mg/dL
***
**
SD
HFHF
HFHF + CORM A-1
(C)
s-LDL
200
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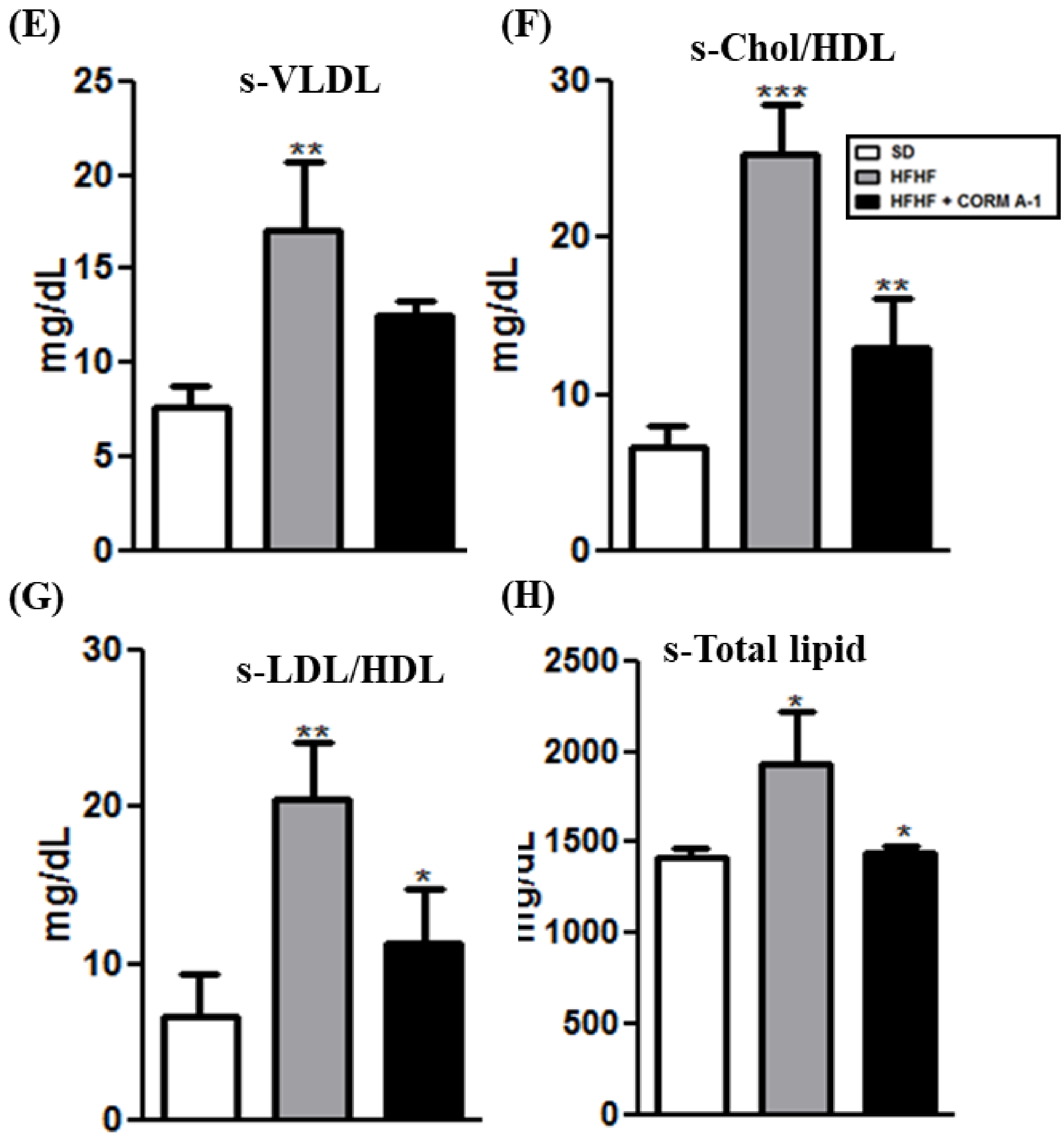


**Fig.3.6.** Mice treated with CORM-A1 maintained a healthier metabolic profile on HFHF diet. serum lipid profiles showing (A) s-TG (B) s-Chol (C) s-LDL Chol (D) s-HDL Chol (E) s-VLDL (F) s-Chol/HDL (G) s-LDL/HDL and (H) s-Total lipids. Results are expressed as mean±S.E.M. *$p<0.05$, **$p<0.01$ or ***$p<0.001$ is when HFHF compared to SD and HFHF + CORM-A1 is compared to HFHF group.

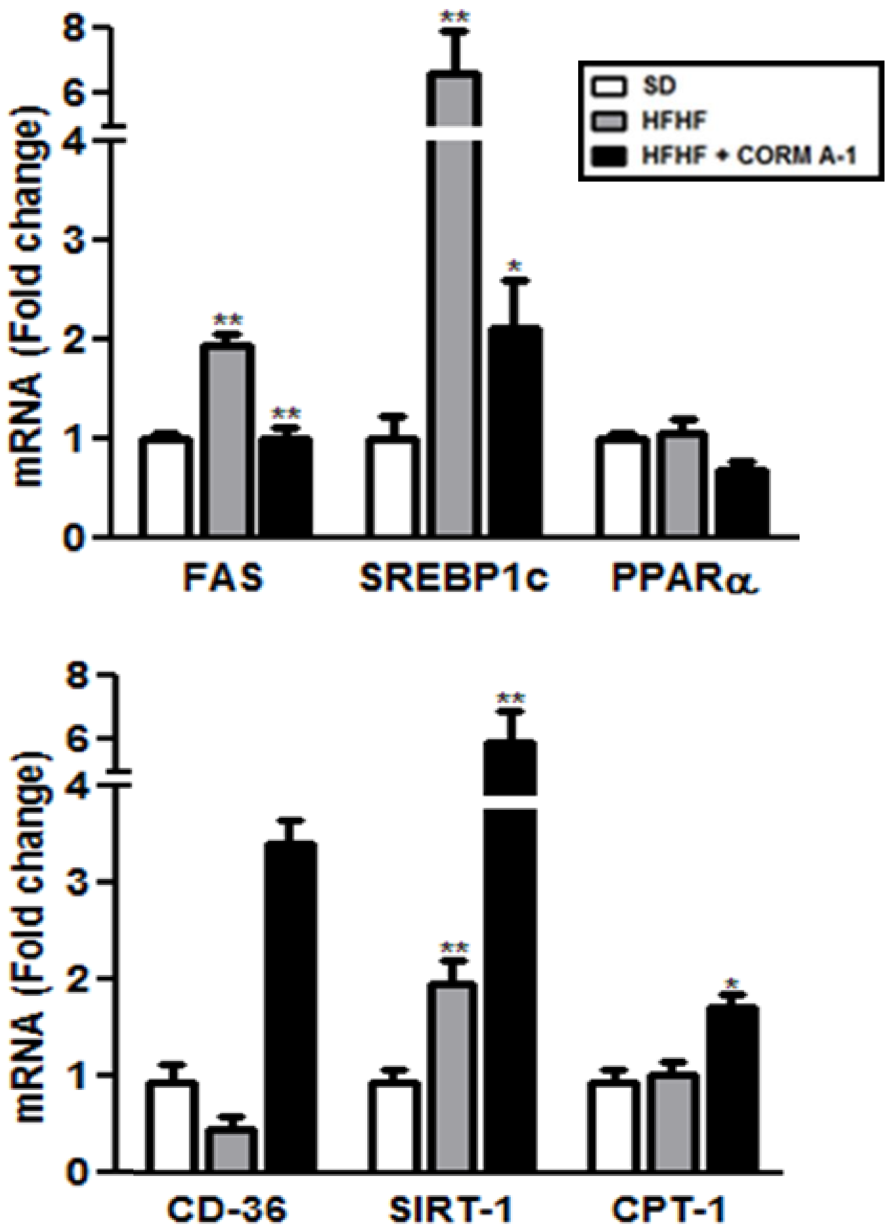


**Fig.3.7**. Changes in mRNA expression of genes governing hepatic lipid metabolism in liver. Results are expressed as mean±S.E.M. *p<0.05, **p<0.01 or ***p<0.001 is when HFHF compared to SD and HFHF + CORM-A1 is compared to HFHF group.

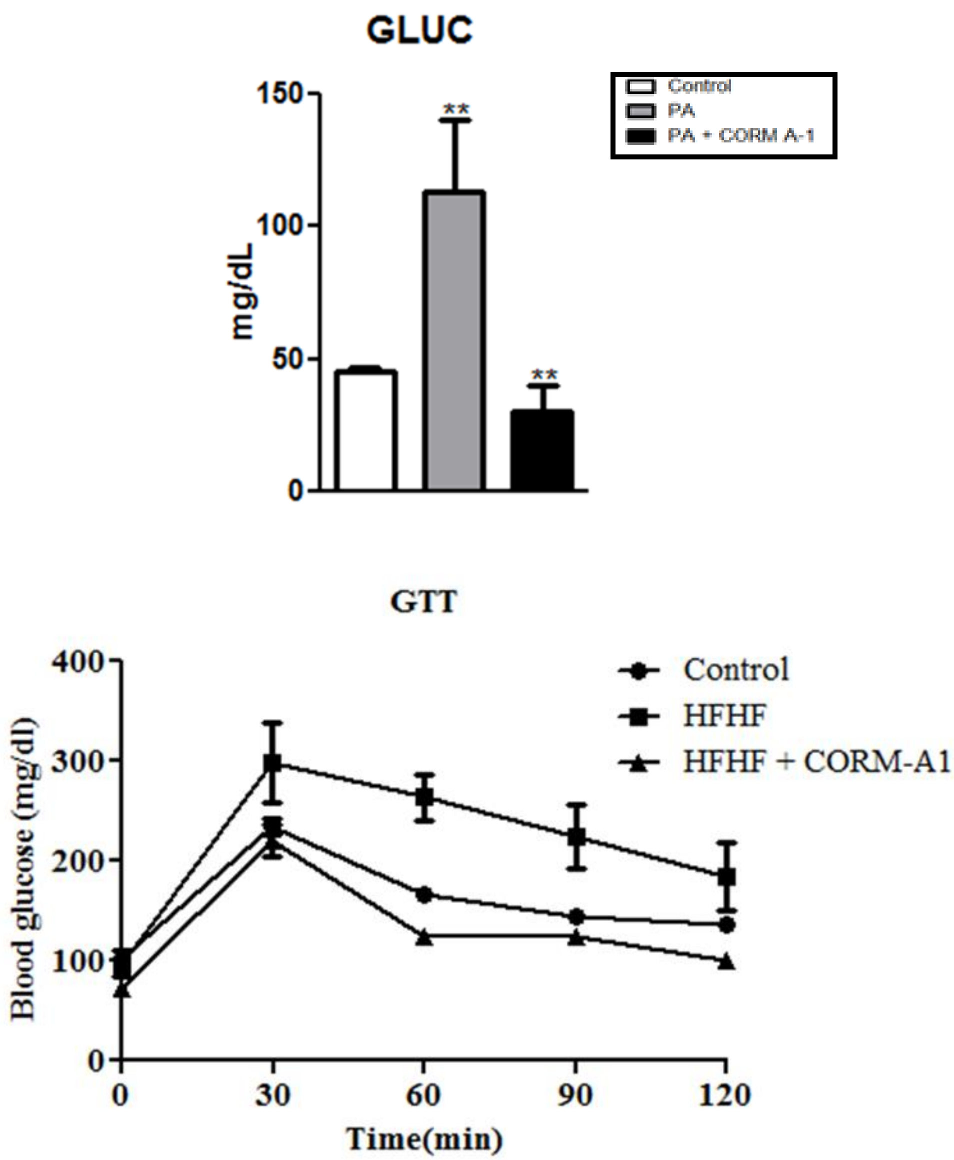


**Fig.3.8.** Fasting serum glucose and IPGTT test at the end of 16 weeks experiments. Results are expressed as mean±S.E.M. *p<0.05, **p<0.01 or ***p<0.001 is when HFHF compared to SD and HFHF + CORM-A1 is compared to HFHF group.

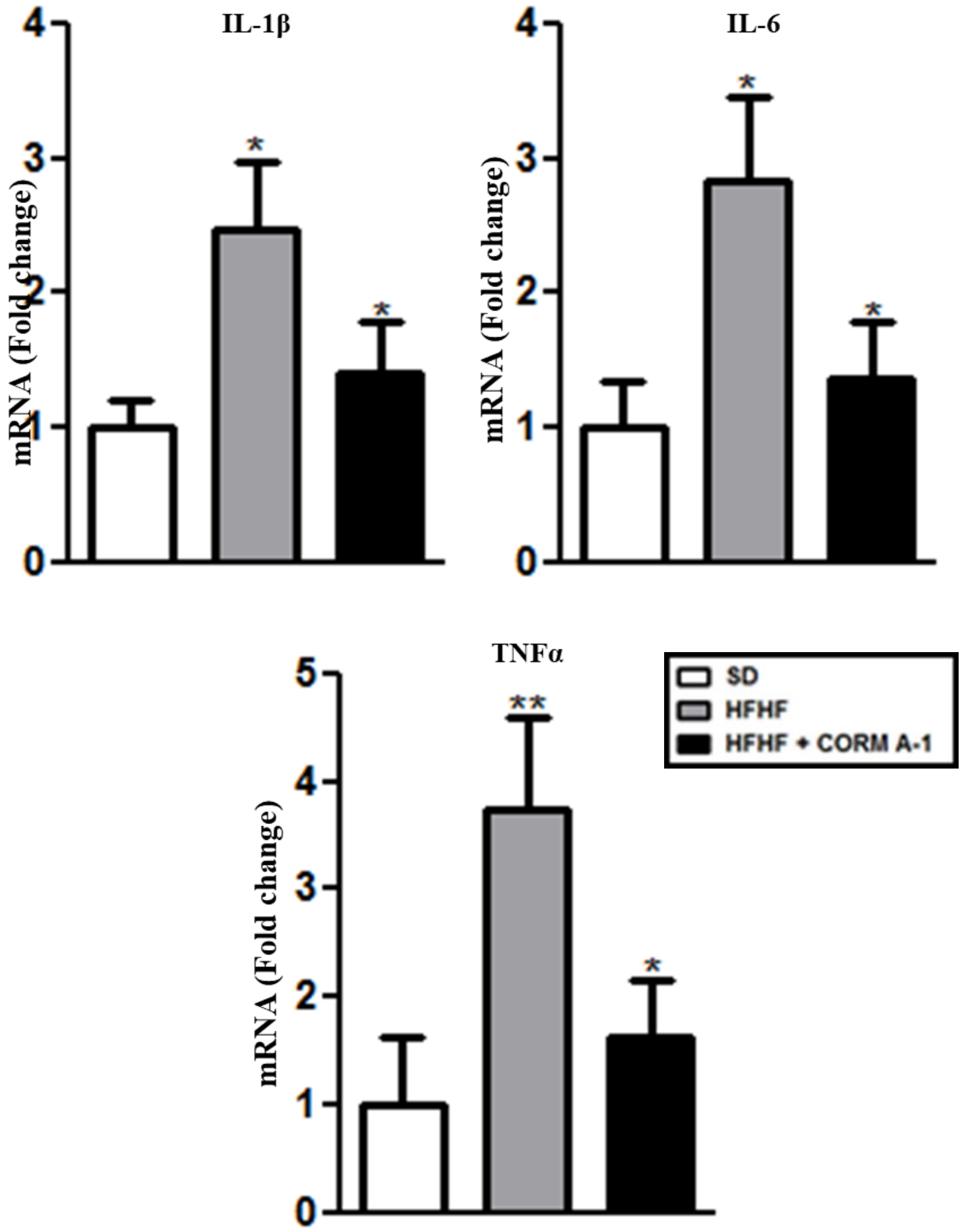


**Fig.3.9.** mRNA expression of inflammatory genes IL-1β, IL-6, TNF-α. Results are expressed as mean±S.E.M.. *p<0.05, **p<0.01 or ***p<0.001 is when HFHF compared to SD and HFHF + CORM-A1 is compared to HFHF group.

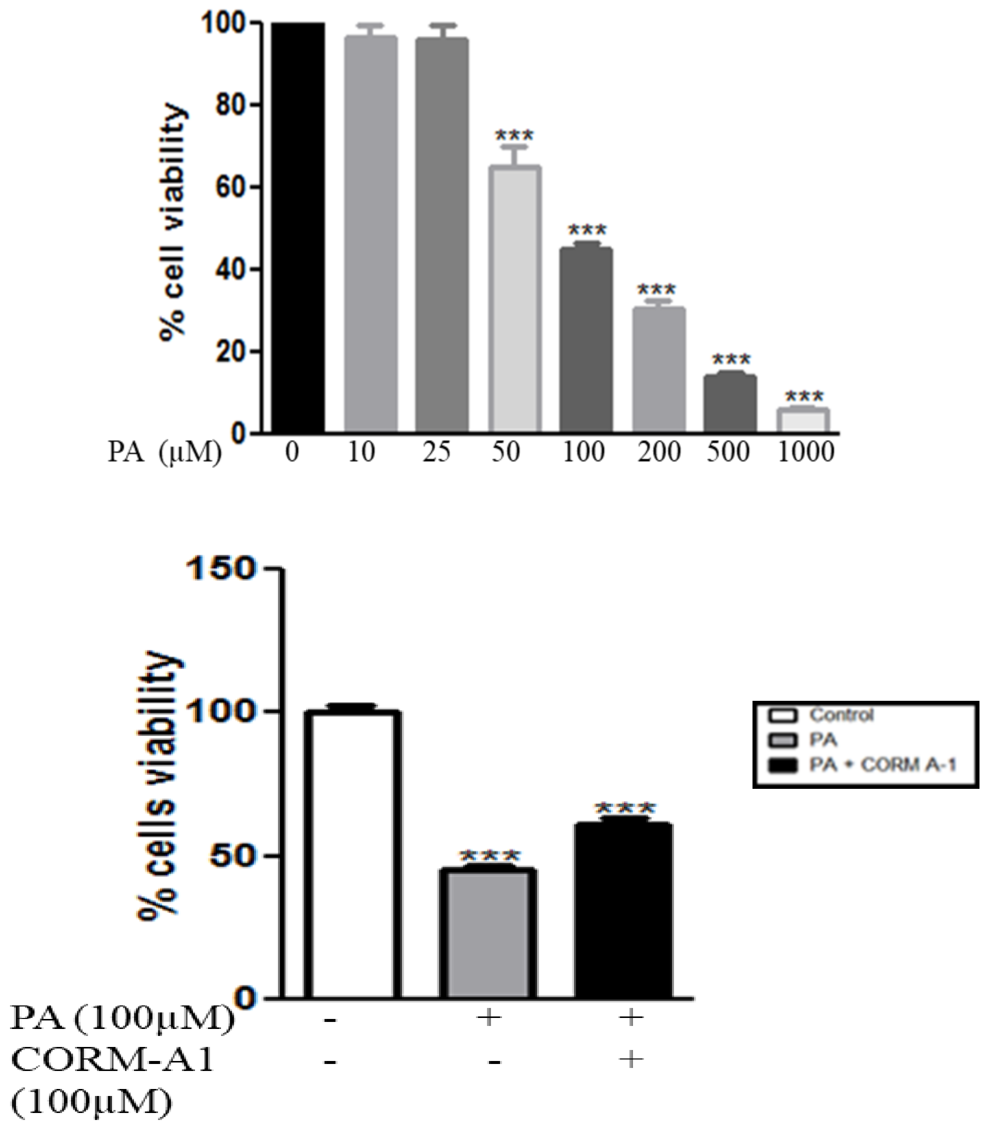


**Fig.3.10.** Cytotoxicity assessment using MTT assay. (A) % cell viability of PA treated HepG2 cells and (B) CORM-A1 co-supplementation to PA treated HepG2 cells. Results expressed as mean±S.E.M. *p<0.05, **p<0.01 or ***p<0.001 is when PA treatment is compared to control cells and PA + CORM-A1 is compared to PA alone group.

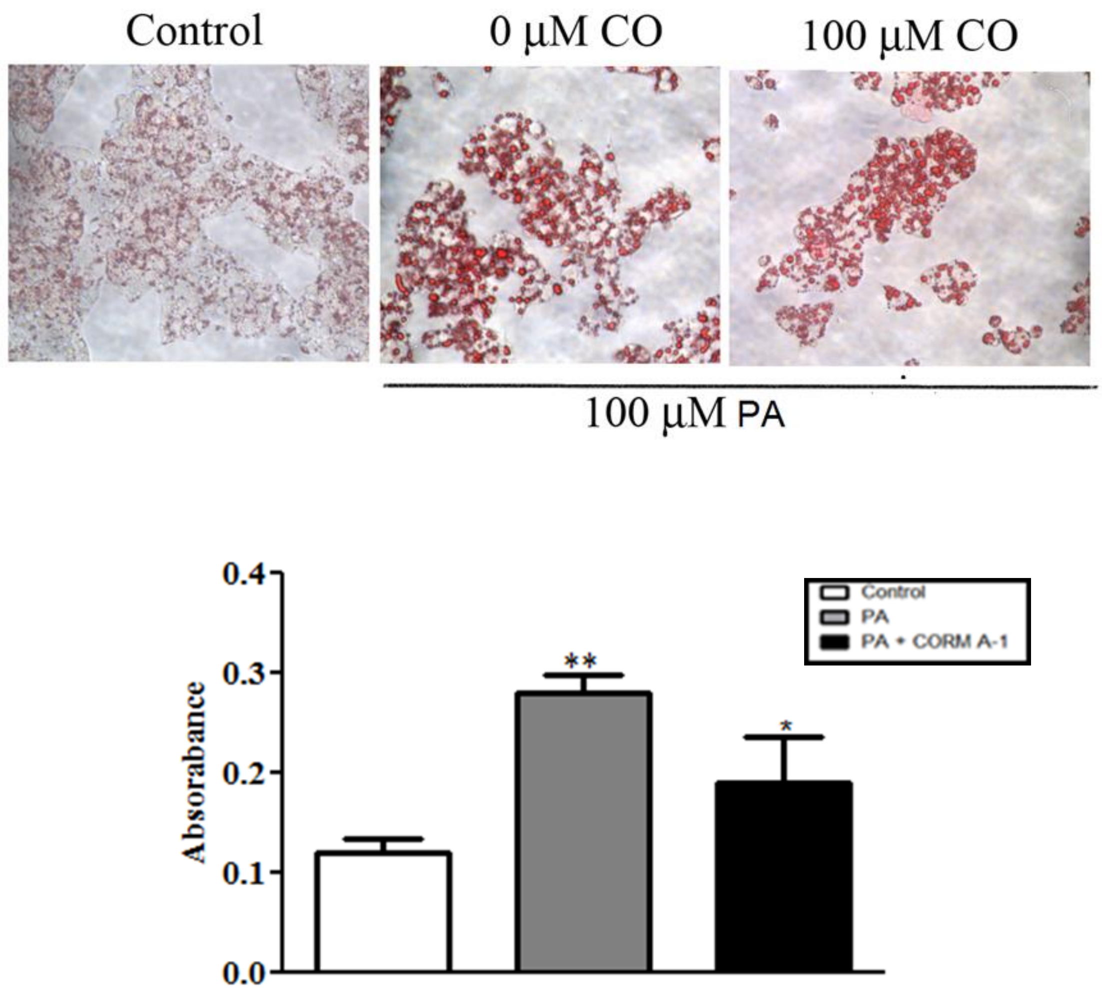


**Fig.3.11.** Qualitative and quantitative assessment of NASH in HepG2 cell. Results expressed as mean±S.E.M. *p<0.05, **p<0.01 or ***p<0.001 is when PA treatment is compared to control cells and PA + CORM-A1 is compared to PA alone group.

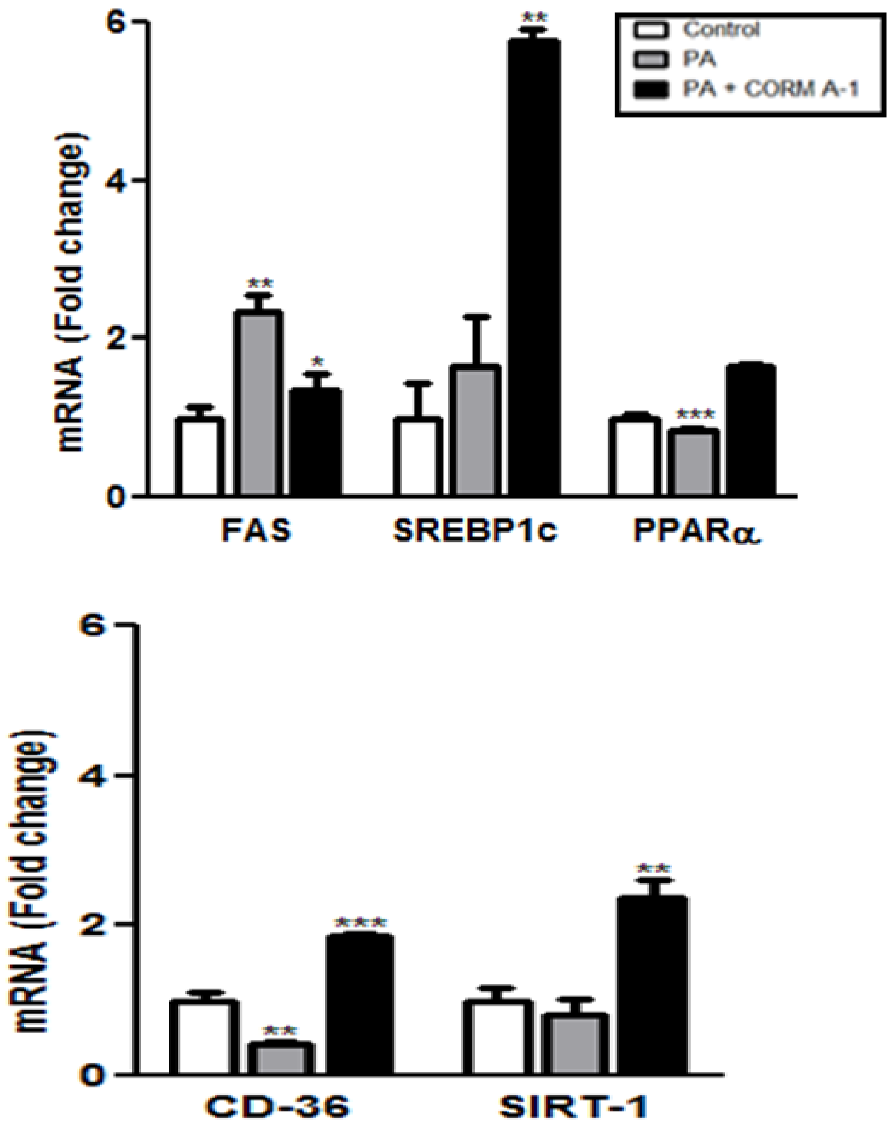


**Fig.3.12.** CORM-A1 regulates lipid mRNAs of PA treated HepG2 cells. Results are expressed as mean±S.E.M. *p<0.05, **p<0.01 or ***p<0.001 is when PA treatment is compared to control cells and PA + CORM-A1 is compared to PA alone group.

**Discussion**

CO is endogenously produced by HO-1 mediated heme cleavage and is detectable in the exhaled air of a healthy human. About two decades ago, involvement of CO was reported in several cellular process including oxidative stress, inflammation, cytoprotection and vasodilation (Motterlini *et al.*, 2010). Protective or detrimental effects of CO have been attributed to its critical dosage thus challenging its clinical use. However, scientific wisdom on its precise dose and delivery is imperative for its clinical applications. In this regard, CORMs have gained popularity amongst scientific fraternity. Till date, CORMs have been investigated successfully along with its limitations and advantages in diabetes, myocardial infarction, posterior uveitis and neurogenesis (Almeida *et al.*, 2016, Fagone *et al.*, 2015, Nikolic *et al.*, 2015). Nasal instillation of CO to MCD diet fed C57BL/6J mice (NASH model) have been reported to alleviate NASH (Kim *et al.*, 2017). However, the use of CO at the organismal level is debated as it poses a risk of toxicity at higher doses. Also, efforts to ensure sustained release/bioavailability raises the challenge of a possible sub-physiological dose that may not be sufficient to elicit a therapeutic response. CORM-2 is metal based releasing molecule that has been reported for induction of SESN-2 and PERK-elF2α-ATF4 pathways in AML-12 cells and with therapeutic effect in NASH (Kim *et al.*, 2017). However, a metal-based releasing molecule such as CORM-2 is known to cause higher cellular toxicity due to fast release of CO resulting in undesirable effects such as mitochondrial COX inhibition and hypoxic condition (Zuckerbraun *et al.*, 2007). These problems have been overcome in CORM-A1 as it has a non-metallic boron core that make it water soluble and a slow releaser of CO. In this study we report on the protective mechanisms of CORM-A1 in NASH wherein, we observed alleviation of HFHF diet induced steatotic changes in liver.

NASH is a complex condition marked by systemic inflammation, metabolic imbalance and related fatty manifestation in liver. Chronic feeding of HFD develops NASH in murine models, but the time required and the degree of fatty manifestation in liver vary commensurate dietary composition and/or strain of mice (Takahashi *et al.*, 2012). In our study HFHF diet has been fed to C57BL/6J mice to mimic fructose and fat rich foods consumed by humans. Fructose feeding facilitates transition of NAFLD to NASH in HFD fed C57BL/6J mice. In these studies, HFHF fed mice recorded significant increment in body weight, abdominal fat and liver: body weight ratio. These observations are in accordance with other studies on HFD fed mice and fulfill prerequisite for development of NASH (Van Herpen *et al.*, 2008). CORM-A1 treatment to HFHF fed mice showed lowered indices of said parameters. The metabolic perturbations due to oxidative stress, inflammation and lipid peroxidation are the key for pathogenic transition towards NASH. In our study, significantly elevated titer of AST, ALT and ALP along with pathological score of liver of HFHF fed mice confirm hepatocyte damage and steatotic changes. CORM-A1 treated mice showed relative decrement in said parameters. These results were in agreement with microscopic observation of liver. Estimation of serum lipid profile further, substantiate improvement of NASH in CORM-A1 treated mice. Thus, minimizing impact of “first hit”. These results are possibly due to elevation of lipid catabolic enzymes and reduced intestinal absorption of dietary lipids. These results are in agreement with the previous study where hepatoprotective potential of CORM-A1 was reported in t-BHP induced oxidative stress in HepG2 cells and APAP induced liver injury in swiss albino mice [Chapter1&2] (Upadhyay *et al.*, 2018).

Subsequent observation reveled that CORM-A1 accounted for observed decrement in mRNAs of FAS, Srebp1c and PPAR-α in hepatic tissue. SIRT-1 is a known regulator of hepatic lipid metabolism wherein; development of fatty liver was found to be prevented by augmented lipid clearance (Purushotham *et al.*, 2009). In this study, liver of CORM-A1 treated mice recorded transcriptional activation of SIRT-1 that possibly was the reason for an improved hepatic lipid profile and histoarchitecture. Upregulation of CD-36 has been implicated in augmented fatty acid uptake in hepatocytes (Wilson *et al.*, 2015). Herein, it is tentatively surmised that CORM-A1 induced upregulation of CD-36 and SIRT-1 mRNA in liver and HepG2 cells caused clearance of systemic lipid load. Also, upregulation of CPT-1 is a rate limiting step in mitochondrial fatty acid oxidation wherein, Nrf2 silenced 293T cells have been reported to have lowered CPT-1 and CPT-2 expressions (Shen *et al.*, 2008). Significantly upregulated CPT-1 following CORM-A1 treatment implies towards healthy catabolic changes in steatotic liver.

Low grade inflammation is hallmark of NASH pathology. Second hit involves inflammation which is a major step in transition of NAFLD to an irreversible NASH condition. NF-κB is the transcription factor that plays a vital regulatory role in the inflammatory and immune pathways, and its activation results in the progression of steatosis to NASH (Locatelli *et al.*, 2013). In this study, higher levels of inflammatory mRNA confirm the NASH condition in HFHF fed mice. Also, the steatotic changes were more pronounced because of fructose in diet as compared to SD.

HepG2 cells are known to develop lipid accumulation on PA treatment and leads to morphological and biochemical transformations. These set of changes are similar to formation of fatty liver in humans making it an ideal model for studying and quantifying experimentally induced NASH (Cui *et al.*, 2010). In vitro studies on HepG2 cells is undertaken essentially to draw confirmatory evidence in favor of in vivo observations and to confirm the efficacy of CORM-A1. Cytotoxicity of PA on HepG2 cells is characterized by reduced cell viability. However, presence of CORM-A1 along with PA significantly prevented lipid accumulation and cell mortality. These results are indicative of protective role of CORM-A1 against PA induced lipid accumulation and cytotoxicity. Overall it can be concluded from the present study that, CORM-A1 was effective against experimentally induced NASH in HFHF fed C57BL/6J mice or PA treated HepG2 cells by regulating expression of lipid metabolizing genes.