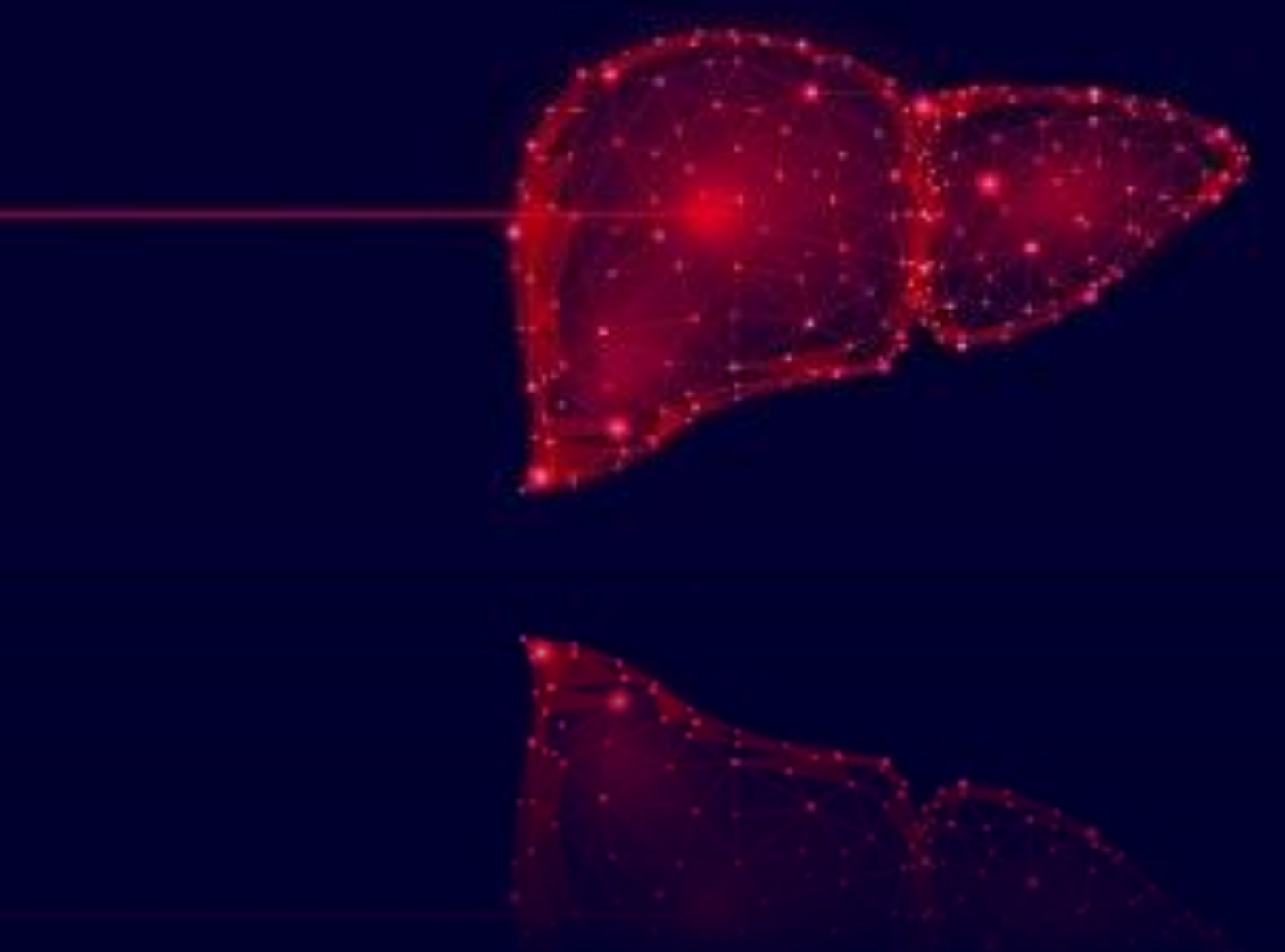
Chapter-4B

Alleviation of experimentally induced Nonalcoholic steatohepatitis (NASH) by CORM-A1: Targeting Mitochondrial biogenesis and cellular respiration.



Introduction

Mitochondria are crucial organelle that plays a vital role in the generation of energy in form of ATP, from nutrient oxidation. In addition to the role of mitochondria in the oxidation of glucose and fat to produce energy, it plays an important role in the generation of ROS. Under normal conditions, about 1%–2% of mitochondrial oxygen consumption results in ROS production, this amount is easily combated by the cell (Jones, 1986). Problem arises with excessive production of ROS that exceeds the cell's antioxidant capacity and result in damaging the cellular components such as lipids, proteins and nucleic acids (particularly mtDNA) leading to oxidative stress and ultimately triggering cellular apoptosis. This can be observed in conditions of increased oxidation of FFA such as in NASH (Begriche *et al.*, 2006).

Lately, scientific fraternity has unveiled critical role of mitochondria in NASH. Mitochondrial dysfunction affects hepatic lipid homeostasis, promotes ROS production and lipid peroxidation, cytokine release and might also result into cell death, with all this it largely contributes to NASH pathology. ROS production sources lipid peroxidation of mitochondrial membranes which can contribute to impaired mitochondrial function and perpetuate the ROS generation. Cellular oxidative stress is coupled with production of inflammatory cytokines, causing inflammation and fibrogenic response. All of these surmount in the development of NASH.

Nrf2 is known to play an important role in maintenance of mitochondrial homeostasis and structural integrity. Nrf2-mediateds up regulation of

cytoprotective responses and influences the total health and survival of the cell and the organism. It becomes even more crucial under stress condition wherein cell is experiencing oxidative, electrophilic, inflammatory stress together. Nrf2 exhibits broad cytoprotective mechanisms by orchestrating activation of transcriptional machinery of ARE genes. Oxidative stress, inflammation, and mitochondrial dysfunction lay a common symptomatic ground for wide number of diseases, specially lifestyle disorders (Tang *et al.*, 2014). In such cases, pharmacological activation of Nrf2 holds promising candidature for preventive measures and treatment. Comprehensive understanding of precise mechanisms of Nrf2 imparting effect on mitochondrial function is essential for rational design of future clinical trials and may offer new biomarkers for monitoring therapeutic efficacy.

CO is known to control mitochondrial functioning and oxidative metabolism which is also helpful in improving cellular energetics. Modulation of COX activity, oxygen consumption, mitochondrial biogenesis and ROS generation are major events orchestrated by CO. Additionally, CO also prevents cell death: (i) by directly targeting mitochondria and inhibiting mitochondrial membrane permeabilization, (ii) by increasing antiapoptotic gene expression, such as Bcl-2, (iii) by interacting with the apoptosis-inducing cytochrome c-cardiolipin complex and inhibiting caspase activation. Based on the reported credentials of CO this study investigates the efficacy of CORM-A1 in improving mitochondrial function in HFHF fed C57BL/6J

mice or HepG2 cells that are stressed due to treatment of PA; a condition similar to fatty liver.

Results

CORM-A1 treatment improves hepatic mitochondrial biogenesis and function in HFHF fed mice

PGC-1α and Nrf1 mRNA directly regulate the mitochondrial number as well as other metabolic events in steatotic mice. We recorded a non-significant increment in mRNA and protein levels of PGC-1α and Nrf1 in HFHF fed mice but the same were significantly elevated in CORM-A1 treated mice. Regulators of mitochondrial fission (Drp-1) and mitochondrial DNA (TFAM) showed moderate to significant increment in mRNA levels in liver of HFHF fed and CORM-A1 treated mice respectively (Fig4B.1). Mitochondrial DNA content in liver of HFHF fed mice was moderately higher than SD mice but, CORM-A1 treatment accounted for more than 2-fold increment than HFHF fed or SD mice. Also, the functional status of mitochondria was studied by assaying the production of total hepatic ATP. Data showed that ATP content of liver of HFHF fed mice was significantly lower than SD mice (Fig4B.2). CORM-A1 treatment was instrumental in increasing the ATP production significantly to match the control levels.

CORM-A1 improves mitochondrial mass and integrity in PA treated HepG2 cells.

CORM-A1 co-supplementation with PA reduced cellular and mitochondrial oxidative stress, improved mitochondrial membrane potential (MMP) and mitochondrial DNA copy number. Mitotracker dye is known to stain mitochondria irrespective of MMP and hence the fluorescence intensity is indicative of the total

mitochondrial mass. Weak fluorescence (~50000) recorded in PA treated HepG2 cells was in contrast to prominent fluorescence that recorded in CORM-A1 treated cells (Fig4B.3). Mitochondrial specific ROS was accessed by MitoSOX staining of HepG2 cells. Photographic evidence and FACS analysis revealed prominent red fluorescence (higher ROS) in PA treated HepG2 cells whereas; a weaker florescence (lower ROS) was recorded in CORMA-1 co-supplemented group (Fig4B.4). In JC-1 stained cell mitochondrial depolarization is indicated by a decrease in red/green fluorescence intensity ratio due to J- aggregates (red color). We recorded a shift in fluorescence intensity ratio in PA treated HepG2 cells as compared to control cells. CORM-A1 co-supplementation accounted for higher indices of fluorescence (red/green ratio) suggesting improved membrane potential of cellular mitochondria. FACS analysis of TMRE staining with PA treated HepG2 cells recorded a significant decrement in absorbance as compared to the control cells. Whereas, CORM-A1 co-treatment improved $\Delta\Psi m$ as evidenced by significant increment in fluorescence intensity of HepG2 cells (Fig4B.5).

CORMA-1 treatment improves mitochondrial biogenesis in PA treated HepG2 cells.

To confirm the findings of in vivo experiments, we studied the mRNA and protein contents of mitochondrial biogenesis regulatory genes in HepG2 cells. mRNA and protein levels of PGC 1-α in PA treated HepG2 cells recorded a significant decrement but presence of CORM-A1was instrumental in improving their levels (Fig4B.6). However, the Nrf1 mRNA levels showed a decrement, but the protein

showed an increment in PA treated HepG2 cells. CORM-A1 treatment recorded a moderate non-significant increase in mRNA and significant increase in protein levels of Nrf1. Other key genes controlling mitochondrial fission and DNA copy number viz Drp1 and TFAM were significantly down regulated as evidenced by their mRNA levels. Though, CORM-A1 did not induce major changes in TFAM mRNA, a significant increment was observed in Drp-1 (Fig4B.6). Further, relative mitochondrial DNA content (copy number) recorded significant decrement in PA treated HepG2 cells but CORMA-1 treatment improved the same (Fig4B.7). These findings are in agreement with the result obtained in in vivo studies.

CORM-A1 improves mitochondrial function in PA treated HepG2 cells.

The increased expression of mitochondrial protein and copy number was correlated with mitochondrial respiration and ATP production in PA and PA +CORM-A1 treated HepG2 cells. Mitochondrial respiration was assessed by seahorse XF extracellular flux analyzer as oxygen consumption rate (OCR) whereas; the glycolytic activity was assessed as resultant lactic acid production and extracellular release (ECAR). The value of basal respiration rate was significantly higher in CORM-A1 treated group as compared to PA treated or control group. Subsequently, addition of oligomycin resultant in respiration linked ATP production and proton leak. Addition of FCCP maximized mitochondrial respiratory capacity whereas rotenone block complex I and inhibited oxidative phosphorylation, the same enabled detection of spare respiratory capacity of HepG2 cells. Oligomycine exposure lead to a significant decrement in ATP

production in PA treated cells but the CORM-A1 treatment accounted for a significant increment. Though, the values obtained in proton leak showed similar pattern of decrement and increment in PA and PA +CORM-A1 treated cells respectively, the changes were non-significant. PA treatment resulted in significant decrement in indices of maximum cellular respiration and spare respiratory capacity. CORM-A1 treatment significantly improves maximum respiration and spare respiration capacity of PA treated HepG2 cells (Fig4B.8A&B).

The extracellular acidification rate is indicative of glycolytic activity in absence of mitochondrial respiration. Maximum ECAR or glycolytic capacity observed in PA treated cell was significantly less and in agreement with indices of glycolysis and glycolytic reserve. Though, CORM-A1 treatment improves the glycolytic capacity and the reserve, the maximum ECAR value as well other indices showed a non-significant increment. Similar pattern was observed in indices of non-glycolytic acidification with PA treated cells showing a moderate non-significant increment (Fig4B.8C&D).

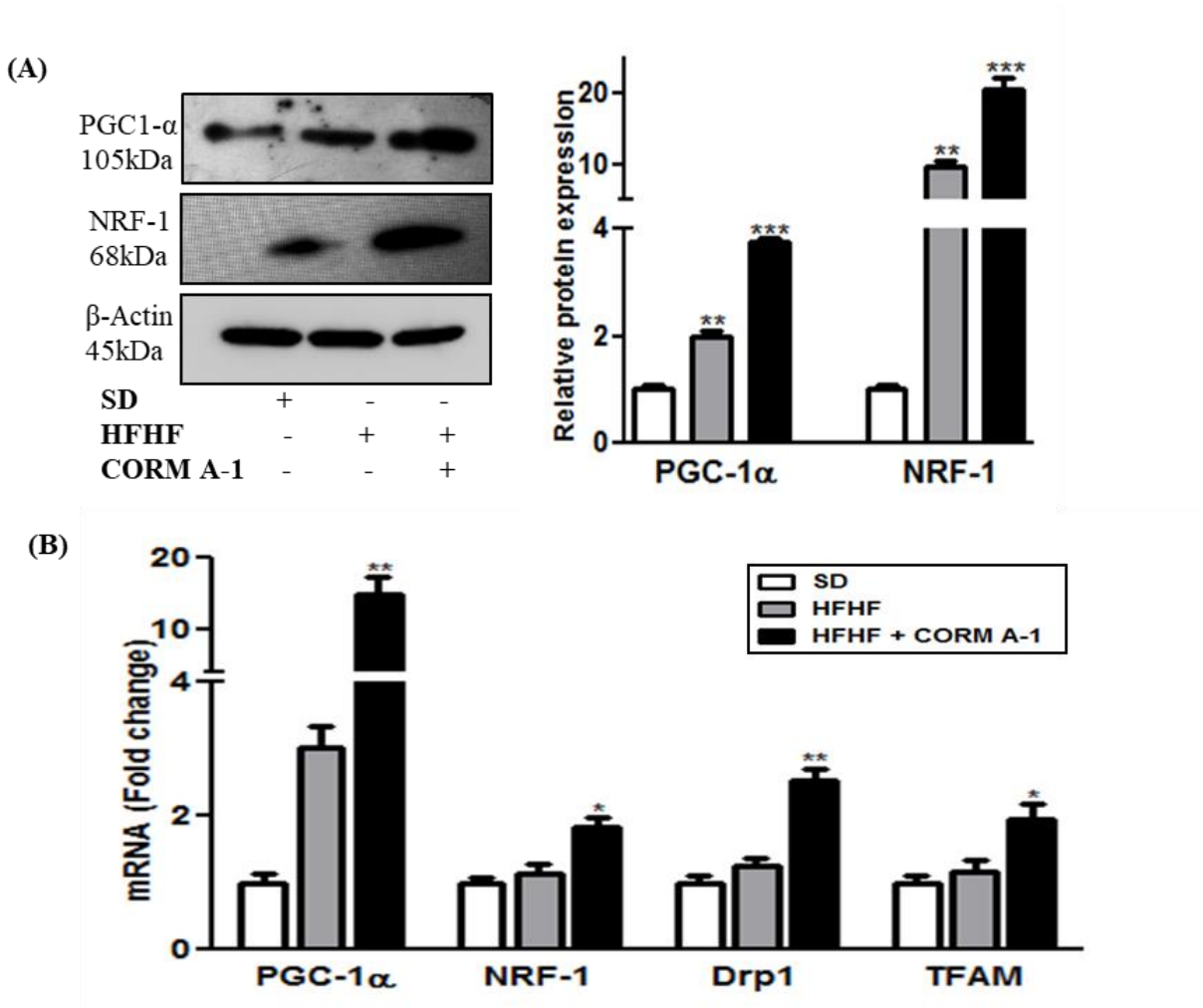


Fig.4B.1. CORM-A1 treatment improves hepatic mitochondrial biogenesis. (A) Western blot image of mitochondrial biogenesis regulator and their quantification and (B) Hepatic mRNA expression of genes related to mitochondrial biogenesis. Results are expressed as mean ± S.E.M. *p<0.05, **p<0.01 or ***p<0.001 is when HFHF compared to SD and HFHF +CORM-A1 is compared to HFHF group.

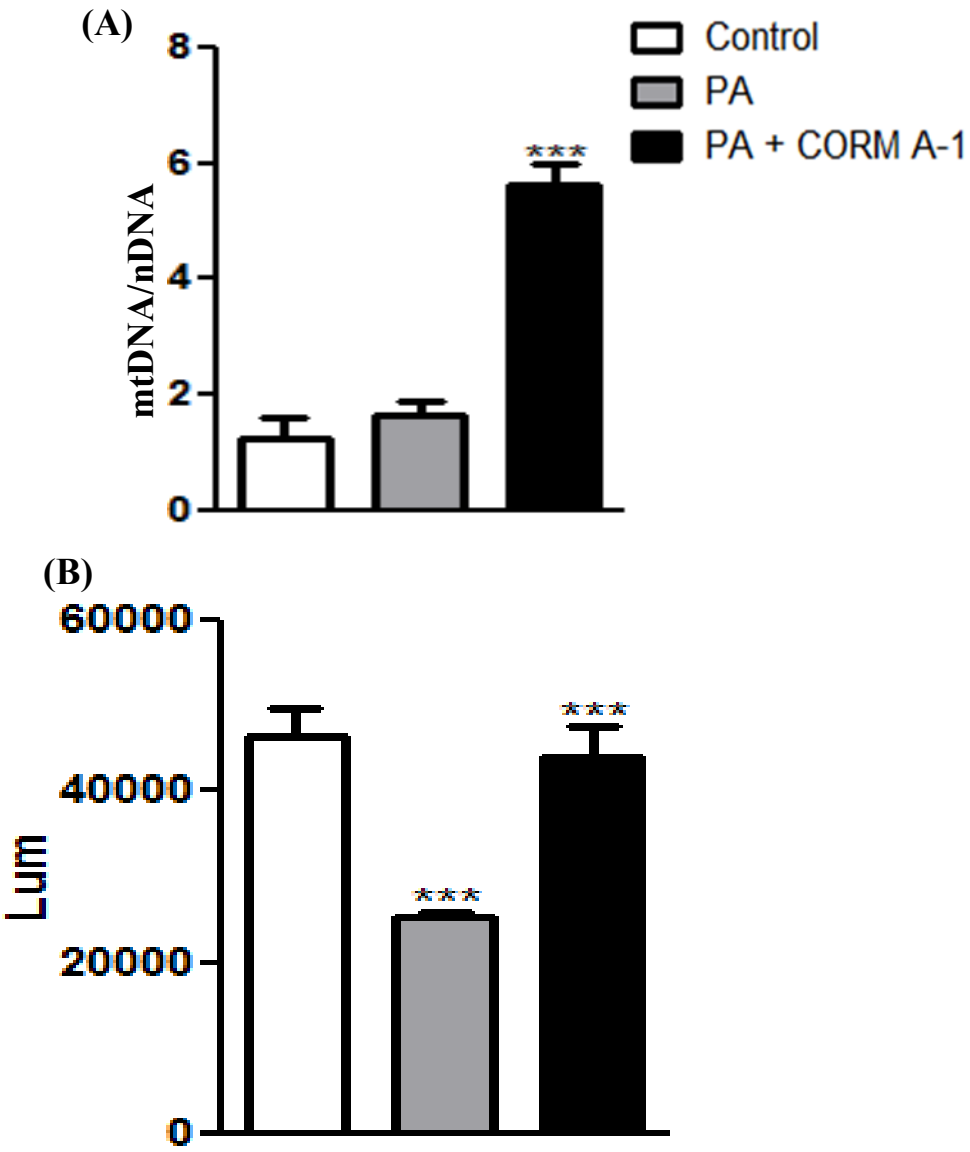


Fig.4B.2. CORM-A1 treatment improves (A) mitochondrial DNA content (B) cellular ATP levels. Results are expressed as mean ± S.E.M. *p<0.05, **p<0.01 or ***p<0.001 is when HFHF compared to SD and HFHF + CORM-A1 is compared to HFHF group.

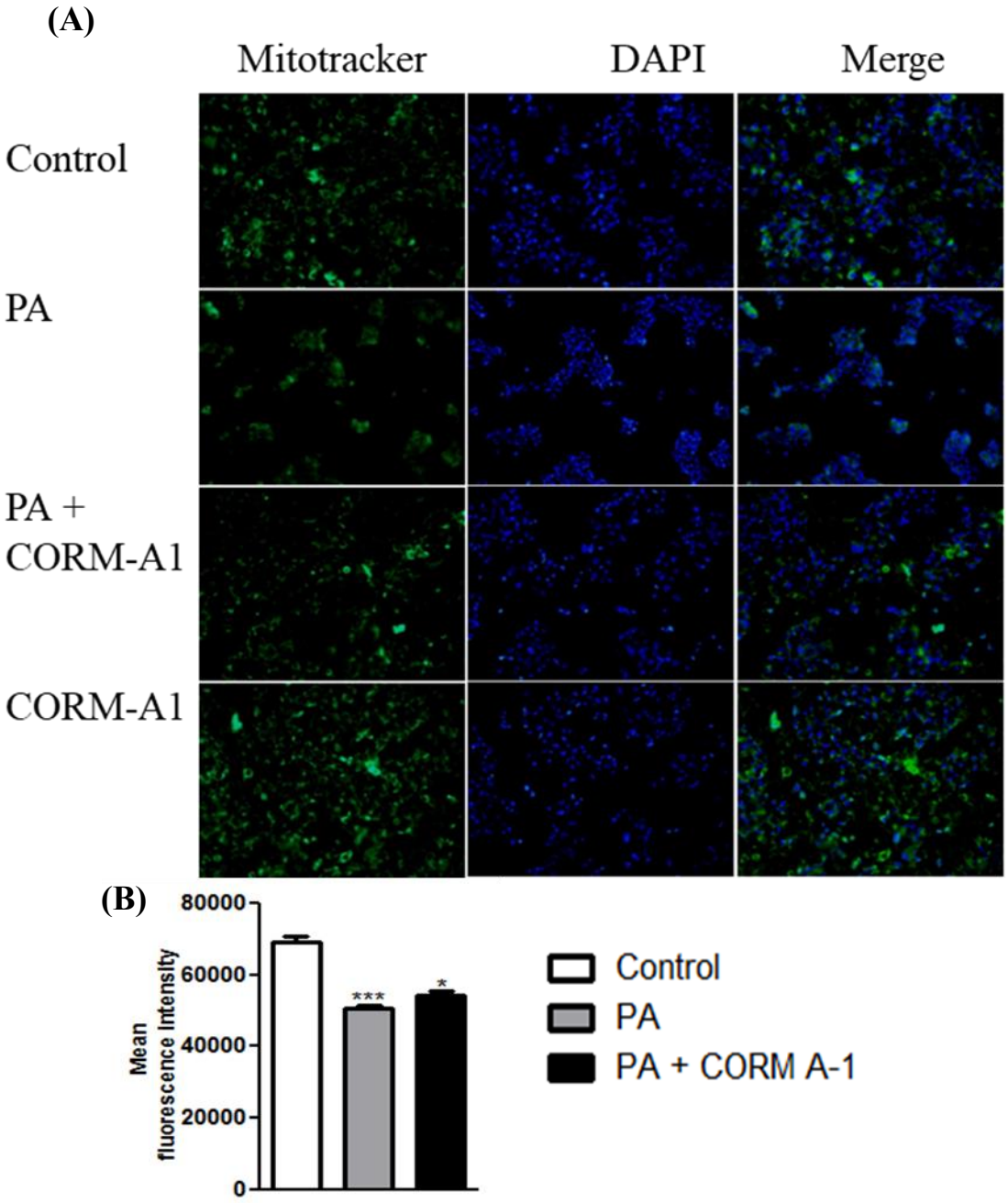


Fig.4B.3. CORM-A1 improves mitochondria mass in PA treated HepG2 cells. (A) Mitotracker staining and (B) their fluorescence intensity quantified using FACS analysis. Results are expressed as mean ± S.E.M. *p<0.05, **p<0.01 or ***p<0.001 is when PA treatment is compared to control cells and PA + CORM-A1 is compared to PA alone group.

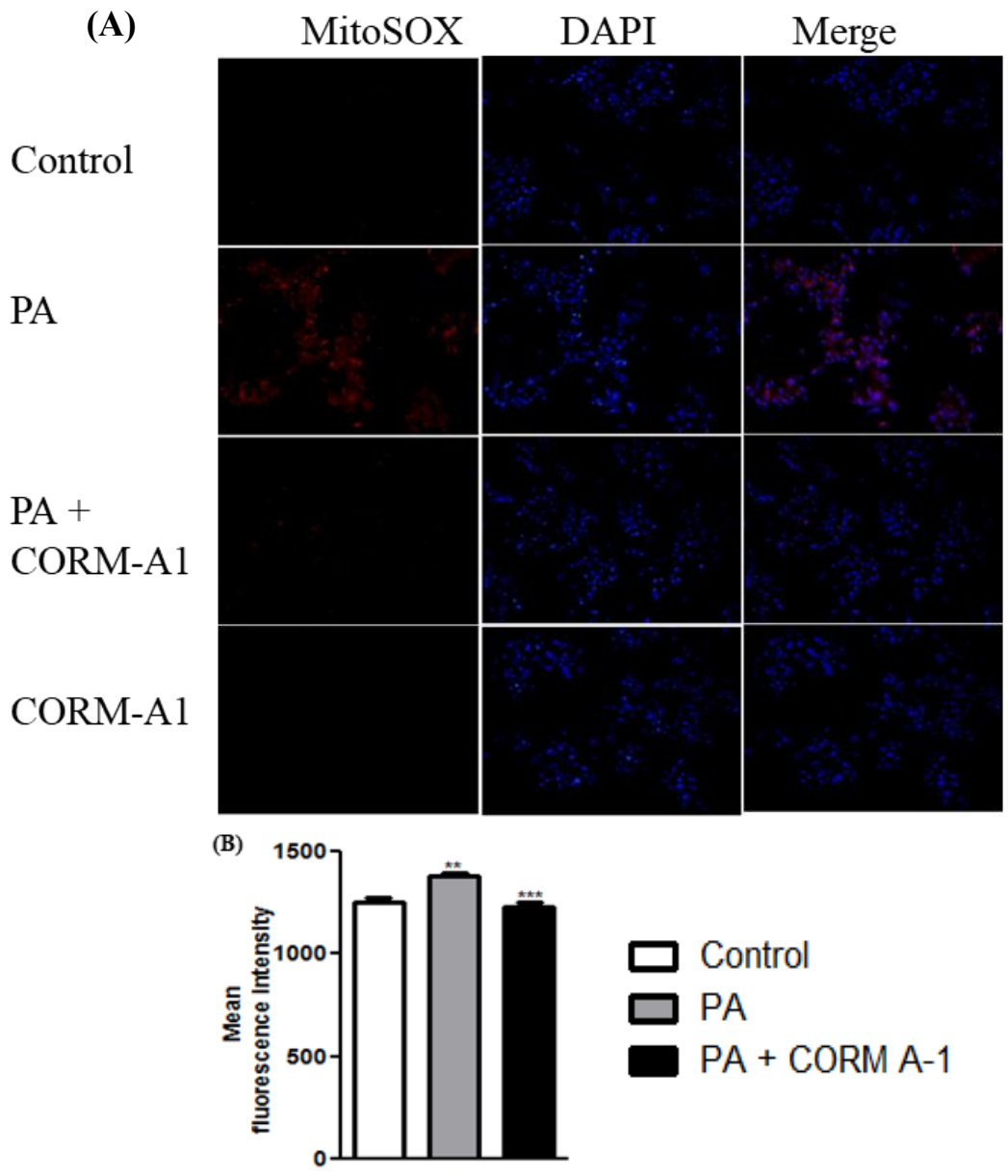


Fig.4B.4. CORM-A1 improves mitochondrial oxidative stress. Cells stained with (A) MitoSOX and (B) their florescence intensity. Results are expressed as mean ± S.E.M. *p<0.05, **p<0.01 or ***p<0.001 is when PA treatment is compared to control cells and PA + CORM-A1 is compared to PA alone group.

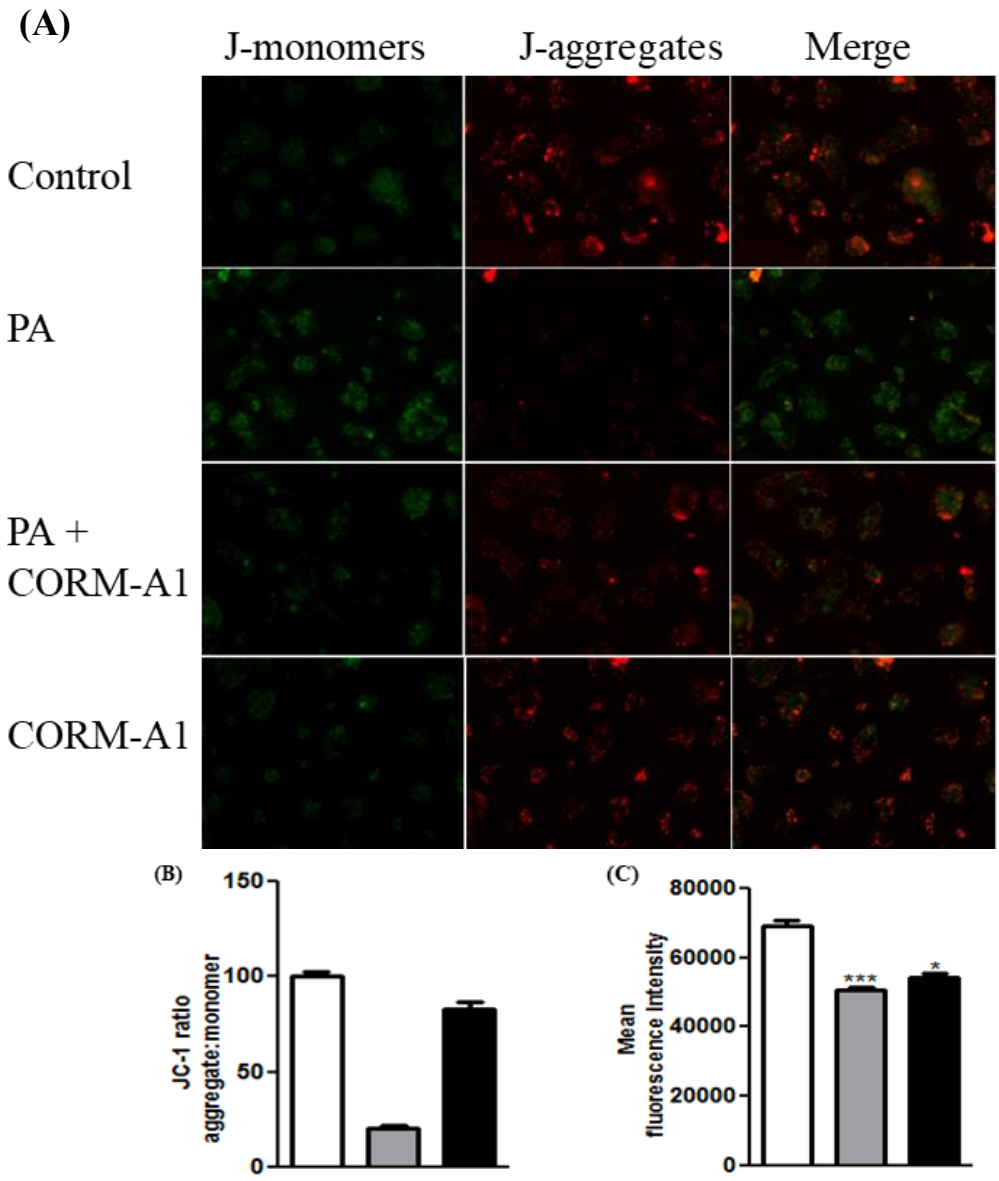


Fig.4B.5. CORM-A1 improves membrane potential in PA treated HepG2 cells. (A) JC-1 staining for measuring MMP and (B) its florescence intensities measured using FACS analysis and (C) florescence intensities of TMRE stain. Results are expressed as mean ± S.E.M. *p<0.05, **p<0.01 or ***p<0.001 is when PA treatment is compared to control cells and PA + CORM-A1 is compared to PA alone group.

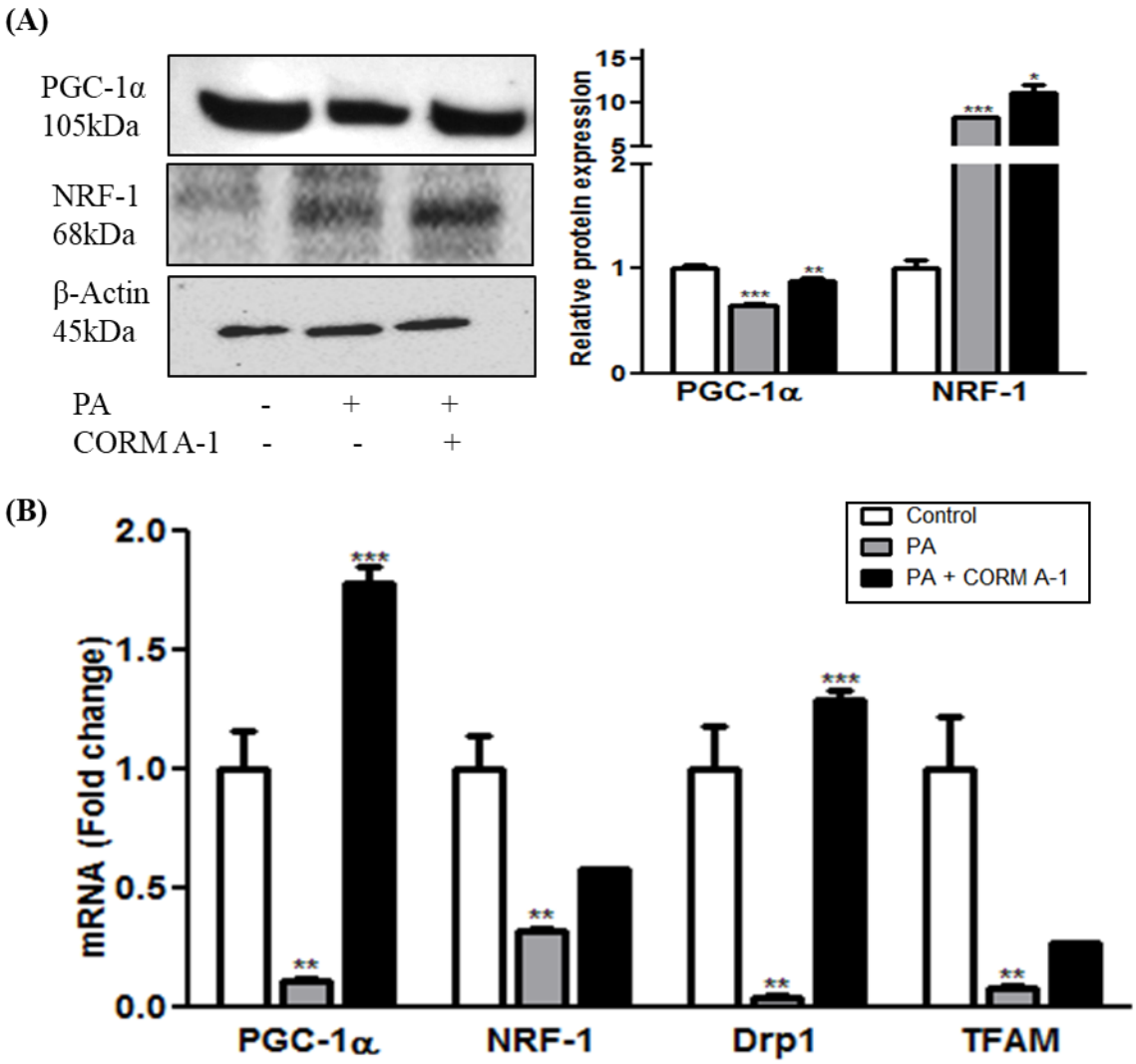


Fig.4B.6. CORM-A1 improves (A) western blot image and quantification (B) mRNA expression of regulatory genes for mitochondrial biogenesis. Results are expressed as mean ± S.E.M. *p<0.05, **p<0.01 or ***p<0.001 is when PA treatment is compared to control cells and PA + CORM-A1 is compared to PA alone group.

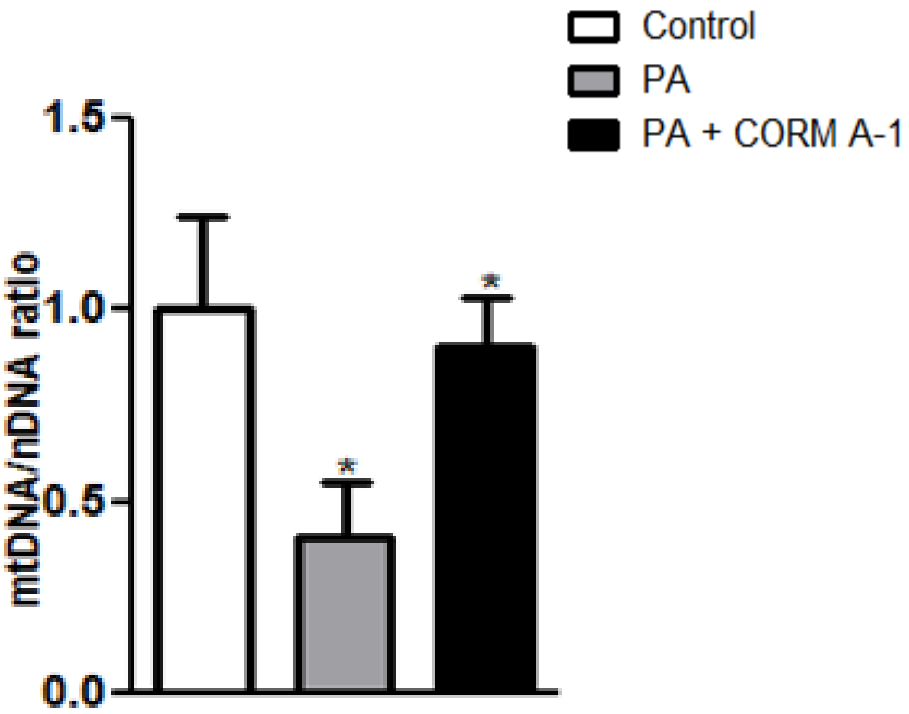
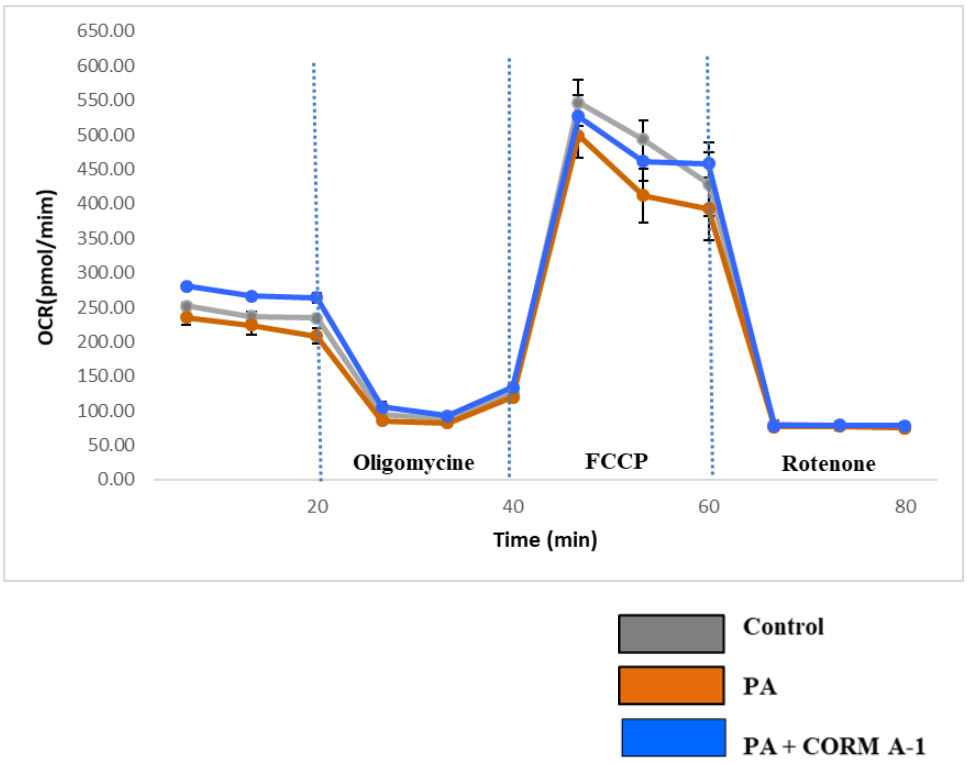
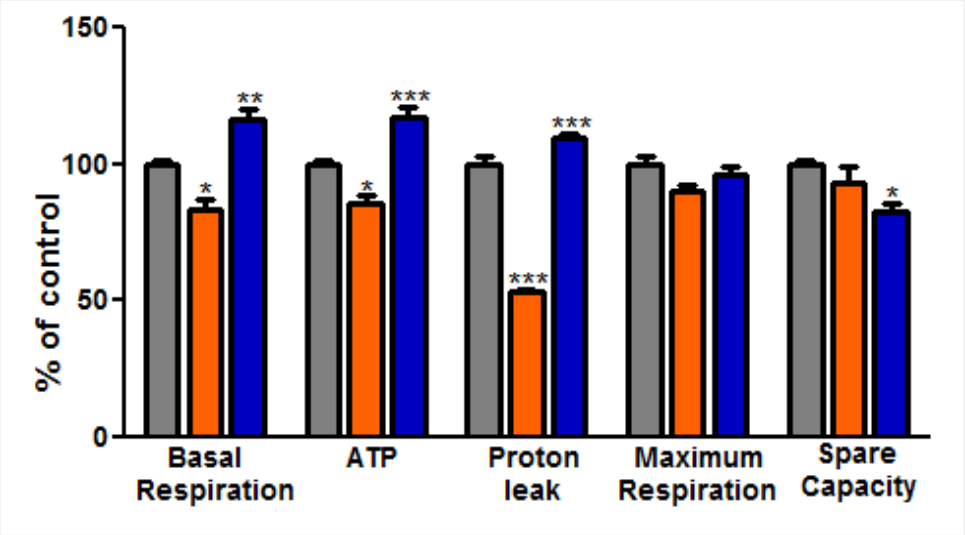


Fig.4B.7. CORM-A1 increase mitochondrial DNA content in PA treated HepG2 cells. Results are expressed as mean ± S.E.M. *p<0.05, **p<0.01 or ***p<0.001 is when PA treatment is compared to control cells and PA + CORM-A1 is compared to PA alone group.

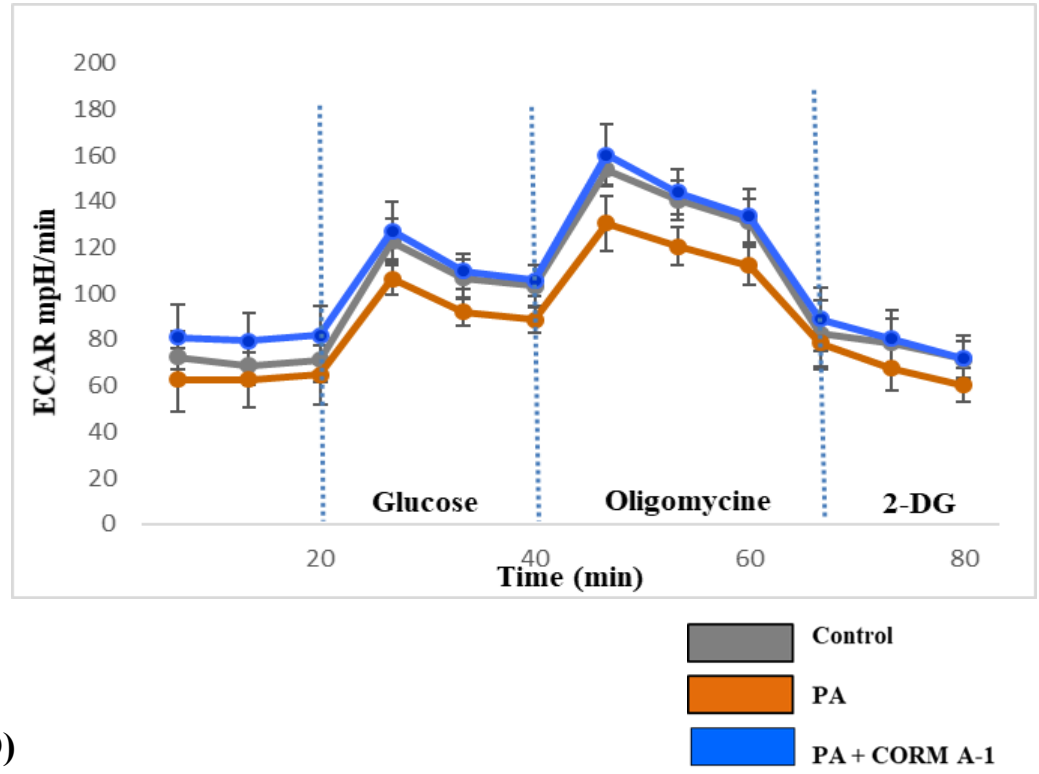
(A)


650.00
600.00
550.00
500.00
450.00
400.00
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
OCR(pmol/mim)
Oligomycine
FCCP
Rotenone
20
40
60
80
Time (min)
Control
PA
PA + CORM A-1

(B)


150
100
50
0
% of control
*
**
***
***
***
*
Basal Respiration
ATP
Proton leak
Maximum Respiration
Spare Capacity

(C)



(D)

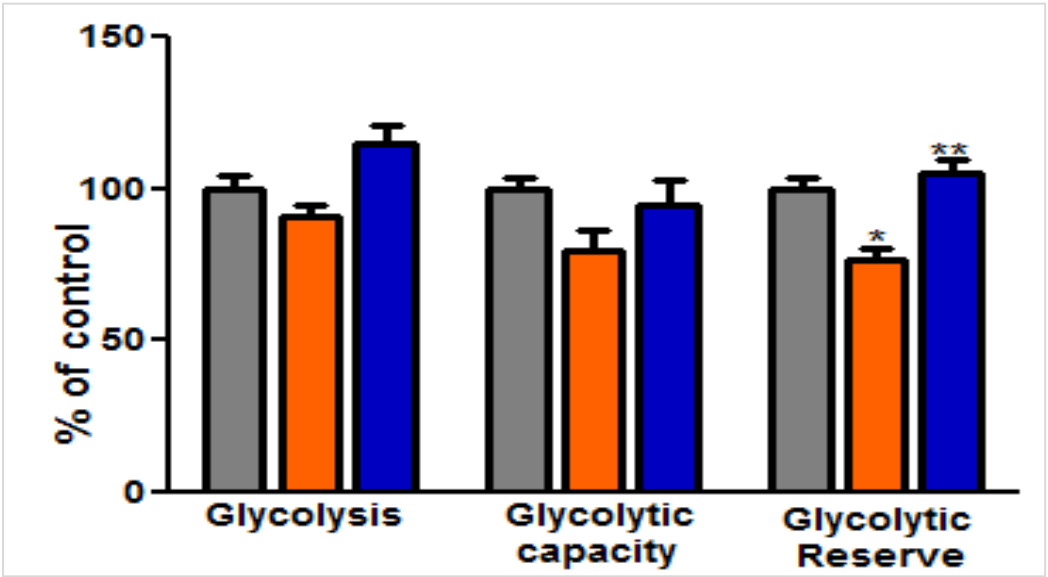


Fig.4B.8. CORM-A1 improves PA-abrogated respiration in HepG2 cells. Cell respiration were measured as Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) and quantification was done using Seahorse XFe96

Metabolic Flux Analyzer. (A) mitochondrial respiration measured as OCR (B) quantification of other respiratory parameters (Basal respiration, ATP, Proton leak, maximum respiration and spare capacity) using OCR data. (C) non-mitochondrial respiration measured as ECAR (D) calculated values for glycolysis, glycolytic capacity and reserves from ECAR data. 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

Mitochondria generates 90% of cellular ATP and serve an integrating platform between nuclear genome and cellular signaling pathways (Lehninger *et al.*, 2005). In NASH, mitochondrial dysfunction plays a crucial role because of the involvement of mitochondria in β-oxidation of fatty acids. The same has also been implicated in first and second hits of NASH (Kovac *et al.*, 2015). Poly-unsaturated fatty acids are implicated in mitochondrial dysfunction and pathogenesis because of impaired fat metabolism, increased oxidative stress, cytokine production and inflammation culminating in cell death. PA treatment to HepG2 cells have been reported to decrease mitochondrial DNA encoded polypeptides and reduce syntheses of OXPHOS subunits. Also, PA has been preferred over stearic and oleic acid more for its ability to induced mitochondrial dysfunction in HepG2 cells (García-Ruiz *et al.*, 2015). We utilized PA treated HepG2 cells to decipher effect of CORM-A1 on mitochondrial ROS, functional status of mitochondria and its biogenesis.

Nrf2 is capable of maintaining mitochondrial function and promoting its biogenesis (Kitteringham *et al.*, 2010) and function of Nrf2 is suppressed in mitochondrial related disorder including NASH (Pessayre, 2007). In our study, CORM-A1 co-supplementation reduced intracellular and mitochondrial specific ROS. Also, mitochondrial membrane potential of HepG2 cells showed a significant increase. Since MMP has been implicated as an indicator of metabolic state of cell an improved MMP (JC-1 and TMRE) are attributed to Nrf2 mediated control of cellular redox homeostasis (Kovac *et al.*, 2015). This process has been quoted as

compensatory step and can be implicated for the observed CO mediated cytoprotection. Further, wild type Nrf2 knockout mice have been reported for lower mitochondrial content (Kitaoka *et al.*, 2016) whereas exogenous administration of Nrf2 activator promotes mitochondrial biogenesis in 3T3L1 adipocytes (Shen *et al.*, 2008). In this study we observed increase in mitochondrial DNA copy number in CORM-A1 treated HepG2 cells in PA or HFHF fed mice respectively, which is attributed to Nrf2 activation resulting in mitochondrial biogenesis. Reports have elaborated Nrf2 dependent translational activation of Nrf1 that further accounts for cytoprotection and mitochondrial biogenesis (Piantadosi *et al.*, 2008). The second class of nuclear transcriptional regulators that are equally important in mitochondrial biogenesis are transcriptional co activator viz. PGC-1α and PGC1-β (Scarpulla, 2011). The role of Nrf2 in maintaining level of Nrf1 and PGC-1α have been emphasized in liver of Nrf2 knockout mice (Dinkova-Kostova *et al.*, 2015). In our study CORM-A1 induced significant increment in mRNA and protein of PGC-1α in liver samples and HepG2 cells is a key finding that forms the bases of our claim on CORM-A1 mediated improvement in mitochondrial biogenesis. TFAM is a mitochondrial specific transcription factor that is associated with replication of mitochondrial genome resulting in higher mitochondrial DNA copy number (Pohjoismäki *et al.*, 2006). Drp1 is a protein that regulates mitochondrial fission and has been implied as potential target for intervention in metabolic diseases (Chang *et al.*, 2010). In our study, CORM-A1 treatment accounted for significantly higher level of TFAM and Drp1 mRNA that corroborates the observed

increment in mitochondrial mass and mitochondrial DNA copy number in liver and HepG2 cells.

PA has been reported to cause ROS production and hyper-polarization of mitochondria. This scenario is mitigated at cellular level by increasing proton leak (Brookes, 2005, Nakamura *et al.*, 2009). Also, PA mediated decrease in basal and maximal mitochondrial respiratory rate shifts the OCR to ECAR by increasing glycolytic flux and results in accumulation of pyruvate (Wallace, 2012). In our study, an improved basal respiration in PA + CORM-A1 treated HepG2 cell and significant increment in indices of proton leak justify higher ATP production. A possible high metabolic flux in hepatocyte to compensate PA induced depressed ATP levels and abrogated mitochondrial respiration is also in agreement with our observations on lowered indices of cell viability. An increment in non-mitochondrial ATP generation through glycolysis in early phase has been reported by other research groups but in advanced stage of hepatic steatosis the ATP reserves are depleted (Nishikawa *et al.*, 2014). These observations are in agreement with the results obtained in ECAR in PA treated HepG2 cells. CORM-A1 co-supplementation accounted for higher indices of glycolytic reserves. Thus, results obtained in our study imply towards a healthy state of mitochondria and less dependency on non-mitochondrial respiration.

Taken together, our study highlights CORM-A1 mediated prevention of NASH and steatotic changes in HepG2 cells by improving OXPHOS activity and overall mitochondrial function. This inference is born from the fact that Nrf2 knockout have lower ATP levels, higher dependency on cytosolic ATP production and

decreased oxygen consumption rate. Gasotransmitters (NO and H_2S) including CORM-A1 have been implicated in improving mitochondrial function but their role in restoring steatotic liver is unclear. Hence this study on CORM-A1 mediated improvement of mitochondrial function and favorable changes orchestrated via Nrf2 in steatotic liver is the first report that establishes the anti-NASH potential of CORM-A1.