Materials and Methods

Chemicals and reagents

Cell culture requirements, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine Serum (FBS), trypsin phosphate versene glucose (TPVG), phosphate buffered saline (PBS), Bovine serum albumin (BSA) and antibiotic-antimycotic solution were purchased from Hi-media Laboratories (Mumbai, India). ENZOPAK AST, ALT, ALP and kit for lipid detection were procured from Reckon Diagnostics (Vadodara, Gujarat). Molecular biology reagents, TRIzol and SYBR select master mix were procured from Invitrogen (CA, USA). iScript cDNA synthesis kit was procured from Bio-Rad (CA, USA). RNA-later stabilizing solution was purchased from Ambion Inc. (USA). Antibodies NRF-2 (12721S), HO-1 (70081S), β-actin (4970S) and 2˚ Antibody (7074P2) were purchased from cell signaling Technology (Danvers, MA). PGC 1α (ab54481), Keap1 (ab139729) Nrf1 (ab175932) were purchased from Abcam (Cambridge, MA, USA). CORM-A1, DAPI stain, Hematoxylin, Eosin and Palmitic acid (PA) were purchased from Sigma Aldrich St. Louis, MO, USA). Methanol, dimethyl sulphoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), tert-Butyl hydroperoxide (t-BHP) were purchased from Sisco Research Laboratory Pvt. Ltd. (Mumbai, India).

Invitro studies

Maintenance of HepG2 cells

Human Hepatoma (HepG2) cells were procured from National Centre for Cell Science (NCCS, Pune, India). Cells were cultured in T25 flasks in DMEM growth media, supplemented with 10% FBS and 1% antibiotic antimycotic solution. The flasks were maintained in CO_2 incubator (Thermo scientific, forma series II 3110, USA) at 37°C temperature and 5% CO_2. Cell passaging was at 80% confluency using 1X TPVG. Once the cells detached from the surface, they were collected in 2mL centrifuge tube and spin down at 5000 rpm for 5 min. Cell pellet was resuspended in media and seeded into fresh T25 flask. For maintenance regular media change was done on every third day.

Treatment with Palmitic acid conjugated BSA and CORM-A1

Palmitate stock solution was prepared as described previously (Cousin *et al.*, 2001). Briefly, 100 mM PA was conjugated with 10% BSA to obtain PA (10 mM FFA/1% BSA) stock solution. PA was further diluted with media to obtain 100uM working solution and the same was used for treatment of HepG2 cells.

Cytotoxicity assessment

For cytotoxic assessment MTT assay was performed. MTT assay is dependent on mitochondrial respiration and indicated number of viable cells in the system. HepG2 cells (10^4 cells/well) were seeded in 96 well plate in DMEM growth medium. After 24h of seeding the plate was used for dosing of different target

compounds. The target compounds were prepared in an incomplete media with desired concentrations and was dosed 200uL/well. Cells were incubated with the test compounds for 24h. Following 24h of dosing, media was removed carefully, without disturbing the cells and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5mg/ml) was added. Mitochondrial succinate dehydrogenase reduces MTT to purple colored, water insoluble formazan crystals. The plate was incubated in dark for 4h at 37ºC. Resultant formazan crystals were dissolved in DMSO (150µL/well) and kept for about 5 minutes till the crystals were completely dissolved. Absorbance was measured at 540 nm using Synergy HTX Multimode Reader.

Determination of Oxidative stress in HepG2 cells

2',7' –dichlorofluorescin diacetate (DCFDA)

For detection and quantification of cellular ROS, DCFDA assay was performed. 2',7' –dichlorofluorescin diacetate (DCFDA) is deacetylated by esterases to a non-fluorescent compound, Cellular ROS oxidises it to 2', 7'-dichlorofluorescein (DCF) which is highly fluorescent and used for detection. HepG2 cells were seeded in 6 well plate in DMEM growth medium with 10% FBS. Cells were allowed to grow till 70% confluency and were treated with 25 & 50 µM t-BHP in presence and absence of 100 µM CORM-A1. After 2h of treatment cells were incubated with 10 µM DCFDA for 30 min. After 30 min cells were washed with 700uL PBS for about 3-4 times till the background noise was minimum. Images were taken on

FLoid Cell Imaging Station using green filters. Intensity of green fluorescence is directly proportional to intracellular cellular ROS.

CellROX

CellROX is a fluorogenic probe indicative of oxidative stress in live cells. It gets oxidised, by intra-cellular ROS, to bright green photostable fluorescing compound that subsequently binds to DNA and can be captured at 520nm. For this assay HepG2 cells were seeded in 6 well plate in DMEM growth medium with 10% FBS. Cells were allowed to grow till 70% confluency and were treated with 100 µM PA or 100 µM PA + 100 µM CORM-A1 or 100 µM CORM-A1 alone. After 12h of treatment cells were incubated with 5 µM of CellROX and Nucleus was counterstained with 0.6 µg/ml of DAPI for 30 min. post-incubation cells were washed with 1X PBS till the background noise was cleared. Images were taken on Zeiss Axioplan-2 imaging florescence microscope.

Dihydroethidium (DHE)

DHE assay is done to analyze cellular oxidative stress. DHE gets oxidize to form ethidium bromide that eventually intercalates with DNA and results into fluorescence. For performing this assay HepG2 cells were seeded in 6 well plate in 10% DMEM growth medium. After 24h when the cells were 70% confluent, they were dosed with 100 µM PA or 100 µM PA + 100 µM CORM-A1 or 100 µM CORM-A1 alone in an incomplete media for 24h. After 24h 10 µM DHE was added for about 10 min. Nucleus was counterstained with 0.6 µg/ml of DAPI. post-

incubation cells were washed twice with 1X PBS and the images were taken on Zeiss Axioplan-2 imaging florescence microscope.

MitoSOX

MitoSOX Red mitochondrial superoxide indicator specifically targets mitochondria in live cells. It readily gets oxidized by mitochondrial superoxide but not by cellular ROS or RNS. For this assay HepG2 cells were seeded in a 6 well plate in 10% DMEM growth medium. On 70% confluency, cells were dosed with 100 µM PA or 100 µM PA + 100 µM CORM-A1 or 100 µM CORM-A1 alone for 24h. After 24h media was removed, and cells were washed with 1X PBS twice. Following that 5 µM of MitoSOX was added for 30 min. Nucleus was counterstained with 0.6 ug/ml of DAPI. At the end of incubation, cells were again washed with PBS, mounted using fluoroshield mounting medium with DAPI and photographed in Zeiss Axioplan-2 imaging florescence microscope.

Determination of GSH

GSH was estimated from HepG2 cell and mice liver. For cellular estimation HepG2 cells were seeded in 6 well plate and were dosed with 100uM PA or 100µM PA and 100µM CORM-A1. Cells were collected and lysed by homogenizing. For invivio GSH determination, 10% (w/v) liver homogenate was prepared using homogenizer. Homogenate was centrifuged at 12000 rpm for 10 min at 4^{o}C for removing debris. Fresh lysate was used for GSH determination assay. Lysate was added with dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent) to form yellow

anions of TNB. This was quantified spectrophotometrically at 412nm wavelength. The GSH content was calculated as µg/mg protein using a standard GSH curve. Protein content was determined using Bradford reagent (BIO-RAD CA, USA) and was measured in multimode reader at 590nm.

Molecular docking study

Molecular Docking of CO against Keap1 protein was performed using open source software Auto Dock Vina (Trott, and Olson, 2010). Docking pose analysis was done with Discovery studio visualizer 4.5. Cryo-crystallized structure of mouse Keap1 was obtained from the protein data bank (PDB; 5CGJ) and possible binding mode of CO at the kelch domain of Keap1 was investigated. These results were compared with a small molecule (3S)-1-[4- [(2,3,5,6- tetramethylphenyl) sulfonylamino] -1-naphthyl] pyrrolidine-3-carboxylic acid (RA839). All other docking parameters were default unless otherwise stated.

Mitochondrial membrane potential by JC-1 and TMRE staining

JC-1 and TMRE stains are used to analyze mitochondrial membrane potential. JC-1 is a cationic carbocyanine dye that accumulates in mitochondria. It exists in two forms; at lower concentration in a monomeric form exhibiting green fluorescence and at higher concentration it forms J aggregates exhibiting red fluorescence. HepG2 cells were seeded in a 6 well plate and treated with 100 µM PA or 100 µM PA + 100 µM CORM-A1 or 100 µM CORM-A1 alone. Control and treated cells were washed with 1X PBS and incubated with 5µg/ml JC-1 in pre-warmed 1X PBS

for 30 minutes at 37°C. post incubation cells were washed with 1X PBS thrice and were photographed using Evos FLoid cell imaging station and fluorescent intensity was quantified using ImageJ software.

The same set of groups was used for TMRE staining. TMRE is a cationic dye that is readily sequestered by active mitochondria. Hence it is an indicative marker of active mitochondria in cellular system. Cells were stained with 100 nM of TMRE stain in 1X PBS for 30 min at 37°C. Later, Cells were trypsinized, collected in 1.5 mL of centrifuge tubes and centrifuged at 1000g for 5 minutes. Cells were re-suspended in pre-heated 1X PBS (37°C). Fluorescence was recorded using a flow cytometer (FACScalibur, BD Biosciences).

Fluorescence activated cell-sorting (FACS) analysis

HepG2 cells treated with 100µM PA or 100µM PA and CORM-A1 were stained with 5 µM MitoSOX or 50 nM MitoTracker in incomplete DMEM growth medium and incubated for 30 min at 37°C in dark. After washing twice, cell samples were collected and analysed using a flow cytometer (FACScalibur, BD Biosciences).

Total ATP content

The fresh lysate was used for ATP content determination using ATP Determination Kit (A22066, Molecular Probes). Equal volume of lysate was used for each reaction. The reaction mixture constituted of 1.25 mg/ml firefly luciferase, 50 mM D-luciferin, and 1 mM dithiothreitol in 1 reaction buffer and liver homogenate. After 15-min incubation luminescence was measured in Synergy HTX Multimode

Readers (Bio-Tek instruments, Inc., Winooski, VT). Results were expressed as arbitrary units of luminescence compared to that of the control group.

Mitochondrial respiration (Seahorse XF analyzer)

The Seahorse XF Analyzer (Seahorse Biosciences, North Billerica, MA) was used according to the manufacturer's protocol to measure OCR and ECAR of the cells. HepG2 cells were seeded in Seahorse Flux Analyzer mini plates (10000 cells/well) and incubated overnight at 37˚C. Later, cells were treated as per *in vitro* treatment schedule mentioned earlier. Thereafter, the culture medium was changed to XFp base medium minimal DMEM (Seahorse Biosciences) and placed in a non-CO2 incubator for at 37˚C. Mitochondrial function was assessed using the Seahorse XFp Analyzer by monitoring changes in OCR and ECAR as previously described. Three OCR measurements were obtained under basal conditions and upon sequential injection of 2 µM oligomycin, 2 µM fluoro-carbonyl-cyanide phenylhydrazone (FCCP), and 0.5 µM rotenone plus 0.5 µM antimycin A. OCR values were calculated from 3-min measurement cycles. The OCR measurements were adjusted to cell numbers. Glycolysis was assessed by analyzing ECAR in hepatocytes cultured in glucose-free medium after sequential addition of 10 mM glucose, 2 µM oligomycin, and 100 mM 2-deoxyglucose. The final data was obtained using the Seahorse XFp software and calculated as per the instruction of manufacturer.

In vivo studies

Animal Ethical Statements

Mice were purchased 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) 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.

Animal Studies

Animals were fasted overnight, and mild anesthesia was given in form of Isoflurane. Whole blood was collected by retro-orbital sinus puncture under anesthetized condition. Whole blood was centrifuged 3000 rpm at 4°C for 10 min and serum was collected and stored at -20^{o}C till further used for analysis. Later, mice were sacrificed, and liver and visceral fat was collected. These tissue samples were divided into 3 parts and stored in 10% formalin for histopathology, in RNAlater for gene expression studies or at -80°C for protein and other analysis.

Diet Composition

Table-1. Composition of experimental diets (Bose *et al.*, 2008).

Standard Diet	
corn	48%
soybean meal	20%
wheat bran	10%
flour	10%
fish meal	8%
farina	2%,
vegetable oil	1%

High Fat Diet	
moisture	4.10%
Protein	18.25%
Fat	35.30%
Fiber	2.75%
Calcium	1.19%
Phosphorous	0.51%
Total ash	4.35%

Serum biochemical parameters

Collected serum samples were used for detecting circulating titers of AST, ALT and ALP indicating liver function. Lipid profiling, Total Lipid (TL), Total Cholesterol (TC), triglycerides (TG) was done in serum samples using kits (Reckon Diagnostic kits, Vadodara, Gujarat, India). Low density lipoproteins (LDL), very low-density lipoprotein (VLDL) were determined applying Friedwald's formula. Further, cholesterol to HDL ratio (CHL/HDL), low density lipoprotein to high density lipoprotein ratios (LDL/HDL) were determined from theoretical calculations.

Liver histopathology

Tissue samples, liver and adipose tissues were fixed in formalin (n=6). Tissue was further dehydrated and embedded in paraffin wax blocks and cut into 5µ thick sections cryotome. These sections were stained with haematoxylin and eosin (H&E). For that the sections were deparaffinized and hydrated by passing decreasing concentration of alcohol on the slide. Put hematoxylin stain on the slide for about 5 minutes and wash it under running water. Further differentiate it in 1% acid and 70% alcohol for about 5 min followed by washing it under running water. Next, put 1% eosin stain and incubate it for about 5 min and wash under running water. Further dehydrate it again in increasing percentage of alcohol solution. Finally, mount the slide in mounting media. The slides were then observed and photographed under Leica DM 2500 microscope. Investigators blinded to this study conducted scoring of liver sections of control and treated mice. Adipose tissue

section was observed, photographed and morphometric scoring was done for the same.

Scoring of liver

H&E staining was used to perform section evaluation. Semi-quantitative scoring system was used to access hepatocyte necrosis and intrahepatic haemorrhage (0-none, <10% of total area-1, <30% of total area-2, less than 40% of total area-3, more than 50% of total area-4 (Gujral *et al.*, 2002). Evaluation of NASH was done using steatotis scoring of liver (0-5%, 1-5 to 33%, 2-34 to 66%, 3>66%) and ballooning hepatocytes (0-none, 1- few ballooned, 2- many ballooned). The evaluation was done by two investigators blinded to the study (Liang *et al.*, 2014).

Intra-peritoneal glucose tolerance test (IPGTT)

After 12 h of overnight fasting, A drop of whole blood was applied on test strip by amputation of tail tip. Blood glucose was measured using accu-chek active glucometer (Roche Diabetes care GmbH). Later, glucose solution was administered intraperitoneally (2 g/kg) body weight. Further glucose tolerance test was performed at 0, 30, 60, 90, and 120 min after administration of glucose load.

Survival studies

Swiss albino mice (n=30) were randomly divided into three groups (n=10 each). Group I: Control, Group II: APAP treated (600mg/kg) and Group III: APAP + CORM-A1 treated (600mg/kg APAP i.p. followed by 20mg/kg i.p. after a gap of

1h). Mice were given free access to food after APAP injection. Observations were made at an interval of every hour for initial 12 h following APAP injection. The subsequent observations of 12-24 h were recorded at an interval of 4 h till the end of experiment. Cumulative percentage survival was plotted using Kaplan-Meier survival curves and was generated in Prism 5.0 (GraphPad Software, San Diego, CA).

Mitochondrial DNA Copy number

Mitochondrial DNA (mtDNA) was used to determine mitochondrial density using q-PCR[R]. Total DNA was isolated from liver tissue or HepG2 cells using GeneJET genomic DNA purification kit (Thermo Scientific, USA) as per manufacturer's instructions. Isolated DNA was then used as template for quantification of cytochrome b and Nuclear 18s rRNA using PCR. Primers for the same were designed using NCBI BLAST and procured from IDT. Further, mitochondrial DNA copy number was calculated from the ratio of Cytochrome b (mitochondrial encoded gene) to Nuclear18s rRNA (nuclear encoded gene).

Quantitative real-time polymerase chain reaction (qPCR) analysis

Total RNA content from control and treated liver and HepG2 cells was isolated using TRIzol reagent (Invitrogen, CA, USA). RNA isolation through TRIzol reagent contains four steps viz. Homogenization, Phase separation, RNA precipitation and RNA wash. Pellet of RNA was dissolved in DEPC water and the purity of same was measured by analyzing A260/A280 ratio in UV-Vis

spectrometer (PerkinElmer, USA). RNA samples with A260/A280 ratio, ranging from 1.9 to 2.1 was used for the analysis. Further, RNA samples were checked for its integrity on agarose gel electrophoresis. OD at 260 was used to calculate concentration of RNA (µg/µl) in sample using the formula given below:

$$\frac{\boldsymbol{OD\ at\ 260\ nm} \times \mathbf{40} \times \text{Dilution factor}}{\mathbf{1000}}$$

Where, 40 = extinction coefficient (1=40 µg/ml of RNA); 1000 = factor for conversion from µg/ml to µg/µl cDNA was synthesized using 1 µg of total RNA via iScript cDNA Synthesis kit (BIO-RAD CA, USA). mRNA levels of candidate genes were quantified by qPCR analysis using QuantStudio-3 real time PCR (Life Technologies, CA, USA) using SYBR Select Master Mix. The data were normalized to the internal control GAPDH and analyzed using 2-ΔΔCT method.

Table 2. List of Primers for Real-time PCR of Human

Target	Primer	Sequence (5'→3')
HO-1	FP	TCTTGGCTGGCTTCCTTACC
	RP	GGATGTGCTTTTCGTTGGGG
Nrf2	FP	CTGCCAACTACTCCCAGGTT
	RP	TGACTGAAACGTAGCCGAAGA
GCLC	FP	AGATTAGGCTGTCCTGGGTT
	RP	TAAGGTACTGAAGCGAGGGT
GCLM	FP	GCGAGGAGGAGTTTCCAGATG
	RP	CTGTGCAACTCCAAGGACTGA
NQO-1	FP	GTTTGGAGTCCCTGCCATTCT
	RP	GCAGAGAGTACATGGAGCCAC
Nrf1	FP	TGGAACAAAATTGGGCCACG
	RP	TGCCACCACCTGTTAAGCG
PGC-1α	FP	AGTTCACTCTCAGTAAGGGGC
	RP	CCAGCTCCTGAATGACGCC
TFAM	FP	ACCAAAAAGACCTCGTTCAGC
	RP	CGAGTTTCGTCCTCTTTAGCA
Drp1	FP	CAAAGCAGTTTGCCTGTGGA
	RP	TCTTGGAGGACTATGGCAGC
GAPDH	FP	GAGTCAACGGATTTGGTC
	RP	GACAAGCTTCCCGTTCTC
Cytochrome b	FP	GCCCTCGGCTTACTTCTCTT
	RP	AGTGATTGGCTTAGTGGGCG
Nuclear 18s rRNA	FP	ACGGACCAGAGCGAAAGCA
	RP	GACATCTAAGGGCATCACAGAC
Keap1	FP	CTCCCCAACCGACAACCAAGA
	RP	AAGATAAGCAACACCACCACCT

FP, Forward Primer; RP, Reverse Primer

Table 3. List of Primers for Real-time PCR For Mice

Target	Primer	Sequence (5'→ 3')
HO-1	FP	ACATCGACAGCCCCACCAAGTTCAA
	RP	CTGACGAAGTGACGCCATCTGTGAG
Nrf2	FP	CGAGATATACGCAGGAGAGGTAAGA
	RP	GCTCGACAATGTTCTCCAGCTT
GCLC	FP	AACACAGACCCAACCCAGAG
	RP	CCGCATCTTCTGGAAATGTT
GCLM	FP	GCCCGCTCGCCATCTCTC
	RP	GTTGAGCAGGTTCCCGGTCT
NQO1	FP	CAGATCCTGGAAGGATGGAA
	RP	TCTGGTTGTCAGCTGGAATG
Nrf1	FP	GCAGAGGTGCAATCAAATGGA
	RP	ATCGGTGGCGTTTCTCACTC
PGC-1α	FP	AGTCTTCGGCTGTTTGGTGA
	RP	TGGAAGAACAGATGTGCCCC
SIRT1	FP	GATACCTTGGAGCAGGTTGC
	RP	CTCCACGAACAGCTTCACAA
FAS	FP	GGAGGTGGTGATAGCCGGTAT
	RP	TGGGTAATCCATAGAGCCCAG
TFAM	FP	GGTCGCATCCCCTCGTCTA
	RP	CCCCTGCCATGTGTTCTCCT
PPARα	FP	TGCAAACTTGGACTTGAACG
	RP	TGATGTCACAGAACGGCTTC
CD36	FP	TGAATGGTTGAGACCCCGTG
	RP	TAGAACAGCTTGCTTGCCCA
Drp1	FP	GGGCACTTAAATTGGGCTCC
	RP	TGTATTCTGTTGGCGTGGAAC

SREBP1c	FP	GCAGCCACCATCTAGCCTG
	RP	CAGCAGTGAGTCTGCCTTGAT
CPT-1	FP	CGATCATCATGACTATGCGCTACT
	RP	GCCGTGCTCTGCAAACATC
GAPDH	FP	TGTGAACGGATTTGGCCGTA
	RP	ACTGTGCCGTTGAATTTGCC
Cytochrome b	FP	CCACTTCATCTTACCATTTA
	RP	ATCTGCATCTGAGTTTAATC
Nuclear 18s rRNA	FP	GGGAGCCTGAGAAACGGC
	RP	GGGTCGGGAGTGGGTAATTT
TNF-α	FP	GTGGAACTGGCAGAAGAG
	RP	AATGAGAAGAGGCTGAGAC
IL-1β	FP	TCTATACCTGTCCTGTGTAATG
	RP	GCTTGTGCTCTGCTTGTG
IL-6	FP	TGGATGCTACCAAACTGGAT
	RP	CCTCAAAGCCAAGATGAGAA
CYP2E1	FP	TTTCCCTAAGTATCCTC CGTGACT
	RP	GCTGGCCTTTGGTCTTTTTG
Keap1	FP	CTGCCCAATTCATGGCTCACA
	RP	CTTAGGGTGGATGCCTTCGAT
NF-kB	FP	GAGGTCTCTGGGGGTACCAT
	RP	AAGGCTGCCTGGATCACTTC

FP, Forward Primer; RP, Reverse Primer

Co-immunoprecipitation assay

The interaction of Nrf2 with Keap1 following CORM-A1 treatment to HepG2 cells was assayed by co-immunoprecipitation (co-IP) assay according to the manufacturer's protocol (Pierce Classic Magnetic IP/Co-IP Kit). 500 µg of protein from different experimental groups was mixed with 10 µg Nrf2 antibody overnight at 4ºC to form the immune complex. Twenty-five microliters of prewashed Pierce Protein A/G Magnetic Beads were placed into the above immune complex and incubated for 1 hour with mixing. Then the beads were washed, and the target antigen was eluted with alternative elution method. Target antigen and the binding proteins were immunoblotted with the indicated antibodies by Western blot assay.

Immunoblot analyses

Control and treated liver samples and HepG2 cells were homogenized with ice-cold lysis buffer (RIPA). Nuclear proteins were isolated as described in NE-PER nuclear extraction kit (Thermo Scientific USA). Total protein was quantified by Bradford assay wherein, 1µL of lysate, 40µL of Bradford reagent and 159 µL of NFW was used for preparation of quantification system. Reading was taken in multimode reader at 590nm. The OD was used to calculate total isolated protein.

Further for western blot assay equal amount (40 µg) of protein was utilized from each sample. Samples were prepared by adding 6X loading dye, water and protein sample such that the concentration of final content was 2ug/µL. It was incubated at 100ºC for 10 min for complete denaturation of proteins, followed by centrifugation at 8000 rpm for 1 min.

6X Loading Dye: 12% SDS

: 30% β Mercaptoethanol

: 60% Glycerol

: 0.012% Bromophenol Blue

: 0.375 M Tris HCl (PH=6.8)

8%, 10%, 12% SDS polyacrylamide gel were prepared based on protein size. Equal amount of protein was loaded in all the wells. Running buffer (30.3g Tris base, 144.10g Glycine, 10g SDS in 1L water for 10X running buffer) was used for PAGE. On completion of run, proteins were transferred on to PVDF membrane (Bio-Rad, USA) by semi-wet transfer in transblot turbo (BioRad). Membrane was stained with ponceau stain for visualizing the efficiency of transfer. Further membrane was washed thrice with TBS till membrane decolorized and 5% milk was added for 1.30h for blocking. After giving a wash with TBS, primary antibodies for Nrf-2/ HO-1/ Keap1/ Nrf1 or PGC 1-α (1:1000) were added and left overnight. The next day secondary anti-rabbit horseradish peroxidase antibody (1:5000) was added for about 2 hours. Blots were stripped using stripping buffer (Thermo Scientific, Wilmington, DE) and re-probed with goat anti-rabbit Lamin B or β-actin antibody (1:5000) to determine equivalent loading. Blots were developed using ECL reagent (Bio-Rad, Hercules, CA) and visualize in iBright Imaging System or on X-ray sheets in dark room.

Statistical Analysis

The data were expressed as mean ± SEM and analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test using Graph Pad Prism 5.0 (CA, USA). *P<0.05, **P<0.001 and ***P<0.0001 were considered to be significant.