Synopsis of the thesis entitled

Epigenetic changes in circadian clock associated miRNAs and their role in atherosclerosis

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Introduction

Lifestyle disorders have become excessively common across the globe. Amalgamation of erroneous dietary habits, sleep-wake cycles, work culture and prolonged exposure to such regime results into manifestation of several diseases. Cardiovascular diseases tops the chart when it comes to mortality. WHO survey states that cardiovascular diseases claim 17.9 million lives annually. Atherosclerosis is a subset of cardiovascular diseases, marked by development of plaque in the arterial wall, obstructing the blood flow. Atherosclerosis is a multifactorial, slow and progressive disorder that can develop at any age. It is a major cause of myocardial infraction, stroke and heart failure. Exact cause of the disease is yet not known. However, obesity, diabetes, chronodisruption, hypertension, high cholesterol, insulin resistance etc. can act as triggers for development the disorder (Ridker *et al.,* 2002). Primarily, intima of the artery gets damaged forming a lesion. The site starts recruiting immune cells and other blood cells to the location. Damaged intima observed excessive ROS at the site. This facilitates oxidation of circulating low density lipoproteins (LDLs) that forms oxidized low density lipoprotein (ox-LDL). Ox-LDL internalize into the intima, followed by internalization of macrophages the eventually forms foam cells. Series of all such events leads to formation of necrotic core and arterial thickening (Gonzalez *et al.,* 2017). Several players like endothelial cells, smooth muscle cells, monocytes, macrophages, etc. are involved in atherogenic pathophysiology.

Circadian rhythm is 24 h day-night cycle that is closely associated to all the lives on the earth. Majority of physiological processes like metabolism, circulation, digestion, regeneration, immune responses, etc are entrained with circadian rhythms. Current life-style with shift jobs, trans-continental travelers, constant exposure to ALAN (artificial light at night) etc. are highly altering the natural circadian rhythm of the body. Studies show close association of atherosclerosis and chronodisruption. Circadian fluctuations in the vessel wall and in the circulation contribute to atherogenesis (McAlpine *et al.,* 2016). Chronodisruption also alters vascular tone and homeostasis. Vasodilation/constriction is associated with NO production also has circadian bases. Major physiological players operational in vessel biology are clock controlled, making circadian regulation an important event in pathophysiology. Circadian association is well established since decades, however the exact modus-operandi and key players in the system yet remains unclear. Herein we hypothesize that miRNAs that regulate several

physiological events are also under circadian control. We aim to investigate these miRNAs and their pin pointed role in atherogenic pathophysiology.

miRNAs (miro RNAs) are about 22-25 nucleotides long, highly conserved sequences of endogenous noncoding RNA that can regulate gene expression. miRNAs bind to 3'UTR of mRNA and can curb the translational process by either degrading the transcript or by inhibiting the assembly of translational machinery (O'Briaen *et al.,* 2018). Human genome hold about more than 1800 miRNAs that potentially regulates about 60% of the transcriptome involving key signaling pathways of cellular processes such as proliferation, differentiation and apoptosis in several life style disorders (Creugny *et al.,* 2018). miRNAs are broadly classifies as intronic, exonic and mirtrons. miRs that are present in the intron of the gene are called intronic miRs, the once present in the exon region are called exonic miRs. Mirtrons are special class of miRs that are located in the intronic region but are processed separately by splicisome.

Circadian control over atherosclerosis is known since a long time now. However the exact mechanism of control and the events are yet not very clear. This study tries to investigate circadian associate miRNA and its role in atherogenic pathophysiology using *in vivo, in vitro* and *in silico* experimental models.

Relevance of the Study

Atherosclerosis is widely prevalent across the world in all set of age groups. However till date no exact cause or the treatment of the same is available. Atherosclerosis is a lifestyle disorder that is multifactorial and is cause by wide number of reasons. Current manner of lifestyle with improper sleep-wake schedule, gobbling on the junk food with carbonated drinks, constant exposure to gadgets and technologies that promote sedentary life has steeply elevated the chances of having atherosclerosis. It becomes imperative to understand and investigate the set of players that are altered by chronodisruption and are involved in manifestation of atherogenic pathophysiology and to understand how we can bring about the corrective changes in the same.

This study tries to investigate miRNAs that are associated with altered biological clock and can manifest proatherogenic changes in the system. Also trying to understand how we can alleviate pro-atherogenic manifestation orchestrated by alteration in clock associated miRNAs. This study will not only unveil clock associated miRNAs but also address a way to manipulate their expression in order to protect from its pro-atherogenic effects.

Objectives

Objective 1: Establishing association of miR34a with core clock genes

Study 1: An *In silico* approach to evaluate clock associated miRNAs using preferential binding studies and functional analysis.

Objective 2: Investigating role of miR 34a-5p in experimentally induced atherogenic models.

Study 1: Establishing association between miR 34a-5p and CO in atherogenic system.

Study 2: Docking studies of CO with transcriptional regulators of miR34a.

Study 3: Exogenous CO lowers miR 34a-5p and improves atherogenic changes in SD rats.

Objective 3: assessing role of miR34a in improving mitochondrial health in atherogenic condition

Study 1: Studies with Human umbilical vein Endothelial cells (HUVEC)

Study 2: Studies with human monocyte derived (THP1) macrophage cells

Observations

Objective 1: Establishing association of miR34a-5p with core clock gene.

Study 1: An *In silico* approach to evaluate clock associated miRNAs using preferential binding studies and functional analysis.

miRNAs binds to the 3'UTR of mRNA and curb the translational process and gene expression. Herein, we were interested in the genes that alter the clock gene expression at the systemic level. For this we employed the insilico approach. At present there are multiple target prediction tools available with customized data bases like miRwalk, miRanda, miRDB, TargetScan etc. herein we used miRDB [http://mirdb.org/] for screening the miRNAs that potentially altered the clock

gene expression. miRDB extracts and encompass database from miRWalk, miRecords and miRDIP. This potentially covers all the existing data required for target prediction and data mining.

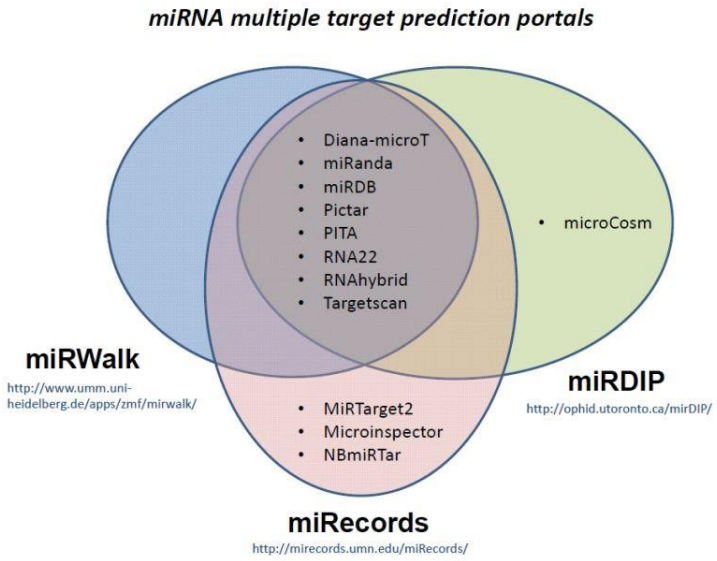


Fig1: Pictorial representation of target prediction software and the data bases utilized for target mining and interaction analysis

Target mining was done for the same using the following inclusion criteria:

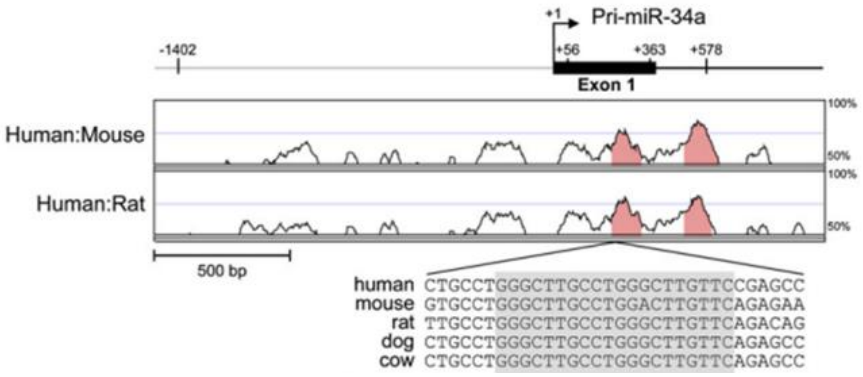
1. Include functional miRNAs
2. Gene targets with target score more than 70 was only considered.
3. miRNAs with more than 2000 predicted targets in genome were considered.

The following data was taken from both human and mice functional miRNA database. miRNAs were deciphered based on the hit score they were allotted based on the 3'UTR gene compatibility. Higher hit score was assigned to miRNA showing higher compatibility and lower ΔG values for bond formation and significantly stable complex formation. Clock is the key gene that regulates central and peripheral circadian rhythms. Alteration in expression of the same leads to chronodisruption. Herein we mined for the miRNAs that can plausibly alter the expression of the clock gene. We got 41 miRNAs that had more than 90 hit score, 38 miRNAs

with hit score 80-89 and 31 miRNAs with score 70-79. Fig 2 shows the miRNAs green with score 90-99, pink 80-89 and blue 70-79.

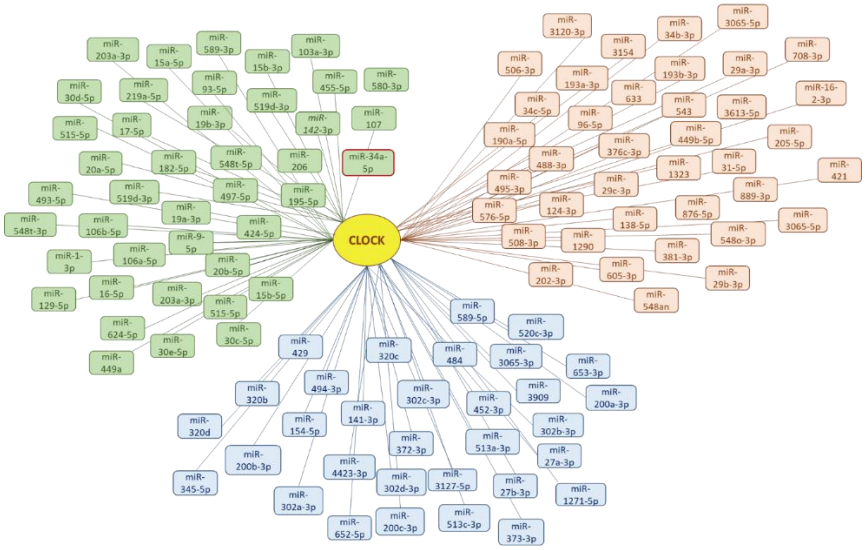
All the target miRNAs were further analyzed in regards to their target genes and related cellular function. Out of all the miRNAs, miR 34a-5p was selected for further investigation based on the atherogenic genes targeted by the miR 34a. About 50% of the genes targeted by miR 34a are potentially functional in atherogenic pathophysiology and cardiovascular biology. Fig2 (c) shows the data mined from miRDB for investigating gene targets of has miR 34a-5p, with the hit score above 70. miR 34a is localized on chromosome 1 p36.22 and is independently expressed with its own promoter. miR 34a is also conserved across the species that makes its functionality a significant in biological system. A lot has been investigated with miR 34a in cancer pathology but a major lacunae exists when it comes to atherosclerosis. miR 34a plays active role in several cellular function that are crucial in atherosclerotic pathology. miR 34a is further processed to form miR 34a-5p and miR 34a-3p. Overall functionality of 5p arm of miRNA is considered to be more functional than the 3p. In silico investigation shows that the 5p arm of miR 34a is compatible to 3'UTR of clock gene with 90 hit score and Per2 gene with the score of 73. Along with that miR 34a-5p shows interaction with atherogenic target genes like SIRT-1, KLF4, MDM4, IL6R, NOTCH1, CDH4, MRPL10, CASP2, DLL4, etc.

(a)



Chang *et al.,* 2007

(b)



(c)

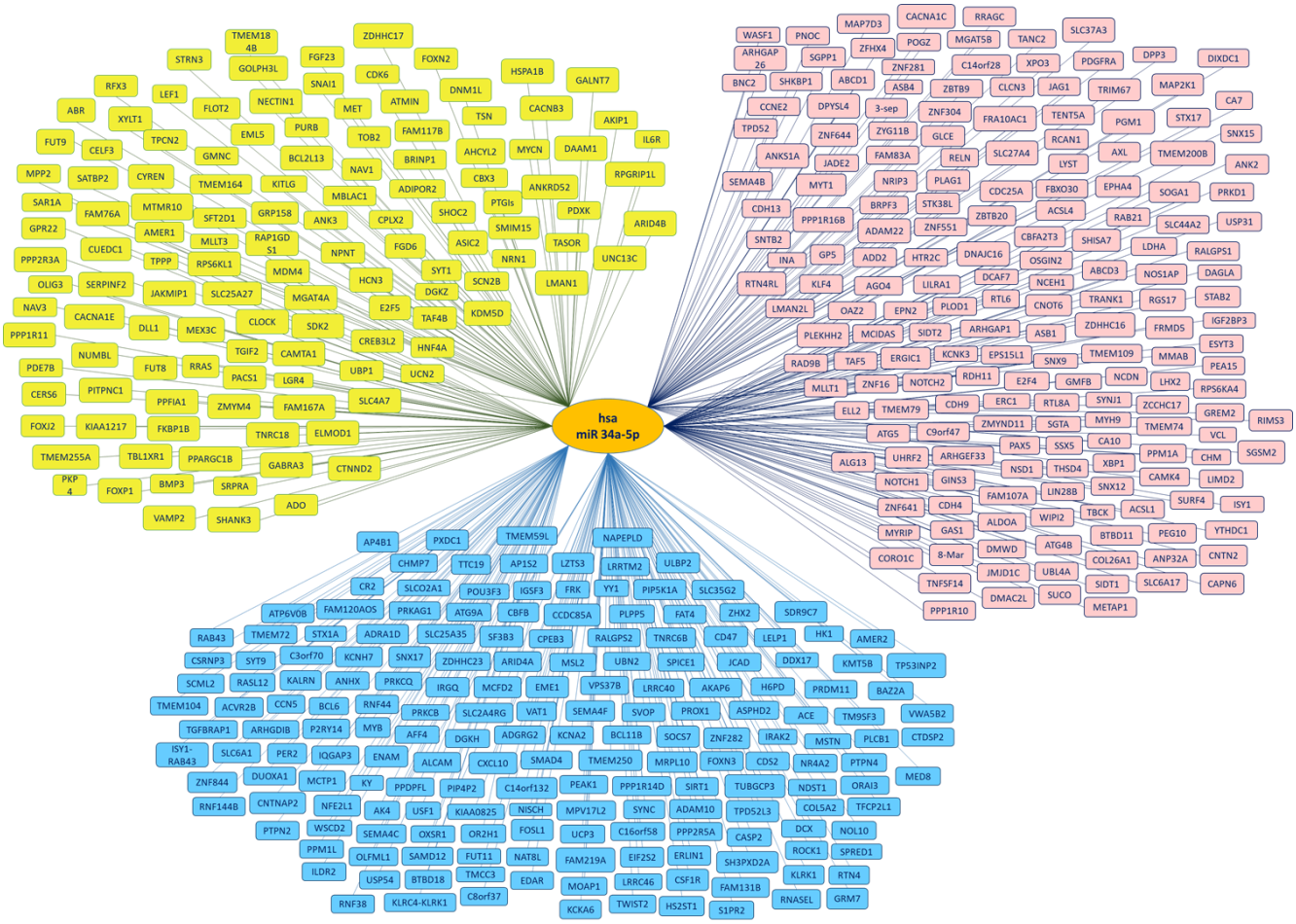


Fig2: (a) Diagrammatic representation of conserved sequence of miR 34a across species. (b) *Insilico* target prediction for all the miRNAs compatible to 3'UTR of CLOCK gene; Hit Scores, Green: 90-99; Pink: 80-89; Blue: 70-79. (c) Targets predictions of all the genes potentially docked by miR34a-5p; Hit Scores, Yellow: 90-100; Pink: 80-89; Blue: 70-79

Objective 2: Investigating role of miR 34a-5p in experimentally induced atherogenic models.

Study 1: Establishing association between miR 34a-5p and CO in atherogenic system.

An in vivo atherogenic model was developed by feeding atherogenic diet to male Sprague Dawley (SD) rats of about 8-10 weeks of age, for 8 weeks. A study by Oiu and group reported that the levels of CO elevated in exhalation of patients with cardiovascular disorders. The group proposes to use CO as a potential biomarker for investigating the cardiovascular risks (Oiu *et al.*, 2020). In our study atherogenic rats showed elevated CO levels as compared to control rats. CO is an endogenous gasotransmitter that is formed as a by-product in the process of heme degradation, along with biliverdin in equal proportion. Biliverdin is immediately reduced to bilirubin. Herein were have quantified bilirubin as a proxy indicator for investigating CO levels in the serum. Levels of bilirubin in serum of atherogenic SD rats were found to be elevated as compared to the control rats. CO is known to play a crucial role in vessel biology. But a little in known about the functional mechanism of the same in atherogenic disease pathophysiology. Here in we try to investigate if CO can modulate the miRNA expression levels in atherogenic pathophysiology and alter atherogenic manifestations.

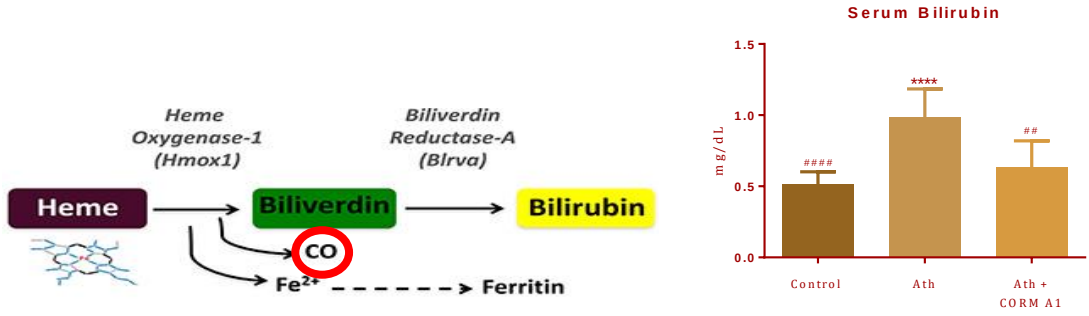


Fig 4: (a) pictorial representation of production of carbon monoxide and biliverdin and its reduction to bilirubin. (b) Serum bilirubin levels in SD rats feed on atherogenic diet and supplemented with CORM A1 (n=7).

On further investigation we found that the levels of miR 34a-5p in aorta of atherogenic rats were high. In order to investigate the correlation between miR 34a-5p and CO, Carbon monoxide

releasing molecule A1 (CORM A1) was used. Cytotoxic evaluation of CORM A1 was done on human umbelical vein endothelial cells (HUVEC) and Thp-1 derived macrophages by MTT assay. A range of concentration from 10 to 200 μM was used for the assay and it was non-toxic at all the concentrations. We checked for gene and miRNA expression at concentration of 40, and 100 μM of CORM A1. Interestingly, the lower dose of 40 μM the levels of miR 34a-5p reduced to almost the half of control cells and the same increased 4 fold at concentration of 100 μM of CORM A1 respectively. We also checked levels of miR 155 that is inflammation specific miRNA that increases in pro-inflammatory conditions. Similar pattern was observed in expression of miR 155 as seen in miR 34a-5p. miR 34a-5p binds to the 3'UTR of SIRT1 transcript and stops its translational process as seen in *insilico* target prediction and literature available (yamakuchi *et al.,* 2008). Herein, we found that the levels of SIRT-1 were elevated 4 folds at 40 μM. Likewise levels of PGC-1α were highly elevated at 40 μM concentration of CORM A1, than at 100 and 200 μM. This concluded that CORM A1 elevated SIRT-1 and PGC-1α expression by lowering expression of miR 34a-5p.

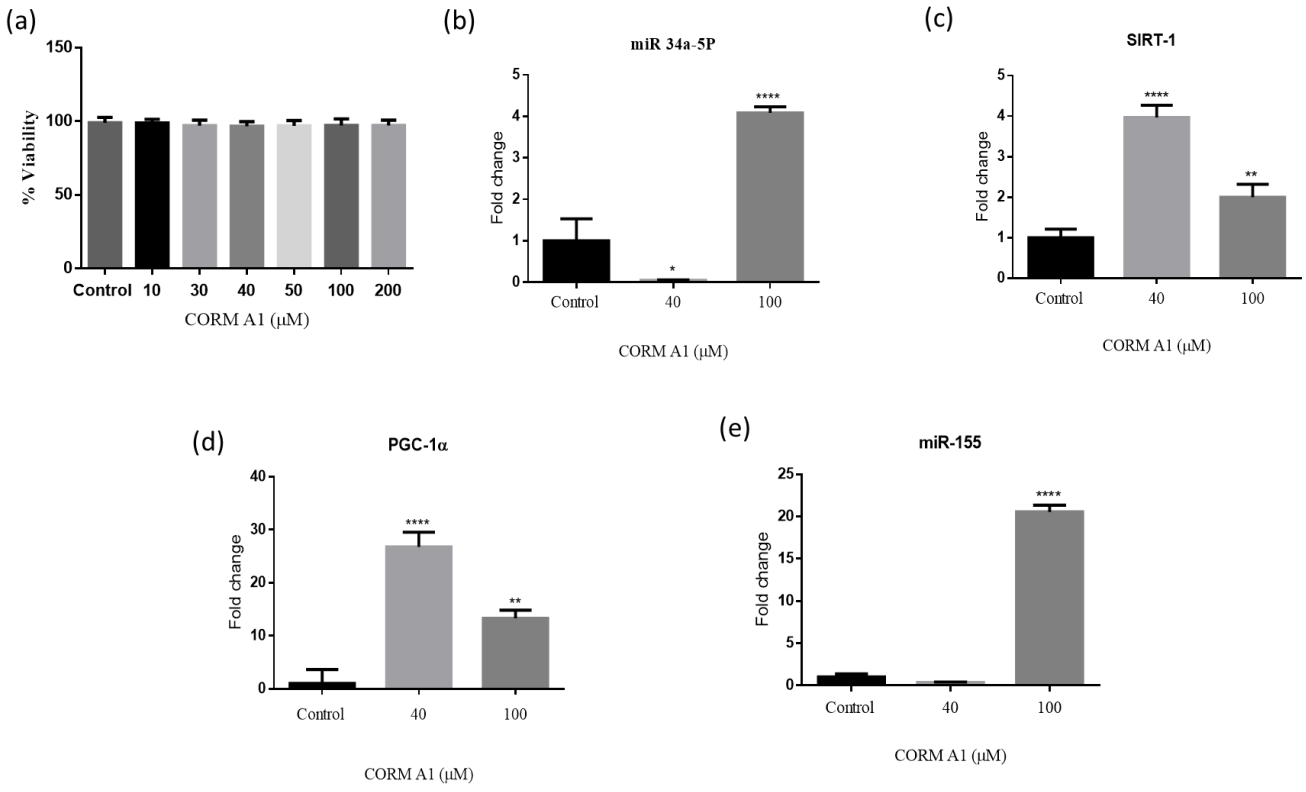


Fig 5: Cell viability assay accessing cytotoxicity of CORM A1 on (a) HUVEC. HUVEC cells treated with 40 and 100 μM of CORM A1, evaluating expression of (b) miR 34a-5p, (c) SIRT-1, (d) PGC-1α and (e) miR 155.

In vitro atherogenic model was developed by treating cells with ox-LDL. It was interesting to know if CORM A1 could inhibit LDL oxidation process itself. TBARS assay was performed for investigating the LDL oxidation kinetics. nLDL was treated with 40 μM of $CuSO_4$ along with 10, 20, 40, 60 and 80 μM of CORM A1. Results showed no change in oxidation status of nLDL in presence of CORM A1.

Further we checked effect of CORM A1 on atherogenic HUVEC. For that HUVEC cells were dosed with 80 μg/mL of ox-LDL in presence and absence of 40 μM of CORM A1 for 24h. Gene expression showed that levels of miR 34a-5p lowered in CORM A1 treated group and the levels of SIRT-1 increased in the same. This concludes that CORM A1 alleviates levels of miR 34a-5p in atherogenic HUVEC and increases SIRT-1 expression.

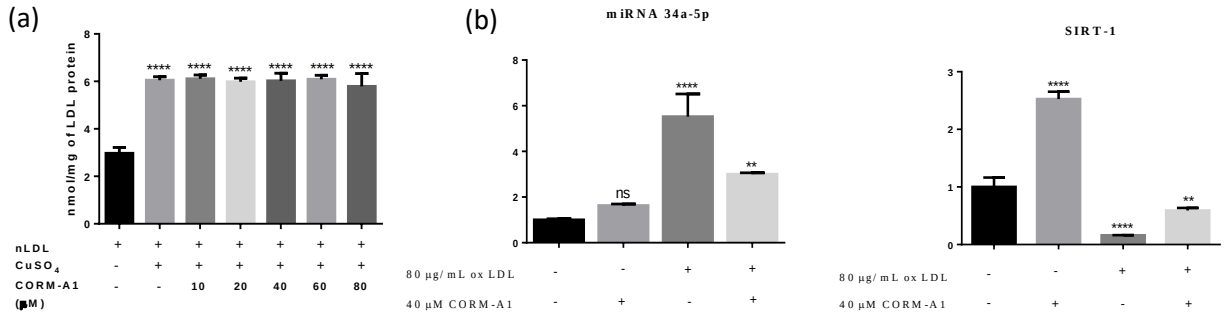
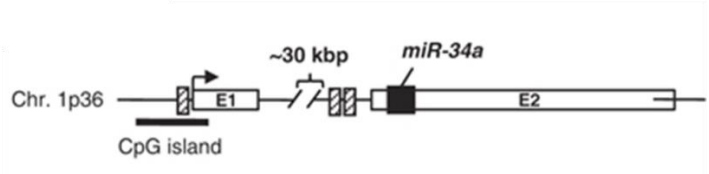


Fig 6: (a) LDL oxidation kinetics in presence of different concentrations of CORM A1 as analyzed with TBARS assay. (b) Evaluating levels of miR 34a-5p and SIRT-1 in atherogenic HUVEC co-treated with CORM A1.

Study 2: Docking studies of CO with transcriptional regulators of miR34a-5p.

miR 34 is a family that comprises of three miRNAs; miR 34a, miR 34b and miR 34c, that have their own promoter region and can transcribe independently. P53 is major transcriptional regulator of miR 34a. P53 binds to the promoter region of miR 34a and initiates the transcription process. However proteins Zeb-1 and Snai1 bind the E-box region of miR 34a and can curb the transcription process.



Herein, we were interested to know if CO can directly impact the binding of transcription factors or inhibitors at promoter or the E-box region of miR 34a. Primarily we analyzed the interaction of the proteins with CO molecule. Data for the same has been listed in the table below. Following that we did a dock score analysis wherein we found CO showing potential binding with P53 and Snai1, whereas it was unable to dock with Zeb1 protein. These data concluded that CO can possibly be docking to DNA binding site of P53 and inhibit miR 34a transcription.

(a) Interaction Profile

Protein Name	Interaction Part			Bond length(Ångstrom)
	Amino Acid Name	Atom	Ligand Atom	
P53	LYS 164	N	C	5.00
SNAI1	GLU 256	O	O	3.92
ZEB1	LYS 19	N	C	3.93

(b) Dock Score table

Protein Name	Glide Score(kcal/mol)	Glide Emodel
P53	-3.039	-6.265
SNAI1	-2.912	-8.656
ZEB1	-2.803	-6.182

(c)

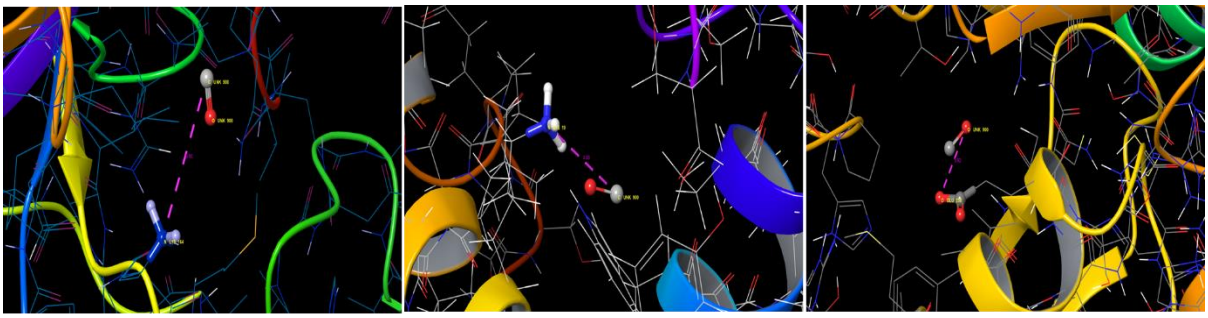
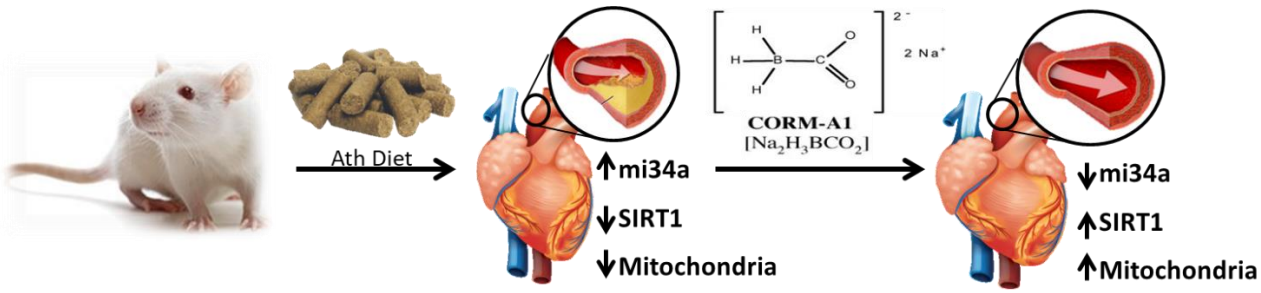


Fig 7: Tabular representation of (a) interaction profile and (b) docking score. (c) Pictorial representation of interaction of CO with P53, Zeb-1 and Snai1 protein structures.

Study 3: Exogenous CO lowers miR 34a-5p and improves atherogenic changes in SD rats.



32 male Sprague Dawley (SD) rats of 8-10 weeks of age were procured from Sun Pharmaceutical Ltd. Vadodara. Rats were allowed to acclimatize for 2 weeks at the animal house of department of Zoology faculty of Science, with approved CPCSEA animal house facility (827/GO/Re/S/04/CPCSEA) and approved animal ethical protocol (MSU-Z/IAEC03/03-2019). Animals were randomly divided into 4 groups (n=8/group) for experiments.

Group 1: Std Diet

Group 2: Ath Diet

Group 3: Ath Diet + iCORM A1

Group 4: Ath Diet + CORM A1

Animals of group 2, 3 and 4 were given single dose of 600000 IU Vit D3 on the 1st day of experiment for development of atherogenic model (Jadeja *et al.*, 2012). All the animals were given food and water *ad libidum.* Animals of group 4 received intra-peritoneal injection of 2 mg/kg of CORM A1 every day from 4th week and group 3 received iCORM A1 likewise. Data for iCORM A1 showed no changes and hence not shown further herein.

At the end of experimentation animals were sacrificed and blood serum and aorta was collected for evaluation. Gasotransmitter, carbon monoxide (CO) was measured with an indirect method. Levels of serum cholesterol, LDL and total lipid elevated in rats fed with atherogenic diet, but the same showed minimal increase in CORM A1 supplemented group, which showed higher

HDL levels than the atherogenic diet fed rats. Atherogenic index of plasma (AIP) is a logarithmically transformed ratio of molar concentrations of triglycerides to HDL-cholesterol. The strong correlation of AIP with lipoprotein particle size may explain its high predictive value. AIP is logarithm of molar ratio of triglyceridemia to high-density lipoprotein cholesterol (TG/HDL-cholesterol) (Onat *et al.*, 2010) (James *et al.*, 2016). AIP showed no difference in control and CORM A1 group but was significantly high in atherogenic group. LDL/HDL and Chol/HDL ratios also pointed towards antiatherogenic effects of CORM A1.

$$\text{Atherogenic index of plasma} = \log_{10}\left(\frac{\text{Triglycerides}}{\text{High density lipoprotein -- Cholesterol}}\right)$$

En face assay was done for gross evaluation of whole thoracic aorta, the atherosclerotic group showed plaque development and stiffened aorta, however the same was not observed in CORM A1 supplemented rats. Histomorphological evaluation of aortic elastin showed elastin derangement in ath-diet fed rat that was highly improved in CORM A1 supplemented rats. Elastin fragmentation was also evaluated for the aorta of all the groups. High fragmentation was observed in atherogenic aorta. Fragmentation in CORM A1 supplemented group was comparable to the control group.

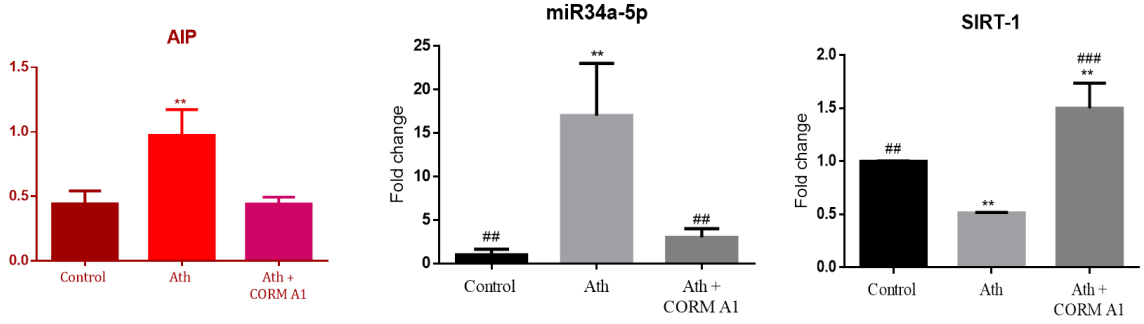


Fig 8: Atherogenic index of plasma was evaluated from plasma profile of SD rats fed on atherogenic diet for 8 weeks along with supplementation of 2mg/kg of CORM A1 for 4 weeks. Expression levels of miR34a-5p and SIRT-1 gene.

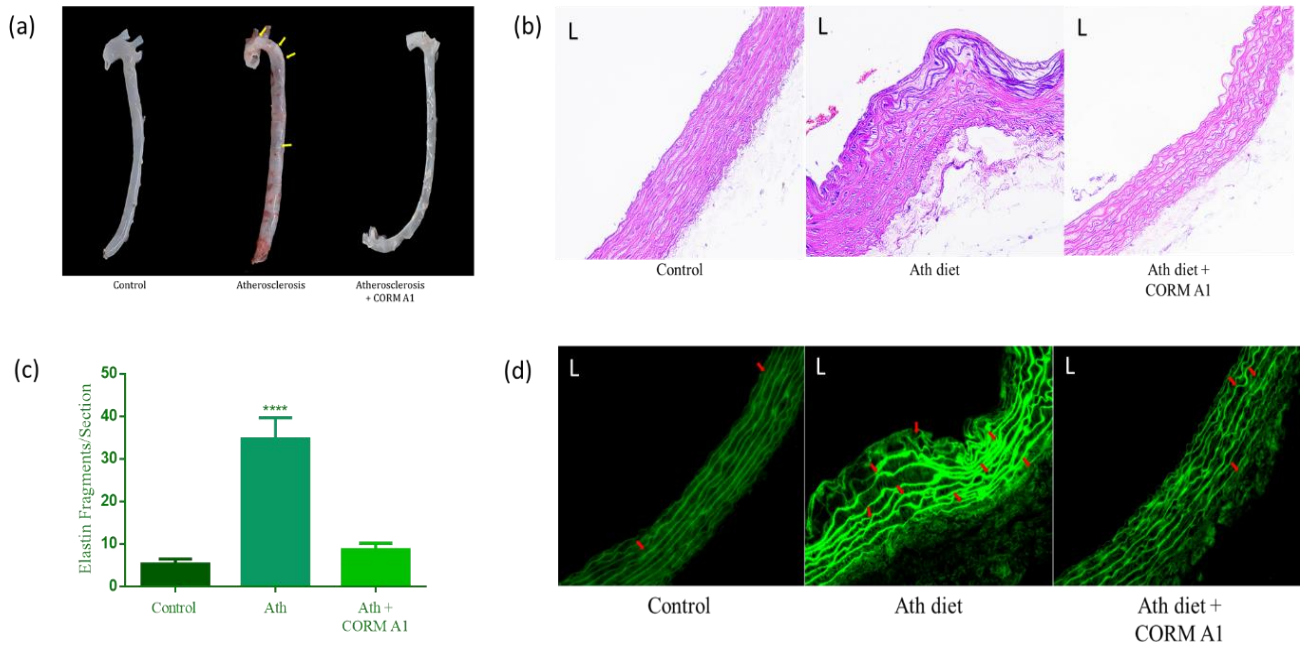
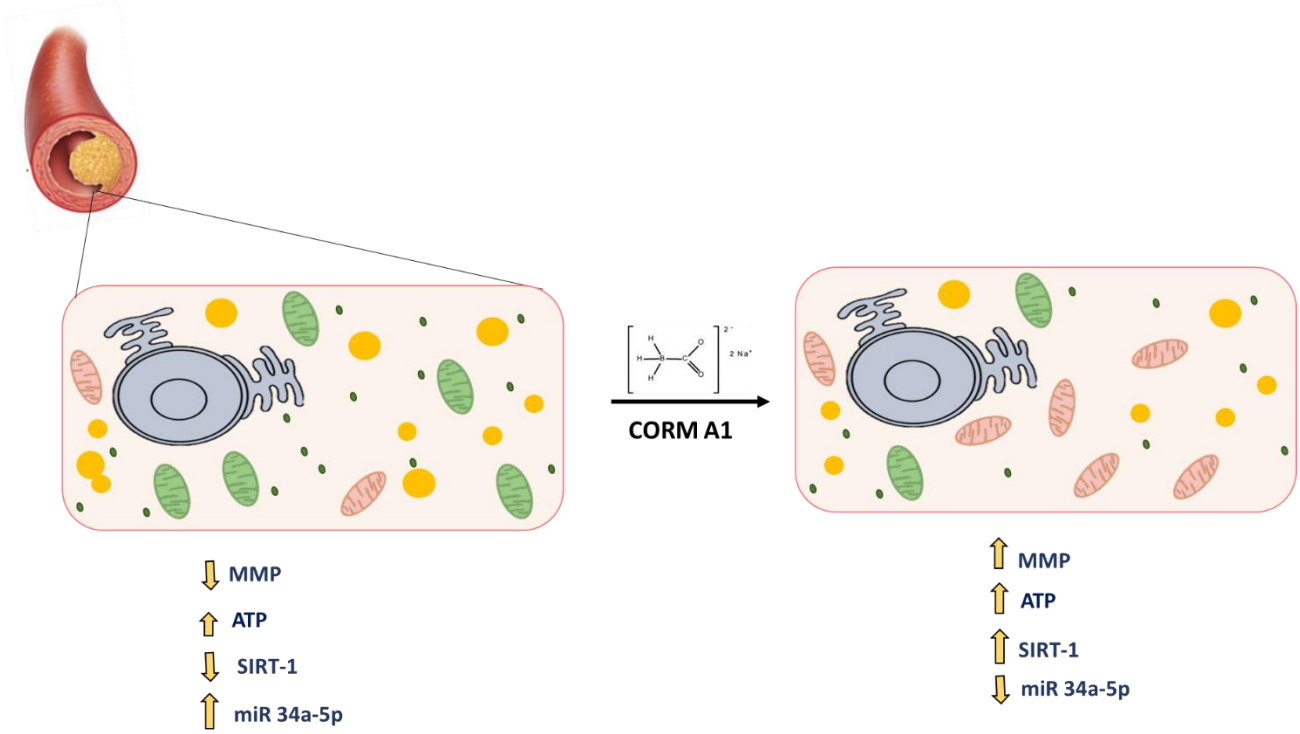


Fig 9: (a) En face assay with whole aorta; yellow arrow shows atherogenic plaques in the aorta. (b) H&E staining for transverse section of aorta; L shows the lumen region. (d) Elastin Imaging; red arrows show elastin fragmentation. (c) Quantification of elastin fragmentation/section of aorta.

Objective 3: Alleviation of miR34a-5p improves mitochondrial health in atherogenic condition

Study 1: Studies on Human umbilical vein Endothelial cells (HUVEC)



Mitochondria plays a vital role in cellular maintenance in case of atherosclerosis. Mitochondrial health is crucial to combat against lipid load and increased cellular ROS. Mitochondrial dysfunction promotes pro-atherogenic changes and apoptosis that eventually culminates into plaque rupture (Madamanchi *et al.,* 2007). Elevated levels of miR 34a-5p and can curb protein expression of SIRT-1 by binds to 3'UTR of its mRNA, as observed in objective 2, study1. Levels of PGC-1α also showed inverse correlation to that of miR 34a-5p expression. SIRT-1 is known to be crucial in several metabolic process going on in the cell. In this study we wanted to see if alleviation of miR 34a-5p via CORM A1 orchestrates any impact on cellular mitochondria. For that we used HUVEC cells that were dosed with 80 µg/mL of ox-LDL and were co-treated with 40 µM of CORM A1 for 24h. Cells were then stained with JC-1 stain for evaluation of mitochondrial membrane potential (MMP). The J aggregates in red showed healthy mitochondria with polarized membrane potential and the J-monomers in green showed poor membrane potential and mitochondrial health. Cells co-treated with CORM A1 showed much lower

concentration of J-monomers as compared to the cells treated with ox-LDL. The data suggest that CORM A1 improved mitochondrial membrane potential and cellular health.

Gene expression of SIRT-1, PCG-1α and Drp-1 was also done. Wherein levels of all the genes increased in with lowered levels of miR 34a-5p in presence of CORM A1, indicating increased mitochondrial biogenesis.

Further we also checked for mitochondrial functionality in lower miR 34a-5p conditions. Functional analysis of mitochondria was done by ATP assay wherein total ATP of the cells were determined. The cells treated with CORM A1 showed elevated levels of ATP as compared to the disease conditions.

Surmising that lower miR 34a-5p expression via CORM A1 can improve mitochondrial biogenesis, function and MMP.

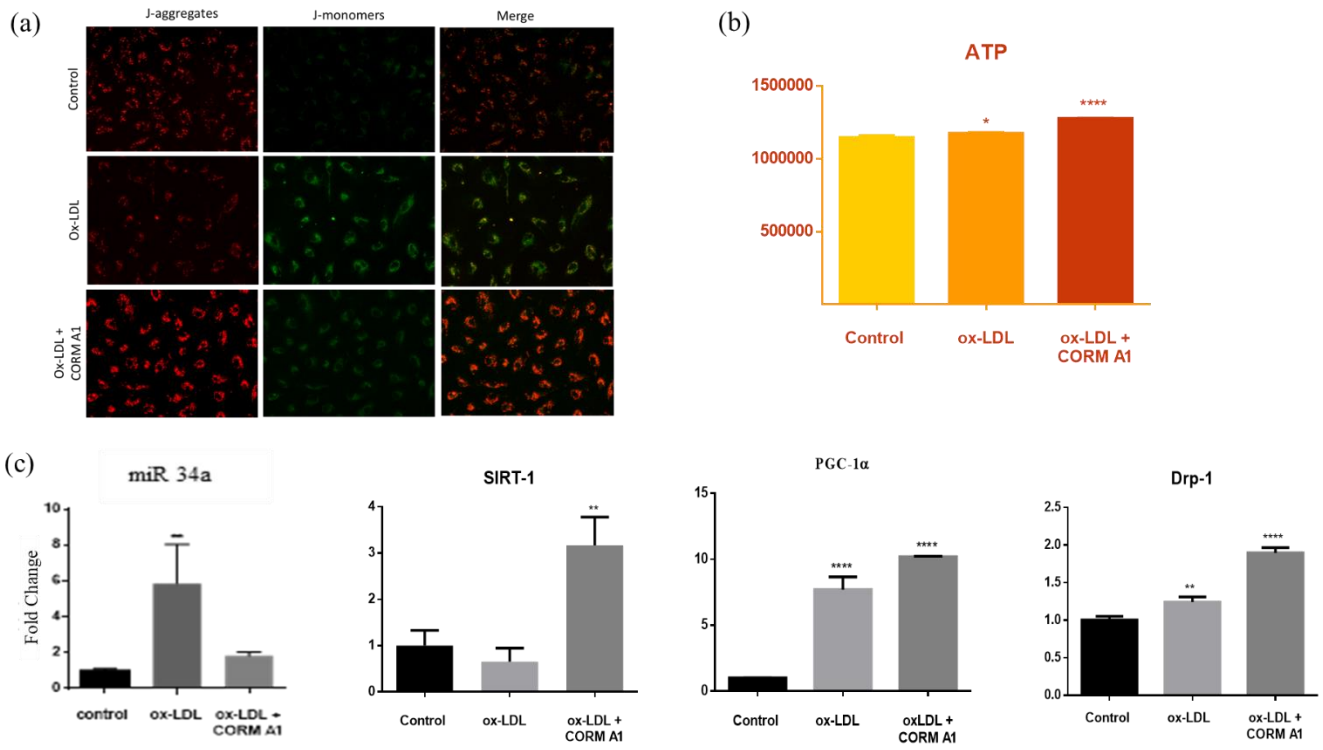
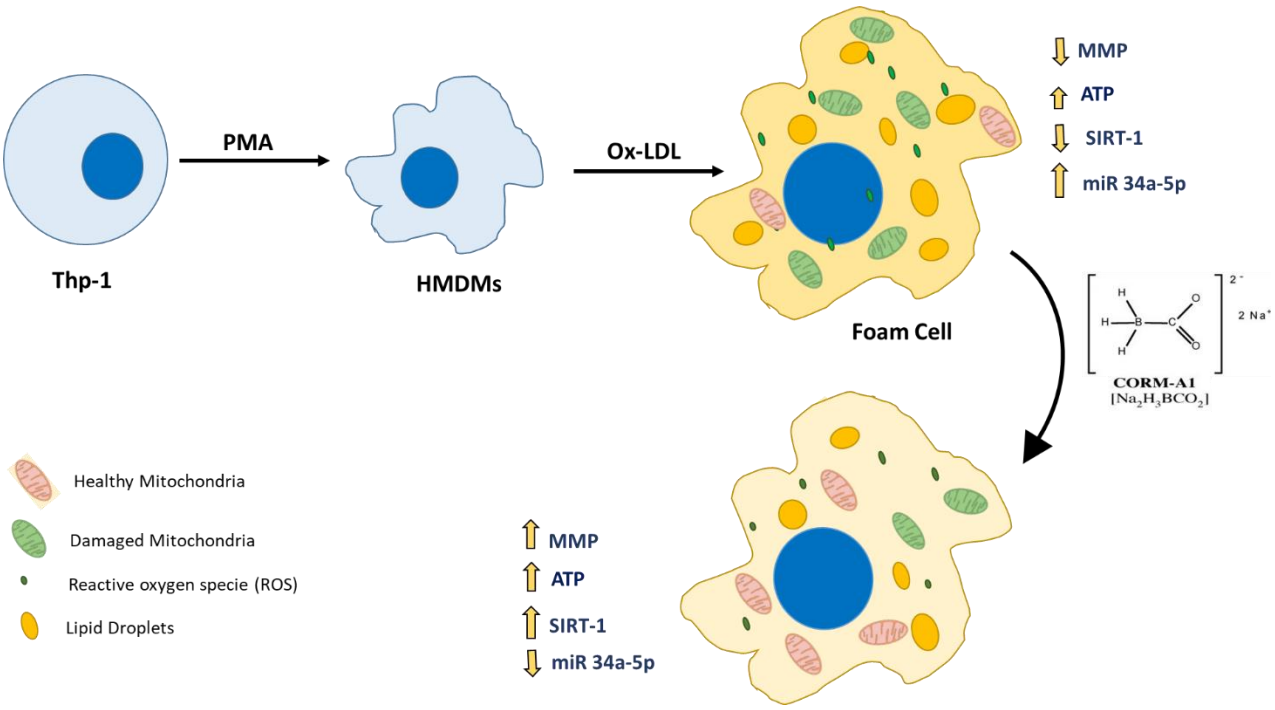


Fig 10: JC-1 staining for HUVEC cells. (b) Quantitative evaluation of ATP assay (c) miRNA and gene expression in atherogenic HUVEC cells co-treated with CORM A1.

Study 2: Studies on human monocyte derived (THP1) macrophage cells



Thp-1 were differentiated to macrophages by treating them with 50 nM of PMA for 24h. Cells were then washed once with PBS and further dosed with ox-LDL and/or CORM A1. Gene expression of SIRT-1, PGC-1α and Drp-1 was done, results indicated the trend as observed in HUVEC in study 1. Levels if all the three genes increased in presence of CORM A1 facilitating mitochondrial biogenesis.

Further JC-1 staining also showed higher J-monomers in ox-LDL treated macrophages as compared to the co-treated cells, suggesting higher mitochondrial health and membrane potential in CORM A1 co-treated cells with lower miR 34a-5p expression.

Mitochondrial functional analysis was also done by performing ATP assay as in HUVEC. ATP levels were increased in CORM A1 treated group as compared to the ox-LDL treated cells.

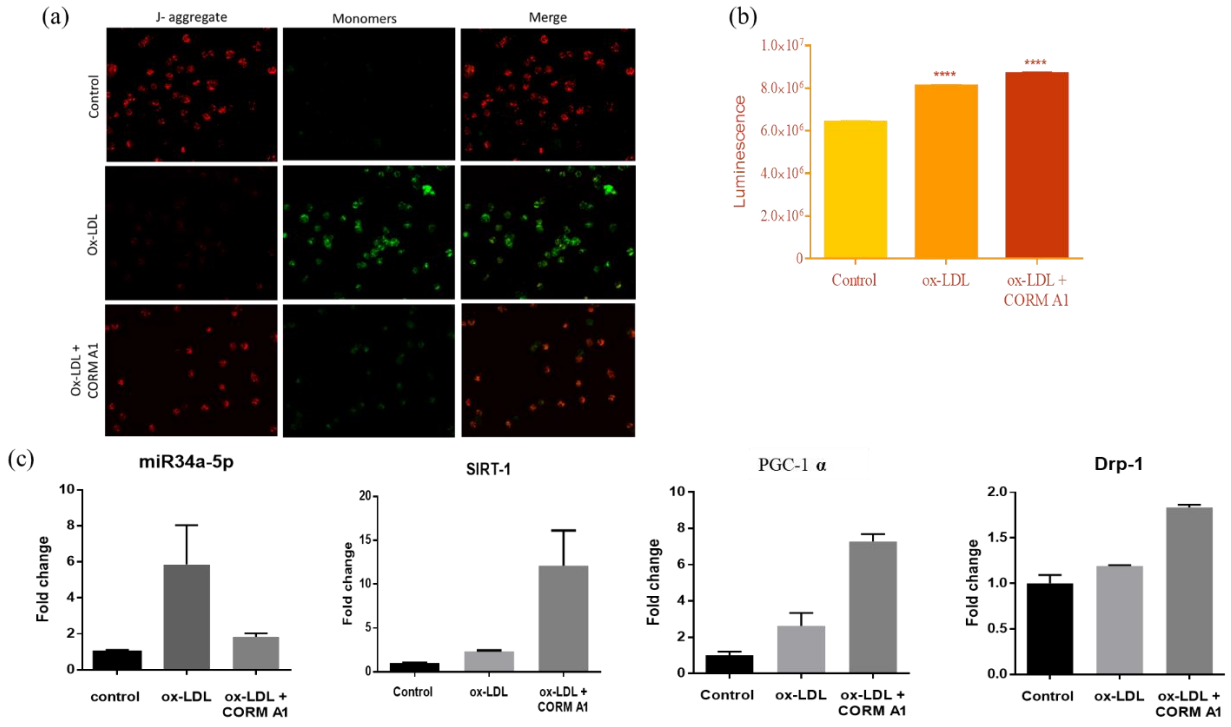


Fig 11: JC-1 staining for thp-1 derived macrophages. (b) Quantitative evaluation of ATP assay (c) miRNA and gene expression in atherogenic macrophages co-treated with CORM A1.

Key Findings:

1. miR 34a-5p potentially binds to 3'UTR of clock gene and also targets several metabolic genes functional in atherogenic pathophysiology.
2. Endogenous levels of Carbon monoxide increases in atherogenic model developed in SD rats.
3. Expression of miR 34a-5p alleviates on exogenous supplementation of CORM A1 in atherogenic conditions and promotes expression of target gene, SIRT-1.
4. CO has anti-atherogenic potential as evidenced by lower miR 34a-5p levels, reduced plaque development, improved serum lipid profile and arterial construct.
5. Lower miR 34a-5p promotes SIRT-1 expression and improved mitochondrial biogenesis, function and MMP in HUVEC and thp-1 derived macrophages in atherogenic conditions.

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Publications

1. **Vyas, Hitarthi**, Kapil Upadhyay, and Ranjitsinh Devkar. "Carbon Monoxide Releasing Molecule A1 (CORM A1) Modulates miRNA 34a-5p Expression via Zeb1 and Snai1 Proteins and Improves Mitochondrial Function in Atherogenic HUVEC." *Free Radical Biology and Medicine* 159 (2020): S101.
2. **Vyas, Hitarthi S**., Kapil K. Upadhyay, and Ranjitsinh V. Devkar. "miRNAs Signatures In Patients With Acute Liver Injury: Clinical Concerns and Correlations." *Current molecular medicine* 20, no. 5 (2020): 325-335.
3. Upadhyay, Kapil K., Ravirajsinh N. Jadeja, **Hitarthi S. Vyas**, Bhaumik Pandya, Apeksha Joshi, Aliasgar Vohra, Menaka C. Thounaojam, Pamela M. Martin, Manuela Bartoli, and Ranjitsinh V. Devkar. "Carbon monoxide releasing molecule-A1 improves nonalcoholic steatohepatitis via Nrf2 activation mediated improvement in oxidative stress and mitochondrial function." *Redox biology* 28 (2020): 101314.
4. Nariya, Pratik, Falguni Shukla, **Hitarthi Vyas**, Ranjitsinh Devkar, and Sonal Thakore. "Synthesis and characterization of Mannich bases of lawsone and their anticancer activity." *Synthetic Communications* 50, no. 11 (2020): 1724-1735.

Conference and Workshop

1. **SfRBM 2020** 27th annual Conference, 18th- 20th Nov 2020, Organized by Society for Redox Biology and Medicine, USA. (Poster Presentation)
2. **B4 Young Scientist Program**, Workshop on Bioimaging: 2019. Organized by Harvard University, at IISER Pune, Pune, Mahrastra.
3. **CRISPER Cas workshop**: 2019, Organized by IIT Mumbai, at The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat.
4. **International Conference on Reproduction, Endocrinology and development** (ICRED), 2018. Organized by Navrachna University, Vadodara, Gujarat. 2019.
5. **Plant and microbial biotechnology conference**, organized by Dr. Bharat Chattoo Genome Research Center (BCGRC), The Maharaja Sayajirao University of Baroda, Vadoara, Gujarat. 2017.

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